A Chlamydia trachomatis strain with a chemically generated amino acid substitution (P370L) in the cthtrA gene shows reduced elementary body production

James W. Marsh¹, Bryan A. Wee¹, Joel D.A. Tyndall², William B. Lott¹, Robert J. Bastidas³, Harlan D. Caldwell⁴, Raphael H. Valdivia³, L. Kari⁴ and Wilhelmina M. Huston¹*

Abstract

Background: Chlamydia (C.) trachomatis is the most prevalent bacterial sexually transmitted infection worldwide and the leading cause of preventable blindness. Genetic approaches to investigate C. trachomatis have been only recently developed due to the organism’s intracellular developmental cycle. HtrA is a critical stress response serine protease and chaperone for many bacteria and in C. trachomatis has been previously shown to be important for heat stress and the replicative phase of development using a chemical inhibitor of the CtHtrA activity. In this study, chemically-induced SNVs in the cthtrA gene that resulted in amino acid substitutions (A240V, G475E, and P370L) were identified and characterized.

Methods: SNVs were initially biochemically characterized in vitro using recombinant protein techniques to confirm a functional impact on proteolysis. The C. trachomatis strains containing the SNVs with marked reductions in proteolysis were investigated in cell culture to identify phenotypes that could be linked to CtHtrA function.

Results: The strain harboring the SNV with the most marked impact on proteolysis (cthtrA-P370L) was detected to have a significant reduction in the production of infectious elementary bodies.

Conclusions: This provides genetic evidence that CtHtrA is critical for the C. trachomatis developmental cycle.

Keywords: Chlamydia, HtrA, Genetics, Heat shock, Intracellular

Background

Chlamydia trachomatis is the most prevalent bacterial sexually transmitted infection worldwide and the leading cause of preventable blindness [1, 2]. If left untreated, chlamydial infection can lead to serious and costly conditions including infertility, ectopic pregnancy, epididymitis, and pelvic inflammatory disease. As intracellular bacteria, the Chlamydiae are defined by their unique development cycle where small, infectious elementary bodies (EB) invade a host cell and differentiate into large, non-infectious reticulate bodies (RB) that replicate by binary fission [3]. Due to the absence of several biosynthesis pathways that are common to most other bacteria, Chlamydia has a reduced genome size (~1 Mb) that requires the organism to rely on the host cell for nutrients and survival [4]. As a result, Chlamydia’s obligate intracellular development cycle had hampered progress towards genetic techniques and the organism’s pathogenic mechanisms have not been fully elucidated.

HtrA is a critical protease and chaperone for many bacteria and has been implicated in several functions including stress response, protein quality control, outer membrane protein (OMP) localization and assembly, host cell manipulation, and virulence [5–9]. The distinct biochemical functions of proteolysis and chaperone activity are mediated by the allosteric activation of the protein to oligomers ranging from trimers to hexamers to at least 24-mers [7, 10]. Using homology modeling and biochemical methods, we have

* Correspondence: w.huston@qut.edu.au
1 Institute of Health and Biomedical Innovation (IHBI), Queensland University of Technology (QUT), 60 Musk Avenue, Kelvin Grove, QLD 4059, Australia
Full list of author information is available at the end of the article
previously suggested that *C. trachomatis* HtrA (CHtrA) proteolytic activation and oligomerization are mediated by distinct structural pathways, and that specificity of the substrate binding may differentiate protease and chaperone activity [11, 12]. HtrA is highly conserved in *Chlamydia* and the application of a chemical inhibitor of CHtrA protease activity indicated that CHtrA is important for the replicative phase of the chlamydial developmental cycle, important for the recovery from penicillin-induced chlamydial persistence, and has a critical role during heat stress [13–18]. These data, relying on both biochemical and inhibitor-based approaches, enabled significant insight into CHtrA function and the role of specific parts of the amino acid sequence for these functions, and provided the foundation for this attempt to use genetics to investigate CHtrA’s physiological role for *C. trachomatis*.

In the absence of genetic techniques such as targeted gene replacement and transposon-based mutagenesis, the field has instead relied on genomic, proteomic, and transcriptomic methods for chlamydial research. However, functional validation requires genetic evidence. In response, several molecular strategies have recently proven successful including small molecule inhibitors [15, 19], random chemical mutagenesis [20], lateral gene transfer [21], and shuttle vector transformations [22]. Given our existing data on the structure and function of CHtrA, genetic approaches such as random chemical mutagenesis are a promising approach to for the characterisation of CHtrA in *C. trachomatis*.

Chemical mutagenesis is the use of chemical compounds to increase the frequency of chromosomal mutations above the spontaneous rate [23]. The types of DNA lesions produced include DNA adducts, intercalation, inter-strand cross-linking, alkylation, and/or base modifications, which result in base-pair substitutions, frameshift mutations, and/or deletions in the nucleotide sequence. In 2011, Kari et al. presented the development of a reverse genetics approach using chemical mutagenesis to generate isogenic mutants of *C. trachomatis* [24], demonstrating that EMS is a viable mutagen for genetic studies in *Chlamydia*. EMS is an alkylating agent that specifically acts at nitrogen positions in nucleotide bases to produce GC to AT transition mutations [25]; based on the GC content of the *C. trachomatis* L2 434/Bu genome, there are 429,520 potential sites that could theoretically be mutated by EMS. Thus, single nucleotide variants (SNV), induced by the EMS treatment of *C. trachomatis*, can be identified in a gene of interest and subjected to a genetic characterization of its function. Given our awareness of the residues and structural elements that are important for CHtrA’s protease and chaperone activity [11, 12, 17, 18], we investigated several SNVs in the chtrA gene to examine the protein’s physiological function for *C. trachomatis*.

### Methods

**Chlamydal culture conditions**

*Chlamydia trachomatis* was routinely cultured in McCoy B or HEP-2 cells in the presence of DMEM supplemented with 10 % fetal calf serum (FCS) at 37 °C, 5 % CO₂. Experiments were typically conducted at an MOI of 0.3. For the calculation of infectious yield, cultures harvested in SPG buffer (10 mM sodium phosphate, 250 mM sucrose, 5 mM glutamic acid, pH 7.4) were serially diluted and cultured in fresh McCoy B cell monolayers until 30 h post-infection, when they were fixed with methanol and stained for microscopy. The infectious yield was determined by the number of inclusion forming units from each milliliter of the original culture.

**Chemical mutagenesis**

Chemical mutagenesis of *C. trachomatis* L2 was performed by the Valdivia Laboratory at Duke University, NC, USA [21]. Vero cells were infected with Rif⁸ *C. trachomatis* serovar LGV biovar L2 434/Bu at an MOI of 5.0 for 18 h and exposed to 20 mg/mL ethyl methanesulfonate (EMS) in PBS for 1 h, as described [21]. EMS was prepared in phosphate buffered saline (PBS) with 0.9 mM calcium chloride and 0.49 mM magnesium chloride [26]. The cells were washed three times in PBS + CaCl₂/MgCl₂ to remove residual mutagen and incubated in DMEM/5 % FCS at 37 °C in a 5 % CO₂ humidified incubator for 48–72 h to allow the bacteria to recover and complete their developmental cycle. EBs were harvested by hypotonic lysis of infected cells with sterile water, followed by addition of 5× SPG media to a final concentration of 1× SPG, titered for IFUs, plaque-purified as described [21] and stored at –80 °C. The frequency of mutagenesis was determined by inducing rifampicin resistance, followed by the plaque purification using 7 × 10-fold serial dilutions of bacteria in the presence of 200 ng/mL rifampicin [26]. The frequency of rifampicin resistance was defined as the number of rifampicin-resistant plaques divided by the total number of bacteria plated [26]. *C. trachomatis* L2 chlamydial mutant strains containing SNVs in chtrA were identified from whole genome sequences generated from a collection of 934 chemically mutagenized *C. trachomatis* L2 strains (sourced from the Valdivia laboratory for this study, investigated in the Huston Lab in Australia). Isolates from this collection containing chtrA variants were further purified by plaque isolation (in Australia).

*C. trachomatis* D was mutagenized four consecutive times in the Caldwell Laboratory, NIAID Rocky Mountain Laboratories, Montana. At each round, infected McCoy cells were exposed to 5–7 mg/mL EMS 19 h post-infection for 1 h and chlamydiae were harvested 40–44 h post-infection. A library of sub-populations were built to screen for chtrA mutants. McCoy B cells in 96-well tissue culture plates were infected with 8
inclusion-forming units per well. Chlamydiae were harvested at 48 h post-infection and were used to reinfect McCoy B cells in 96-well tissue culture plates. Infected cells were harvested and passaged for a third time, and DNA extracted from the third passage was used to PCR amplify cthtrA. Amplicons were heat denatured, slowly reannealed, and digested by CEL I endonuclease. Digestion products were visualized by DNA agarose gel electrophoresis. Mutations detected by CEL I digestion were identified by Sanger sequencing (these strains and this work to identify the mutations was conducted in the Caldwell laboratory, characterization of the strains was conducted in the Huston laboratory).

**In silico analysis of cthtrA SNVs**

CtHtrA sequence alignments, 2D structural motifs, and homology models for the hexameric and 24-meric oligomers were used for the characterisation of EMS mutants, as previously described [11]. Each mutation was examined in both oligomeric models of CtHtrA with the PyMOL Molecular Graphics System, Version 1.7.4 Schrödinger, LLC, according to secondary structure localisation and potential impact on CtHtrA function.

**Site-directed mutagenesis and recombinant protein expression**

The previously generated CtHtrA recombinant expression construct was used for this study [17]. Site-directed mutagenesis was used to introduce point mutations corresponding to the EMS mutations identified in the chlamydial strains using a QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene) using the primers A240Vsdm, E47Ksdm, G268Rsdm, G475Esdm, P370Lsdm, and R55Qsdm (Additional file 1: Table S4). All mutations were confirmed by Sanger sequencing. Heterologous recombinant protein expression and purification of CtHtrA recombinant mutated proteins were completed as previously published [11, 12, 17].

**Protease assays**

Full-length β-casein cleavage assays were initially used to test the proteolytic activity of wild type CtHtrA and the mutants. Up to 2 mg of recombinant CtHtrA was incubated with 10 mg of β-casein in 50 mM Tris, 20 mM MgCl₂ and examined with SDS-PAGE as previously described [17]. The gel bands were quantified by densitometry using a Li-Cor Odyssey 9120 Infrared Imaging System. The rate of proteolysis was tested with a peptide substrate labelled with 7-methoxycoumarin-4-acetic acid (MCA; fluorophore) and 2,4-dinitrophenyl (DNP; quencher) in black plates at 37 °C using a POLARStar Optima D77656 fluorimeter. Substrate specificity was analysed with an xMARK microplate spectrophotometer. All protein and peptide substrates and allosteric activators are listed in Additional file 1: Table S5. All peptides and activators were synthesised by Mimotopes (Melbourne, VIC, Australia) to 95 % purity and were re-suspended in 50 % isopropanol or DMSO. Statistical analyses were conducted using an unpaired t-test, calculated with Prism software. All assays were conducted on two separate occasions, in triplicate.

**Oligomerization assays**

The ability of CtHtrA or the mutants to oligomerize to higher oligomers was examined by crosslinking oligomers with glutaraldehyde as previously described [12]. The samples were separated on 3–8 % Tris-Acetate gradient polyacrylamide gels and examined by silver staining. All assays were conducted in triplicate on three separate occasions. HiMark pre-stained molecular weight marker (Life Technologies, USA) was included on each gel.

**Heat shock of chlamydial culture**

Heat shock assays were performed by infecting HEp-2 cells in a 48-well plate with CtL2wt and each mutant at an MOI of 0.3 in DMEM at 37 °C, 5 % CO₂. Cultures were heat shocked for 4 h at 42 °C, 5 % CO₂ beginning at 20 h post-infection, before returning the cultures to 37 °C for the remainder of the development cycle, prior to the measurement of infectious progeny [14].

**Chlamydia growth curves**

Growth curve assays were performed by infecting McCoy B cells in a 96-well plate with CtL2wt and each of the mutants at an MOI of 0.3. The cells were incubated in DMEM at 37 °C, 5 % CO₂ for up to 48 h. At each time point the cultures were harvested by media removal, the addition 200 μL SPG, and storage at −80 °C. The plates were thawed and the McCoy B cells lysed by pipette disruption and cell lysates for each time point were serially diluted and used to re-infect fresh McCoy B cell monolayers in DMEM + 1 μg/mL cycloheximide on 96-well plates, in triplicate. The infectious yield was determined as described above.

**Morphological analysis and immunocytochemistry**

*Chlamydia* cultures were visualised with immunofluorescence as previously described [16, 27]. Briefly, confluent McCoy B cells seeded on 8 mm coverslips in a 48-well plate were infected with *Chlamydia* at an MOI of 0.3 in DMEM media and were stained with the *Chlamydia* Cel LPS product (Cellabs, Brookvale, NSW, Australia) as per the manufacturer’s instructions. Primary antibody was incubated for 1 h in 1 % BSA followed by two washes in PBS. Secondary antibodies were Alexa Fluor 488 goat anti-rabbit (Life Technologies, USA) and were used at 1/600
dilution in 1% BSA for 1 h. Coverslips were mounted using Prolong Gold antifade (Life Technologies, USA) and viewed under a Leica TCS SP5 confocal laser scanning microscope (Leica Microsystems). Images were captured using the Leica application suite for advanced fluorescence.

Genetic analysis of C. trachomatis strains with EMS generated mutations
CtL2\text{wt} and mutants were semi-purified and genomic DNA was extracted using the phenol-chloroform method as previously described [28]. Whole genome sequencing was performed commercially by the Micromon sequencing facility on the Illumina MiSeq platform, using theTruSeq v3 chemistry and the NexteraXT Library Preparation Kit (Monash University, Victoria, Australia). SNV analysis was performed using Nesoni (https://github.com/Victorian-Bioinformatics-Consortium/nesoni) with the C. trachomatis L2 434/Bu complete genome (RefSeq accession: NC_010287) serving as the reference sequence.

Lateral gene transfer
Spectinomycin-resistant (\text{Spc}^R) C. trachomatis L2 isolates and rifampicin-resistant (\text{Rif}^R) mutant isolates were generated by repeated passage on McCoy B cells in the presence of sub-inhibitory concentrations of the antibiotic. Passaging continued until maximum resistance was reached in the presence of 200 ng/mL rifampicin or 200 μg/mL spectinomycin. Lateral gene transfer between the \text{Spc}^R CtL2\text{wt} and \text{Rif}^R mutant was induced by co-infecting each strain at an MOI of 4.0 in 12 wells of a 24-well plate (total MOI of 8.0). Positive controls were included by infecting duplicate wells in the presence of rifampicin only or spectinomycin only. Negative control wells with no antibiotics were also included. The cell lysates from each well were serially diluted and used to infect a fresh McCoy B cell monolayer on 6-well plates for plaque purification cultures [21].

Results
Chemical mutagenesis allows identification of SNVs that impact on C\text{HtrA} function
Two existing libraries of C. trachomatis mutants were screened for mutations in the c\text{htrA} gene, one based on C. trachomatis serovar L2 (CTL0195) and the other on C. trachomatis serovar D (CT_823). The screening of two libraries was performed to enable higher coverage of the c\text{htrA} gene, while enabling the reduction of possible SNV bias given that the libraries were prepared using different methods. The C. trachomatis L2 and D serovars share 99.5% nucleotide identity and 99.4% amino acid identity at the c\text{htrA} locus and thus are not expected to exhibit functional differences.

Firstly, we determined that there were 797 C/G to T/A mutations possible in c\text{htrA}; 601 would be non-synonymous, 160 synonymous, and 36 nonsense. We have previously used a chemical inhibition approach to demonstrate that C\text{HtrA} is critical for the survival of C. trachomatis [15], so it was expected that strains harboring null mutations in c\text{htrA} would be nonviable. However, strains containing c\text{htrA} alleles with non-synonymous mutations in functionally important residues could potentially be used to investigate C\text{HtrA} physiological function(s).

Three C\text{HtrA} mutations have an impact on in vitro proteolysis but not oligomerization
The c\text{htrA} EMS mutations were examined using molecular models of the inactive hexamer and active 24-mer oligomers of C\text{HtrA}, according to 2D and 3D structural motifs known to have a role in C\text{HtrA} protein function [11, 12, 17, 18]. Mutations were identified throughout the entire c\text{htrA} gene, with SNVs identified in the signal peptide, protease domain, PDZ1 domain, and PDZ2 domain (Additional file 1: Table S1). Six mutations that resulted in amino acid substitutions were selected for characterization based on \textit{in silico} prediction of a structural and/or functional impact: E47K (G − A), R55Q (G − A), A240V (C − T), G268R (G − A), P370L (C − T), and G475E (G − A) (Fig. 1a).

The E47K and R55Q mutations were located at the N-terminus of the protease domain, upstream from loop LA, and had the potential to disrupt the trimeric protease domain interface by forming a steric clash with an adjacent protease domain loop (Fig. 1b). A240V represented a minor substitution from alanine to valine, two small and hydrophobic amino acids, however this residue is situated on loop L1 near to the catalytic serine and any conformational shift that may occur as a result of this mutation, however minor, would likely impact C\text{HtrA} proteolytic activity (Fig. 1b). G268R was situated in the protease domain active site in loop L2
and potentially results in a steric clash with loop L2 from an adjacent protease domain, also potentially disrupting proteolysis (Fig. 1b). P370L was located in the PDZ1 domain at the base of the ‘carboxylate binding loop’ and is likely to disrupt the conformational turn of this loop, which may impact the binding of the substrate C-terminus to the PDZ1 domain and the subsequent activation cascade (Fig. 1b). G475E was located in the PDZ2 domain and resulted in a potential steric clash with a nearby PDZ2 domain loop, as well as an adjacent PDZ1 domain in the oligomeric state, thus potentially affecting the oligomerization mechanism (Fig. 1b). These six mutations were then generated in vitro in our CtHtrA recombinant protein expression construct using site-directed mutagenesis for biochemical characterization of the recombinant mutated proteins.

The proteolysis and oligomerization activities of these recombinant proteins were compared to the wild-type recombinant protein (CtHtrA). All recombinant proteins were able to cleave full-length β-casein at 37 °C, with proteins CtHtrA_E47K, CtHtrA_G268R, and CtHtrA_R55Q displaying similar proteolytic activity to the wild-type (Fig. 2a). The rate of proteolysis was slower for the CtHtrA_A240V and CtHtrA_G475E proteins compared to the wild type, while the proteolysis activity of the CtHtrA_P370L was substantially reduced (Fig. 2a; Additional file 2: Figure S1). Similarly, in the presence of a peptide substrate, the CtHtrA_A240V and CtHtrA_G475E proteins demonstrated a reduction in proteolytic rate, respectively, compared to the wild-type, while no proteolytic activity could be detected for the CtHtrA_P370L protein (Fig. 2b). The proteolytic rate of the CtHtrA_G268R and CtHtrA_R55Q proteins were not substantially different from the wild-type. We have previously shown that the addition of a second peptide based on the 12 C-terminal residues of β-casein (Act1) can activate or increase the proteolytic activity of CtHtrA [11, 12]. Thus, the activation of proteolysis by the recombinant proteins was investigated and a 1.4–2.3-fold increase in the rate of proteolysis was observed for CtHtrA_E47K, CtHtrA_G268R, CtHtrA_R55Q, CtHtrA_A240V, and CtHtrA_G475E. Alternatively, CtHtrA_P370L displayed no
detectable proteolytic activity against this peptide substrate (Fig. 2b), which may be due to this mutation causing a conformational change to the `PDZ1 activation cleft,' impacting the correct binding of Act1. Substrate specificity of the proteins was also examined using different para-Nitroanilide (pNA)-labeled substrates that differ in length and sequence (P1 – P4). No differences in substrate specificity were observed for any of the proteins compared to the wild type, suggesting that the mutations do not appreciably change the conformation of the catalytic domain (at least using these substrates; Fig. 2c). The CttHtrA<sub>P370L</sub> protein again displayed no proteolytic activity. CttHtrA has been previously shown to oligomerize from a resting hexamer to an activated 12-24-mer in the presence of both peptide and protein substrates [11, 12]. To investigate whether any of the recombinant mutated protein disrupted or impaired the activation of oligomerization, each were incubated with full-length β-casein at 37 °C and cross-linked with glutaraldehyde. In the presence of substrate, each mutant formed particles that are consistent with a higher order oligomer of CttHtrA (such as a 24-mer), with no evidence of the hexameric or trimeric forms that are observed when the oligomerization mechanism has been disrupted [12, 31]. While this method cannot detect differences in oligomeric structure or the exact number of monomers present, oligomeric activation to some form of oligomer appears to be unperturbed for each of the mutants (Fig. 2d) [11]. These data demonstrated that the CttHtrA<sub>A240V</sub>, CttHtrA<sub>G475E</sub>, and CttHtrA<sub>P370L</sub> mutations resulted in an appreciable disruption to the in vitro proteolytic activity of CttHtrA and therefore isolates with these three mutations were selected from the libraries for further in vitro analysis of their phenotypic impact on chlamydial growth.
The C. trachomatis isolate harboring a P370L substitution in cthtrA displays increased susceptibility to heat shock

The role of bacterial HtrA during heat shock has been widely reported [32–34], and ChtHtrA in particular has been shown to be upregulated during heat stress conditions [16] and critical for heat stress survival when the chemical inhibitor against ChtHtrA was used [14]. Therefore, Chlamydia strains with functionally disruptive mutations in cthtrA are likely to be more severely impacted by heat stress. HEp-2 cells were infected with each strain and subjected to 42 °C heat shock during the replicative phase (20 h post-infection) for 4 h, prior to restoration to 37 °C for the remainder of the development cycle. The impact of heat shock treatment on the mutants was determined by calculating the subsequent infectious yield from cultures harvested at 44 h post infection. The cthtrA_{A240V} mutant had an infectious yield that was comparable to the wild-type, while both the cthtrA_{G475E} and cthtrA_{P370L} mutants exhibited a significant reduction in infectious yield following heat shock, relative to the wild-type (Fig. 3). Notably, the cthtrA_{P370L} mutant resulted in a 32.5-fold reduction in infectious EB yield following heat shock compared to the wild type ($p < 0.0001$).

**The cthtrA_{P370L} inclusions are smaller in size**

The impact of the mutations on the chlamydial inclusion morphology was examined using immunocytochemistry and confocal laser scanning microscopy. Cultures were examined at 24 h post-infection (log phase) and 40 h post-infection (stationary phase; Fig. 4). At 24 h post-infection, the inclusion size of each mutant appeared smaller compared to the wild-type, while in the cthtrA_{P370L} strain there appeared to be fewer chlamydial cells within the inclusions. At 40 h post-infection, the inclusion sizes appeared to be more comparable to the wild-type, if slightly smaller. The inclusion sizes were measured to allow statistical comparison of the mutants against the wild-type, confirming that the mutant inclusion sizes were appreciably smaller compared to the wild-type at 24 h post-infection (Fig. 5). The difference was less pronounced at 40 h post-infection, where the cthtrA_{G475E} inclusion sizes were not significantly different compared to the wild-type. Alternatively, in the cthtrA_{A240V} and, to a greater extent, cthtrA_{P370L} strains, inclusion sizes were significantly smaller than the wild-type ($p < 0.0001$; Fig. 5).

**Genomics and lateral gene transfer to isolate chlamydial SNVs for further characterization resulted in a ctl0738_null but not a cthtrA_{P370L} isogenic strain**

While these observed phenotypes are consistent with our previous observations for ChtHtrA function, it is likely that the additional SNVs present in these mutant genomes also contribute to the phenotype. As a result,
the genome sequences of the cttrA<sub>A240V</sub>, cttrA<sub>G475E</sub>, and cttrA<sub>P370L</sub> mutants were determined by whole genome sequencing (WGS). When compared to the Ctl2<sub>wt</sub> genome sequence, the mutants consisted SNVs at nine loci that were consistent in all three isolates (cttrA<sub>A240V</sub>, cttrA<sub>G475E</sub>, and cttrA<sub>P370L</sub>): ctl0103, nusA, ctl0518, clpC-1, clpC-2, rpoB, pykF, and pmpC, which were confirmed to originate from the Ctl2<sub>wt</sub> strain used to generate the library (Additional file 1: Table S2) and were thus not expected to contribute to the observed phenotypes. Alternatively, we observed 13 unique SNVs in the cttrA<sub>A240V</sub> isolate (including the A240V C – T transition at position 247526 in cttrA), 19 unique SNVs in the cttrA<sub>P370L</sub> isolate (including the P370L C – T transition at position 247916 in cttrA), and eight unique SNVs in the cttrA<sub>G475E</sub> isolate (including the G475E G – A transition at position 248231 in cttrA; Additional file 1: Table S3), which could potentially contribute to the observed phenotypes.

Given that the isolate with the cttrA<sub>P370L</sub> mutation displayed the most marked phenotypes and was severely impaired during CHtrA recombinant protein in vitro protease assays, it was reasoned that this isolate will be the most informative for determining the physiological function of CHtrA and was selected for further genetic

---

**Fig. 4** Confocal microscopy images of Ctl2<sub>wt</sub> and mutants at 24 h and 44 h post infection. The Chlamydia isolates (Ctl2<sub>wt</sub> and mutant) are shown to the left of the panels and the time point is shown above the panels. The second and fourth images for each isolate are enlarged representations of single inclusions. The image colours are, green: LPS (FITC anti-chlamydial LPS); red: host cells (Evans blue). The scale bars (bottom right) indicate 50 μm and 25 μm for the enlarged images.
characterization. Of the 19 unique SNVs in the genome of the ctl0738
mutant isolate, 11 were non-synonymous, five were synonymous, and three were located in intergenic regions. Notably, there was a null mutation in ctl0738, a putative DNA methyltransferase. Of the eleven non-synonymous mutations, three resulted in a change to a similar amino acid (i.e. polar, hydrophobic etc.) and are therefore unlikely to have a functional effect (these were SNVs found in ctl0493, metG, and ctl0220). Consequently, a total of eight SNVs in the following cthtrA
isolate were identified as potentially significant, found on the following locus: recB, murC, incA, ydhO, ctl0738 (putative DNA methyltransferase), ctl0791 (putative membrane protein), ctl0885 (conserved hypothetical protein), and cthtrA
(Fig. 6, Additional file 1: Table S3).

In an effort to separate the cthtrA
SNV from these remaining SNVs, a lateral gene transfer approach was utilized, by co-infecting host cells with the rifampicin resistant cthtrA
strain and a spectinomycin resistant Ctl2_wt strain to generate recombinant isolates [21]. Positive recombinants were selected by plaque purification in the presence of both antibiotics. PCR and Sanger sequencing was conducted on 56 plaque-purified double resistant recombinant strains for the cthtrA
SNV and ctl0738
DNA methyltransferase), in addition to the other SNVs identified as potentially significant. The cthtrA
SNV was not detected in any of the 56 plaque purified recombinant isolates, while the ctl0738
null SNV was variously distributed among the recombinants. One recombinant isolate (ctl0738
null) contained the ctl0738
SNV and none of the other seven SNVs, allowing the potential for characterization of the DNA methyltransferase null mutation in the absence of the cthtrA
and remaining SNVs.

Characterization of the ctl0738
null mutation suggests that the cthtrA
SNV is the major contributor to the reduced infectious progeny phenotype

The lateral gene transfer experiments did not enable the generation of an isogenic cthtrA
mutant isolate. The SNV that could be contributing to the phenotype observed in the cthtrA
strain was the null mutation in the DNA methyltransferase, ctl0738. This mutation was able to be transferred to the genome of C. trachomatis L2 spectinomycin-resistant strain (CtL2spc) in the absence of the eight other EMS mutations that were considered potentially significant on the cthtrA
isolate genome. Therefore, further analysis of the isogenic strain containing the DNA methyltransferase null (ctl0738_null) was conducted to examine the role of this mutation for the phenotypes observed in the cthtrA
strain. Growth curve and heat shock experiments were conducted using the wild type strains (CtL2wt and CtL2spc), the original cthtrA
mutant strain, and the ctl0738
null strain. The cthtrA
strain has the same severely impacted reduced infectious progeny yield (8–25-fold reduction in infectious progeny; Fig. 7a) and the ctl0738
null strain showed infectious progeny production similar to the wild type. Notably, by including additional time-points at the beginning of the replicative phase (16–22 h post infection), it was observed that both the cthtrA
and ctl0738
strains displayed a delayed start to the log growth phase implying slowed RB to EB conversion. Somewhat unexpectedly, the heat stress phenotype was similar in the cthtrA
and ctl0738
strains with a significant reduction in infectious progeny (CtL2wt: 4.5 × 10⁷ EBs, cthtrA
: 1.2 × 10⁶ EBs; ctl0738
null: 1.4 × 10⁶ EBs; p < 0.0001), corresponding with a 30–40-fold reduction in infectious progeny after heat stress relative to CtlL2_wt (Fig. 7b).

Discussion

Here we present genetic evidence that cthtrA has a role in the production of infectious EBs. There are a number of possible conclusions that could be hypothesized based on the results presented here. Firstly, we did not find cthtrA alleles with null mutations in either library, so it is tempting to speculate that such a mutation would not be viable, however, considerably greater numbers of mutant strains would need to be screened before we could conclude with confidence that null mutations in cthtrA are not permissible. Although, we have previously demonstrated that a CHtrA specific chemical inhibitor of its protease activity is completely lethal when added during
the replicative phase of the developmental cycle [15], which supports that it may not be possible to isolate strains with null mutation in \textit{cthtrA}.

We were able to identify a strain with a SNV resulting in an amino acid substitution (P370L) in the \textit{cthtrA} sequence, that resulted in a severe reduction in proteolytic activity in the recombinant protein bearing the substitution. However, we expect there is some proteolytic activity by this form of CtHtrA (\textit{cthtrA}_{P370L}) in vivo. Firstly, our in vitro assays are based on model substrates requiring a second peptide for allosteric activation of maximal protease activity and thus may not accurately reflect in vivo activity. Secondly, the assay against the full-length $\beta$-casein protein demonstrated in vitro protease activity for this mutated protein, suggesting that in vivo activity is likely to be present. Given that the strain is still viable and that chemical inhibition of the proteolysis activity of CtHtrA was found to be lethal [15], we suspect some CtHtrA proteolysis activity is occurring in vivo in this strain (\textit{cthtrA}$_{P370L}$). While causality will require additional genetic evidence, this mutation to be a major contributor to the severe defect in the production of infectious EBs we observed in the \textit{cthtrA}$_{P370L}$ mutant strain (20–40-fold reduction), that was not contributed to by the \textit{ctl0738} SNV (as this was not observed in the \textit{ctl0738}$_{null}$ strain generated by lateral gene transfer).

The \textit{cthtrA}$_{P370L}$ strain had 18 SNVS compared to 11 and 8 SNVs detected in the \textit{cthtrA}_{A240V}, \textit{cthtrA}_{G475E} strains respectively, and hence, while it is tempting to attribute the phenotypes observed to the P370L mutation, it could in fact be a consequence of a combination of mutations or one of the other mutations alone. However, we can be confident that the reduced infectious EB production phenotype is not a consequence of the \textit{ctl0738}$_{null}$ mutation, based on the data in Fig. 7a. It was surprising to observe that the \textit{ctl0738}$_{null}$ strain showed similar reduced infectious EB yield after heat stress as the \textit{cthtrA}$_{P370L}$ isolate, as this model of stress is not expected to induce DNA damage that would require the repair enzyme predicted to be encoded by this locus [35].

Perhaps the most important result is that after lateral gene transfer experiments and the screening of 56 plaque purified isolates, no strain was identified containing the P370L mutation (C $\rightarrow$ T at nucleotide position 247916). This could be random chance or could suggest that the

---

Fig. 6 Circular representation of the reference L2/434/Bu genome (1.04 Mbp) showing the position of SNVs found on protein coding genes and the annotation of those genes. Each mutant strain is represented by a single ring layer (from inner to outer: \textit{cthtrA}$_{P3470L}$, \textit{cthtrA}$_{G475E}$, \textit{cthtrA}$_{A240V}$). Blue labels correspond to synonymous SNVs and black labels indicate non-synonymous SNVs. Figure generated using BRIG [37].
The P370L mutation in isolation is responsible for a severe growth defect that prevented us from isolating any strains during these experiments. Alternatively, it is possible that one of the other SNVs is suppressing the P370L mutation in the cthtrAP370L strain. Given that we expect ChtHtrAs function to impact the integrity of several chlamydial cell envelope proteins, including major outer membrane protein (MOMP), Porin B (PorB), and the polymorphic membrane proteins (Pmps) [12], ChtHtrAs chaperone and protease activity as a stress response and housekeeping protein important for outer membrane protein assembly could be indirectly driving the observed phenotypes [31, 36].

The smaller inclusion sizes observed in the cthtrAP370L strain corresponds with the reduced infectious EB yield suggesting a growth defect (possibly relating to optimal replication) could be a consequence of reduced ChtHtrA function, likely indirectly due to the function of a substrate that requires ChtHtrA for correct assembly or maintenance.

Overall, we have provided genetic evidence that C. trachomatis with a genetic mutation (C – T at position 247916 in cthtrA) likely resulted in reduced production of infectious EB, potentially correlating with a negative impact of this mutation on ChtHtrA in vitro proteolytic activity. While genetic causality could not be confirmed, these results provide further evidence that ChtHtrA has a critical role during the developmental cycle of Chlamydia.

Conclusions
HtrA is known to be an important protease for many bacterial pathogens. Analysis of Chlamydia trachomatis genetic mutants possessing variants of the cthtrA gene that firstly showed an impact on proteolytic function by in vitro analysis demonstrated that these mutants had reduced infectious progeny. Thus HtrA has a critical role in formation of chlamydial infectious progeny.

Availability of supporting data
Supplementary tables are provided. The draft assemblies of all genome sequences generated during this project are available through the EMBL ENA database under the Study number PRJEB9044: http://www.ebi.ac.uk/ena/data/search?query=PRJEB9044.

Additional files

Additional file 1: Table S1. EMS library mutants with confirmed non-synonymous SNVs in the cthtrA gene. Table S2. List of bacterial strains used in this study. Table S3. Total SNVs present in the cthtrA247916, cthtrA247916, and cthtrA247916 mutants as confirmed by whole genome sequencing. Table S4. Sequences and associated annealing temperatures for the PCR primers used in this study. Table S5. Active site substrates and activators used for proteolysis and oligomerisation assays. (DOCX 45 kb)

Additional file 2: Figure S1. SDS-PAGE gels of full length β-casein cleavage by wild-type ChtHtrA and mutants over a 60 min time course. A. Wild-type ChtHtrA; B. ChtHtrA247916; C. ChtHtrA247916; D. ChtHtrA247916; E. ChtHtrA247916; F. ChtHtrA247916; G. ChtHtrA247916. Lanes are labelled: M: protein molecular weight marker (Bio-Rad); 0 min; 10 min; 20 min; 30 min; 40 min; 50 min; 60 min; H.
ChlrA only; βc: β-casein only. The molecular masses of standard proteins are indicated by arrows next to the gels. (DOC 21 kb)

Abbreviations
DNP: 2,4-dinitrophenyl; EB: Elementary body; EMS: Ethyl methanesulfonate; FCs: Fetal calf serum; MCA: 7-methoxycoumarin-4-acetic acid; MOMP: Major outer membrane protein; DMP: Outer membrane protein; Pmp: Polymorph membrane protein; pNMA: p-Nitroaniline; RB: Reticulate body; RifR: Rifampicin-resistant; SNV: Single nucleotide variant; SpcR: Spectinomycin-resistant; TILLING: Targeting induced local lesions in genomes; WGS: Whole genome sequencing.

Competing interests
The authors declare that they have no competing interests.

Author's contributions
JM carried out the experimental work and drafted the manuscript. BW conducted the data analysis of the genome sequences and manuscript drafting. JT helped interpret and analyze the data and also contributed to draft the manuscript. BL helped interpret and analyze the data and also contributed to drafting the manuscript. RB screened for and provided the chemical mutants for L2, participated in the data interpretation, and helped draft the manuscript. RV screened for and provided the chemical mutants for either L2, participated in the data interpretation, and helped draft the manuscript. UK screened and provided the chemical mutants for D, participated in the data interpretation, and helped draft the manuscript. WH conceived of the study, worked on the technical and experimental support from Amba Lawrence throughout the study, and participated in its design, participated in data analysis and interpretation, and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Authors’ information
Not applicable.

Acknowledgements
Most of the work on Chlamydia HtrA in the Huston group has been supported by NHMRC Project Grant 553020 and an ARC Linkage Project in Conjunction with The Wesley Research Institute. WH has previously received support from an NHMRC Peter Doherty Fellowship. Queensland University of Technology and Institute of Health and Biomedical Innovation funding supported several components of this study. JM wishes to acknowledge the technical and experimental support from Amba Lawrence throughout the experimental work of this study.

Author details
1Institute of Health and Biomedical Innovation (IHBI), Queensland University of Technology (QUT), 60 Muske Avenue, Kelvin Grove, QLD 4059, Australia. 2National School of Pharmacy, University of Otago, PO Box 56, Dunedin 9054, New Zealand. 3Department of Molecular Genetics and Microbiology, Center for Microbial Pathogenesis, Duke University Medical Center, Durham, NC 27710, USA. 4Laboratory of Intracellular Parasites, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, MT 59840, USA.

Received: 18 May 2015 Accepted: 25 September 2015
Published online: 30 September 2015

References
1. Cartiller LHH. Global Data on Visual Impairments. WHO. 2010;2010:1–17.
2. Rowley J, Toskin I, Nduwa F. Global Incidence and Prevalence of Selected Curable Sexually Transmitted Infections. Sex Reprod Health. 2008;11:1–28.
3. Schachter J. The intracellular life of Chlamydia. Curr Top Microbiol Immunol. 1988;138:109–39.
4. Stephens RS. Genome sequence of an obligate intracellular pathogen of humans: Chlamydia trachomatis. Science. 1998;282:754–9.
5. Lipinski BB, Sharma S, Georgopoulos CC. Sequence analysis and regulation of the htrA gene of Escherichia coli: a Δσ70-independent mechanism of heat inducible transcription. Nucleic Acids Res. 1988;16:10053–67.
6. Metzger M, Hasenbein S, Hauske P, Kucz N, Merdanovic M, Grau S, et al. Allergic activation of HtrA protease DegP by stress signals during bacterial protein quality control. Angew Chem Int Ed. 2008;47:1332–4.
7. Krojer T, Sawa J, Schäfer E, Sabili HR, Ehrmann M, Clausen T. Structural basis for the regulated protease and chaperone function of DegP. Nature. 2008;453:885–90.
8. Wu X, Lii L, Gong S, Chen D, Flores R, Zhong G. The chlamydial periplasmic stress response serine protease chlrA is secreted into host cell cytosol. BMC Microbiol. 2011;11:87.
9. Jones CH, Bolken TC, Jones KF, Zeller GO, Hruby DE. Conserved DegP protease in Gram-positive bacteria is essential for thermal and oxidative tolerance and full virulence in Streptococcus pyogenes. Infect Immun. 2003;69:5538–45.
10. Krojer T, Garido-Franco M, Huber R, Ehrmann M, Gausen T. Crystal structure of DegP (HtrA) reveals a new protease-chaperone machine. Nature. 2002;416:455–9.
11. Marsh JW, Lott WB, Tyrndall JDA, Huston WM. Proteolytic activation of Chlamydia trachomatis HtrA is mediated by PDZ1 domain interactions with protease domain loops L3 and LC and beta strand (B5. Cell Mol Biol Lett. 2013;18:522–37.
12. Huston WM, Tyrndall JDA, Lott WB, Stansfield SH, Timms P. Unique residues involved in activation of the multistalking protease/chaperone HtrA from Chlamydia trachomatis. PLoS ONE. 2011;6:e25457.
13. Patel P, De Boer L, Timms P, Huston WM. Evidence of a conserved role for Chlamydia HtrA in the replication phase of the chlamydial developmental cycle. Microbes Infect. 2014;16:690–4.
14. Ony VA, Marsh JW, Lawrence A, Aban JA, Timms P, Huston WM. The protease inhibitor JO146 demonstrates a critical role for HtrA for Chlamydia trachomatis reversion from penicillin persistence. Front Cell Infect Microbiol. 2013;3:1–10.
15. Goeckli S, Ony VA, Patel P, Tyrndall JDA, Timms P, Beagley KW, et al. Identification of a serine protease inhibitor which causes inclusion vacuole reduction and is lethal to Chlamydia trachomatis. Mol Microbiol. 2013;89:676–90.
16. Huston WM, Theodoropoulos C, Mathews SA, Timms P. Chlamydia trachomatis responds to heat shock, penicillin-induced persistence, and IFN-gamma persistence by altering levels of the extracytoplasmic stress response protease HtrA. BMC Microbiol. 2008;8:1–16.
17. Huston WM, Svedberg JE, Harris JM, Walsh TP, Mathews SA, Timms P. The temperature activated HtrA protease from pathogen Chlamydia trachomatis acts as both a chaperone and protease at 37 °C. FEBS Lett. 2007;581:3382–6.
18. Goeckli S, Tyrndall JDA, Stansfield SH, Timms P, Huston WM. The active site residue V266 of chlamydial HtrA is critical for substrate binding during both in vitro and in vivo conditions. J Mol Microbiol Biotechnol. 2012;22:10–6.
19. Muschiel S, Bailey L, Gyffle A, Sundin C, Hulteney K, Bergstrom S, et al. A small-molecule inhibitor of type III secretion inhibits different stages of the infectious cycle of Chlamydia trachomatis. Proc Natl Acad Sci U S A. 2006;103:14566–71.
20. Kari L, Goheen MM, Randall LB, Taylor LD, Carlson JH, Whitmore WM, et al. Generation of targeted Chlamydia trachomatis null mutants. Proc Natl Acad Sci U S A. 2003;103:14566–70.
21. Nguyen BD, Valdivia RH. Virulence determinants in the obligate intracellular pathogen Chlamydia trachomatis revealed by forward genetic approaches. Proc Natl Acad Sci U S A. 2012;109:1263–8.
22. Wang Y, Kehane S, Cutcliffe LT, Skilton RJ, Lambden PR, Clarke IN. Development of a transformation system for Chlamydia trachomatis: restoration of glycogen biosynthesis by acquisition of a plasmid shuttle vector. PLoS Pathog. 2011;7:e1002258.
23. Kodym A, Alfa R. Physical and chemical mutagenesis. Methods Mol Biol. 2003;236:189–204.
24. Bivare PA, Sandoz KM, Omsland A, Rockey DD, Heinzen RA. Advances in genetic manipulation of obligate intracellular bacterial pathogens. Front Microbiol. 2011;2:1–13.
25. Segue GA. A review of the genetic effects of ethyl methanesulfonate. Mutat Res. 1986;134:113–42.
26. Kokes M, Dunn JD, Graneck J, Nguyen BD, Barker JR, Valdivia RH, et al. Integrating chemical mutagenesis and whole-genome sequencing as a platform for forward and reverse genetic analysis of Chlamydia. Cell Host Microbe. 2015;17:776–25.
27. Sturdevant GL, Kari L, Gardner DJ, Olivarres-Zaavaleta N, Randall LB, Whitmore WM, et al. Frameshift mutations in a single novel virulence factor alter the in vivo pathogenicity of Chlamydia trachomatis for the female murine genital tract. Infect Immun. 2010;78:3660–8.
28. Merdanovic M, Mamant N, Meltzer M, Poepsel S, Auckenthaler A, Melgaard R, et al. Determinants of structural and functional plasticity of a widely conserved protease chaperone complex. Nature. 2010;17:837–43.

29. Lipinska BB, Fayet O, Baird L, Georgopoulos CC. Identification, characterization, and mapping of the Escherichia coli HtrA gene, whose product is essential for bacterial growth only at elevated temperatures. J Bacteriol. 1989;171:1574–84.

30. Strauch KL, Beckwith J. An Escherichia coli mutation preventing degradation of abnormal periplasmic proteins. Proc Natl Acad Sci U S A. 1988;85:1576–80.

31. Spiess CC, Beil AA, Ehrmann MM. A temperature-dependent switch from chaperone to protease in a widely conserved heat shock protein. Cell. 1999;97:339–47.

32. Lindahl T, Sedgwick B, Sekiguchi M, Nakabeppu Y. Regulation and expression of the adaptive response to alkylating agents. Annu Rev Biochem. 1988;57:133–57.

33. Ge X, Wang R, Ma J, Liu Y, Ezemaduka AN, Chen PR, et al. DegP primarily functions as a protease for the biogenesis of β-barrel outer membrane proteins in the Gram-negative bacterium Escherichia coli. FEBS J. 2013;281:1226–40.

34. Nguyen BD, Valdivia RH. Forward genetic approaches in Chlamydia trachomatis. J Vis Exp. 2013;80:e50636.

35. Huston WM, Gloeckl S, de Boer L, Beagley KW, Timms P. Apoptosis is induced in Chlamydia trachomatis-infected HEP-2 cells by the addition of a combination innate immune activation compounds and the inhibitor wedelolactone. Am J Reprod Immunol. 2010;65:460–5.

36. Myers GSA, Mathews SA, Erpinger M, Mitchell CM, O’Brien KK, White OR, et al. Evidence that human Chlamydia pneumoniae was zoonotically acquired. J Bacteriol. 2009;191:7225–33.

37. Alikhan N, Petty NK, Ben Zakour NL, Beatson SA. BLAST Ring Image Generator (BRIG): simple prokaryote genome comparisons. BMC Genomics. 2011;12:402.