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The HtrA-like protease CD3284 modulates virulence of *Clostridium difficile*

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Infection and Immunity 2014

Abstract

In the past decade, *Clostridium difficile* has emerged as an important gut pathogen. Symptoms of *C. difficile* infection range from mild diarrhea to pseudomembranous colitis. Besides the two main virulence factors Toxin A and B, other virulence factors are likely to play a role in the pathogenesis of the disease. In other Gram positive and Gram negative pathogenic bacteria conserved High temperature requirement A (HtrA)-like proteases have been shown to have a role in protein homeostasis and quality control. This affects the functionality of virulence factors and the resistance of bacteria to (host-induced) environmental stresses.

We found that the *C. difficile* 630 genome encodes a single HtrA-like protease (CD3284; *htrA*) and have analyzed its role *in vivo* and *in vitro* through the creation of an isogenic ClosTron-based mutant of *htrA* in *C. difficile* strain 630Δ*erm* (wild-type). In contrast to the attenuated phenotype seen with *htrA* deletion in other pathogens, this mutant showed enhanced virulence in the Golden Syrian hamster model of acute *C. difficile* infection. Micro-array data analysis showed a pleiotropic effect of *htrA* on the transcriptome of *C. difficile*, including up-regulation of the toxin A gene. In addition, *the htrA* mutant showed reduced spore formation and adherence to colonic cells. Together, our data show that *htrA* can modulate virulence in *C. difficile*.

Introduction

The bacterial pathogen *Clostridium difficile* is the leading cause of infectious nosocomial diarrhoea (1-3). *Clostridium difficile* infection (CDI) can cause disease with a wide variety of symptoms, ranging from mild diarrhea to severe pseudomembranous colitis (1,4,5). Since 2003, the global prevalence of reported CDI cases has escalated (5-8). In addition, the severity of the disease and the mortality has increased (1,4,9).

The main virulence factors of *C. difficile* are the two large clostridial toxins, Toxin A (TcdA) and Toxin B (TcdB) (10-12). These toxins are glycosyltransferases that inactivate Rho, Rac and Cdc42, thereby disrupting the cytoskeleton and tight junctions of the colon epithelial cells, resulting in activation of the inflammasome and cellular apoptosis (10,12). A third toxin, binary toxin, is produced by certain strains (e.g. PCR ribotype (RT) 027 and 078) that have been associated with increased levels of mortality and morbidity (1,9). It has been suggested that binary toxin may contribute to disease in hamsters (13). *In vitro* assays have demonstrated that the binary toxin affects adhesion of *C. difficile* to cells through protrusion formation of the target cells (14).

Besides the three toxins, little is known about other virulence factors and their role in colonization and establishment of an infection in the host. Presently, several surface proteins have been identified or hypothesized to play a role in colonic adhesion. These include the fibronectin-binding protein A (15), S-layer proteins (16), Cwp84 (17), flagellar proteins (18) and CD1581 (19).

Alongside colonization factors, adaptation to stresses in the host (including the antibacterial response, elevated temperatures, extreme pH and osmotic stress) are likely to play a vital role in the establishment of an infection. These stresses can result in the accumulation of (partially) unfolded proteins that are non-functional or form poisonous aggregates (20). The well-conserved family of bacterial High temperature requirement A (HtrA) proteases plays an important role in the protein quality control and homoeostasis by combining proteolytic and chaperone activities in a variety of microorganisms (21-23).

HtrA-like proteases are generally composed of three structurally distinct domains: a trypsin-like serine protease domain, one or two PDZ domains and a trans-membrane domain or a signal peptide (22-24). The PDZ domain(s) are highly flexible domains and are involved in oligomerization, substrate recognition and/or the regulation of protease activity (21,24). Membrane anchored HtrA-like proteases are active as trimers and soluble HtrA-like proteases form larger active oligomers (21,22).

In this study we describe the identification and characterization of a *C. difficile* HtrA-like protease (CD3284; hereafter HtrA). We show that an *htrA* null mutant

has enhanced virulence in the Golden Syrian hamster model of acute *C. difficile* infection. This contrasts data reported for other pathogens, in which mutation of *htrA* results in attenuation (25-27). Furthermore, the *htrA* null mutant displayed a pleiotropic effect in a transcriptomic analysis. Several differentially expressed genes were validated and are discussed in the context of *C. difficile* virulence.

Material & Methods

Bacterial strains and growth conditions

The *C. difficile* and *E. coli* strains and plasmids used in this study are listed in Table 1. *E. coli* strains were grown in Luria Bertani medium (LB, USB cooperation) supplemented with appropriate antibiotics when required. *C. difficile* strains were grown anaerobically in a microaerobic cabinet (Don Whitley DG 250) at 37 ºC in pre-reduced TTY medium (30 g/L Bacto Trypton (BD), 20 g/L Yeast extract (Difco) and 0.1% thioglycolate pH 7.4) or Brain Heart Infusion broth (Oxoid) supplemented with 5 g/L yeast extract and 0.01% L-cysteine (Sigma) (BHIS) (28). Logarithmic growth phase bacteria (OD_{600} 0.4 ± 0.08) from overnight pre-cultures were used to inoculate pre-reduced TTY broth at a starting OD_{600} of 0.05 (±0.01). Optical density readings were taken hourly until the stationary growth phase (8 hour post inoculation) and at 24 and 48 hour post inoculation. Cultures of *C. difficile* strains harboring an expression plasmid were induced with anhydrotetracycline (100 ng/mL) at one hour post inoculation. We routinely monitored the purity of cultures by performing control PCRs to confirm identity of mutant strains and stability of conjugated expression plasmids.

Protein purification of His10-Δ(1-30)-HtrA and His10-Δ(1-30)-HtrA-S217A

To facilitate purification from *Escherichia coli*, CD3284 was cloned without the N-terminal 30 amino acids into pET-16B, resulting in a fusion protein with an N-terminal 10xhis-tag but lacking the predicted transmembrane helix. To generate this clone, the CD3284 open reading frame was amplified using primers CD3284F2 and CD3284R2 using Pfu polymerase (Fermentas) and chromosomal DNA from strain 630Δ*erm* (29) as a template. The resulting product was digested with NdeI and XhoI and ligated into similarly digested pET-16B (Novagen), yielding pVW001. After sequence verification using conventional Sanger sequencing, the plasmid was transformed into *E. coli* C43(DE3), in which expression of toxic proteins/proteases is feasible (30). To induce expression of His10-Δ(1-30)-HtrA, an overnight culture grown in LB with 100 μg/mL ampicillin was diluted 1/100 in 500 mL fresh medium to an optical density at 600 nm of 0.6 after which 1mM of IPTG was added for

3 hour. Hereafter, cells were harvested by centrifugation (10 minutes 10 rpm 4 °C) and the protein was purified essentially as described for Spo0A (31), with the following modifications: an 0.5 mL TALON Superflow column was used, and the stepwise washing/elution was carried out with 8, 4, 4, 4, and 4 column volumes (20mM, 50mM, 100mM, 250mM, 500mM, respectively). After the addition of glycerol to a final concentration of 8%, protein was quantified by measuring the absorption at 280nm on a Nanodrop ND-200 machine (ThermoScientific), using an extinction coefficient (ε) of 14440 M-1 cm-1 and a molecular weight of 36.77 kDa, based on analysis of the protein sequence using the ProtParam tool (http://web.expasy.org/protparam/). Protein not used immediately was stored at -80°C. To generate a catalytic mutant of HtrA, we mutated the conserved Ser217 to an alanine on the basis of published mutants of the *E. coli* DegS protease. To do so, oligo-directed mutagenesis was performed using primers CD3284S217AF and CD3284S217AR on plasmid pVW001, yielding plasmid pJV001. The mutant protein (His10-Δ(1-30)-HtrA-S217A) was purified and quantified as described for His10-Δ(1-30)-HtrA.

Table 1: Strains and plasmids used in this study.

Strains	Description/Genotype	Origin
Escherichia coli		
DH5α		Laboratory stock
CA434		(34)
C43		(30)
Clostridium difficile		
WKS1241	630Δ*erm*	(29)
DB0051	630Δ*erm*, pRPF185	This study
DB0002	630Δ*erm*, c*d3284::*(*ClosTron*, *ermB*)	This study
DB0052	630Δ*erm*, c*d3284::*(*ClosTron*, *ermB*) pRPF185 (*catP*)	This study
DB0047	630Δ*erm*, *cd3284::*(*ClosTron, ermB*) ,pDB0031 (*catP*)	This study
WKS1237	630Δ*erm*, *spo0A::*(*ClosTron*, *ermB*)	(32)
WKS1710	630Δ*erm*, *tcdA::*(*ClosTron*, *ermB*) *tcdB::*(*ClosTron*, *catP*)	(40)
Plasmids		
pMTL007	*catP*, ClosTron GroupII Intron	(32)
pDB0001	pMTL007::*cd3284*-162s, *catP*	This study
pRPF185	*tetR*, *Ptet-gusA*, *catP*,	(35)
pDB0031	*tetR, Ptet-cd3284, catP*	This study
pVW001	*PT7-His10-*Δ(*1-30*)*-htrA, bla*	This study
pJV001	*PT7-His10-*Δ(*1-30*)*-htrA-S217A, bla*	This study

Table 2: Primers and probes used in this study.

Oligos	Sequence (5'- 3')	Description	origin
IBS-cd3284	AAAAAAGCTTATAATTATCCTTAGGTAACAGTCAAGTGCGCCCAGATAGGGTG	ClosTron	This study
EBS2-cd3284	TGAACGCAAGTTTCTAATTTCGATTTTACCTCGATAGAGGAAAGTGTCT	ClosTron	This study
EBS1d-cd3284	CAGATTGTACAAATGTGGTGATAACAGATAAGTCAGTCAAAATAACTTACCTTTCTTTGTGT	ClosTron	This study
EBS universal	CGAAATTAGAAACTTGCGTTCAG TAAA	ClosTron/Control PCR	
oDB0067	CTGAGCTCCTGCAGTAAAGGAGAAAATTTTATGTCAAGAAGAAAGAAAGG	Complementation	This study
oDB0068	TAGGATCCGGTTAGAAATTCACATTTATTGTT	Complementation	This study
oDB0121	CCAGCTTTTTCTGCTGATGA	Control PCR/seq.	This study
oDB0123	GAAGTTCTACGCCGATAGTT	Control PCR/seq.	This study
oDB0124	AATACCTACAACAGATGGCG	Control PCR	This study
NF_794	CACCGACGAGCAAGGCAAGACCG	Control PCR/ seq.	(23)
NF_1323	CTGGACTTCATGAAAAACTAAAAAAAATATTG	sequencing	(23)
oWKS-1177	ATCTAGCTAGCGCCAGGAGAGTTGTTGATTC	Control PCR	This study
rspJ Forward	GATCACAAGTTTCAGGACCTG	qPCR	(4)
rspJ Reverse	GTCTTAGGTGTTGGATTAGC	qPCR	(4)
tcdA Forward	AATCCAATACAAGCCCTGTAG	qPCR	(4)
tcdA Reverse	TATCAGCCCATTGTTTTATGTATTC	qPCR	(4)
tcdA probe	FAM-TCACTGACTTCTCCACCTATCCATACAA-BHQ-1	qPCR	(4)
oDB0117	ACTCAAAGCGCAATAAATCTAGGAGC	qPCR	This study
oDB0118	ACTGGTCTAGGTTTTTGGCTCAACTTGT	qPCR	This study

Protease assay

Protease activity of the purified proteins was assayed as follows. In a 100 **µ**L reaction, 2.5 **µ**M of His10-Δ(1-30)-HtrA or His10-Δ(1-30)-HtrA-S217A was incubated with 20 **µ**M of casein or beta-casein (molecular weight ~24kDa) in 1xHNE buffer (150mM NaCl, 0.1mM, 5mM HEPES, pH7.4, 0.1 mM EDTA), with the pH adjusted to pH5.5 by the addition of 0.1x MES/A buffer (0.01M MES, 0.01M acetic acid, pH5.0). Reactions were incubated on ice (0°C) or at 37°C for 18h with a no-protein control included. After incubation, the reaction was terminated by the addition of 25 **µ**L 5xSDS sample buffer (10% SDS, 10% beta-mercaptoethanol, 50% glycerol, 0.1% bromophenolblue, 250mM Tris-HCl pH6.8, 50mM EDTA) and heating to 96°C for 2 minutes. 20 **µ**L volumes of the samples were loaded on a 12% SDS-PAGE gel and separated by electrophoresis at 80mA. Gels were stained with Coomassie solution (0.1 % Coomassie Brilliant Blue R-250, 20 % (v/v) methanol, 10% (v/v) acetic acid) and destained with 50% methanol -10% (v/v) acetic acid.

Generation of an *htrA* (*cd3284*) mutant strain

An *htrA* mutant was generated by insertional inactivation of the *cd3284* gene in 630Δ*erm* (which will be referred to as wild type in the rest of this paper) using ClosTron technology (32,33). Briefly, the Perutka algorithm on the ClosTron website (http://www.clostron.com) was used to design primers (supplemental Table 2) for retargeting the Group II intron (Sigma; Targetron). The retargeted intron was cloned using the restriction enzymes BsrGI and HindIII into plasmid pMTL007 and the constructs were verified by sequencing (33). The sequenced verified plasmid (pDB001) was transformed to *E. coli* CA434 and transferred via conjugation to *C. difficile* (29,34). The selection of *C. difficile* transconjugants was achieved by sub-culturing on pre-reduced BHIS agar supplemented with thiamphenicol (Sigma; 10 **µ**g/mL) and *C. difficile* selective supplement (Oxoid). After this, several rounds of sub-culturing on pre-reduced BHIS agar supplemented with lincomycin (Sigma; 20 **µ**g/mL) and *C. difficile* selective supplement were used to promote integration of the GroupII intron into the gene of interest. Chromosomal DNA isolated from the transconjugants (QIAamp blood kit, Qiagen) was analyzed by conventional PCR and sequencing of the PCR product to confirm the insertional inactivation of the *cd3284* gene. Primers used for cloning and sequencing are listed (Table 2). In addition, Southern blot analysis with an *ermB* probe was performed to verify a specific single integration into the genome as described previously (28) (data not shown).

Generation of strains carrying a plasmid based inducible *htrA*

Chromosomal DNA from the wild type strain (WKS1241) was used to PCR amplify the open reading frame of *cd3284* with primers oDB0067 and oDB0068. The resulting amplicon was digested with *BamHI* and *SacI* and cloned into pRPF185 thereby replacing the *gusA* gene (35). The sequence of the resulting inducible plasmid (pDB0031) was verified with primers NF_794, NF_1323 (35), oDB0067 and oDB0068. Plasmid pDB0031 was transferred into *E. coli* CA434 and conjugated into the *htrA* null mutant, resulting in a *htrA* mutant that can be complemented by inducible expression of a plasmid located copy of *htrA*. To address potential effects of the conjugated plasmid (pDB0031) in the phenotypic assays we generated control strains by conjugating the pRPF185 plasmid into wild type (DB0051) and *htrA* mutant (DB0052) strains (35). These strains served as controls in the *in-vitro* assays. There was no significant difference between strains with and without pRPF185 (data not shown).

Animal experiments

All procedures were conducted in strict accordance to the Animals (Scientific Procedures) Act 1986 approved by the Home Office, U.K. (project license number PPL60/4218). Female Golden Syrian hamsters that weighed approximately 100g (bred in-house) were housed individually and given water and food *ad libitum*. Telemetry chips (VitalView Emitter) were inserted by laparotomy into the body cavities of the animals at least 3 weeks before infection with *C. difficile*. Once the wounds healed, the animals were placed on receiver pads, and the body temperature and activity were monitored (Vital View software). Each animal received orogastrically 30 mg/kg of clindamycin phosphate in a single dose five days before infection. Six animals per group were inoculated by oral gavage with 10^4 spores of *C. difficile*. Animals were carefully monitored and culled when core body temperature dropped below 35 °C (28).

Statistical analyses were performed using the GraphPad Prism 5.03 (GraphPad Prism Software). A Mantel-Cox Log-rank statistical test was used to determine significant differences in hamster survival times between *C. difficile* strains. P values ≤0.05 were considered significant.

Minimum Inhibitory concentration (MIC) determination

The MIC of *C. difficile* wild type and *htrA* null mutant to erythromycin and clindamycin was determined by the doubling dilution method described previously (26). Briefly, rows of pre-conditioned BHI broth (90 µl) were supplemented with a concentration range of 1024-0.06 µg/mL of either antibiotic. Wells were inoculated with ~$5x10^3$ spores/100 µl and incubated at 37 °C for 48 hour anaerobically.

Wells to allow positive and negative evaluation of growth were included in which either no antibiotic or no spores were added. After incubation, plates were visually inspected and compared to the controls; the MIC value was determined as the lowest concentration of antibiotic at which growth was no longer visible. Results are given as the median MIC from three biological repeats.

RNA extraction

Five mL of the *C. difficile* cultures were diluted with ice cold methanol (1:1) and stored overnight at -80°C. Bacterial pellets, obtained by centrifugation (20 min, 3000 x g, 4°C), were resuspended in 200 µL lysis buffer (100 mM EDTA, 200 mM Tris-HCl pH 7.0, 50 mg/mL lysozyme) and incubated for 1 hour at 37°C. RNA was isolated using Tri-pure reagent (Roche) as previously described (28). The RNA was treated twice with a TurboDNase (Ambion) according to the manufacturer's instruction, followed by another Tri-pure RNA isolation. The Agilent Bioanalyzer (Agilent Technologies Netherlands BV) and a ND-1000 spectrophotometer (NanoDrop Technologies) were used to analyze the quality and purity of the extracted RNA.

DNA Microarray Analysis

Random nonamers were used to convert total RNA into amino allyl-modified cDNA using a Superscript III reverse transcriptase kit (Life Technologies) and purified with Nucleospin Gel and PCR clean-up kit (Machery-Nagel). The synthesized cDNA was labeled with DyLight 550 and DyLight 650 Amine/Reactive Dyess (Pierce Biotech USA) and purified again with the Nucleospin Gel and PCR clean up kit. The Agilent DNA micro-arrays (G2509F Custom Microarray GE 8x15K 60mer) were designed based on the available genome sequence of *C. difficile* 630 (36,37). The custom made micro-array slides consist of 8 sub-arrays each with 15.208 60-mer probes (37). The Agilent microarray slides were competitively hybridized with 300 ng labeled cDNA made from RNA isolated from both wild type and *htrA* mutant (DB0002) during logarithmic phase (OD_{600} 0.5 ±0.1) and stationary phase growth (12 hour post inoculation) . The microarray slides were scanned with a GenePix scanner and the fluorescent intensities were quantified using GenePix 6.1. The raw data files were analyzed with a LimmaR package via the Genome2D pipeline (http://genome 2d.molgenrug.nl) (38) using the following settings; i) the microarray data was normalized using LOESS normalization, followed by quantile normalization between the slides ii) a weight factor was determined based on the quality of each DNA microarray slide. For each time-point (logarithmic or stationary phase) 3 biological replicates were used. The complete data set, experimental raw data and analyzed data, was submitted to the GEO database (accession number GSE55926).

Reverse Transcriptase Quantitative PCR

For Reverse Transcriptase quantitative PCR (RT-qPCR) the RevertAid™ H Minus Reverse Transcriptase kit (Fermentas) was used to synthesize cDNA according to the manufacturer's instruction. Random hexamers were used to convert 750 ng RNA into cDNA. The synthesized cDNA was treated with RNase (Qiagen) for 1 hour at 37°C and stored at -20°C. The software program Molecular Beacon (Premier Biosoft) was used to design primer pairs (Table 2) for the quantitative PCRs (qPCR), based on the available genome sequence of *C. difficile* strain 630 (36).

All primer pairs were first tested by conventional PCR to confirm specificity and amplicon sizes. The RT-qPCR reactions were then performed using a CFX96 real-time PCR detection system (Biorad). The amplification efficiencies of the genes were determined using serially diluted genomic DNA. Expression levels of the *tcdA* and *spo0A* genes were normalized using the amplification efficiencies and the expression level of the reference gene *rpsJ* (39). The qPCR for *tcdA* and *rpsJ* was performed as previously described (28). The qPCR for the *spo0A* gene contained 25 µL Hotstar mastermix (Qiagen), forward (oDB0117) and reverse primers (oDB0118) (80 nm each primer), 2.5 mM $MgCl_2$, 0.06 % SYBRgreen (Sigma) and 2 µL synthesized cDNA. The qPCR protocol included an enzyme activation step for 15 min at 95 °C, followed by 50 cycles of amplification; 95 °C for 30 sec, 52 °C for 30 sec and 72 °C for 30 sec.

To determine the specificity of the fluorescence, the fluorescent data was converted into melt curve peaks. For each biologic replicate (n=3) four technical replicates were tested. An independent student t-test was employed to compare the strains at different time points.

Western blot analysis of TcdA and Spo0A

Quantitative western blots for TcdA and Spo0A were performed as follows. For TcdA, filtered (0.45 µm) bacterial supernatants from 48 hour cultures were analyzed by SDS-PAGE (6% polyacrylamide) and transferred onto nitrocellulose membranes. For Spo0A protein, bacterial pellets from cultures in the logarithmic phase (OD_{600} nm 0.5 ±0.1), and at 8, 12 and 24 hour post inoculation were collected by centrifugation (10 min, 11,000 g, 4 °C). Pellets were resuspended in PBS containing protease inhibitor cocktail (Sigma) and lyzed with a TissueLyser LT bead beater (50 Hz, 10 min, Qiagen) using acid wash glass beads (≤ 106 µm, Sigma). Equal amounts (OD_{600} nm corrected) of the lyzed bacterial pellets were separated on 15 % SDS-PAGE gels and transferred onto nitrocellulose membrane.

For TcdA quantification the membranes were probed with a mouse monoclonal anti-TcdA antibody (TCC8, tgcBiomics, Bingen, Germany) and for Spo0A quantification the membranes were probed with a mouse polyclonal Spo0A serum

(31). The probed membranes were analyzed by using a secondary anti-mouse biotin-labeled antibody (Dako) and a tertiary anti-biotin antibody labeled with Cy3. A Typhoon 9410 scanner (GE Healthcare) was used to measure the fluorescence intensity. *C. difficile* strains WKS1237 (*spo0A* null mutant) and WKS1710 (*tcdA* and *tcdB* null mutant) were used as control strains (32,40). The software package ImageQuant TL (Amersham Biosciences) was used to quantify fluorescence intensity.

Relative quantification of toxin expression

Quantification of the total toxin production was performed on filtered (0.45 μM) 24 and 48 hour post-inoculation supernatants, using a Vero cell based cytotoxicity assay (28). The end-point titer was defined as the first dilution at which Vero cell morphology was indistinguishable from the neutralized 200 fold diluted supernatant and untreated cells (28). *C. difficile* strain WKS1710 (*tcdA* and *tcdB* null mutant) was used as a negative control (40). Each experiment was performed in duplicate on three separate occasions. An independent student t-test was employed to compare the strains at different time points.

Relative quantification of sporulation efficiency

Sporulation efficiency was determined by analyzing culture samples of 24 and 48 hour post inoculation. Serially diluted non-heat shocked and heat shocked (20 min , 65 °C) culture samples were plated onto BHI plates supplemented with 0.1 % taurocholate to stimulate germination and enhance spore recovery (41). Plates were incubated for 48 hour before colony forming units (CFU) were enumerated. The *C. difficile* strain WKS1237 was included as a negative control for sporulation (32). Sporulation efficiency was normalized using the CFU count of the non-treated sample. Three biological replicates were analyzed in duplicate. An independent student t-test was employed to compare the strains at different time points.

Relative quantification of adhesion to Caco-2 Cells

Adhesion of *C. difficile* to Caco-2 cells was performed essentially as described previously with minor modifications (42). Briefly, Caco-2 cells were grown in RPMI (GE healthcare), 10 % fetal calf serum, Penicillin (100 μg/mL) and Streptomycin (100 U/mL) in a humidified 5 % CO_2 atmosphere at 37 °C. Caco-2 cells were seeded into a 24 wells plate at a density of 1x 10^5 cells per well and incubated overnight at 37 °C and 5 % CO_2. Confluent Caco-2 monolayers were washed once with PBS before transfer into the anaerobic cabinet.

Mid-logarithmic phase bacterial cultures (OD_{600} 0.5 ± 0.08) were collected by centrifugation (1 min, 4000 x g, 4 °C) and washed once with pre-reduced PBS. To

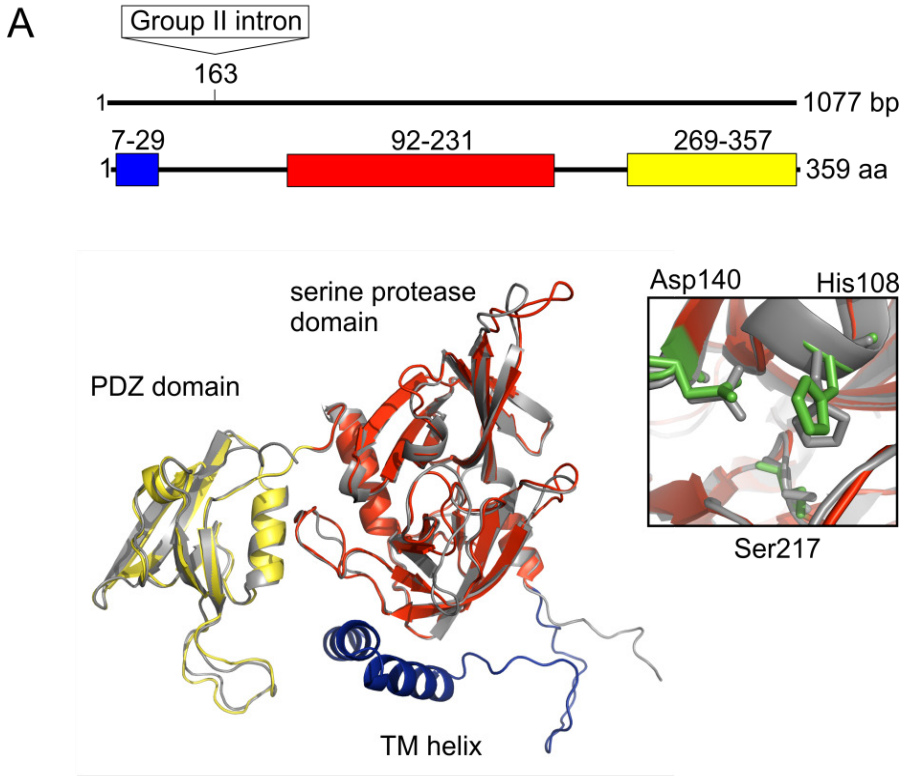

Figure 1: HtrA is a protease. **(A)** Schematic representation of the predicted structural domains of HtrA. The box on the top line indicates the position (nucleotide 163) at which the group II intron has inserted to create the *htrA* null mutant (not on scale). The predicted N-terminal transmembrane helix is shown in blue, the serine protease domain in red and the PDZ domain in yellow. The inset shows the conserved catalytic triad (in green) of the serine protease domain. The predicted structure of *C. difficile* HtrA is overlaid with the structure of *E.coli* DegS (1TE0A) in grey. **(B)** HtrA shows protease activity towards β-casein. 2.5µM of the indicated protein was incubated for 18 hour with 20 µM of beta-casein at 0°C or 37°C. wt = His10-Δ(1-30)-HtrA, S217A is the catalytic mutant His10-Δ(1-30)-HtrA- S217A. The asterisk indicates a proteolytic product of β-casein.

each well of Caco-2 approximately 1x 10^8 bacteria were added. Exact numbers of viable bacteria within the inoculums for each well were calculated by serial dilution and plating of cultures on BHIS plates. After 3 hour of incubation under anaerobic conditions, the Caco-2 cell monolayers with the adhered bacteria were washed 5 times with pre-reduced PBS. Adherent bacteria were released using 100 µL 1x trypsin solution (PAA laboratories, Coelbe, Germany) and serially diluted and plated on pre-reduced BHIS plates. After 48 hour colony forming units were enumerated to determine the percentage of adherent *C. difficile* relative to the initial inoculum. Each experiment was carried out in duplicate and repeated three times. An independent sample t-test was employed to compare the strains at different time points.

Results

Bioinformatics analysis of CD3284

In this study we identify a single HtrA-like protease in *C. difficile* 630Δ*erm*. CD3284 was identified using the BlastP (http://blast.ncbi.nlm.nih.gov/Blast.cgi), Smart (http://smart.embl-heidelberg.de/) and Merops (http://merops.sanger.ac.uk/) databases (36,43-45). Further bioinformatics analyses were performed with a conserved domain prediction webserver (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml), the trans-membrane prediction webserver TMHMM2.0 (http://www.cbs.dtu.dk/services/TMHMM/) and the signal peptide cleavage site webserver SignalP4.1 (http://www.cbs.dtu.dk/services/SignalP/). These analyses revealed the presence of a trans-membrane domain (Amino Acid (AA) 7-29), a trypsin-like serine protease domain (AA 92-231, E-value $3.96x10^{-29}$) and a PDZ domain (AA 269-357, E-value $2.17x10^{-19}$) (Figure 1A). The N-terminus of CD3284 was predicted by TMHMM to be highly hydrophobic, most likely transmembrane, whereas SignalP analysis revealed no signal sequence, suggesting that the protease and PDZ domains are located extracellularly, but membrane associated (46). Comparison of the CD3284 protein sequence of our wild type strain with the NCBI non-redundant protein sequences database using BlastP, resulted in identical protein sequence hits (Query coverage 100%, E-value 0.0) in other epidemic (e.g. RT027) and non-epidemic (e.g. RT001) *C. difficile* strains.

As protein 3D-structure is more conserved in evolution than its equivalent amino acid sequence, we validated the homology of CD3284 using the I-TASSER (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) and Phyre2 three-dimensional structure prediction server (http://www.sbg.bio.ic.ac.uk/phyre2/) (47,48). These analyses revealed with 100% confidence, over 81% alignment coverage and 40%

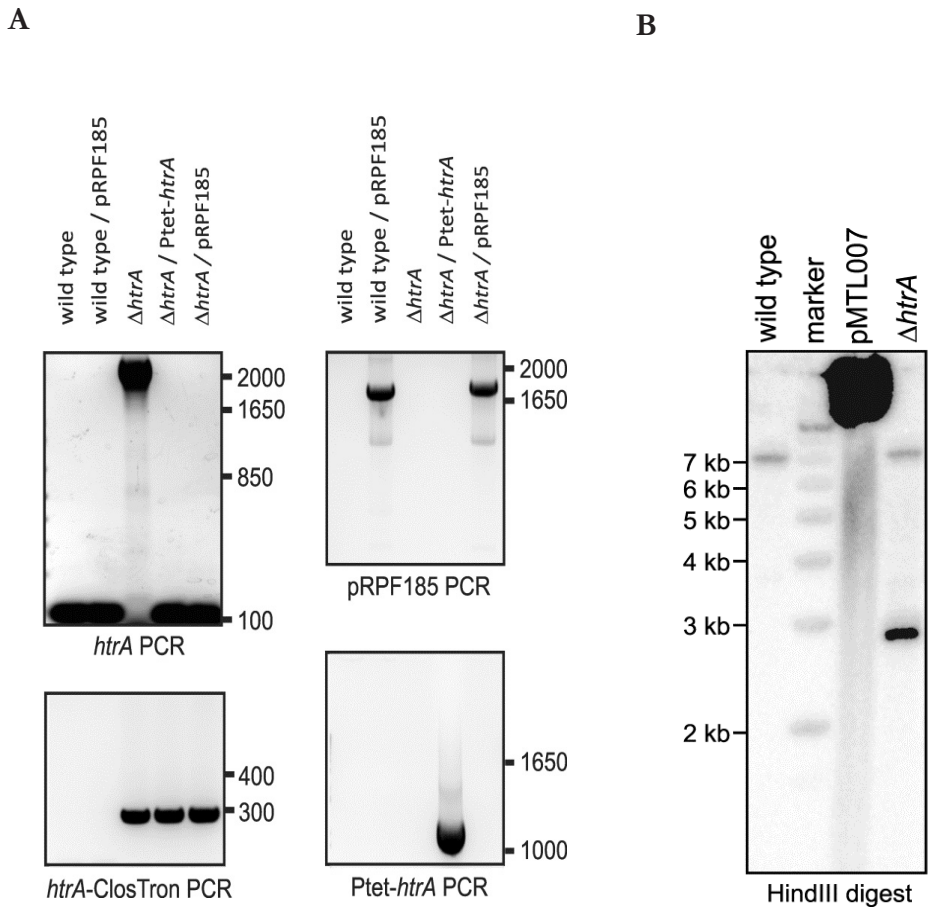

Figure 2: (A) PCR screening of the strains used in this study. *HtrA* PCR: The primers oDB0123 and oDB0124 generated a PCR product of approximately 2200 bp in the *htrA* mutant (Δ*htrA*, DB0002) and a PCR product of 150 bp in the wild type (WKS1241), wild type/pRPF185 (DB0051), complemented *htrA* mutant (Δ*htrA*/*htrA*+, DB0047) and Δ*htrA*/pRPF185 (DB0052). pRPF185 PCR: The primers oWKS-1177 and NF794 generated a PCR product of approximately 1800 bp in the DB0051 and DB0052 strains. *htrA*-ClosTron PCR: The primers oDB0123 and EBSuni generated a PCR product of approximately 280 bp in DB0002, DB0047 and DB0052 strains. Ptet-*htrA* PCR: The primers oDB0123 and NF794 generated a PCR product of approximately 1200 bp in DB0047. **(B)** Southern blot analysis of C. difficile wild type and Δ*htrA*. Chromosomal DNA digested of wild type (WKS1241) and Δ*htrA* (DB0002) with HindIII was hybridized with an *ermB* specific probe. Note that wild type shows a single band, indicative of the *ermB* element that is still present in this strain (29), Δ*htrA* contains an additional element, pMTL007 is shown as a positive control.

amino-acid identity, the structural homology of this protein to the HtrA-like protease DegQ (49,50), and the predicted structure of *C. difficile* HtrA overlaid with the structure of *E.coli* DegS demonstrated a high level of structure homology (Figure 1A). Homologs of DegQ and DegS are generally referred to as HtrA-like proteases in Gram-positive prokaryotes. Considering the above, we will refer hereafter to the CD3284 encoding gene as *htrA*, and the CD3284 protein as HtrA.

C. difficile HtrA (CD3284) shows protease activity towards β-casein

To investigate if *C. difficile* HtrA possesses proteolytic activity similar to that of other HtrA-like proteases (51) we performed a protease assay. Wild type HtrA (His10-Δ(1-30)-HtrA) and a catalytic HtrA mutant (His10-Δ(1-30)-HtrA-S217A) protein were incubated with β-casein, a highly unfolded protein substrate (Figure 1B). After 18 hour of incubation at 37 °C casein was only degraded by wild type HtrA and not by the catalytic HtrA mutant. These results demonstrate the proteolytic activity of *C. difficile* HtrA.

Construction of an *htrA* mutant

To determine the contribution of HtrA to *C. difficile* virulence we constructed an *htrA* mutant using ClosTron technology in the 630Δ*erm* (wild type) (29) strain by insertion of a Group II intron upstream of the sequence encoding the predicted trypsin-like serine protease domain (Figure 1A). Insertional inactivation of genes using the ClosTron technology results in stable mutants and non- functional proteins (32,40). The genotype of the insertional inactivation was confirmed by PCR (Figure 2A), sequence analysis and Southern blot (Figure 2B), which verified that the ClosTron group II intron was inserted at a single site. Initially, the impact of the mutation on growth *in vitro* was considered. This revealed that post inoculation

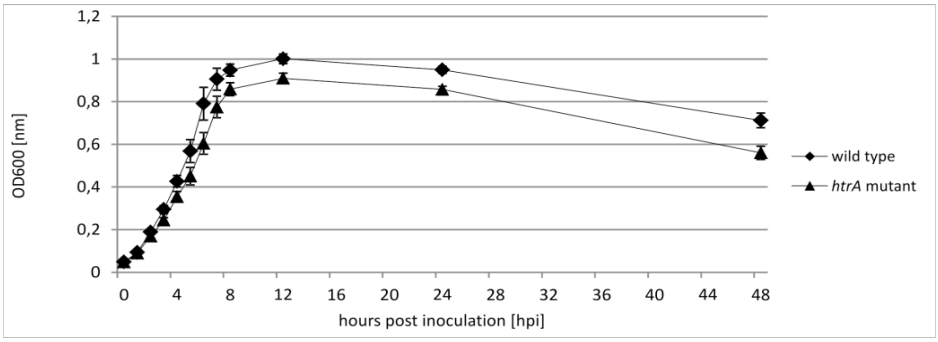

Figure 3: Growth curves of the wild type and the *htrA* mutant strain. The absorbance (OD_{600}) was measured over 48 hours of growth in TTY medium. The error bars indicated the standard error of the mean of three experiments.

both mutant and wild type strains showed logarithmic growth for the first 8 hour and by 12 hour post inoculation both strains had entered stationary growth phase (Figure 3). Though growth kinetics were not significantly different from wild type cells, we noted that the *htrA* mutant strain generally showed a slight growth delay in logarithmic phase and reached lower optical density in stationary phase (Figure 3).

The *htrA* null mutant is more virulent in hamsters

HtrA like proteases have been shown to be important for the virulence of pathogenic bacteria (25,52,53). To determine the contribution of HtrA in *C. difficile* virulence we performed *in vivo* experiments with the *htrA* mutant and wild type in the commonly used Golden Syrian hamster model (54,55). Six clindamycin pre-treated hamsters were orally infected either with wild type or the *htrA* mutant. The mean time to death for hamsters challenged with wild-type was 53 h and 39 min (± *5 h and 3 min; n=6*). Hamsters infected with the *htrA* mutant revealed a significant decreased survival time (31 h 20 min ± 44 min, n=6, p=0.0005) compared to wild type (Figure 4), suggesting that disruption of *htrA* causes enhanced virulence in this strain. Our experience evaluating a number of mutants in the hamster model suggests that animals challenged with other mutants created using ClosTron

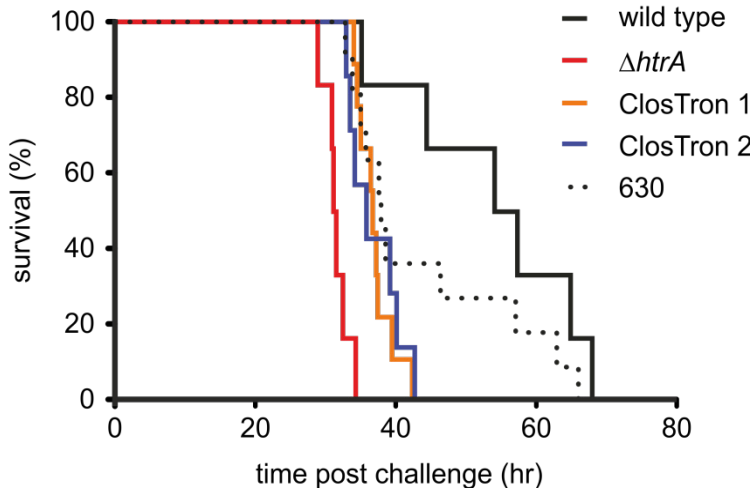

Figure 4: The *htrA* null mutant has increased virulence in a hamster model of infection. Survival curve of hamsters challenged with either 10^4 spores of *C. difficile* 630Δ*erm* (solid black line), *htrA* null mutant (red line), ClosTron mutant 1 & 2 (blue & green lines, respectively) or 630 wild-type (dashed black line). Hamsters challenged with *htrA* mutant (31 h 20 min, n=6) showed a significantly decreased survival time compared to those challenged with 630Δ*erm* (53 h 39 min, n=6, p=0.0005), ClosTron mutant 1 (36 h 41 min, n=7, p=0.049), ClosTron mutant 2 (36 h 47 min, n=9, p<0.0001) or 630 (43 h 43 min, n=11, p<0.0001). Hamsters were culled when body temperature declined below 35°C (clinical end point) and data analysed by Mantel-Cox Log-rank statistical tests.

technology have a shorter survival time compared to wild type (unpublished data). We hypothesize that this observation is due to the greater resistance to clindamycin (as a consequence of ClosTron insertion) and hence greater survival rates *in vivo* following pretreatment of animals with clindamycin to induce disease. Indeed, we find that the *htrA* mutant (and other ClosTron mutants) *in vitro* is more resistant to clindamycin (MIC >256 μg/mL) than the wild type (630Δ*erm*; MIC = 16 μg/mL).

Nevertheless, if we compared the *htrA* mutant to the original parental 630 isolate (Wust *et al*., 1982) (43 h 43 min, n=11, $p<0.0001$) or to other mutants (ClosTron 1, 36 h 41 min, n=7, $p=0.0049$; ClosTron 2, 36 h 47 min, n=9, $p<0.0001$) infected in the same way, the enhanced mortality rate remains significant. We therefore conclude that in the Golden Syrian hamster model the *htrA* null mutant of *C. difficile* demonstrates an enhanced virulence phenotype.

Comparative transcriptional micro-array data analysis of *htrA* mutant and wild type strains

To understand the enhanced virulence of the *htrA* mutant in the Golden Syrian hamster model, we performed comparative transcriptome analysis of the wild type and the *htrA* mutant. The *htrA* mutant displayed a pleiotropic effect in the transcriptomic analysis. The most interesting genes that were differentially regulated in the *htrA* mutant were *tcdA*, *spo0A*, and several genes encoding surface associated proteins.

The transcriptional profiles of the *htrA* mutant strain and wild type strain were studied at two different time points, the logarithmic and stationary growth phase respectively. The DNA micro-array raw data sets and the normalized ratio data are available from the NCBI GEO database (http://www.ncbi.nlm.nih.gov/geo/, accession number GSE55926) (56). In the logarithmic growth phase and in the stationary growth phase 551 genes and 567 genes were significantly differentially expressed, respectively (fold change ≥1.5, $p \leq 0.05$). In total 248 genes in the logarithmic growth phase and 263 genes in the stationary growth phase were up-regulated. Two-hundred and eight genes were up-regulated both in logarithmic and stationary growth phase. Among these, several genes were involved in stress adaptation response, like the *ctsR* and *hrcA* genes (57). The *tcdA* gene encoding the well-characterized virulence factor Toxin A was 3.3-fold and 3.0-fold up-regulated in the logarithmic and stationary growth phase, respectively.

A total of 303 genes in the logarithmic growth phase and 304 genes in the stationary growth phase were down-regulated in the *htrA* mutant. Two-hundred and sixty five genes were down-regulated both in logarithmic and stationary growth phase. Among these are genes involved in cell metabolism, like ribosomal proteins, ABC transporters and PTS transporters. The *spo0A* gene, encoding the

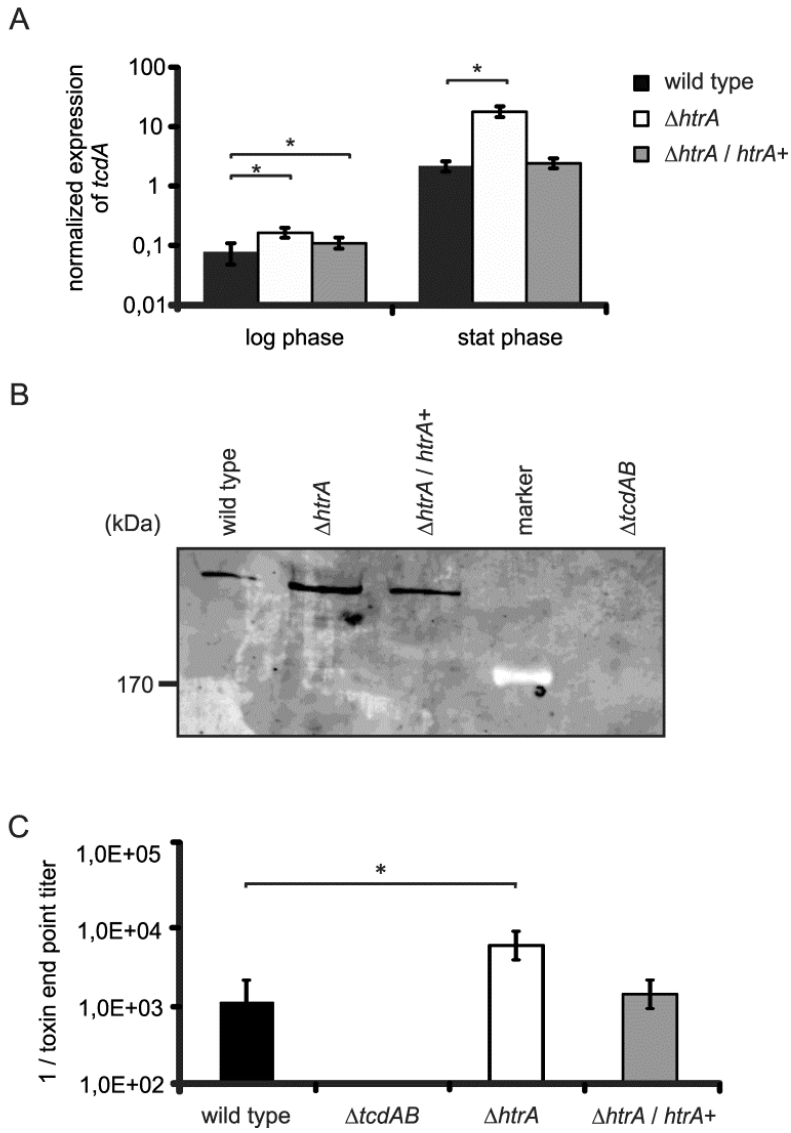

Figure 5: Increased TcdA production of *htrA null* mutant compared to wild type. **(A)** The relative *tcdA* gene expression profiles of *C. difficile* strains in logarithmic and stationary phase. The asterisks (*) indicate a significant difference between wild type and *htrA* mutant ($p<0.000001$) and complemented *htrA* mutant ($p<0.000001$). **(B)** Quantitative Western blot analysis of TcdA in culture supernatant of wild type, *htrA* null mutant and complemented *htrA* mutant at 48 hour post inoculation. **(C)** The toxin end point titers of the various *C. difficile* strains. The asterisk (*) indicate a significant difference between wild type and *htrA* null mutant ($p=0.001$) at the same time point. The error bars indicate the standard error of the mean of three experiments. Wild type is WKS1241, *htrA* mutant is DB0002, complemented *htrA* mutant is DB0047 and *tcdA/B* mutant is WKS1710.

master regulator of sporulation (58,59), was 2.4-fold and 2.3-fold down-regulated in the logarithmic and stationary growth phase, respectively, and many other sporulation genes showed a similar effect. Furthermore, several genes encoding surface-associated proteins (like *slpA* and 5 other paralogues of *slpA*) (16), flagella associated genes (18,60), and CD1581 (a gene with a potential effect on adhesion and intestinal colonization) (19) were significantly down regulated.

Overall, transcriptional profiling of the *htrA* null mutant showed a plethora of differences compared to wild type. The most prominent differentially regulated genes were *tcdA*, *spo0A* and several genes encoding surface associated proteins that were further investigated phenotypically.

Increased TcdA production of *htrA* mutant compared to wild type

Golden Syrian hamsters are exquisitely sensitive to *C. difficile* toxins and increased levels of *tcdA* transcription might explain the observed enhanced virulence. To validate the increased level of *tcdA* transcription in the comparative DNA microarray analysis we determined the *tcdA* transcription by RT-qPCR, quantitative Western blot analysis of TcdA and a cytotoxicity assay.

First, we confirmed the effect on transcription of the *tcdA* gene in the *htrA* mutant. We also complemented the *htrA* mutant (DB0047) to confirm that the observed effect on *tcdA* transcription in the *htrA* mutant was specific. RT-qPCR was used to compare the normalized *tcdA* transcription profiles in wild type, *htrA* mutant and the complemented *htrA* mutant strains in the logarithmic and stationary growth phase. In the logarithmic phase and the stationary phase the normalized *tcdA* transcription of the *htrA* mutant was significantly increased, 2.1-fold ($p<0.000001$) and 8.1-fold ($p<0.000001$), respectively (Figure 5A). Expressing HtrA from a plasmid in the mutant strain (DB0047) partially restored *tcdA* transcript levels in logarithmic phase (1.4-fold, $p=0.0022$) towards wild type level, whilst in stationary phase the *tcdA* transcript level was indistinguishable from wild type levels (Figure 5A). These data suggest that reduction in transcription of *tcdA* in the *htrA* mutant is a consequence of the *htrA* mutation. Second, we determined if the increased level of *tcdA* transcription was reflected in elevated levels of TcdA protein. Quantitative Western blot analysis with a TcdA specific monoclonal antibody on filtered supernatants of 48 hour post inoculation cultures showed a 7-fold increase of TcdA in the *htrA* mutant compared to wild type (Figure 5B). Similar to *tcdA* transcription, complementation of the *htrA* mutant resulted in TcdA levels that were partially restored towards the wild type (2-fold, Figure 5B). The signals detected were specific for TcdA as they were absent in the supernatant of the control strain WKS1710 (40).

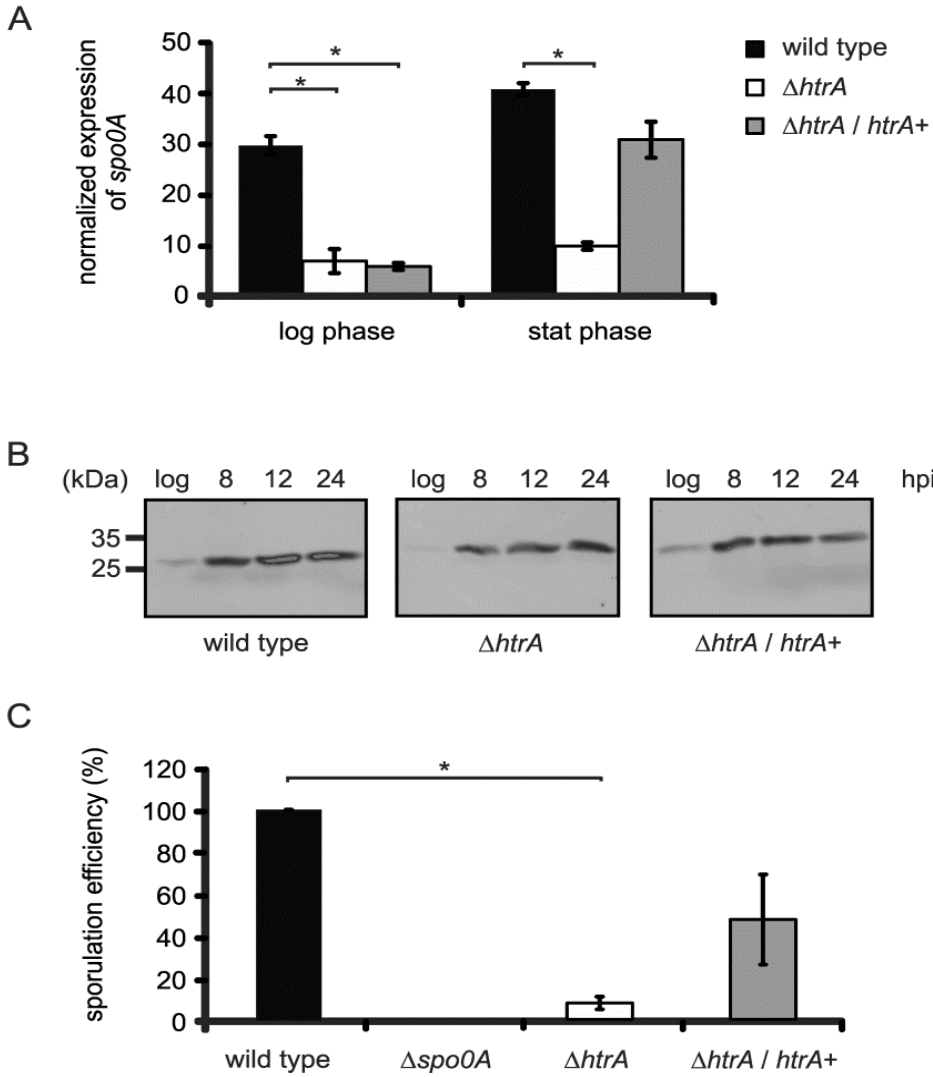

Figure 6: Decreased sporulation of *htrA* mutant compared to wild type. **(A)** The relative *spo0A* gene expression profiles of *C. difficile* strains in logarithmic and stationary phase. The asterisks (*) indicate a significant difference between wild type and *htrA* mutant (*p<0.000001*) and the complemented *htrA* mutant (*p<0.000001*) at the same time point. **(B)** The quantitative Western blot analysis of Spo0A in wild type and *htrA* mutant and the complemented *htrA*mutant in time. **(C)** Normalized sporulation efficiencies (heat resistant CFU compared to wild type) of the different strains at 24h post inoculation. The asterisk (*) indicates a significant difference between wild type and *htrA* mutant (*P=0.0006*). The error bars indicate the standard error of the mean of three experiments. Wild type is WKS1241, *htrA* mutant is DB0002, complemented *htrA* mutant is DB0047 and the *spo0A* mutant is WKS1237.

We also determined the effect of the *htrA* mutant in a cytotoxicity assay on Vero cells, which detects the cumulative effects of both Toxin A and B (28,40) (Figure 5C). We observed a 5-fold increase in toxin end point titer for the *htr*A mutant (p=0.001) compared to the wild type (Figure 5C). Complementing the *htrA* mutant with an inducible copy of *htrA* on a plasmid (DB0047) restored the toxin end point titer to near-wild type levels. To determine these effects as toxin specific, Vero cells were exposed to filter sterilized supernatant of strain WKS1710 (630 ⊿*tcd*A⊿*tcdB*) (40), which as expected did not result in cytotoxic effect. Moreover, pre-incubation of the filter sterilized bacterial supernatants with an anti-toxin (Techlab, Blacksburg, VA, USA) resulted in complete neutralization of the observed cytotoxic effects on the Vero cell monolayers (data not shown). Thus, the effects of the supernatants on Vero cells were Toxin A and/or Toxin B specific.

In summary, we found increased *tcdA* transcription levels, increased TcdA expression levels and elevated toxin end point titers of supernatants derived from *htrA* mutant cells compared to wild type. Taken together, these data contribute to an explanation for the enhanced virulence of the *htrA* mutant strain *in vivo*.

The *htrA* mutant displays decreased sporulation compared to wild type

Our transcriptome analysis revealed pleiotropic effects that included a decreased transcription of *spo0A*, the master regulator of sporulation, in the *htrA* null mutant compared to wild type (58,59). Sporulation is highly relevant for transmission, persistence and biofilm formation of *C. difficile* (61,62). To validate the microarray results, we performed RT-qPCR, quantitative Western blot analysis of Spo0A and a sporulation assay.

We used a RT-qPCR to determine the normalized *spo0A* transcript levels in wild type, *htrA* mutant and the complemented *htrA* mutant strains in the logarithmic and stationary growth phase. The *spo0A* transcript level in the *htrA* mutant was significantly decreased in the logarithmic phase (4.3-fold, p<0.000001) and stationary phase (4.1-fold, p<0.000001) compared to wild type (Figure 6A). The complemented *htrA* mutant failed to reach wild type levels of *spo0A* transcript in the log phase (5.2 fold difference, p<0.000001), but in the stationary phase the complemented strain reached wild type levels of *spo0A* transcript (Figure 6A). Therefore, we believe that the reduced *spo0A* transcript level in the *htrA* mutant is partially, if not completely, due to the *htrA* mutation.

Next, we quantified the decreased Spo0A levels by quantitative Western blot analysis (31). In wild type cells the Spo0A levels increased during growth and signals were absent from the *spo0A* mutant strain as previously reported (Figure 6B) (31). In the *htrA* mutant, Spo0A levels were on average 3-fold lower compared to wild type (Figure 6B). Complementation of the *htrA* mutant restored Spo0A protein levels throughout the growth to near-wild type levels (Figure 6B).

We also investigated the sporulation efficiency of the various strains by determining heat resistant colony forming units (CFU). The efficiency of heat treatment on spore recovery was initially tested using cultures of mutant (32). No colonies could be recovered using this strain, indicating that this is a good way to measure actual spore formation. At 24 hour post inoculation we observed (12-fold) decreased sporulation efficiency for the *htrA* mutant compared to wild type (significant, p=0.0006) (Figure 6C). Expressing *htrA* from a plasmid in the *htrA* mutant strain (DB0047) partly restored sporulation efficiency to wild type levels (Figure 6C). In contrast, sporulation efficiency at 48 hour post inoculation appeared comparable in all strains tested, including the *htrA* mutant (data not shown).

In summary, we show that the *htrA* mutant has a decreased *spo0A* transcript level, a delayed expression of Spo0A protein and a significant reduction in the number of spores formed at 24h post inoculation.

Decreased adherence of *htrA* mutant to Caco2 cell monolayer compared to wild type

In addition to spores, it has been suggested that surface proteins on vegetative cells are important factors for adherence of *C. difficile* to the colonic epithelium and colonization of the intestine (16,19,63). Prompted by the down-regulation of several important cell surface proteins in our transcriptome analyses, we determined the capability of the *htrA* mutant strains to adhere to colon epithelial cells.

We incubated the various strains on a monolayer of Caco-2 cells and determined the fraction of bacterial cells that remained attached after rigorous washing. We observed only 10% adherence of the *htrA* mutant to the Caco-2 cell monolayer (p=0.000001) compared to wild type (Figure 78). Inducing DB0047 resulted in a

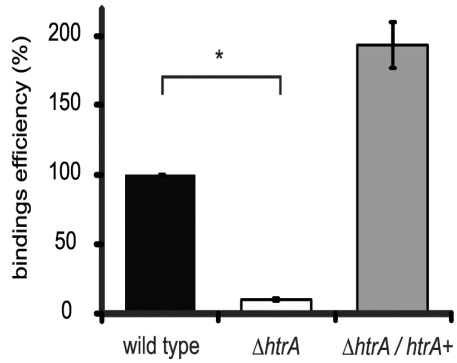

Figure 7: Decreased adhesion of *htrA* null mutant to Caco2 cells. The asterisk (*) indicates a significant difference between wild type and *htrA* mutant (*p=0.000001*). The error bars indicate the standard error of the mean of three experiments. Wild type is WKS1241, Δ*htrA* mutant is DB0002, Δ*htrA*/*htrA*+ is the complemented mutant (DB0047).

slightly increased, but not significant (p=0.17), capability to adhere to a monolayer of Caco-2 cells compared to wild type levels, indicating that the adherence defect of the *htrA* mutant is specific.

Discussion

Role for HtrA in virulence of *C. difficile*

In addition to the prevalence, the severity and mortality of disease caused by *C. difficile* infections has increased significantly in the last decade (1,6,9). It is unclear whether this is a consequence of more diligent screening or a consequence of acquisition of additional virulence traits by the bacteria. The retractable nature of the organism to genetic manipulation has limited opportunities to investigate the role of many genes in the control of virulence traits including the two large clostridial toxins, the binary toxin and other factors that contribute to the virulence of *C. difficile,* such as those affecting colonization and survival of the bacteria in the host (15,16,18,19). In multiple organisms HtrA-like foldase/proteases play a considerable role in virulence by controlling protein homeostasis (21,22,26,52,64). Here, we find that *C. difficile* encodes a single HtrA-like protease. In contrast to observations in other organisms, we have found that a *C. difficile htrA* mutant shows enhanced virulence in the Golden Syrian hamster model of acute *C. difficile* infection (Figure 4).

The observed increase in virulence is likely to be a consequence of elevated toxin levels. Toxin measurements from the hamsters showed no differences in measurement of toxin production in filtered gut samples, however these samples were taken at the endpoint of infection when toxin levels reflect significant symptoms. *In vitro*, we find increased transcription of *tcdA*, increased levels of TcdA protein in the *htrA* mutant, and increased cytotoxicity of the filtered supernatant derived from *htrA* mutant cells, all of which could be substantially reversed by prolonged induction of a plasmid-located copy of *htrA* (Figure 5). Shorter induction did not result in full complementation, most likely because expression levels or the temporal pattern of expression does not match that of wild type.

Our transcriptome analysis did not reveal a significant change in *tcdB* levels in the *htrA* mutant. This has been observed before, and is probably due to lower transcription levels of *tcdB* compared to *tcdA* in general though our data cannot exclude differential regulation of the two toxin genes (28,65,66).

We did notice changes in transcription of several other virulence-associated genes. We validated a number of phenotypes that could be related to these genes experimentally, including a reduction in sporulation frequency and decrease in

colon epithelial cell adherence. In agreement with the observation for TcdA, these effects could be reversed by complementation (Figures 6 and 7). Whilst the most significant impact of this mutation in the hamster model appears to be the change of toxin expression, this does not reflect the potential role of HtrA more globally on gene expression.

The delay in sporulation, as measured by enumeration of colonies on taurocholate containing plates after heat shock, does not necessarily reflect a block in sporulation, but could also be the result of a defect in germination. However, the complementary observation of decrease in *spo0A* transcript level in the mutant strain would suggest the impact to be on sporulation. In addition, this delay in sporulation could result in extending the time spentin the vegetative state during which more toxin than wild type cells could be produced.

Structure of *C. difficile* HtrA

The HtrA family of proteases can be distinguished from other serine proteases by the presence of at least one PDZ domain (21,23). PDZ domains are involved in the regulation of the activity of the protein through protein-protein interactions or binding of specific substrates (22,50). HtrA proteins in other organisms can either be membrane associated or secreted (49,50). A detailed bioinformatics analysis of *C. difficile* HtrA suggests that the protein is a membrane-associated protein with a single PDZ domain (Figure 1A).

HtrA-like proteases of *E. coli* are among the best-characterized members of the family. DegP and DegQ consist of single protease domains with two associated PDZ domains (22,49). Both proteins are foldase/proteases that have relatively broad substrate specificity. DegS is a protease with a single PDZ domain, that acts specifically as a site-1 protease in a regulatory pathway for the extracytoplasmic stress response (49). Considering the domain structure, one might expect HtrA of *C. difficile* to be a site-1 protease. However, we would argue against this based on three findings; first, our protease assay with a non *C. difficile* related unfolded protein suggests a non-specific proteolytic activity. Second, our transcriptome analyses suggest a pleiotropic effect, rather than a specific pathway to be affected. Third, homology modeling of the predicted structure of *C. difficile* HtrA using I-TASSER and Phyre2 suggests that the overall fold of the catalytic domain and PDZ domain is more similar to the DegQ/P subfamily of HtrA-like proteases (22,24,49,50). Finally, site-1 proteases in Gram-positive bacteria generally belong to the PrsW family (67-69). Indeed, in *C. difficile* PrsW (CD0552) is involved in the activation of the extracytoplasmic function sigma factor CsfT (70).

HtrA and virulence

In the majority of pathogenic bacteria, HtrA is directly or indirectly implicated in virulence. In many bacteria *htrA* null mutants show a complete loss of virulence, or at least a significant level of attenuation (21,22,25,26,52). To our knowledge, our study is the first example of increased virulence for an *htrA* null mutant.

Only a few studies have found little or no effect of *htrA* on virulence in animal models. In *Staphylococcus aureus* COL, the deletion of *htrA* led to minimal changes in surface protein expression and virulence (71). In *Brucella abortis* the *htrA* mutant was not found to be attenuated in a BALB/c mouse model (72). For *Porphyromonas gingivalis* only late effects were observed in a competition assay in mice (73). For *Streptococcus pyogenes* no effects were seen in a murine model of subcutaneous infection (74). In these cases, *htrA* mutant strains did display an increased sensitivity to stresses, or an altered expression of virulence factors. However, this may reflect the choice of model and environmental niche tested.

It should be noted that the effects of HtrA can be strain dependent (26,71), and may be masked by additional *htrA* like genes that can compensate in site specific deletion mutants (75). In the case of *C. difficile*, we have not identified any additional HtrA-like genes (data not shown), but we cannot exclude the possibility that other (serine) protease(s) might be able to substitute for certain functions of HtrA. The contribution of the sporulation and adhesion phenotypes of the *htrA* mutant of *C. difficile* to virulence therefore awaits validation in a model other than the hamster, in which toxin production is so dominant that observation of changes to other traits can be limited. For example, analysis of this mutant in mice, which can be colonized but are less susceptible to toxin production, may be a good alternative to study the effects of *htrA* on colonization, transmission and persistence (15,35).

Mode of action of HtrA

In most pathogens, attenuation as a consequence of mutation in *htrA* appears to be the result of a reduced capacity of the cells to deal with environmental or host-dependent stresses (e.g. heat or oxidative stress), a reduced level of virulence factors that depend on HtrA for processing or activation, and/or a reduced invasiveness of the bacterium (21,24,50). In some cases, the effects of HtrA have not been traced to a specific protein or pathway (76,77).

Our study revealed broad effects of the mutation of *htrA* on multiple pathways. Interestingly, several of these pathways have been linked to each other in previous work. For instance, it was noted that the asporogenic mutant of *spo0A*, showed elevated levels of toxin production in PCR ribotype 027, but not in 630Δ*erm* (61,78). Similarly, there is a clear link between motility and toxin production as the alternative sigma factor SigD is required for both (79). Interestingly, mutants

in the flagellar operon showed changes in both toxin levels (80) and their capacity to adhere to Caco-2 cells (60). Finally, more general effects of stress (nutrient depletion, temperature) on toxin levels were noted (81,82).

Our study shows that *htrA* of *C. difficile* can modulate toxin levels, the formation of spores and the adhesion to colonic cells, all of which are highly relevant for virulence. However, the high connectivity of the networks regulating these processes does not allow us to draw any conclusions as to the direct targets of HtrA in *C. difficile*. Future research will be aimed at identifying which proteins are direct targets of the foldase/protease activity of *C. difficile* HtrA.

Acknowledgments

Wiep Klaas Smits was supported by Veni grant 016.116.043 from NWO-ZonMW and an LUMC Gisela Thier fellowship. Anthony M. Buckley is funded by a Wellcome Trust grant 086418. We thank N. Minton and S. Kuehne for providing the ClosTron system and the toxin A/B mutant. We thank R. Fagan and N. Fairweather for providing the pRF185 plasmid.

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