

# The HtrA Protease of *Campylobacter jejuni* Is Required for Heat and Oxygen Tolerance and for Optimal Interaction with Human Epithelial Cells

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***Campylobacter jejuni* is a predominant cause of food-borne bacterial gastroenteritis in the developed world. We have investigated the importance of a homologue of the periplasmic HtrA protease in *C. jejuni* stress tolerance. A *C. jejuni* *htrA* mutant was constructed and compared to the parental strain, and we found that growth of the mutant was severely impaired both at 44°C and in the presence of the tRNA analogue puromycin. Under both conditions, the level of misfolded protein is known to increase, and we propose that the heat-sensitive phenotype of the *htrA* mutant is caused by an accumulation of misfolded protein in the periplasm. Interestingly, we observed that the level of the molecular chaperones DnaK and ClpB was increased in the *htrA* mutant, suggesting that accumulation of nonnative proteins in the periplasm induces the expression of cytoplasmic chaperones. While lack of HtrA reduces the oxygen tolerance of *C. jejuni*, the *htrA* mutant was not sensitive to compounds that increase the formation of oxygen radicals, such as paraquat, cumene hydroperoxide, and H<sub>2</sub>O<sub>2</sub>. Using tissue cultures of human epithelial cells (INT407), we found that the *htrA* mutant adhered to and invaded human epithelial cells with a decreased frequency compared to the wild-type strain. This defect may be a consequence of the observed altered morphology of the *htrA* mutant. Thus, our results suggest that in *C. jejuni*, HtrA is important for growth during stressful conditions and has an impact on virulence.**

*Campylobacter jejuni* is a gram-negative human pathogen and is recognized as a major cause of bacterial food-borne infections worldwide. The symptoms of campylobacteriosis are malaise, fever, severe abdominal pain, and diarrhea. In rare cases, *C. jejuni* triggers the Guillain-Barré syndrome, which is an autoimmune disorder of the peripheral nervous system (32). *C. jejuni* is a common and efficient gut colonizer of many animals, and fecal contamination of poultry meat during slaughtering is considered to be the major source of *Campylobacter* organisms that cause campylobacteriosis in humans. The optimum growth temperature of *C. jejuni* is 42°C, which may reflect adaptation to the core temperature of the avian host. *C. jejuni* does not normally multiply on food products (35) because most strains grow only between 31 and 45°C and require reduced oxygen tension as well as 5 to 10% carbon dioxide for growth. In contrast, *C. jejuni* survives well in food products at low temperature (5, 26), and due to a low infective dose (6), even few surviving *Campylobacter* organisms can present a health risk to humans.

During transmission from the intestinal tract of poultry to the human gut, *C. jejuni* experiences unfavorable conditions which may lead to protein misfolding and loss of protein function. Since misfolded proteins tend to form potentially harmful aggregates, removal of nonnative proteins is required in all

cellular compartments. The HtrA protease was first identified in *Escherichia coli* as being required for growth at 42°C (28) and for degradation of misfolded protein in the periplasm (49). Later it was shown that HtrA degrades heat-denatured proteins in vivo and in vitro (25), and because only a few native proteins have been demonstrated to be in vivo substrates for HtrA proteolytic activity (2, 8, 20), it is suggested that the major biological role of HtrA is the removal of nonnative misfolded protein from the cellular envelope. In *E. coli*, the HtrA protease is located at the periplasmic side of the inner membrane (29, 43) and is derived from a pre-HtrA precursor protein by cleavage of the 26 N-terminal amino acids during export (29). The HtrA protein contains a catalytic triad (His<sub>105</sub>-Asp<sub>135</sub>-Ser<sub>210</sub>) that is required for proteolytic activity (44) and two PDZ domains that are responsible for oligomerization of the protein complex, substrate recognition, and binding (42, 46). In addition to the proteolytic activity, *E. coli* HtrA also possesses chaperone activity in vitro at low temperatures (47), where a conformational change of the protein masks the proteolytic residues (24). At elevated temperatures, the catalytic residues are accessible and the proteolytic activity of HtrA predominates (24, 47).

HtrA homologues are found in most bacteria, and even though the protein is well conserved in evolution, the impact on bacterial physiology differs among the gram-negative bacteria. In contrast to *E. coli*, HtrA is not essential for growth at high temperatures in *Salmonella enterica* serovar Typhimurium (19). Interestingly, the *S. enterica* serovar Typhimurium *htrA* mutant showed a reduced virulence in the murine salmonello-

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sis model (9) and reduced survival in macrophages (19). Phenotypic characterization of the *S. enterica* serovar Typhimurium *htrA* mutant furthermore revealed a decreased tolerance to oxidative stress, which may explain the reduced survival in macrophages, where reactive oxygen intermediates are released during the oxidative burst (19). *htrA* mutants of other gram-negative pathogenic bacteria, such as *Yersinia enterocolitica*, *Klebsiella pneumoniae*, and *Brucella abortus*, are sensitive both to high temperatures and oxidative stress (10, 11, 27, 58). Furthermore, *htrA* mutants of *Y. enterocolitica* and *B. abortus* show reduced virulence in murine models (12, 58). It is unclear why *htrA* mutants of gram-negative bacteria are attenuated in virulence. However, since they are more susceptible to stress than the parental strains, the mutants may also be less viable in host tissues. In addition, it has been speculated that the chaperone and protein processing functions of HtrA are needed for the folding of secreted proteins or that HtrA might be involved in the oligomerization and export of virulence factors (36).

In general, bacteria that are exposed to stressful conditions such as heat shock induce synthesis of proteases and chaperones, which are involved in degradation and refolding of unfolded protein substrates, respectively. Using a whole-genome DNA microarray, it was shown that transcription of some protease genes (*lon*, *clpB*, and *hslU*) and several chaperone genes (*groEL*, *groES*, *grpE*, *dnaK*, and *dnaJ*) of *C. jejuni* NCTC11168 were up-regulated in response to a temperature up-shift from 37°C (human body temperature) to 42°C (temperature of the chicken core) (48). In addition, transcription of the *lon*, *clpB*, and *dnaK* genes as well as the *groELS* operon was found to be induced by heat shock (48°C) (50–53), and recently, we have shown that *clpB*, *dnaK*, and *groELS* are negatively regulated by HspR (1). Experiments suggest that heat shock proteins play a role in vivo, since a *C. jejuni dnaJ* mutant is unable to colonize chickens (22). In contrast, the HtrA protease does not seem to be important in the ability of *C. jejuni* to colonize chickens (7). The aim of the present study was to examine the role of HtrA in *C. jejuni* stress tolerance by analyzing growth and survival of a constructed *htrA* mutant. In addition, the importance of HtrA for interaction between *C. jejuni* and the human host has been investigated.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** *C. jejuni* NCTC11168 (National Collection of Type Cultures) and *C. jejuni* NCTC11168  $\Delta$ *htrA::cat* (LB1281; this study) were routinely grown on blood agar baseII (Oxoid) supplemented with 5% calf blood (base II) or in brain heart infusion (BHI) broth (Difco) in a microaerobic environment provided by CampyGen (Oxoid). *E. coli* DH5 $\alpha$  [ $\phi$ 80*lacZ* $\Delta$ M15  $\Delta$ (*lacZYA-argF*) U169 *recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1*] was grown in Luria-Bertani broth or on Luria-Bertani agar (Difco). When appropriate, media were supplemented with ampicillin (100  $\mu$ g/ml), chloramphenicol (20  $\mu$ g/ml), or kanamycin (50  $\mu$ g/ml).

**Transformation.** *C. jejuni* NCTC11168 was transformed by electroporation essentially as described by Wassenaar and coworkers (57). To produce competent cells, *C. jejuni* NCTC11168 was harvested from overnight-incubated agar plates with 2 ml ice-cold wash buffer (272 mM sucrose, 15% glycerol) and subjected to four repeated centrifugations (4°C, 10,000 rpm, 10 min) and wash steps. After the last centrifugation, the cells were resuspended in 1/10 volume wash buffer, resulting in a concentration of 10<sup>9</sup> CFU/ml. Cell volumes of 50  $\mu$ l were electroporated (1.80 kV, 200  $\Omega$ , 25 F) with 1 to 5  $\mu$ l plasmid DNA. Immediately after electroporation, 1 ml of recovery broth (10% glycerol, Mueller-Hinton broth [2/3], brucella broth [1/3]) was added, and cells were plated on nonselective plates. After incubation overnight at 37°C under microaerobic conditions, the cells were harvested with recovery broth and plated on selective

plates. *E. coli* DH5 $\alpha$  was transformed by electroporation using standard methods.

**Primers used in this study.** The primers used in this study were as follows (PstI restriction enzyme sites are underlined, and bases complementary to Ht2 are indicated in bold): Ht1, 5'-AATTTAATGGTTTCGCCTTG-3'; Ht2Ps, 5'-CTG CAGTGCAGCAAATAAAGCACTGC-3'; Ht3Ps, 5'-GCITTAATTGCTGCA CTGCAGGAATTTACCAAAGTTTGGGT-3'; Ht4, 5'-AGCTTATAACCTAT TCCACG-3'; HtrA-up, 5'-CATCATCTTCTCTGTGTAA-3'; HtrA-down, 5'-TTCACGTATAACTCCTGC-3'; htrA-start, 5'-GCTAACTCCAAGAGTTTC G-3'; htrA-end, 5'-GAATATTTGTCATAGTTTTCC-3'; htrA-intF, 5'-GTAA AGATCCAAAACAGATT-3'; htrA-intR, 5'-ACTAAATCTCCACGCTTAA CG-3'.

**Construction of a *C. jejuni htrA* mutant.** Chromosomal DNA from *C. jejuni* NCTC11168 was used as a template for amplification of DNA fragments containing either the 5' end of the *htrA* gene and upstream sequences or the 3' end of the *htrA* gene and downstream sequences. For the upstream fragment, primers Ht1 and Ht2Ps (731 bp) were used, while primers Ht3Ps and Ht4 (799 bp) were used for the downstream fragment. In a second round of PCR, the *htrA* fragments were joined by using the splicing-by-overlap-extension PCR method (16), creating a PCR fragment containing an in-frame deletion of 1,308 bp in *htrA* and introducing a PstI site between the upstream and downstream fragments. The  $\Delta$ *htrA* PCR fragment was cloned in the TOPO TA cloning vector pCR2.1 (Invitrogen), resulting in plasmid pLB217. Subsequently, pLB225 was constructed by cloning the  $\Delta$ *htrA* PCR fragment from pLB217 into the EcoRI site of pGEM-7Zi(+) (Promega). Finally, the *cat* gene (obtained from pRY109) was cloned into the PstI site of pLB225, resulting in pLB229. The *cat* gene was transcribed in the same direction as the *htrA* gene.

*C. jejuni* NCTC11168 was transformed with pLB229, and several chloramphenicol-resistant colonies were isolated. Chromosomal DNA was isolated from four different chloramphenicol-resistant colonies and used as templates in PCRs. By using primers htrA-up and htrA-down, which anneal to chromosomal sequences upstream and downstream of the region cloned in pLB229, respectively, it was verified that the  $\Delta$ *htrA::cat* mutation was transferred to the chromosome of *C. jejuni* NCTC11168 in all four isolates. Furthermore, PCR using primers htrA-up and htrA-down demonstrated that no wild-type *htrA* allele was present in the genome, showing that a double-crossover event had occurred. In the present report, the *C. jejuni* NCTC11168 *htrA::cat* mutant is named LB1281.

**Complementation of an *E. coli htrA* mutant.** The *htrA* gene of *C. jejuni* NCTC11168, including 231-bp upstream sequences, was amplified in a PCR using chromosomal DNA extracted from *C. jejuni* NCTC11168 as a template and the primers htrA-start and htrA-end. The 1,740-bp fragment was cloned in the TOPO TA cloning vector pCR2.1 (Invitrogen), resulting in plasmid pLB262. *E. coli htrA20* (BL20) and *E. coli* wild-type BL78 (28) were transformed with pLB262 and the parental plasmid pCR2.1. By selecting for kanamycin resistance, colonies that carried the plasmids were isolated. Single colonies of each strain were streaked on three different Luria-Bertani agar plates containing kanamycin, and after incubation overnight at 30, 39, or 42°C, the plates were inspected for growth. The experiment was repeated three times.

**Adherence and invasion assay.** Adherence and invasion assays were performed with INT407 cell monolayers growing in Eagle's minimal essential medium (EMEM) supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. Approximately 10<sup>8</sup> bacterial cells in EMEM were added to a monolayer consisting of 5 × 10<sup>5</sup> epithelial cells (multiplicity of infection, 200) and incubated for 2 h. For determination of adherence, the monolayers were washed three times with 10 mM phosphate-buffered saline (PBS), pH 7.2, and epithelial cells were lysed by adding 0.1% Triton X-100. The adhered bacteria were enumerated by plate count. For determination of invasion, the monolayers were subsequently incubated with EMEM containing gentamicin (250  $\mu$ g/ml) for 2 h at 37°C in 5% CO<sub>2</sub> to kill all extracellular bacteria. The monolayers were washed three times with PBS, epithelial cells were lysed by adding 0.1% Triton X-100, and the internalized bacteria were enumerated by plate count. The values represent the mean counts  $\pm$  standard deviations derived from four wells. One representative experiment of four is shown.

**Motility assay.** *C. jejuni* strains were grown overnight on base II agar plates at 37°C under microaerobic conditions. Bacterial cells were harvested using BHI broth, and the optical density at 600 nm (OD<sub>600</sub>) was adjusted to 0.1. One microliter of the cell suspension was deposited at the center of each heart infusion broth (Difco) plate containing 0.25% agar. After 48 h of microaerophilic incubation at 37°C, the ability of the strain to move in the soft agar was evaluated. Three independent experiments were performed.

**Autoagglutination assay.** The ability to autoagglutinate was investigated essentially as described by Misawa and Blaser (30). Bacterial cells were harvested from base II agar plates with MilliQ water and washed once before the OD<sub>600</sub>

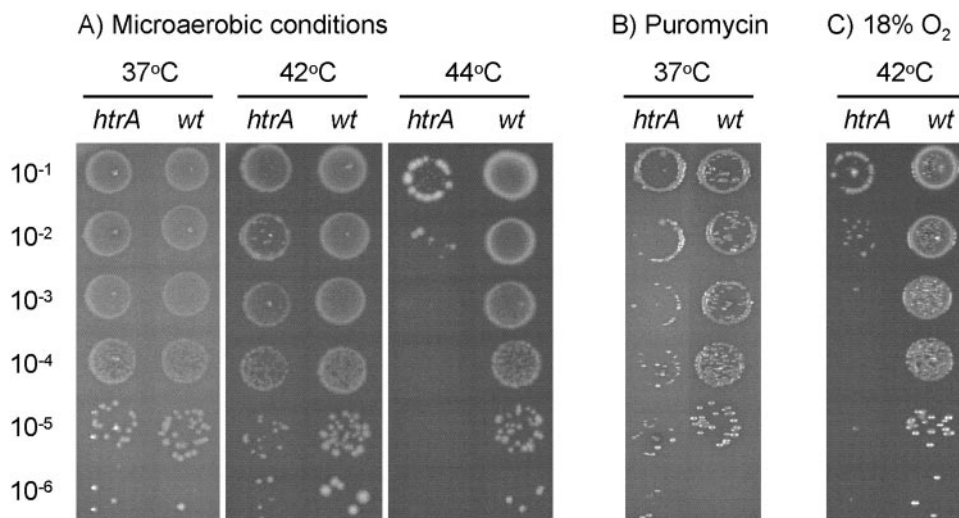


FIG. 1. Effect of temperature, oxygen concentration, and puromycin on growth of the *C. jejuni htrA* mutant on solid surfaces. Serial dilutions ( $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$ ,  $10^{-4}$ ,  $10^{-5}$ , and  $10^{-6}$ ) of *C. jejuni* NCTC11168 (wt) and LB1281 (*htrA*) cultures with an  $OD_{600}$  of 0.1 were spotted in 10- $\mu$ l volumes onto base II agar plates. The plates were incubated at 37, 42, or 44°C under microaerobic conditions (A), at 37°C under microaerobic conditions on a base II agar plate containing 5  $\mu$ g/ml puromycin (B), or at 42°C in a candle jar providing 17 to 18% oxygen (C). One representative of three independent experiments is presented.

was adjusted to 1 in 10 mM PBS, pH 7.2. Cell suspensions of 4 ml were incubated at 25°C for 24 h, and the  $OD_{600}$  was measured by carefully removing 1 ml from the top phase. Bacterial cells which have autoagglutinated fall to the bottom of the tube, and the  $OD_{600}$  diminishes. The percentage decrease in  $OD_{600}$  was calculated, and the values represent the mean counts  $\pm$  standard deviations derived from three independent assays.

**Growth on plates.** *C. jejuni* NCTC11168 and LB1281 were grown overnight on base II agar plates at 37°C under microaerobic conditions. Bacterial cells were harvested using BHI broth, and the  $OD_{600}$  was adjusted to 0.1. Serial dilutions were made, and 10- $\mu$ l volumes of the  $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$ ,  $10^{-4}$ ,  $10^{-5}$ , and  $10^{-6}$  dilutions were spotted onto four base II agar plates, which were incubated under microaerobic conditions using CampyGen (Oxoid) for 3 days at 37, 42, or 44°C or at 42°C in the presence of a lit candle. Alternatively, 10- $\mu$ l volumes of the  $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$ ,  $10^{-4}$ ,  $10^{-5}$ , and  $10^{-6}$  dilutions were spotted onto a base II agar plate containing 5  $\mu$ g/ml puromycin and incubated under microaerobic conditions for 4 days at 37°C. Experiments were repeated at least three times.

**Protein analysis.** *C. jejuni* NCTC11168 and LB1281 were grown in BHI broth at 37°C or 42°C under microaerobic conditions. Cells (50 ml of a culture with an  $OD_{600}$  of 0.3) were harvested, washed with 0.9% NaCl, and resuspended in TM buffer (5 mM  $MgCl_2$ , 10 mM Tris-HCl [pH 7.5]) to the same protein concentration. A NuPage 4 to 12% Bis-Tris gel (Invitrogen) was used to analyze samples of protein extracts, followed by Coomassie staining with Safe Stain (Invitrogen). To detect DnaK immunologically, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Invitrogen) and probed with rabbit polyclonal anti-DnaK (*E. coli*) (Abcam). Bound antibody was detected with the Western-Breeze chemiluminescent anti-rabbit antibody kit (Invitrogen).

**Electron microscopy.** *C. jejuni* NCTC11168 and LB1281 were grown overnight in sterile filtered Mueller-Hinton broth (Oxoid) under microaerobic conditions at 37°C. Bacteria were negatively stained with saturated aqueous uranyl acetate and photographed in a JEOL EX/B transmission microscope at 80 kV.

**Northern blotting.** *C. jejuni* NCTC11168 was grown under microaerobic conditions in BHI broth to an  $OD_{600}$  of approximately 0.3 at 37°C; cells were cooled on ice and harvested by centrifugation for 5 min at 10,000 rpm at 4°C, and the pellet was resuspended in Tri agent (Sigma). Another portion of the cells was exposed to 46°C for 15 min, before the cells were harvested. RNA was isolated as described by Andersen and coworkers (1). Four micrograms of RNA was denatured at 75°C for 10 min and subsequently separated on 1% agarose gels in 10 mM sodium phosphate buffer as described by Pelle and Murphy (39). RNA was blotted onto PVDF membranes, and finally the RNA was fixed to the membrane by exposure to UV light. An internal 536-bp *htrA* fragment (obtained by PCR using primers *htrA-intF* and *htrA-intR*) was used as a probe. The probe was labeled with [ $\alpha$ - $^{32}P$ ]dCTP and Ready-To-Go labeling beads from Amersham

Biosciences, and unincorporated nucleotides were subsequently removed by using ProbeQuant G-50 micro columns. Hybridization was performed overnight at 65°C in hybridization buffer (0.5 M sodium phosphate buffer, pH 7.2, 7% [wt/vol] sodium dodecyl sulfate [SDS]) after 1 h of prehybridization under the same conditions. To remove unspecific binding of the probes, the membranes were washed three times (15, 10, and 10 min) with 20 mM sodium phosphate buffer (pH 7.2)–1% (wt/vol) SDS. Membranes were autoradiographed at  $-80^\circ\text{C}$ .

## RESULTS

**HtrA is expressed from a monocistronic transcript.** The open reading frame *cj1228c* of the *C. jejuni* NCTC11168 genome (accession number NP\_002163) encodes an HtrA homologue. The amino acid sequence of the *C. jejuni* HtrA protein has the highest degree of identity to HtrA of *H. pylori* (49% identity) but also shows homology to HtrA proteins of other bacteria, e.g., *E. coli* (38% identity). By using the program SignalP V1.1 (34), we predicted that the *C. jejuni* HtrA protein possesses a 16-amino-acid leader peptide, suggesting a periplasmic location similar to that of *E. coli* HtrA (29, 43). The *htrA* gene (1,416 bp) of *C. jejuni* NCTC11168 is preceded by a Shine-Dalgarno sequence (AAGGAA) and two putative *C. jejuni* promoters that are located 27 and 82 bp upstream of the translational start site (data not shown). Because the upstream gene (*cbpA*) is divergently transcribed and a putative rho-independent terminator (ATAACGGAAGGATTTTCCTTT CGTTAT;  $\Delta G = -13$  kcal/mol) is located downstream of the *htrA* gene, the *htrA* gene is suggested to be not in an operon with the neighboring genes. This possibility was verified by Northern blot analysis using an internal *htrA* fragment as a probe, as the only hybridization found was to a transcript that corresponded in size to an mRNA containing *htrA* (data not shown).

**HtrA is required for growth under conditions that increase the level of nonnative protein.** With the aim of studying the role of HtrA in stress tolerance and virulence in *C. jejuni*, we



constructed an *htrA* mutant in which the main part of the *htrA* (*cj1228c*) gene was replaced with a selective marker. A total of 436 amino acids, including the catalytic triad (His<sub>120</sub>-Asp<sub>151</sub>-Ser<sub>225</sub>) and the two PDZ domains, were removed (for details, see Materials and Methods). Previous studies of HtrA homologues in various bacterial species have pointed to a role of HtrA in heat tolerance in some organisms but not in others (10, 11, 19, 27, 28, 58). In *C. jejuni* we found that while the *htrA* mutant formed colonies with the same frequency as the wild type did at 37°C and 42°C, the ability of the *htrA* mutant to form colonies at 44°C was greatly reduced (10<sup>4</sup>-fold) (Fig. 1A). In contrast, the wild type formed colonies with the same frequency at all three temperatures (Fig. 1A). To determine if HtrA is required generally for growth under conditions in which the nonnative proteins accumulate, we compared the abilities of the wild type and the *htrA* mutant to form colonies in the presence of puromycin. This antibiotic increases the level of misfolded proteins by competing with charged tRNAs for the ribosomal A-site, thus leading to premature termination of protein synthesis (14). When growth was investigated using plates containing 5 µg/ml puromycin, we found that the *htrA* mutant formed fewer and smaller colonies than the wild type (Fig. 1B). Thus, our results suggest that in *C. jejuni*, HtrA is required for growth under conditions in which misfolded proteins accumulate.

**Complementation of an *E. coli htrA* mutant.** Our results suggest that HtrA of *C. jejuni* may retain the same function as *E. coli* HtrA, since an *E. coli htrA* mutant is also temperature and puromycin sensitive (28). To investigate whether *C. jejuni* HtrA was able to complement an *E. coli htrA* mutant, we supplied a fragment containing the *C. jejuni htrA* gene and the putative promoter on a plasmid (pLB262) and examined growth at 30, 39, and 42°C (Fig. 2). While the *E. coli htrA* mutant containing the vector plasmid pCR2.1 was able to form colonies only at 30°C, we found that the sequences present in pLB262 were able to complement the temperature-sensitive phenotype of the *E. coli htrA* mutant at 39°C but not at 42°C (Fig. 2). The incomplete suppression may be due to an inappropriate expression level of the *C. jejuni htrA* gene or that the HtrA protein of *C. jejuni*, to some extent, is different from *E. coli* HtrA (38% identity). However, the partial suppression of the temperature-sensitive phenotype of the *E. coli htrA* mutant indicates that *htrA* of *C. jejuni* is a functional homologue of the *htrA* gene from *E. coli*.

**The cytoplasmic heat shock response is induced in the absence of HtrA.** During our initial characterization of the *C. jejuni htrA* mutant, we observed that the pattern of proteins extracted from *htrA* mutant cells was different from that of wild-type cells. In particular, protein bands with approximate sizes of 100, 70, and 60 kDa were more intense in cells lacking HtrA than in the wild-type cells (Fig. 3A). Protein sequence analysis revealed that the 100-kDa protein corresponds to the ClpB chaperone, which in *E. coli* is required for the resolution of protein aggregates (31). The 70-kDa band specifically reacted with DnaK antibodies raised against *E. coli* DnaK, thus identifying the 70-kDa band as DnaK (Fig. 3B). DnaK is a chaperone, which in *E. coli* refolds nonnative proteins and, in cooperation with ClpB, disaggregates protein aggregates (3). The predicted molecular sizes of *C. jejuni* ClpB and DnaK are 95.6 and 67.4 kDa, and these sizes correspond well to the

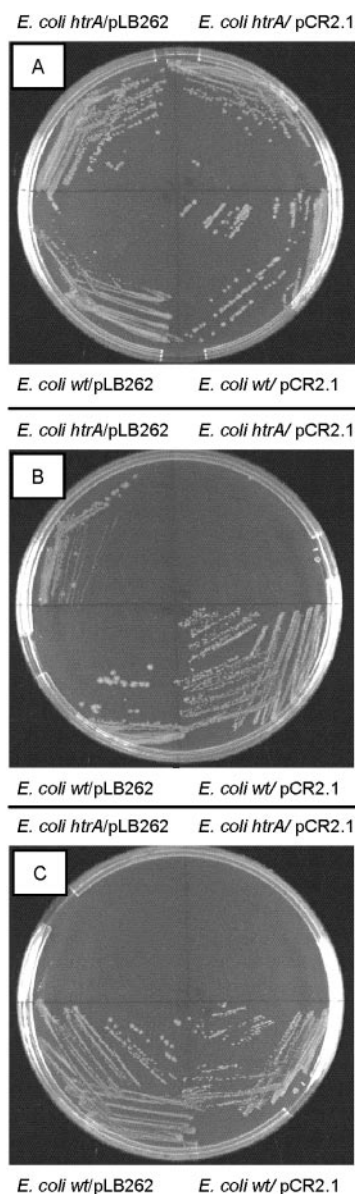


FIG. 2. Complementation of an *E. coli htrA* mutant. *E. coli* strains (*wt* or *htrA21* mutant) containing pLB262 (*C. jejuni htrA*) or pCR2.1 (vector) were streaked on Luria-Bertani agar plates containing kanamycin (50 µg/ml) and incubated at 30°C (A), 39°C (B), or 42°C (C) overnight.

observed sizes. Furthermore, the Western blot revealed that in wild-type cells, equal amounts of DnaK were present at 37 and 42°C (Fig. 3B, lanes 1 and 3). In contrast, growth at 42°C resulted in an increase in the amount of DnaK in the *htrA* mutant compared to that at 37°C (Fig. 3B, lanes 1 and 3). The protein present in the 60-kDa protein band was not identified; however, the size corresponds to the predicted size of the *C. jejuni groEL* protein product (58 kDa) which encodes a third chaperone.

**The *htrA* mutant is sensitive to oxygen but not to H<sub>2</sub>O<sub>2</sub>, paraquat, or cumene hydroperoxide.** In previous studies, HtrA has been shown to be important for oxidative stress tolerance in several gram-negative pathogenic bacteria (10, 12, 19, 27,

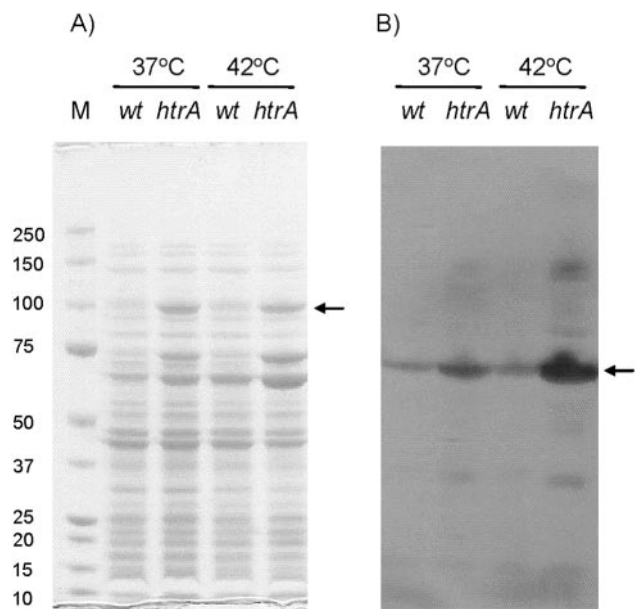


FIG. 3. Effect of the *htrA* mutation on protein composition of *C. jejuni*. Proteins were extracted from *C. jejuni* NCTC11168 (wt) and LB1281 (*htrA*) cells growing in exponential phase ( $OD_{600} = 0.3$ ) at 37 or 42°C. (A) Extracted proteins were separated on a SDS-polyacrylamide gel electrophoresis gel, and the 100-kDa band (arrow) was cut out and identified by mass spectrometry as ClpB. Lane M, molecular weight markers. (B) In a Western blot, proteins were transferred to a PVDF membrane and probed with rabbit polyclonal anti-DnaK (*E. coli*). An arrow indicates the DnaK band.

58). Therefore we compared the sensitivities of the *C. jejuni htrA* mutant and wild-type cells to oxidative stress inducers by using a disk diffusion assay. When exposed to H<sub>2</sub>O<sub>2</sub>, cumene hydroperoxide, or paraquat, the mutant and wild-type cells were equally sensitive, and this finding was confirmed by survival assays (data not shown).

Since reactive oxygen species may also be produced from oxygen via metabolism, we investigated oxygen sensitivity by incubation of *htrA* and wild-type cells in an atmosphere with a lit candle, in which the oxygen concentration is around 17 to 18% (55). After 3 days of incubation at 42°C, we found that the *htrA* mutant formed colonies at a lower frequency than the wild type did (Fig. 1C), suggesting that the *htrA* mutant is oxygen sensitive. In addition, the viability of the *htrA* mutant was reduced approximately 500-fold compared to that of the wild type when liquid cultures were exposed to vigorous shaking in atmospheric air for 6 h, thus confirming that the *htrA* mutant has a reduced oxygen tolerance. Since iron catalyzes reactions that lead to formation of reactive oxygen species, we investigated whether the reduced oxygen tolerance was dependent on iron. However, the presence of the iron chelator desferal did not eliminate the inhibitory effect of oxygen, nor did additional iron decrease the oxygen tolerance further (data not shown). These results suggest that the toxic effect of oxygen on the *htrA* mutant is mediated mainly via an iron-independent reaction.

**HtrA is important for adherence to and invasion of INT407 epithelial cells.** Although *C. jejuni* is considered to be primarily an extracellular pathogen, the ability to adhere to and invade epithelial cells is also believed to be part of its pathogenesis

(23). With the aim of determining whether HtrA plays a role in the interaction between *C. jejuni* and human epithelial cells, we compared the abilities of *htrA* mutant cells and wild-type cells to adhere to and invade the human epithelial cell line INT407. We found that when HtrA was absent, both adhesion to and invasion of the epithelial cells were reduced compared to those of the wild-type strain of *C. jejuni* (Fig. 4A and B). The sensitivity to gentamicin and Triton-X of the *htrA* mutant was not significantly affected compared to that of the wild type (data not shown). Even though the reduced adherence and invasion of the *htrA* mutant may be an indirect effect of *C. jejuni* lacking HtrA, our results show that HtrA is important for both adherence to and invasion of INT407 epithelial cells.

To investigate whether the lack of HtrA affects the human response mediated by the complement system, we measured the viabilities of the *htrA* mutant and the wild type after incubation in human serum. The complement system present in human serum is part of the human defense system against infection by gram-negative bacteria, which are killed through interaction with specific components of the complement. While a *Klebsiella pneumoniae htrA* mutant has decreased resistance to human serum (10), we found that the viability of the *htrA* mutant in human serum was not significantly different from that of the wild-type *C. jejuni* strain NCTC11168, regardless of whether the complement was inactivated by heat treatment (data not shown).

**HtrA is not required for autoagglutination and motility, but lack of HtrA alters the morphology of *C. jejuni*.** Since the ability to self-associate or autoagglutinate has been associated with virulence in some gram-negative pathogenic bacteria such as

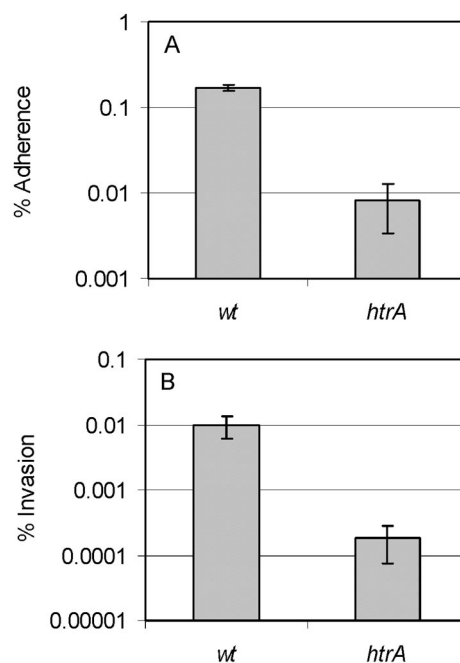


FIG. 4. Effect of the *htrA* mutation on the ability of *C. jejuni* to adhere to and invade cultured INT407 intestinal cells. Adhered (A) and invaded (B) cells are expressed as percentages of those in inocula. The values represent the means (grey bar) and standard deviations (thin lines) of results for four wells. One representative of three independent experiments is presented.

enteropathogenic *E. coli* (4) and *Y. enterocolitica* (41), we determined the abilities of the *C. jejuni* wild type and *htrA* mutant to autoagglutinate. Because bacterial cells in suspension fall to the bottom of the tube when they autoagglutinate, the reduction in OD<sub>600</sub> in a suspension of cells adjusted to an OD<sub>600</sub> of 1 after 24 h of static incubation at 25°C is a measure of autoagglutination. However, a lack of HtrA did not affect the ability of *C. jejuni* to autoagglutinate, since the OD<sub>600</sub> of the *htrA* mutant was reduced to 9.1% ± 0.4%, a result similar to that of the wild type (9.7% ± 1.7%).

Previous studies have shown that lack of the major flagellar subunit FlaA of *C. jejuni* reduces adherence and invasion of *C. jejuni* into INT407 epithelial cells, suggesting that motility is associated with virulence (56, 59). When investigating motility in a soft agar assay, we found that the *htrA* mutant and wild-type cells were equally motile, indicating that neither motility nor chemotaxis is affected by deletion of *htrA* (data not shown). This notion was confirmed by phase-contrast microscopy, in which the *htrA* mutant and wild-type *C. jejuni* appeared equally motile (data not shown). However, when we examined the morphology of the *htrA* mutant cells by using electron microscopy, we repeatedly observed that the *htrA* mutant cells were longer and less curved than the wild-type cells but carried flagella resembling those of the wild type (data not shown).

## DISCUSSION

The food-borne pathogen *C. jejuni* continues to be a public health problem all over the world. The persistence of *C. jejuni* in the different environments that the bacterium experiences during the contamination cycle may be a consequence of a significant ability to adapt to and survive these conditions. In the present study, we have investigated the role of the HtrA protease in the stress tolerance of *C. jejuni*. We showed that the HtrA protease is required for both heat and oxygen tolerance, and our results furthermore indicate that HtrA is important for interaction with human epithelial cells, suggesting that HtrA contributes to the successful survival of *C. jejuni* during the contamination cycle. In contrast to data presented here, it was previously noted that a *C. jejuni htrA* mutant did not display any obvious phenotype (7, 36). However, since there are no details available concerning construction of this mutant and the physiological conditions used, it is not possible to pinpoint the reason for this apparent discrepancy between our results and those of previous studies.

In the present communication, we show that in *C. jejuni*, HtrA is required for growth at high temperatures and that the *htrA* mutant is impaired in growth in the presence of puromycin. Since we predict that HtrA of *C. jejuni* is located in the periplasmic space, we propose that the temperature sensitivity of the *htrA* mutant is a result of the accumulation of misfolded protein in the periplasm and that *C. jejuni* HtrA, like *E. coli* HtrA (29, 43, 49), is involved in the degradation of nonnative proteins in this cellular compartment. Thus, we suggest that HtrA of *C. jejuni* is a functional homologue of HtrA in *E. coli*, which is supported by the complementation of the temperature-sensitive phenotype of an *E. coli htrA* mutant. Interestingly, we found that a lack of HtrA increased the cellular content of the cytoplasmic chaperones ClpB and DnaK, which are conserved chaperones involved in the refolding of heat-

denatured proteins and solubilization of larger protein aggregates by the expenditure of ATP in *E. coli* (3, 31). In general, the heat shock response is induced in response to an unfolded protein signal, and the increased levels of the ClpB and DnaK chaperones in the *htrA* mutant thus indicate that a nonnative protein signal is elicited in the absence of HtrA in *C. jejuni*. In contrast, increased amounts of ClpB and DnaK are not, to our knowledge, found in an *E. coli htrA* mutant, suggesting that the heat shock response is not induced. While *C. jejuni* lacks homologues of the heat shock sigma factor and the sigma factor responding to envelope stress (37), the genome of *C. jejuni* encodes two homologues of the negative heat shock regulatory proteins HrcA and HspR that are found mostly in gram-positive bacteria (33). Analysis of a *C. jejuni hspR* mutant has revealed that the expression of *dnaK*, *groEL*, and *clpB* is controlled by HspR (1), while binding sites for HrcA are found only upstream of *dnaK* and *groEL*, suggesting that the nonnative protein signal elicited in the absence of HtrA might be communicated to the heat shock genes via HspR.

Questions arise as to whether the accumulation of misfolded proteins in the periplasm increases the level of cytoplasmic chaperones and how exactly the signal is transmitted from periplasm to cytoplasm. The fact that a *clpB* mutation could not be transduced to an *E. coli htrA* mutant stresses the biological requirement for ClpB in the absence of HtrA (25). In *E. coli*, blocking of protein export leads to an intracellular accumulation of precursor molecules of the envelope proteins, which was found to induce heat shock proteins (17). Similarly, when *C. jejuni* lacks HtrA, nonnative proteins that accumulate in the periplasm may inhibit the protein translocation systems located in the inner membrane, leading to stalling of unfolded precursor proteins in the cytoplasm and induction of the heat shock proteins, including ClpB and DnaK. Alternatively, the accumulation of misfolded proteins in the periplasm is sensed and communicated via specific signal transduction systems to the cytoplasm of *C. jejuni*. In *E. coli*, the presence of misfolded proteins in the periplasm is sensed by the two-component systems Cpx or Bae or by the RseA/DegS system (40, 60). However, examination of the *C. jejuni* genome failed to reveal homologues of RseA or the HtrA-like protease DegS. In addition, we were unable to find close homologues of the Cpx or Bae two-component systems. Thus, further work to identify the molecular mechanisms by which the nonnative protein signal is transmitted from periplasm to cytoplasm will be needed.

In contrast to the growth and survival of bacterial *htrA* mutants of other pathogenic gram-negative bacteria (11, 19, 27, 58), those of the *C. jejuni htrA* mutant were not reduced by oxidative stress inducers. Conversely, the *C. jejuni htrA* mutant showed reduced oxygen tolerance, a phenotype that, to our knowledge, has not been previously associated with bacterial *htrA* mutants. Even though *C. jejuni* is a microaerobic organism, it is able to perform aerobic respiration (45), and an increase in oxygen tension might result in the accumulation of oxidatively damaged proteins in the periplasm that require HtrA for removal. Also a *C. jejuni* strain with a mutation in the *fdxA* gene, encoding a putative ferredoxin that promotes one-electron transfer from a highly negative potential, shows reduced oxygen tolerance but no sensitivity towards H<sub>2</sub>O<sub>2</sub> or cumene hydroperoxide (54). A lack of ferredoxin may interfere with the respiration process, as the small iron-sulfur proteins



may be involved in electron transfer in the respiratory pathways.

By the use of tissue cultures of human epithelial cells (INT407), we demonstrated that both adherence to and invasion of the *htrA* mutant were severely reduced compared to those of the wild-type *C. jejuni*, showing that HtrA has an impact on the interaction with human epithelial cells in vitro. Although we did not include a *C. jejuni* mutant containing a *cat* gene in a neutral position in the assay, it is our experience (based on other *cat* mutants) that the impact on invasion and adherence is not likely an effect of the *cat* gene itself. In *C. jejuni*, several factors have been shown to be important for in vitro adherence and invasion. *C. jejuni* mutants lacking the major flagellar subunit FlaA are less motile and show a reduced ability to adhere to and invade intestinal cells in vitro (15, 18, 56). However, the impaired adherence and invasion of the *htrA* mutant cannot be explained by reduced motility, since the *htrA* mutation does not affect these parameters. But surface components other than flagella, for example, the outer membrane proteins PebA1 and CadF, seem to be involved in *C. jejuni* adherence to epithelial cells (21, 38). Although we did not observe differences in outer membrane-associated proteins between the *htrA* mutant and the wild-type strain on an SDS-polyacrylamide gel electrophoresis gel, the surface components of the *htrA* mutant may be different (data not shown). Recent results indicate that the morphology of *C. jejuni* affects invasion in INT-407 epithelial cells, as a straight variant invades with a lower frequency than one with a spiral shape (13); thus, the changed morphology of the *htrA* mutant may result in reduced adherence to and invasion of the human epithelial cells.

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