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The HtrA-Like Protease CD3284 Modulates Virulence of *Clostridium difficile*

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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 toxin 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 *htrA* mutant of *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. Microarray data analysis showed a pleiotropic effect of *htrA* on the transcriptome of *C. difficile*, including upregulation 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*.

The bacterial pathogen *Clostridium difficile* is the leading cause of infectious nosocomial diarrhea (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 have 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 ribotypes 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 by 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, adaptations 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 nonfunctional 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 transmembrane domain or a signal peptide (22–24). PDZ domains 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; here HtrA). We show that an *htrA* mutant has enhanced virulence in the Golden Syrian hamster model of acute *C. difficile* infection. These data contrast with those reported for other pathogens, in which mutation of *htrA* results in attenuation (25–27). Furthermore, the *htrA* mutation displayed a pleiotropic effect in a transcriptome analysis. Several differentially expressed genes were validated and are discussed in the context of *C. difficile* virulence.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *C. difficile* and *Escherichia coli* strains and plasmids used in this study are listed in Table 1. *E. coli* strains were grown in Luria-Bertani medium (LB) (USB Corporation) supplemented with appropriate antibiotics when required. *C. difficile*

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TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Description	Reference or 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*, *cd3284*::*[ClosTron, ermB]*	This study
DB0052	630Δ*erm*, *cd3284*::*[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*::*[lsqb]ClosTron, ermB] tcdB*::*[ClosTron, catP]*	40
Plasmids		
pMTL007	*catP*, ClosTron group II 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

strains were grown anaerobically in a microaerobic cabinet (Don Whitley DG 250) at 37°C in prereduced TTY medium (30 g/liter Bacto tryptone [BD], 20 g/liter yeast extract [Difco], and 0.1% thioglycolate, pH 7.4) or brain heart infusion broth (Oxoid) supplemented with 5 g/liter yeast extract and 0.01% L-cysteine (Sigma) (BHIS) (28). Logarithmic-growth-phase bacteria (optical density at 600 nm [OD_{600}], 0.4 ± 0.08) from overnight precultures were used to inoculate prereduced TTY broth at a starting OD_{600} of 0.05 (±0.01). Optical density readings were taken hourly until the stationary growth phase (8 h postinoculation) and at 24 and 48 h postinoculation. Cultures of *C. difficile* strains harboring an expression plasmid were induced with anhydrotetracycline (100 ng/ml) at 1 h postinoculation. We routinely monitored the purity of cultures by performing control PCRs to confirm the 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 (aa) into pET-16B, resulting in a fusion protein with an N-terminal 10×His tag but lacking the predicted transmembrane helix. To generate this clone, the CD3284 open reading frame was amplified using primers CD3284F2 and CD3284R2, *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 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) was added and left for 3 h. Thereafter, cells were harvested by centrifugation (10 min, 10,000 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 (20 mM, 50 mM, 100 mM, 250 mM, and 500 mM, respectively). After the addition of glycerol to a final concentration of 8%, protein was quantified by measuring the absorption at 280 nm on a NanoDrop ND-1000 machine (Thermo Scientific), using an extinction coefficient (ε) of 14,440 M^{-1} cm^{-1} and a molecular mass 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, oligonucleotide-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.

Protease assay. Protease activity of the purified proteins was assayed as follows. In a 100-μl reaction mixture, 2.5 μM His10-Δ(1-30)-HtrA or His10-Δ(1-30)-HtrA-S217A was incubated with 20 μM casein or beta-casein (molecular mass, ~24 kDa) in 1× HNE buffer (150 mM NaCl, 0.1 mM EDTA, 5 mM HEPES, pH 7.4), with the pH adjusted to pH 5.5 by the addition of 0.1× MES/A buffer (0.01 M MES [morpholineethanesulfonic acid], 0.01 M acetic acid, pH 5.0). Reaction mixtures were incubated on ice (0°C) or at 37°C for 18 h with a no-protein control included. After incubation, the reaction was terminated by the addition of 25 μl 5× SDS sample buffer (10% SDS, 10% beta-mercaptoethanol, 50% glycerol, 0.1% bromophenol blue, 250 mM Tris-HCl [pH 6.8], 50 mM EDTA) and heating to 96°C for 2 min. Twenty-microliter volumes of the samples were loaded on a 12% SDS-polyacrylamide gel and separated by electrophoresis at 80 mA. Gels were stained with Coomassie blue solution (0.1% Coomassie brilliant blue R-250, 20% [vol/vol] methanol, 10% [vol/vol] acetic acid) and destained with 50% methanol–10% (vol/vol) acetic acid.

Generation of an *htrA* (*cd3284*) mutant strain. An *htrA* mutant was generated by insertional inactivation of the *cd3284* gene in 630Δ*erm* (here referred to as wild type) using ClosTron technology (32, 33). Briefly, the Perutka algorithm on the ClosTron website (http://www.clostron.com) was used to design primers (see Table S1 in the supplemental material) for retargeting the group II intron (Targetron; Sigma). 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 into *E. coli* CA434 and transferred via conjugation to *C. difficile* (29, 34). The selection of *C. difficile* transconjugants was achieved by subculturing on prereduced BHIS agar supplemented with thiamphenicol (Sigma; 10 μg/ml) and *C. difficile* selective supplement (Oxoid). After this, several rounds of subculturing on prereduced BHIS agar supplemented with lincomycin (Sigma; 20 μg/ml) and *C. difficile* selective supplement were used to promote integration of the group II 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 in Table S1 in the supplemental material. In addition, Southern blot analysis with an *ermB* probe (see Fig. S1 in the supplemental material) was performed to verify a specific single integration into the genome as described previously (28).

Generation of strains carrying a plasmid-based inducible *htrA* gene. 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* mutant, resulting in an *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 with the Animals (Scientific Procedures) Act of 1986 approved by the Home Office, United Kingdom (project license number PPL60/4218). Female Golden Syrian hamsters that weighed approximately 100 g (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 5 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 the core body temperature dropped below 35°C (28).

Statistical analyses were performed using 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 of ≤0.05 were considered significant.

MIC determination. The MICs of the *C. difficile* wild type and *htrA* mutant to erythromycin and clindamycin were determined by the doubling-dilution method described previously (85). Briefly, rows of preconditioned BHI broth (90 μl) were supplemented with a concentration range of 1,024 to 0.06 μg/ml of either antibiotic. Wells were inoculated with ~5×10^3 spores/100 μl and incubated at 37°C for 48 h 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 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 milliliters of the *C. difficile* cultures was diluted with ice-cold methanol (1:1) and stored overnight at −80°C. Bacterial pellets, obtained by centrifugation (20 min, 3,000 × *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 h at 37°C. RNA was isolated using Tri-pure reagent (Roche) as previously described (28). The RNA was treated twice with Turbo DNase (Ambion) according to the manufacturer's instructions, followed by another Tri-pure RNA isolation. The Agilent Bioanalyzer (Agilent Technologies Netherlands BV) and an 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 purification was with the NucleoSpin Gel and PCR cleanup kit (Macherey-Nagel). The synthesized cDNA was labeled with DyLight 550 and DyLight 650 Amine/Reactive Dyes (Pierce Biotech USA) and purified again with the NucleoSpin Gel and PCR cleanup kit. The Agilent DNA microarrays (G2509F custom microarray GE 8x15K 60mer) were designed based on the available genome sequence of *C. difficile* 630 (36, 37). The custom-made microarray slides consist of 8 subarrays each with 15,208 60-mer probes (37).

The Agilent microarray slides were competitively hybridized with 300 ng labeled cDNA made from RNAs isolated from both the wild type and *htrA* mutant (DB0002) during logarithmic-phase (OD_{600}, 0.5 ± 0.1) and stationary-phase (12 h postinoculation) growth. The microarray slides were scanned with a GenePix scanner, and the fluorescence intensities were quantified using GenePix 6.1. The raw data files were analyzed with a LimmaR package via the Genome2D pipeline (http://genome2d.molgenrug.nl) (38) using the following settings: (i) the microarray data were normalized using LOESS normalization, followed by quantile normalization between the slides, and (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.

RT-qPCR. 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 h at 37°C and stored at −20°C. The software program Molecular Beacon (Premier Biosoft) was used to design primer pairs (see Table S1 in the supplemental material) for the quantitative PCRs (qPCRs), 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-qPCRs were then performed using a CFX96 real-time PCR detection system (Bio-Rad). 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 mixture for the *spo0A* gene contained 25 μl HotStar master mix (Qiagen), forward (oDB0117) and reverse primers (oDB0118) (80 nM each primer), 2.5 mM $MgCl_2$, 0.06% SYBR green (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 at 95°C for 30 s, 52°C for 30 s, and 72°C for 30 s.

To determine the specificity of the fluorescence, the fluorescence data were converted into melt curve peaks. For each biological 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 blotting for TcdA and Spo0A were performed as follows. For TcdA, filtered (0.45-μm filter) bacterial supernatants from 48-h 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}, 0.5 ± 0.1) and at 8, 12 and 24 h postinoculation were collected by centrifugation (10 min, 11,000 × *g*, 4°C). Pellets were resuspended in phosphate-buffered saline (PBS) containing protease inhibitor cocktail (Sigma) and lysed with a TissueLyser LT bead beater (50 Hz, 10 min) (Qiagen) using acid-wash glass beads (≤106 μm; Sigma). Equal amounts (OD_{600} corrected) of the lysed bacterial pellets were separated on 15% SDS-polyacrylamide 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 antibiotin antibody labeled with Cy3. A Typhoon 9410 scanner (GE Healthcare) was used to measure the fluorescence intensity. *Clostridium difficile* strains WKS1237 (*spo0A* mutant) and WKS1710 (*tcdA tcdB* mutant) were used as control strains for SpoA and TcdA production, respectively (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 filter) 24- and 48-h-postinoculation supernatants, using a Vero cell-based cytotoxicity assay (28). The endpoint titer was defined as the first dilution at which the Vero cell morphology was indistinguishable from that for the neutralized 200-fold diluted supernatant and untreated cells (28). *Clostridium difficile* strain WKS1710 (*tcdA tcdB* 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 at 24 and 48 h 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 h before 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 nontreated 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-well plate at a density of 1×10^5 cells per well and incubated overnight at 37°C with 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, 4,000 × *g*, 4°C) and washed once with prereduced PBS. To each well of Caco-2 cells, approximately 1×10^8 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 h of incubation under anaerobic conditions, the Caco-2 cell monolayers with the adhered bacteria were washed 5 times with prereduced PBS. Adhered bacteria were released using100 μl 1× trypsin solution (PAA Laboratories, Coelbe, Germany), serially diluted and plated on prereduced BHIS plates. After 48 h, CFU 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.

Microarray data accession number. The complete microarray data set, including experimental raw data and analyzed data, has been submitted to the GEO database (accession number GSE55926).

RESULTS

Bioinformatics analysis of CD3284. In this study, we identified 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 web server (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml), the transmembrane prediction webserver TMHMM2.0 (http://www.cbs.dtu.dk/services/TMHMM/) and the signal peptide cleavage site web server SignalP4.1 (http://www.cbs.dtu.dk/services/SignalP/). These analyses revealed the presence of a transmembrane domain (amino acids [aa] 7 to 29), a trypsin-like serine protease domain (aa 92 to 231; E value, 3.96×10^{-29}), and a PDZ domain (aa 269 to 357; E value, 2.17×10^{-19}) (Fig. 1A). The N terminus of CD3284 was predicted by TMHMM to be highly hydrophobic, most likely transmembrane, whereas SignalP analysis revealed no signal sequence. This suggests 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 nonredundant protein sequence database using BLASTP resulted in identical protein sequence hits (query coverage, 100%; E value, 0.0) in other epidemic (e.g., PCR ribotype 027) and nonepidemic (e.g., PCR ribotype 001) *C. difficile* strains.

As protein three-dimensional (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% 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 (Fig. 1A). Homologs of DegQ and DegS are generally referred to as HtrA-like proteases in Gram-positive prokaryotes. Considering the above, we will refer here to the CD3284-encoding gene as *htrA* and to the CD3284 protein as HtrA.

***C. difficile* HtrA shows protease activity toward 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 (Fig. 1B). After 18 h of incubation at 37°C, casein was degraded only by wild-type HtrA and not by the catalytic HtrA mutant. This effect was temperature dependent, as no degradation was seen when the proteins were incubated at 0°C (Fig. 1B). 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 of 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 using ClosTron technology (Fig. 1A). Insertional inactivation of genes using the ClosTron technology results in stable mutants and nonfunctional proteins (32, 40). The genotype of the insertional inactivation was confirmed by PCR (Fig. 2), sequence analysis, and Southern blotting (see Fig. S1 in the supplemental material), 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 postinoculation both the mutant and wild-type strains showed logarithmic growth for the first 8 h, and by 12 h postinoculation both strains had entered the stationary growth phase (see Fig. S2 in the supplemental material). Though growth kinetics were not significantly different from those of 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 (see Fig. S2 in the supplemental material).

The *htrA* 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-pretreated hamsters were orally infected with either the wild type or the *htrA* mutant. The mean time to death for hamsters challenged with the wild type was 53 h 39 min (±5 h 3 min; $n = 6$). Hamsters infected with the *htrA* mutant revealed a significantly decreased survival time (31 h 20 min ± 44 min; $n = 6$; $P = 0.0005$) compared to that with the wild type (Fig. 3), 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 technology

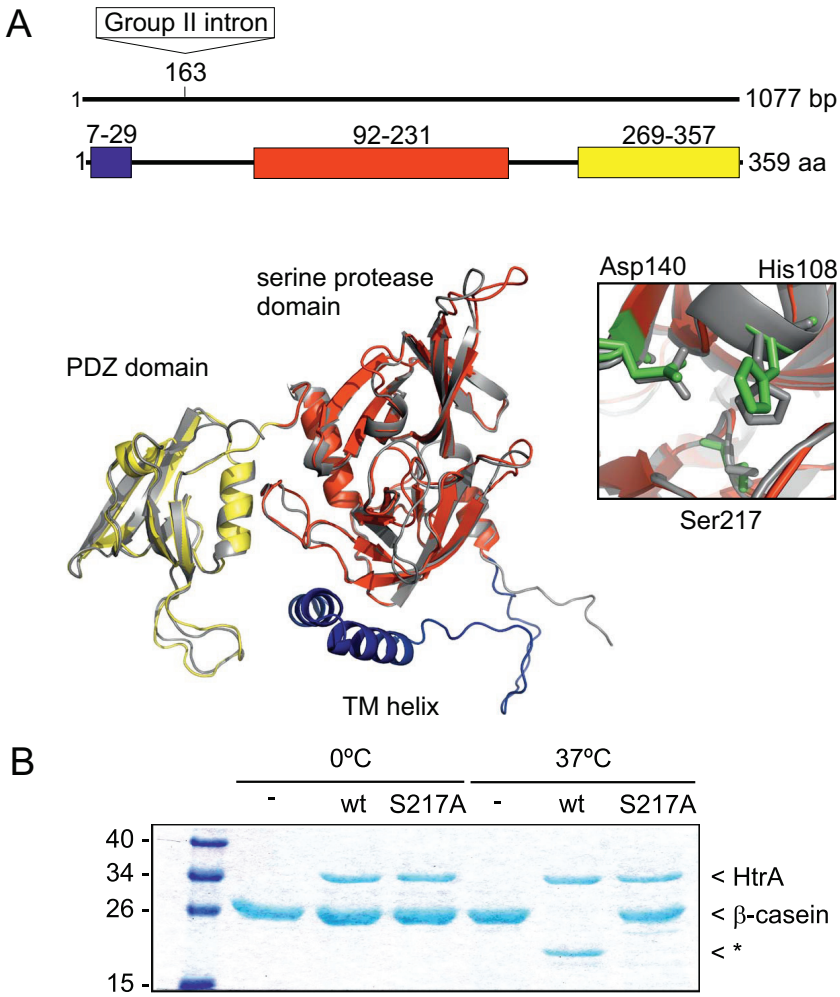

FIG 1 HtrA is a protease. (A) Schematic representation of the predicted structural domains of HtrA. The box on the top line indicates the position (nucleotide 163) at which the group II intron has inserted to create the *htrA* mutant (not to scale). The predicted N-terminal transmembrane helix is shown 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 gray. (B) HtrA shows protease activity toward β-casein. A 2.5 μM concentration of the indicated protein was incubated for 18 h with 20 μM beta-casein at 0°C or 37°C. wt, His10-Δ(1–30)-HtrA; S217A, catalytic mutant His10-Δ(1–30)-HtrA-S217A. The asterisk indicates a proteolytic product of β-casein.

have a shorter survival time than the wild type (unpublished data). We hypothesize that this 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 compare the *htrA* mutant to the original parental 630 isolate (56) (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* mutant of *Clostridium difficile* demonstrates an enhanced virulence phenotype.

Comparative transcriptional microarray 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 analyses of the wild type and the *htrA* mutant. The *htrA* mutant displayed a pleiotropic effect in the transcriptome analyses (see Table S2 in the supplemental material). 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 the wild-type strain were studied at two different time points, the logarithmic and stationary growth phases. The DNA microarray 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) (57). In the logarithmic growth phase and in the stationary growth phase, 551 genes and 567 genes were significantly differentially expressed, respectively (fold change of ≥1.5; $P \leq 0.05$) (see Table S2 in the supplemental material). In total, 248 genes in the logarithmic growth phase and 263 genes in the stationary growth phase were upregulated. Two hundred eight genes were upregulated in both logarithmic and stationary growth

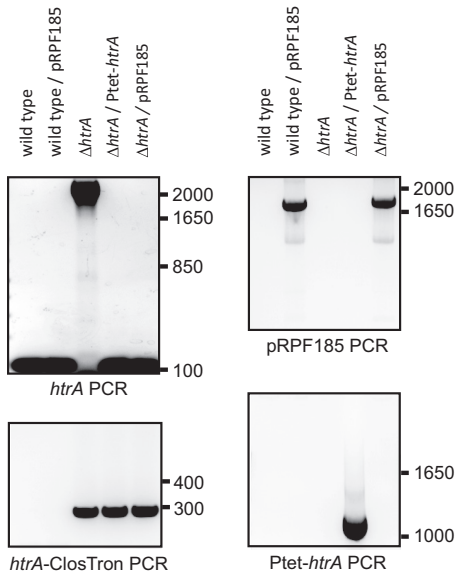

FIG 2 PCR screening of the strains used in this study. For *htrA* PCR, the primers oDB0123 and oDB0124 generated a PCR product of approximately 2,200 bp in the *htrA* mutant (Δ*htrA*, DB0002) and a PCR product of 150 bp in the wild type (WKS1241), wild type/pRPF185 (DB0051), complemented *htrA* mutant (Δ*htrA*/*htrA*+, DB0047), and Δ*htrA*/pRPF185 (DB0052). For pRPF185 PCR, the primers oWKS-1177 and NF794 generated a PCR product of approximately 1,800 bp in the DB0051 and DB0052 strains. For *htrA*-ClosTron PCR, the primers oDB0123 and EBSuni generated a PCR product of approximately 280 bp in the DB0002, DB0047, and DB0052 strains. For Ptet-*htrA* PCR, the primers oDB0123 and NF794 generated a PCR product of approximately 1,200 bp in DB0047.

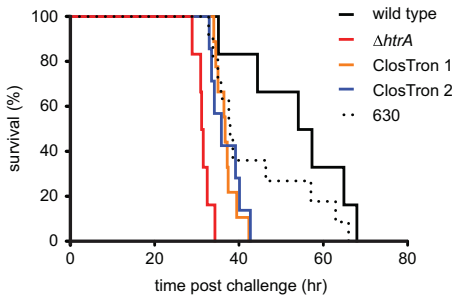

FIG 3 The *htrA* mutant has increased virulence in a hamster model of infection. Survival curves of hamsters challenged with either 10^4 spores of *C. difficile* 630Δ*erm* (solid black line), the *htrA* mutant (red line), ClosTron mutants 1 and 2 (blue and green lines, respectively), or the erythromycin-resistant 630 strain (dashed black line) are shown. Hamsters challenged with the *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; $n = 9$; $P = 0.049$), ClosTron mutant 2 (36 h 47 min; $n = 7$; $P < 0.0001$), or 630 (43 h 43 min; $n = 11$; $P < 0.0001$). Hamsters were culled when the core body temperature declined below 35°C (clinical endpoint), and data were analyzed with Mantel-Cox log rank statistical tests.

phases. Among these, several genes were involved in the stress adaptation response, such as the *ctsR* and *hrcA* genes (58). The *tcdA* gene, encoding the well-characterized virulence factor toxin A, was 3.3-fold and 3.0-fold upregulated in the logarithmic and stationary growth phases, respectively.

A total of 303 genes in the logarithmic growth phase and 304 genes in the stationary growth phase were downregulated in the *htrA* mutant. Two hundred sixty-five genes were downregulated in both logarithmic and stationary growth phases. Among these are genes involved in cell metabolism, such as ribosomal proteins, ABC transporters, and phosphotransferase system (PTS) transporters. The *spo0A* gene, encoding the master regulator of sporulation (59, 60), was 2.4-fold and 2.3-fold downregulated in the logarithmic and stationary growth phases, respectively, and many other sporulation genes showed a similar effect (see Table S2 in the supplemental material). Furthermore, several genes encoding surface-associated proteins (such as *slpA* and 5 other paralogs of *slpA*) (16), flagellum-associated genes (18, 61), and *cd1581* (a gene with a potential effect on adhesion and intestinal colonization) (19) were significantly downregulated.

Overall, transcriptional profiling of the *htrA* mutant showed a plethora of differences compared to the 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 the *htrA* mutant compared to the 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 the wild-type, *htrA* mutant, and complemented *htrA* mutant strains in the logarithmic and stationary growth phases.

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 (Fig. 4A). Expressing HtrA from a plasmid in the mutant strain (DB0047) partially restored *tcdA* transcript levels in logarithmic phase (1.4-fold; $P = 0.0022$) toward the wild-type level, while in stationary phase the *tcdA* transcript level was indistinguishable from wild-type levels (Fig. 4A). These data suggest that the 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-h-postinoculation cultures showed a 7-fold increase of TcdA in the *htrA* mutant compared to the wild type (Fig. 4B). Similar to *tcdA* transcription, complementation of the *htrA* mutant resulted in TcdA levels that were partially restored toward the wild-type level (2-fold) (Fig. 4B). The signals detected were specific for TcdA, as they were absent in the supernatant of the control strain WKS1710 (40).

We also determined the effect of the *htrA* mutant in a cytotoxicity assay on Vero cells, which detects the cumulative effects of both toxins A and B (28, 40) (Fig. 4C). We observed a 5-fold increase in toxin endpoint titer for the *htr*A mutant ($P = 0.001$) compared to the wild type (Fig. 4C). Complementing the *htrA* mutant with an inducible copy of *htrA* on a plasmid (DB0047) restored the toxin endpoint titer to near-wild-type levels. To determine these effects as toxin specific, Vero cells were exposed to filter-

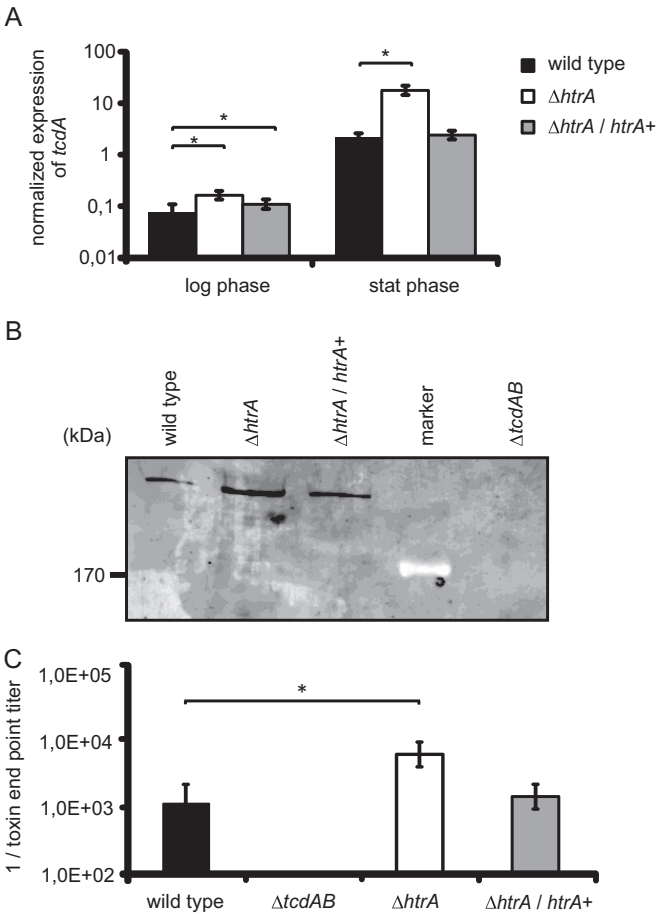

FIG 4 Increased TcdA production by the *htrA* mutant compared to the wild type. (A) Relative *tcdA* gene expression profiles of *C. difficile* strains in logarithmic and stationary phases. The asterisks indicate a significant difference between the wild type and the *htrA* mutant ($P < 0.000001$) and the complemented *htrA* mutant ($P < 0.000001$). (B) Quantitative Western blot analysis of TcdA in culture supernatants of the wild-type, *htrA* mutant, and complemented *htrA* mutant strains at 48 h postinoculation. (C) Toxin endpoint titers of the various *C. difficile* strains. The asterisk indicates a significant difference between the wild-type and *htrA* mutant strains ($P = 0.001$) at the same time point. The error bars indicate the standard errors of the mean from three experiments. The wild type is WKS1241, the *htrA* mutant is DB0002, the complemented *htrA* mutant is DB0047, and the *tcdAB* mutant is WKS1710.

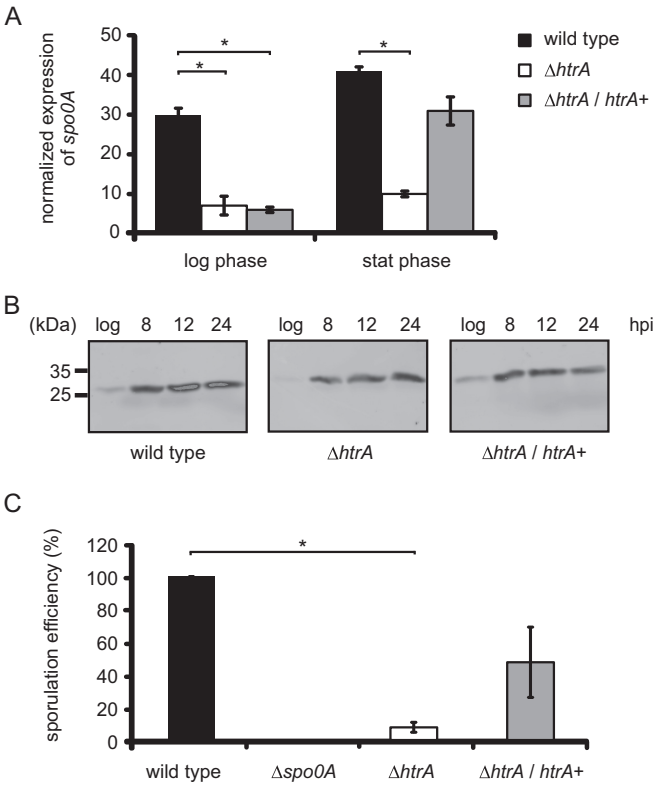

FIG 5 Decreased sporulation of the *htrA* mutant compared to the wild type. (A) Relative *spo0A* gene expression profiles of *C. difficile* strains in logarithmic and stationary phases. The asterisks indicate a significant difference between the wild type and the *htrA* mutant ($P < 0.000001$) and the complemented *htrA* mutant ($P < 0.000001$) at the same time point. (B) Quantitative Western blot analysis of Spo0A in the wild-type, *htrA* mutant, and complemented *htrA* mutant strains over time. (C) Normalized sporulation efficiencies (heat-resistant CFU compared to wild type) of the different strains at 24 h postinoculation. The asterisk indicates a significant difference between the wild type and the *htrA* mutant ($P = 0.0006$). The error bars indicate the standard errors of the mean from three experiments. The wild type is WKS1241, the *htrA* mutant is DB0002, the complemented *htrA* mutant is DB0047, and the *spo0A* mutant is WKS1237.

sterilized supernatant of strain WKS1710, which produces no toxin A and B (40), which as expected did not result in a cytotoxic effect. Moreover, preincubation of the filter-sterilized bacterial supernatants with antitoxin (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 endpoint titers of supernatants derived from *htrA* mutant cells compared to the 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 that of the wild type. Our transcriptome analysis revealed pleiotropic effects that included a decreased transcription of *spo0A*, the master regulator of sporulation, in the *htrA* mutant compared to the wild type (59, 60). Sporulation is highly relevant for transmission, persistence, and biofilm formation of *C. difficile* (62, 63). To validate the microarray results, we performed RT-qPCR, quantitative Western blot analysis of Spo0A, and a sporulation assay.

We used RT-qPCR to determine the normalized *spo0A* transcript levels in the wild-type, *htrA* mutant, and complemented *htrA* mutant strains in the logarithmic and stationary growth phases. 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 that of the wild type (Fig. 5A). 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. (Fig. 5A). 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*

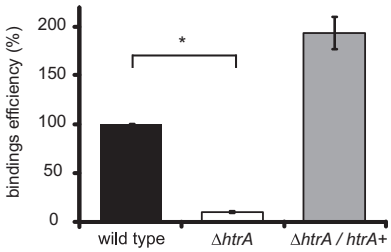

FIG 6 Decreased adhesion of the *htrA* mutant to Caco2 cells. The asterisk indicates a significant difference between the wild type and the *htrA* mutant ($P = 0.000001$). The error bars indicate the standard errors of the mean from three experiments. The wild type is WKS1241, the *htrA* mutant is DB0002, and the complemented *htrA* mutant is DB0047.

mutant strain, as previously reported (Fig. 5B) (31). In the *htrA* mutant, Spo0A levels were on average 3-fold lower than those in the wild type (Fig. 5B). Complementation of the *htrA* mutant restored Spo0A protein levels throughout the growth to near-wild-type levels (Fig. 5B).

We also investigated the sporulation efficiencies of the various strains by determining heat-resistant CFU. The efficiency of heat treatment on spore recovery was initially tested using cultures of the WKS1237 mutant (32). No colonies could be recovered using this strain, indicating that this is a good way to measure actual spore formation. At 24 h postinoculation, we observed (12-fold) decreased sporulation efficiency for the *htrA* mutant compared to the wild type (significant, $P = 0.0006$) (Fig. 5C). Expressing *htrA* from a plasmid in the *htrA* mutant strain (DB0047) partly restored sporulation efficiency toward wild-type levels (Fig. 5C). In contrast, sporulation efficiency at 48 h postinoculation appeared to be comparable in all strains tested, including the *htrA* mutant (data not shown).

In summary, we showed 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 24 h postinoculation.

Decreased adherence of the *htrA* mutant to a Caco-2 cell monolayer compared to the 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, 64). Prompted by the downregulation 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 the wild type (Fig. 6). Inducing DB0047 resulted in a slightly, but not significantly ($P = 0.17$), increased 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 have 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 refractory nature of the organism to genetic manipulation has limited opportunities to investigate the roles 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, 65). Here, we found 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 (Fig. 3).

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* (Fig. 4). Shorter induction did not result in full complementation, most likely because expression levels or the temporal pattern of expression does not match that of the 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* than of *tcdA* in general, though our data cannot exclude differential regulation of the two toxin genes (28, 66, 67).

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 a decrease in colon epithelial cell adherence. In agreement with the observation for TcdA, these effects could be reversed by complementation (Fig. 5 and 6). While 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 in 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 a decrease in the *spo0A* transcript level and a delay in the Spo0A protein level in the mutant strain would suggest that sporulation is most likely affected. Interestingly, the delay in sporulation results in a prolonged vegetative state during which the bacteria can produce more toxin than wild-type cells.

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 be either 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 (Fig. 1A). Consistent with this, we did not identify HtrA in the exoproteome of strain 630Δ*erm* (68).

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 four findings. First, our protease assay with a non-*C. difficile* unfolded protein suggests a nonspecific proteolytic activity. Second, our transcriptome analyses (see Table S2 in the supplemental material) 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 that of 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 (69–71). Indeed, in *C. difficile*, PrsW (CD0552) is involved in the activation of the extracytoplasmic function sigma factor CsfT (72).

HtrA and virulence. In the majority of pathogenic bacteria, HtrA is directly or indirectly implicated in virulence. In many bacteria, *htrA* 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* 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 (73). In *Brucella abortus*, the *htrA* mutant was not found to be attenuated in a BALB/c mouse model (74). For *Porphyromonas gingivalis*, only late effects were observed in a competition assay in mice (75). For *Streptococcus pyogenes*, no effects were seen in a murine model of subcutaneous infection (76). 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 the environmental niche tested.

It should be noted that the effects of HtrA can be strain dependent (26, 73) and may be masked by additional *htrA*-like genes that can compensate in site-specific deletion mutants (77). In the case of *C. difficile*, we have not identified any additional HtrA-like protease genes (data not shown), but we cannot exclude the possibility that another (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 (78, 79).

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 *spo0A* mutant showed elevated levels of toxin production in PCR ribotype 027 but not in 630Δ*erm* (62, 80). Similarly, there is a clear link between motility and toxin production, as the alternative sigma factor SigD is required for both (81). Interestingly, flagellar operon mutants showed changes in both toxin levels (82) and their capacity to adhere to Caco-2 cells (61). Finally, more general effects of stress (nutrient depletion or temperature) on toxin levels were noted (83, 84).

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.

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