**CtHtrA: the lynch pin of the chlamydial surface and a promising therapeutic target**

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***Abstract***

*Chlamydia trachomatis* is the most prevalent sexually transmitted bacterial infection worldwide and the leading cause of preventable blindness. Reports have emerged of treatment failure, suggesting a need to develop new antibiotics to battle *Chlamydia* infection. One possible candidate for a new treatment is the protease inhibitor JO146, which is an effective anti-*Chlamydia* agent that targets the CtHtrA protein. CtHtrA is a lynch pin on the chlamydial cell surface due to its essential and multifunctional roles in the bacteria’s stress response, replicative phase of development, virulence, and outer membrane protein assembly. This review summarizes the current understanding of CtHtrA function and presents a mechanistic model that highlights CtHtrA as an effective target for anti-*Chlamydia* drug development.

***Key words***

HtrA, protease, virulence, intracellular, DegP

***Introduction***

The development of antibacterial therapeutics has not been adequate to compete with the rise of antibiotic resistance for many disease-causing pathogens. Rational development of drugs that target new biological pathways is important to ensure continued disease prevention or treatment [1]. Preferably, new antimicrobials should be bactericidal, have a high therapeutic index, have a targeted spectrum of activity that limits perturbation of the normal host microbiota and have a low potential for developing resistance. Traditional antibiotic targets, including cell wall and DNA/RNA biosynthesis, may be exhausted for new leads, and attention is instead shifting towards other factors for developing novel drug candidates, such as proteases [2]. Chemical biology approaches have proven successful for identifying potentially viable protease inhibitors [3], but their utility as a therapeutic requires an in-depth understanding of both the target protein function and the associated cellular pathways that are critical for pathogen survival and/or pathogenicity [1].

*Chlamydia trachomatis* is a significant human pathogen as the etiological agent for the most commonly reported sexually transmitted infection (STI) worldwide and the leading cause of preventable blindness [4]. Diagnosis rates have increased dramatically over the last decade, but *Chlamydia* can lead to adverse and severe outcomes if left untreated, including pelvic inflammatory disease, ectopic pregnancy, and infertility [5,6]. Treatment with azithromycin and tetracyclines are recommended for *Chlamydia* infection [7], but increasing rates of repeat infections and suspected treatment failures suggest that azithromycin treatment might not be as reliable as previously thought [8-10]. New and novel anti-*Chlamydia* drugs are essential, but the application of genetic techniques to identify the organism’s key pathogenic pathways has only recently become possible.

*Chlamydia* is an obligate intracellular pathogen with a developmental cycle that transitions between an infectious non-dividing form known as the elementary body (EB) and a replicative form called the reticulate body (Figure 1) [11]. EBs initiate an infection by attaching to and invading host cells, and are internalized in a membrane-bound vacuole known as an inclusion. At 8-12 hours post-infection (h PI), EBs differentiate into RBs that undergo binary fission until 18-24 h PI when reversion to the EB form occurs. At 40-72 h PI, the host cell lyses, releasing EBs to infect neighboring cells. In the presence of a variety of stressors, including antibiotics [12] and interferon gamma [13], *Chlamydia* enters a persistent phase by forming large, pleomorphic, non-replicative forms known as aberrant bodies.

Using a screening strategy, we identified a chlamydial serine protease inhibitor, JO146, as a potential candidate for a novel anti-*Chlamydia* therapeutic [14]. JO146 is a chemical inhibitor that is specific for the *C. trachomatis* High Temperature Requirement A (CtHtrA) protein that irreversibly inhibits its protease activity. JO146 was lethal to *C. trachomatis in vitro* when added during the mid-replicative stage of the development cycle and was lethal during reversion or recovery from penicillin persistence and during heat stress [14,15]. Lethality was conserved across a range of cell types, clinical isolates and species, and no obvious toxicity was detected in mouse or human cells [14,16,17]. Furthermore, JO146 was significantly less active against *Escherichia coli* HtrA (also known as DegP) and human HtrA proteins HTRA1 and HTRA2, suggesting unusual specificity [14].

HtrA homologs are common to all domains of life, although only a few examples have been extensively investigated. They appear to primarily function as quality control proteases that degrade un- or mis-folded proteins during stress conditions, but some evidence indicates an additional chaperone and/or transporter role in protein assembly and localization to the cell surface or extracellular sites [18]. HtrAs have also been implicated in the virulence of several bacteria, including *E. coli*, *Bordetella pertussis, Campylobacter jejuni, Legionella pneumophila, Salmonella enterica, Listeria monocytogenes, Staphylococcus aureus, Bacillus anthracis,* and *Helicobacter pylori*, and *htrA*- deletion strains are typically attenuated [19-27]. In bacteria such as *E. coli*, *Shigella flexneri*, *C. jejuni*, and *H. pylori*, HtrAs are also secreted for an extracellular virulence role, promoting host invasion by cleaving the host cell adhesion protein, E-cadherin [27].

HtrAs’ involvement in bacterial pathogenicity has made them attractive targets for anti-virulence therapeutics. Several inhibitors of bacterial HtrA have recently been shown to be effective [27,28], however they are suboptimal against HtrA homologs. This indicates that HtrA inhibitor specificity can be genus or even species specific [29]. This highlights the emerging view that bacterial HtrA proteins are functionally distinct and do not conform to the *E. coli* DegP model as previously thought. Indeed, the extraordinary bactericidal effect of JO146 in *Chlamydia* infection suggests that CtHtrA may play an essential physiological role in this organism, unlike other bacterial HtrAs. This review of the recent developments in our understanding of CtHtrA’s physiological role in *Chlamydia* focuses on the CtHtrA characteristics that make it unique from other bacterial HtrAs and an ideal target for drug development.

***CtHtrA structure and function***

CtHtrA is an ATP-independent serine protease with broad specificity to cleave generally hydrophobic substrates at hydrophobic residues [30-32]. Bioinformatic analysis indicated the presence of a periplasmic secretion signal sequence, and confocal laser scanning microscopy confirmed CtHtrA's localization to the cell envelope [30,33].

In the *E. coli* homolog, DegP, the protease and chaperone activities depend upon the structural interaction between its monomeric protease and two PDZ domains, and the allosteric activation of active and inactive multi-oligomeric forms [34-37]. Crystallographic analysis indicated that *E. coli* DegP exists as a resting hexamer composed of staggered trimeric rings, with the protease domains at the center of a chamber bordered by the PDZ domains [34]. Upon detection of exposed hydrophobic regions of unfolded proteins by the PDZ1 ‘carboxylate-binding loop’, a structural rearrangement of several loops and the formation of higher order oligomers transforms DegP from an inactive hexamer to an active 24-mer [37].

Initial *in vitro* biochemical analyses in *Chlamydia* demonstrated that CtHtrA is also a hexamer under native conditions [30] and that the presence of substrates or activators also induce the formation of higher order oligomers [31]. CtHtrA homology models provided a structural context for these higher oligomers, allowing an examination of the residues and structural elements that are likely to be responsible for CtHtrA activation (Figure 2). A predicted interaction between the PDZ1 domain, loop L3, and loop LD proved critical for the proteolysis activation, but not oligomerization (Figure 2) [31,38]. This suggests a similar activation mechanism to that reported for *E. coli* DegP, in which the C-terminus of a substrate bound to the PDZ1 domain induces a ‘PDZ1 – L3 – LD’ interaction cascade that reorients the active site [39]. However, *E. coli* DegP remained as a hexamer with a disruption to the ‘PDZ1 – L3’ interaction [35], while CtHtrA readily oligomerized to a 24-mer under similar conditions, suggesting that CtHtrA oligomerization can occur in the absence of a correct catalytic site formation. Oligomerization was triggered *in vitro* by peptides based on the C-terminal sequences of both proteolysis (β-casein) and potential chaperone substrates (chlamydial outer membrane proteins), while proteolysis was best activated by peptides derived from the C-terminus of proteolysis substrates [31]. Thus, the substrate binding specificity of the PDZ1 domain may determine whether or not a ‘PDZ1 – L3 – LD’ proteolytic activation cascade develops, suggesting that substrate specificity or sequence features are a key determinant of either protease or chaperone activity. Meanwhile, oligomerization to 24-mer appears to occur independently of substrate specificity.

***CtHtrA as a heat shock response protein***

The heat shock response is a critical HtrA function for most bacteria where it has been studied. Heat shock proteins are found in all domains of life and help maintain cellular organization and homeostasis both under normal conditions and during high temperature or other forms of protein stress. Protein misfolding and aggregation can rapidly compromise cellular survival, and consequently most cells have evolved a suite of proteins to degrade damaged or non-functional proteins or promote their refolding. In initial studies, mutations in *E. coli* DegP altered their ability to degrade heat-denatured proteins [40], and *E. coli* containing *degP* null mutants did not grow at elevated temperatures [41].

While the stress response pathways in *Chlamydia* are significantly less well defined, recent evidence indicates that CtHtrA may play a key role. CtHtrA expression increased during heat stress, while expression of the major outer membrane protein (MOMP) decreased under the same conditions. A smaller number of RBs than usual were identified by transmission electron microscopy amongst increased membraneous-like material within the inclusion [33], and *cthtrA* could heterologously complement the lethal high temperature phenotype of *E. coli* *htrA*- [33]. Additionally, a CtHtrA single nucleotide, chemically induced mutation, P370L (Figure 2), produced a ~140-fold reduction in EBs compared to the wild type following reversion from heat stress. This mutation, which is at the base of the CtHtrA ‘PDZ1 activation cleft’, likely disrupted the initial substrate C-terminus binding. This disruption would negatively impact CtHtrA proteolysis and/or oligomerization activity, and possibly protein binding and chaperone activity as well (Figure 2) [42]. However, it was not clear if the P370L mutation disrupted CtHtrA’s potential homeostatic function throughout the period of heat stress, during the conversion of RBs to EBs following stress removal, or both. Previously, the JO146 CtHtrA inhibitor demonstrated lethality under heat shock conditions or during the post-heat shock recovery, [15]. These data suggest that *C. trachomatis* critically required CtHtrA during and immediately following heat stress, most likely for the proteolytic degradation of damaged or misfolded proteins that are likely to accumulate rapidly during this stress condition.

***Recovery from chlamydial persistence likely involves CtHtrA in some conditions***

Bacterial stress can be caused by alterations in pH, oxidation state, temperature, and membrane integrity, and tightly regulated quality control proteins are required to ensure homeostasis and the continued survival of the cell [43]. In *Chlamydia*, however, it is important to distinguish “heat stress” from chlamydial “persistence”, as CtHtrA has demonstrated divergent roles in each [33,44]. Persistence in *Chlamydia* occurs in response to certain stressors and is characterized by disruption of the developmental cycle (Figure 1) [45]. Bacterial cell division and differentiation to EBs is inhibited during this phase, while DNA replication continues, and elevated levels of stress response proteins are expressed [13,46]. CtHtrA levels increased in the presence of penicillin-induced persistence [33], while treatment with the JO146 CtHtrA inhibitor reduced the yield of infectious progeny during penicillin persistence, and treatment during the recovery from persistence was lethal [15]. These observations indicate a functional role for CtHtrA during reversion from penicillin persistence.

Penicillin acts by irreversibly binding to penicillin-binding proteins (PBPs) and restricts the biosynthesis of peptidoglycan (PG) [47]. PG is a crucial component of the RB cell envelope that provides stability, an anchor for OMPs, and a potential mechanism for RB replication [48]. It is likely that PG biosynthesis and RB replication is inhibited during penicillin persistence, reducing the stability of the PG-anchored OMPs and increasing the permeability of the membrane. Indeed, the loss of OMP-PG covalent cross-links in bacteria such as *Salmonella*, *Vibrio cholerae*, and *E. coli* leads to an increasingly fragile cell envelope, accompanied by the leakage of membrane and periplasmic proteins [49-54]. Thus, when the effect of penicillin is removed and PG biosynthesis can continue, it is conceivable that the exposed hydrophobic sequences from these damaged periplasmic or OMPs activate CtHtrA proteolytic activity to facilitate their removal, concomitant with CtHtrA’s degradative role during and following heat shock.

The CtHtrA response to penicillin persistence, however, does not appear to be conserved for other chlamydial stress conditions. In the presence of IFN-γ persistence, we observed no change to CtHtrA protein levels [33], which was consistent with transcriptional analysis where CtHtrA was shown to be down-regulated in response to and following IFN-γ persistence [13]. In human epithelial cells, IFN-γ induced indoleamine-2,3-dioxygenase to degrade tryptophan, an essential amino acid for both eukaryotic cells and *Chlamydia* [55,56]. The persistent state derived from the depletion of tryptophan was therefore in response to nutrient starvation and was unlikely to trigger changes in the integrity of the outer membrane (OM) or to induce cell envelope stress. Thus, CtHtrA most likely functions primarily in the aftermath of cellular stress (including heat stress), specifically when the outer membrane or outer membrane proteins (OMPs) or periplasm/extracytoplasmic space has been disturbed, suggesting that OMPs are a key substrate for CtHtrA. This sensing leading to degradation and/or assembly of (some) outer membrane proteins is also likely a universal bacterial HtrA function, given the phenotypic evidence in *htrA* null mutants for the loss of virulence [24,26,27,57-59], and the absence of outer membrane proteins [18,60,61] in several bacteria.

***The role of CtHtrA in outer membrane protein assembly***

A major bacterial pathway for OMP biogenesis in bacteria is via passing the cytoplasmic membrane through the general secretory system (Sec) in an unfolded state. The proteins are then translocated to the outer membrane (OM) by the SurA, Skp, and HtrA [62]. In *E. coli*, evidence suggests that these chaperones have functional redundancies, especially Skp and DegP, and that at least one of these is required for cellular viability [60]. While *E. coli* DegP is generally thought to be the back-up OMP chaperone [18], evidence from other bacterial species suggests that OMP translocation and OM biogenesis may be HtrA’s defining function, rather than an ancillary feature. For example, neither Skp nor SurA play a general OMP chaperone role for *Neisseria meningitidis* [61]. In *S. flexneri*, DegP is important in a protease-independent manner for the efficient delivery of the IcsA autotransporters to the bacterial surface [63].

Although CtHtrA is crucial during cell envelope stress conditions in *C. trachomatis*, transcriptional analysis indicated that CtHtrA is expressed throughout the developmental cycle, from ~8 h PI to its highest levels between 24 – 40 h PI [64]. Furthermore, the observation that the JO146 inhibitor was lethal when added during the replicative phase [14] confirmed an essential replicative phase function for CtHtrA. An *in silico* scan of the *C. trachomatis* serovar L2 proteome for C-terminal PDZ domain binding motifs [65] exclusively identified 11 potential periplasmic or outer membrane proteins, including MOMP and polymorphic membrane proteins (Pmp) A, B, C, D, E, F, G, and I (Figure 3). Two conclusions might be drawn from these data. Firstly, the cellular, heat stress, and persistence data above that links OMPs with CtHtrA are consistent with OMPs as key substrates for CtHtrA. Secondly, a CtHtrA binding motif potentially recognizes OMPs, likely as chaperone substrates, combined these data suggest substrate sequence features are a key determinant that triggers either protease or chaperone activity of CtHtrA.

Bioinformatic analysis of the available chlamydial genomes did not identify SurA in *Chlamydia*, but did identify a Skp homologue, OmpH. Transcriptome analysis suggested that *ompH* is transcribed similarly to *cthtrA*, with protein expression detected from ~8 h PI and the highest expression levels observed during the replicative and elementary body formation stages of the chlamydial developmental cycle (24 – 40 h PI) [64]. As the chlamydial genome is small (1 Mb) due to the evolutionary loss of several biosynthesis and regulatory pathways [66], it is likely that Skp (OmpH) and CtHtrA are the dominant chaperones for the *C. trachomatis* periplasm in the absence of SurA.

The chlamydial Pmps are bioinformatically predicted to be autotransporters, which are characterized by their ability to facilitate their own translocation across the periplasm to the OM [67]. However, *S. flexneri* and *E. coli* autotransporters appear to rely on HtrA/DegP for their translocation to the OM via specific binding motifs [60,68], correlating with the specific PDZ binding motifs identified above for the chlamydial Pmps (and other OMPs) (Figure 3a). In support of this potential major chaperone role we have previously reported that CtHtrA oligomerization could be triggered by specific C-terminal sequences of chlamydial OMPs, including PmpC and MOMP peptides, while oligomerization was not triggered by cytoplasmic proteins with C-termini that do not contain the predicted PDZ domain binding motif (e.g. PykC) (Figure 3b) [31]. Biochemical characterization of the CtHtrA PDZ1 domain identified key residues that differentially impact proteolysis and oligomerization (Figure 3c), confirming that the PDZ domain is a key substrate binding site for CtHtrA. Thus, given the critical role for CtHtrA during the replicative phase of chlamydial development, the conserved PDZ domain-binding motifs in chlamydial OMPs, the ability of CtHtrA to distinguish protease and chaperone substrates via their C-terminus, and the co-transcription of CtHtrA and OmpH (Skp homologue), we propose that OMP assembly is a key constitutive function for CtHtrA in *C. trachomatis*. This agrees with data from other bacteria for which the biogenesis of specific OMPs by HtrA has been reported (*S. flexneri*, *E. coli*, and *H. pylori*) [60,62,69].

***A potential role for CtHtrA in RB replication or the conversion to EBs***

Treatment of *C. trachomatis* with the JO146 inhibitor was lethal when added during the replicative stage of development [14], while a *C. trachomatis* mutant with reduced *in vitro* protease activity (P370L) produced a significant reduction in the production of infectious EBs. Thus, two distinct lines of evidence support CtHtrA’s role during the *Chlamydia* replicative cycle [42]. CtHtrA’s predicted role in OMP assembly and degradation, a disruption to OMP integrity when CtHtrA is inhibited is possible. In turn the disrupted OMP integrity would influence the peptidoglycan stability feeding back to the cell division apparatus and leading to obstruction of RB replication. Such a process would explain the lethality of CtHtrA inhibition, as well as the non-lethal but infective progeny reduction phenotype observed for the P370L (*in vitro*) mutant [42].

As an alternative scenario to cell division, an impact on the RB to EB transition process could explain the phenotypes observed from CtHtrA inhibition or the P370L mutation. The collapse of the cell envelope might correlate with the dissociation of the type III secretion apparatus from the inclusion membrane, potentially triggering RB to EB differentiation [70]. Indeed, the breakdown of the disulfide cross-linked membrane proteins in EBs has been associated with the initial reorganization of EBs into RBs [71]. Thus, this parallel yet reverse mechanism defined by the breakdown in OMP-PG interaction might be a key initiator of RB-EB conversion.

During this phase of RB to EB transition, the expression of EB-specific OMPs is upregulated in preparation for insertion into the EB OM. In this context, we propose that these highly expressed OMPs activate CtHtrA via the C-terminal hydrophobic motif (Figure 3), thus enabling the critical OMP biogenesis role of assembling the EB OM proteome. In summary, the experimental evidence indicates that CtHtrA plays a critical role in the replicative phase of chlamydial development, which we speculate is directly linked to either OMP degradation during replication (protease activity), and/or the formation of infectious EBs (chaperone activity).

***CtHtrA as a virulence factor***

CtHtrA’s putative virulence role in *C. trachomatis*islikely associated with the extra-cytoplasmic stress response and the translocation of virulence factors to the cell surface. Although inside a vacuole, this intracellular niche may still have stress conditions requiring CtHtrA’s critical role in protein folding, OMP integrity, and ultimately, cell fate [235]. In addition, CtHtrA’s chaperone activity might be required for the periplasmic translocation and assembly of extracytoplasmic virulence factors, such as the Pmps, providing an additional mechanism for pathogenesis. An association between HtrA’s quality control function and pathogenesis has been reported for several bacteria. HtrA expression in *Bartonella henselae* and *Yersinia enterocolitica* was observed to increase upon the transition from extra- to intra-cellularenvironment [72,73], while *htrA* was shown to be essential for *Brucella abortus*toadapt to the intracellular environment of host macrophages but not essential once aninfection was established [74].

***CtHtrA may have further function as a secreted virulence factor for* Chlamydia**

CtHtrA has been detected in both the chlamydial inclusion lumen and host cell cytosol [75], suggesting that its secretion may involve a function for manipulating the host cell [75,76]. CtHtrA was also reported to be exposed on the surface of EBs, and *C. muridarum* HtrA (CmHtrA) expressed in *E. coli* with a leader sequence for secretion is incorporated into outer membrane vesicles (OMVs). When used to immunize mice, these OMVs induced anti-CmHtrA-specific antibodies that neutralized the *Chlamydia* infection, demonstrating a functional immune response which is consistent with previous reports of high frequency human antibody responses [77]. Thus, CtHtrA secretion likely occurs via a vesicle-mediated mechanism, possibly through outer membrane blebbing [75]. While the production of OMVs is often associated with envelope stress as a mechanism to discard harmful proteinaceous waste [78], the localization of CtHtrA itself to the vesicle, the associated immune response, and vesicle detection during non-stress conditions could suggest an OMV-mediated chlamydial mechanism of host manipulation, analogous to the extracellular virulence role of HtrA in *H. pylori* [27].

***A model for bacterial HtrA function***

Here we propose a model describing CtHtrA’s functions in *C. trachomatis* (Figure 4), based on the currently available data. Bioinformatic, transcriptional, proteomic, and immunocytochemistry data identified CtHtrA as a constitutively expressed protein that localizes to the cell envelope [44,64,79], and is also found in the inclusion lumen, on the EB surface, and in OMVs [30,31,33,38,75]. In the cell envelope, we propose that CtHtrA assumes an inactive hexameric conformation, until its protease or chaperone function is required. Damaged or misfolded substrates activate CtHtrA by binding the CtHtrA PDZ1 carboxylate binding loop to the substrate’s exposed hydrophobic C-terminus. Proteolysis is allosterically activated through a structural cascade that leads to oligomerization into higher order complexes (as much as 24-mers). CtHtrA returns to the hexameric resting state once there are no further unfolded proteins binding to the activation cleft. In addition to this cell envelope stress-related proteolytic activity, CtHtrA also potentially aids in OMP assembly, including the Pmps, via its chaperone activity and recognition via a conserved C-terminal PDZ binding motif. The data also support an essential role during the replicative phase of chlamydial development (8 – 24 h PI) that corresponds with constitutive upregulation of CtHtrA. We propose a possible interplay of CtHtrA OMP substrates, their interaction with peptidoglycan, and feedback of peptidoglycan to the apparatus that drives RB binary fission. Furthermore, as a potential host cell targeting virulence factor, CtHtrA is incorporated into OMVs, which are translocated to the inclusion or cellular cytosol.

In summary, CtHtrA is a multitasking protein in the chlamydial cell envelope that has a critical role during protein stress conditions and a constitutive function as an extracytoplasmic chaperone for OMP assembly. Unique to *Chlamydia*, however,this function may impact RB replication (binary fission) or conversion to EBs (or at least the formation of infectious EBs). CtHtrA also potentially plays a role in virulence or host cell manipulation via its secretion and/or incorporation into OMVs. These diverse functions demonstrate that in *C. trachomatis*, CtHtrA is a multi-functional protein that appears to be the lynchpin of the chlamydial surface.

***Conclusions***

These data highlight a HtrA protein that is functionally distinct from its bacterial homologs. The lethality displayed by treatment with the JO146 inhibitor correlates with mutagenesis studies to highlight a multi-tasking role in several cell envelope pathways, including outer membrane protein assembly, heat shock, virulence, reversion from chlamydial persistence, and EB to RB conversion. As a cell envelope lynch-pin, CtHtrA is a compelling target for antibiotic development.

***Future perspective***

Our insight in *C. trachomatis* supports the growing body of evidence that bacterial HtrA is not primarily a heat shock quality control protein, but an OMP assembly factor and extracytoplasmic virulence factor. The recent advent of chlamydial genetic manipulation techniques [80] will likely accelerate our understanding of CtHtrA's mechanistic role in each of these pathways. A crystal structure for the inactive hexamer, active 24-mer, and a 24-mer in the presence of the JO146 inhibitor will facilitate deeper insight into CtHtrA's structural configuration and oligomerization mechanism, and enable further optimization of JO146 efficiency and specificity.

***Executive summary***

* *Chlamydia trachomatis* is a significant human pathogen, but new drug targets are required.
* JO146 is an effective anti-*Chlamydia* small molecule, but little is known about its cellular target, CtHtrA.
* CtHtrA function is mediated by a distinct structural pathway resulting in oligomerization to a 24-mer.
* CtHtrA functions in response to periplasmic heat stress by degrading misfolded proteins.
* Recovery from chlamydial persistence likely involves CtHtrA in some conditions.
* CtHtrA has a role in OMP assembly.
* CtHtrA has a potential role in RB replication or the conversion to EBs.
* As a multi-tasking periplasmic lynch-pin, CtHtrA is a promising therapeutic target for *Chlamydia*.

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**Figure Captions**

Figure 1. The *Chlamydia* developmental cycle. The *Chlamydia trachomatis* developmental cycle, with relative time-points shown around the center and each stage of the cycle shown in green around the cells. Code: small, open circles: elementary bodies (EB); large, black circles: reticulate bodies (RB); blue circle: host cell; yellow circle: chlamydial inclusion vacuole.

Figure 2. The CtHtrA structural mechanism of proteolytic activation predicted by homology modeling. This figure highlights the key interactions between the PDZ1 domain (green), protease domain (purple), and an adjacent protease domain (light orange) in a CtHtrA oligomer, formed upon proteolytic activation. A C-terminal peptide (grey) allosterically binds to the ‘carboxylate binding loop’ in the ‘PDZ1 activation cleft’ which results in two key interactions that are critical for the activation of proteolysis: PDZ1 – loop L3 (red residues) and loop L3 – LD (green residues). This activation cascade is modulated by supplementary interactions: PDZ1 – loop LC (yellow residues) and PDZ1 – β-strand 5 (blue residues). The proline that was changed to leucine in the P370L mutation is shown in purple.

Figure 3. Predicted determinants of allosteric substrate binding to the CtHtrA PDZ1 domain. (a) The four C-terminal residues of all *C. trachomatis* serovar L2 periplasmic and outer membrane proteins predicted to recognize class II binding motifs of PDZ domains. The protein name is shown with the associated NCBI accession number, and the four C-terminal residues consisting of a PDZ class II (X – Ψ – X – Ψ) binding motif. X represents any amino acid and Ψ is a hydrophobic residue [65]. Hydrophobic residues corresponding with a PDZ class II binding motif are highlighted in green. (b) The four C-terminal residues of previously tested *in vitro* substrates of CtHtrA shown to produce a differential response for proteolysis and oligomerization. Hydrophobic residues corresponding with a PDZ class II binding motif are highlighted in green. Act1 is a peptide based on the C-terminal residues of β-casein; Act2 is a peptide based on the C-terminal residues of elastase. (c) Cartoon outline of the CtHtrA PDZ1 activation cleft and carboxylate binding loop, with experimentally determined key residues shown in red.

Figure 4. A model of the role of CtHtrA as a *C. trachomatis* cell envelope lynch-pin. (a) In the resting state, CtHtrA exists as an inactive hexamer (blue). (b) Unfolded periplasmic and OMPs are passed through the general secretory (Sec) system in an unfolded state. (c) The C-terminus of these unfolded proteins is detected by the CtHtrA PDZ1 domain resulting in the oligomerization of CtHtrA to its 24-meric form, which encapsulates the protein to aid its native conformation to be reached. (d) Misfolded or damaged proteins that appear either during normal conditions or in the presence of heat stress bind to the CtHtrA PDZ1 domain via their C-terminus to activate proteolysis and oligomerization. (e) These misfolded or damaged proteins are proteolytically degraded. (f) Disruptions to the PG layer that occur during binary fission or in the presence of penicillin persistence lead to membrane permeability and instability and unstable or misfolded OMPs that bind to the CtHtrA PDZ1 domain via their C-terminus. (g) This results in the proteolytic activation and oligomerization of CtHtrA. (h) These misfolded or damaged proteins are proteolytically degraded. (i) As a potential virulence mechanism, CtHtrA is incorporated into outer membrane vesicles to potentially mediate host cell interactions. This figure includes the inner membrane (pink), periplasm (yellow), peptidoglycan layer (brown), outer membrane (blue), and lipooligosaccharide (LOS) layer (white). The inner membrane is populated with integral membrane proteins (green) and the general secretory (Sec) system (dark blue). The outer membrane is populated with outer membrane proteins (OMPs) that include polymorphic membrane proteins (Pmps; red), the PorB β-barrel (dark blue), and the major outer membrane protein (MOMP) β-barrel porin (dark green). These OMPs are surface exposed and anchored to the periplasmic via the peptidoglycan layer.

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