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Different Contributions of HtrA Protease and Chaperone Activities to *Campylobacter jejuni* Stress Tolerance and Physiology▽

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The microaerophilic bacterium *Campylobacter jejuni* is the most common cause of bacterial food-borne infections in the developed world. Tolerance to environmental stress relies on proteases and chaperones in the cell envelope, such as HtrA and SurA. HtrA displays both chaperone and protease activities, but little is known about how each of these activities contributes to stress tolerance in bacteria. *In vitro* experiments showed temperature-dependent protease and chaperone activities of *C. jejuni* HtrA. A *C. jejuni* mutant lacking only the protease activity of HtrA was used to show that the HtrA chaperone activity is sufficient for growth at high temperature or under oxidative stress, whereas the HtrA protease activity is essential only under conditions close to the growth limit for *C. jejuni*. However, the protease activity was required to prevent induction of the cytoplasmic heat shock response even under optimal growth conditions. Interestingly, the requirement of HtrA at high temperatures was found to depend on the oxygen level, and our data suggest that HtrA may protect oxidatively damaged proteins. Finally, protease activity stimulates HtrA production and oligomer formation, suggesting that a regulatory role depends on the protease activity of HtrA. Studying a microaerophilic organism encoding only two known periplasmic chaperones (HtrA and SurA) revealed an efficient HtrA chaperone activity and proposed multiple roles of the protease activity, increasing our understanding of HtrA in bacterial physiology.

The Gram-negative pathogenic bacterium *Campylobacter jejuni* is the most common cause of bacterial food-borne infections in developed countries (1), and handling and consumption of contaminated poultry meat products are recognized as the most frequent sources of infection (22). The symptoms of human campylobacter infections are diarrhea, abdominal pain, and fever, which in rare cases are followed by development of more serious complications. *C. jejuni* colonizes the intestinal tract of mammals and birds, but in contrast to infected humans, no apparent symptoms are observed in colonized poultry or other avian species. *C. jejuni* is a microaerophilic bacterium having an optimal growth temperature of 42°C, which may reflect that *C. jejuni* is highly adapted to a commensal lifestyle in the avian cecum. During transmission from the avian host to the intestinal tract of humans, *C. jejuni* is exposed to various stressful conditions, such as temperature and pH fluctuations, osmotic stress, and atmospheric oxygen concentrations. *C. jejuni* is generally considered a fragile organism, because it is killed more readily by environmental stress conditions than other food-borne pathogens (46) and because of its fastidious growth requirements. *C. jejuni* grows only between 30°C and 45°C (16, 59) in a microaerobic atmosphere containing carbon dioxide (5, 30) and fails to grow on sugars and by fermentation (47). Despite these growth limitations, *C. jejuni* is able to survive in the food production chain and is a frequent cause of human disease.

A number of vital processes take place in the periplasmic space of Gram-negative bacteria. In *C. jejuni*, these processes include nutrient transport (33, 39), protein glycosylation (66), protection against oxidative stress (4), and respiration (14). Because the outer membrane allows free diffusion of small molecules (29), periplasmic proteins are more highly exposed to chemical variations in the extracellular environment than cytoplasmic proteins, which are protected by a tight inner membrane. Periplasmic proteins and proteins targeted for the outer membrane are synthesized in the cytoplasm but folded in the periplasm (57). Since protein misfolding and aggregation are accelerated by stress, the native state of periplasmic proteins is constantly challenged by the fluctuating environmental conditions encountered by *C. jejuni* during its transmission to the human host. Therefore, *C. jejuni*, and Gram-negative bacteria in general, depends on periplasmic proteases and chaperones to degrade misfolded or damaged proteins and to promote their folding by protecting them from interactions that lead to aggregation, respectively.

In Gram-negative bacteria, such as *Salmonella* and *Escherichia coli*, the periplasmic chaperones HtrA (DegP), SurA, Skp (OmpH), and FkpA have partly overlapping functions (23, 55) and provide these bacteria with a robust system for protein folding in the periplasm. In contrast, *C. jejuni* carries homologs of only HtrA and SurA and thus depends on these two chaperones for protein folding in the periplasm. A few bacterial HtrA homologs have been investigated *in vitro*, and these experiments have shown that HtrA is a bifunctional protein that, in addition to the chaperone activity, possesses proteolytic activity (24, 28, 63). In general, HtrA homologs are characterized by the presence of a trypsin-like serine protease domain and one or two PDZ domains that recognize substrates and activate the protease function (25, 31). Upon binding of un-

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folded proteins to the PDZ domains, HtrA organizes into larger active oligomers, while reverting to a hexameric resting state after substrate degradation is completed (32). We previously showed that HtrA is required for growth of *C. jejuni* at elevated oxygen tensions and under conditions that induce protein misfolding, such as high temperature and puromycin (6). While bacterial *htrA* mutants show different stress-sensitive phenotypes, all *htrA* mutants examined so far are sensitive to high temperature (9, 10, 34, 35, 48, 69). In addition, several studies have suggested an important role of HtrA in virulence of *C. jejuni*, as an *htrA* mutant invades epithelial cells (6, 44) and kills infected *Galleria mellonella* larvae less efficiently than the wild type (8). However, despite a detailed mechanistic insight into HtrA function (26, 31, 32), only few studies have addressed how the individual HtrA activities contribute to stress tolerance in bacteria. These studies have shown that plasmid-mediated expression of an HtrA mutant protein that lacks protease activity but retains chaperone activity allows growth of *E. coli* and *Salmonella htrA* deletion mutants at high temperature (34, 61, 63). Such experiments indicate that the protease activity may be dispensable if the HtrA chaperone activity is present in elevated amounts; however, they do not show how each of these activities contributes to stress tolerance in bacteria.

With the aim of determining the contribution of the HtrA protease and chaperone activities to heat and oxidative stress tolerance of *C. jejuni*, we used a method described by Hendrixson et al. (18), which employs counterselection for site-directed mutagenesis in *C. jejuni*, to construct a mutant that lacks HtrA protease activity but retains HtrA chaperone activity. Using this mutant, we show that the chaperone activity of HtrA is sufficient for growth under a wide range of heat and oxidative stress conditions, whereas the protease activity is essential only under severe stress conditions. Furthermore, we find that the heat sensitivity of an *htrA* mutant depends on the level of oxidative stress, emphasizing an important role of HtrA in tolerance to oxidative stress. In this study, we provide molecular and phenotypic evidence demonstrating that HtrA chaperone and protease activities participate in both stress tolerance and protein homeostasis in *C. jejuni*, thereby contributing to the success of this apparently fragile zoonotic pathogen. By studying a conserved protein in a new context, namely, a microaerophilic bacterium that encodes a limited number of periplasmic chaperones and regulatory factors, we have shown the chaperone activity of HtrA to play a significant role in stress tolerance, and we propose a regulatory function of the HtrA protease activity.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *C. jejuni* NCTC11168 (National Collection of Type Cultures) and derivatives thereof were routinely grown on blood agar base II (Oxoid) supplemented with 5% (vol/vol) bovine blood or in brain heart infusion (BHI) broth (Oxoid) at 37°C in a microaerobic environment (6% O_2, 6% CO_2, 4% H_2, and 84% N_2). *E. coli* strains were routinely grown in Luria broth (LB) or on Luria agar. When appropriate, media were supplemented with 100 μg ml^{-1} ampicillin, 50 μg ml^{-1} kanamycin, 50 μg ml^{-1} streptomycin, or 20 μg ml^{-1} chloramphenicol.

Construction of *C. jejuni htrA*(*S197A*) mutant. Streptomycin counterselection (18) was used to construct a *C. jejuni htrA*(*S197A*) mutant. Plasmid pLB252 (Δ*htrA*::*cat-rpsL*) was constructed by cloning a *cat-rpsL* SmaI fragment from pDRH265 (18) into the PstI site of pLB225, containing an in-frame deletion in the *htrA* gene with an internal PstI site (6). A PCR fragment containing a site-specific mutation in the *htrA* gene was obtained by the following procedure. First, two PCR fragments were amplified from *C. jejuni* NCTC11168 using primers HT1 (5′-AATTTAATGGTTTCGCCTTG-3′) and HT2 (5′-ATCCACC AAAGCTCCGCCGGCATTTCCTGGATTGAT-3′) or HT3 (5′-ATCAATCC AGGAAATGCCGGCGGAGCTTTGGTGGAT-3′) and HT4 (5′-AGCTTATA ACCTATTCCACG-3′). Primers HT2 and HT3 are overlapping and contain substitutions that change HtrA serine 197 to alanine 197. The PCR fragments were joined by the splicing-by-overlap-extension PCR method (21) using HT1 and HT4 as primers, and the resulting fragment carrying *htrA*(*S197A*) was cloned into pSC-A (Stratagene), resulting in pKB1002. A spontaneous streptomycin-resistant mutant of *C. jejuni* NCTC11168 was isolated by streaking on streptomycin plates. A 0.8-kb fragment containing the mutated *rpsL* gene (*rpsL*^{Sm}) was amplified by PCR using primers 5′-GGGGGCAGCTGCATGGTCTTGAAGA ATATTTAGC-3′ and 5′-GGGGGCAGCTGCTAACGGATTTGTCTG-3′, cloned into vector pCR2.1 (Invitrogen), and transferred to NCTC11168 by electroporation (7), thereby creating LB1238 (*rpsL*^{Sm}). A *C. jejuni* Δ*htrA*::*cat-rpsL* mutant (KB1022) was obtained by transformation of LB1238 (*rpsL*^{Sm}) with pLB252 (Δ*htrA*::*cat-rpsL*) and selection for chloramphenicol resistance. KB1022 was then transformed with pKB1002 [*htrA*(*S197A*)], and Δ*htrA*::*cat-rpsL* located on the chromosome was replaced by *htrA*(*S197A*) by selection for streptomycin resistance. Sm^r colonies were tested for sensitivity to chloramphenicol, and one sensitive clone [KB1025; *C. jejuni htrA*(*S197A*)] was isolated.

Cloning of HtrA expression plasmids. PCR fragments containing *htrA* or *htrA*(*S197A*) were amplified using *C. jejuni* NCTC11168 chromosomal DNA or pKB1002 DNA, respectively, as the template and 5′-TATACCATGGGCGCA AGTATTAATTTTAAC-3′ (HtrA-his-F) and 5′-GTGCTCGAGTTTAAGCAC AAGCAAAGTCGC-3′ (HtrA-his-R) as primers. The resulting fragment contains no *htrA* signal peptide and carries an NcoI site and Met-Gly codons upstream of codon 17 of the *htrA* gene as well as an XhoI site and Leu-Glu codons upstream of the *htrA* stop codon. The NcoI and XhoI sites were used to clone the PCR fragments in frame and upstream of six C-terminal His codons in the pET28a+ vector (Novagen) to give pKB1004 (HtrA-His) and pKB1005 ($HtrA_{S197A}$-His). To express HtrA-His to the periplasm, an NcoI-XhoI fragment encoding HtrA-His was cloned in the pET26b+ expression vector (Novagen), giving rise to pKB1012. Blunt-end-forming DNA polymerase was used to amplify a fragment containing *htrA*(*S197A*) and the six C-terminal His codons by PCR using pKB1005 as the template and the primers 5′-TAGTTATTGCTCAGCG GTGG-3′ and 5′-TTGTGAGCGGATCCCAATTC-3′. This PCR fragment was digested with BamHI and ligated into pJS17 (62), which had been opened with HindIII, blunt ended with T4 DNA polymerase, and redigested with BamHI to give pKB1014, containing *htrA*(*S197A*)-His_6 controlled by the T5 promoter.

Expression and purification of HtrA. Wild-type HtrA-His was expressed from pKB1012 in *E. coli* BL21(DE3) by growing the cells at 37°C to an optical density at 600 nm (OD_{600}) of 0.9, and expression was induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 h. $HtrA_{S197A}$-His was expressed from pKB1014 in *E. coli htrA* strain BL20 (35) by growth at 30°C to an OD_{600} of 0.9, and expression was induced by addition of 1 mM IPTG for 16 h. After induction, cells were lysed on ice in 20 ml lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, 10% [vol/vol] glycerol) by addition of 1 mg ml^{-1} lysozyme, followed by sonication. Lysates were cleared by centrifugation at 35,000 × *g* for 30 min. His-tagged HtrA proteins were purified by nickel affinity chromatography using Ni-nitrilotriacetic acid (NTA) resin (Qiagen) equilibrated with lysis buffer. Unspecifically bound proteins were removed with 50 mM imidazole, and HtrA was eluted with 250 mM imidazole. Eluted proteins were concentrated and dialyzed against 25 mM HEPES, pH 7.5, 50 mM NaCl, 10% (vol/vol) glycerol. Protein bands were excised from the gel and prepared for mass spectrometry (MS) as described previously (12).

Proteolytic activity of HtrA. Proteolytic activity of HtrA or $HtrA_{S197A}$ was determined using casein as the substrate in reaction mixtures containing 50 mM Tris-HCl (pH 8.0), 0.4 mg ml^{-1} β-casein, and 20 μg ml^{-1} HtrA or $HtrA_{S197A}$ at 37°C. At different time points, aliquots were transferred to SDS sample buffer containing dithiothreitol (DTT) and immediately frozen in −80°C ethanol. The samples were separated by SDS-PAGE and stained with Coomassie brilliant blue. Protease activity was also tested using an EnzChek protease activity kit (Molecular Probes) according to the manufacturer's instructions. Briefly, reaction mixtures containing casein labeled with the fluorescent dye BODIPY FL were incubated in a quartz cuvette at different temperatures, and fluorescence was measured using a Perkin-Elmer LS55 luminescence spectrophotometer. Upon hydrolysis of the labeled casein by HtrA, quenching is relieved and the increase in fluorescence is proportional to substrate degradation. Fluorescence was followed upon addition of 4 μg ml^{-1} HtrA, and the slope of the initial linear increase in fluorescence was a measure of proteolytic activity.

Light scattering. Assays were performed essentially as previously described (61). Reaction mixtures containing 40 μM lysozyme, 20 μM $HtrA_{S197A}$, 25 mM HEPES, pH 7.5, and 50 mM NaCl were incubated at different temperatures in quartz cuvettes in a thermostated cell holder to record the light scattering baseline. Then, 4 mM DTT was added and light scattering was measured immediately. Protein aggregation was monitored using a Perkin-Elmer LS55 luminescence spectrophotometer, with excitation and emission wavelengths set to 360 nm.

Lysozyme aggregation. Measurements were performed as previously described (61) in reaction mixtures containing 80 μM lysozyme, 40 μM $HtrA_{S197A}$, 25 mM HEPES, pH 7.5, and 50 mM NaCl incubated at different temperatures. DTT was added to 4 mM, and aliquots were withdrawn at selected time points and centrifuged immediately at 21,000 × *g* for 10 min. Pellets were dissolved in 8 M urea, 100 mM $NaPO_4$, pH 7.5, and protein concentration was determined by staining with amido black. Relative values of protein content were obtained by densitometry. Absolute protein concentration was estimated by spectrophotometric measurements of amido black-stained samples, using native lysozyme as the standard.

HtrA polyclonal antibody preparation. Wild-type HtrA ($HtrA_{wt}$) was purified as described above and separated by SDS-PAGE. The band containing full-length HtrA (determined by mass spectrometry) was cut out and used for the production of rabbit polyclonal antibodies (CovalAB, Lyon, France). Final serum was acquired 67 days after the initial immunization.

Protein analysis and Western blotting. For denatured protein extracts, *C. jejuni* cells were grown in BHI broth at 37°C or 42°C under microaerobic conditions, and proteins were extracted as described by Brøndsted et al. (6), separated by SDS-PAGE, Coomassie stained or blotted onto a polyvinylidene difluoride (PVDF) membrane, and probed with HtrA antibody. For the native protein extracts, *C. jejuni* cells were grown in BHI broth at 37°C under microaerobic conditions to the exponential phase (OD_{600} of 0.1). Native lysates were prepared by washing the harvested cells in 0.9% (wt/vol) NaCl, resuspension in NativPAGE buffer (Invitrogen), and sonication. Native samples were separated by blue native PAGE (58) on a NativPAGE bis-Tris gel (Invitrogen), blotted onto a PVDF membrane, and probed with HtrA antibody. Bound antibody was detected with a WesternBreeze chemiluminescence detection kit (Invitrogen) and quantified densitometrically with GeneTools software (PerkinElmer).

Growth experiments on solid medium. *C. jejuni* cells were grown overnight on base II, 5% (vol/vol) blood agar plates at 37°C under microaerobic conditions and harvested in 0.9% (wt/vol) NaCl, and the OD_{600} was adjusted to 0.1. The cell suspension was serially diluted, and 10 μl of the 10^{-1} to 10^{-5} dilutions was spotted on base II, 5% blood agar plates and incubated for 3 days at 37°C, 42°C, or 44°C under the following conditions. The microaerobic atmosphere was generated by CampyGen (Oxoid), the ca. 18% O_2 atmosphere was generated by the presence of a lit candle in a closed jar (37), and the 1% O_2 atmosphere was obtained in an incubator with 6% CO_2, 4% H_2, and 89% N_2. A mixture of ferrous sulfate, sodium bisulfate, and pyruvate (FBP) was added to 0.025% (wt/vol) each by addition of campylobacter growth supplement (Oxoid).

Disc diffusion assays. Approximately 10^9 *C. jejuni* stationary-phase cells were added to 0.6% Mueller-Hinton top agar and poured on Mueller-Hinton agar plates. Filter discs (13 mm; Whatman) were placed on the top agar, and 10 μl 30% hydrogen peroxide, 5 μl 80% cumene hydroperoxide, or 20 μl 98% paraquat (all from Sigma) was added to the discs. Plates were incubated microaerobically at 37°C or 42°C for 3 days, and inhibition zones were measured.

Protein carbonylation. *C. jejuni* cells were grown in 50 ml BHI broth in 300-ml Erlenmeyer flasks at 42°C under microaerophilic conditions until stationary phase (OD_{600} of 0.7 to 1.4), transferred to aerobic conditions, and shaken at 300 rpm for 1 h. Cells were pelleted at 6,000 × *g* for 10 min, washed in 50 mM Tris-HCl, pH 7.8, and split into two portions. One portion, used for whole-cell extracts, was lysed for 1 h at room temperature in phosphate-buffered saline (PBS) buffer containing 8 M urea, 1% (vol/vol) Triton X-100, 1% (wt/vol) SDS, and 1% (vol/vol) β-mercaptoethanol in order to solubilize proteins and subsequently sonicated briefly to shear DNA and centrifuged (10,000 × *g* for 10 min) to remove cell debris. The second portion was used to extract outer membrane proteins essentially as described previously (38). Briefly, the pellet was resuspended in 0.2 M glycine-HCl, pH 2.2, to an OD_{600} of 10 and incubated at room temperature for 10 min, followed by removal of cells at 6,000 × *g* for 5 min. The protein content in the extracts was quantified by densitometry of amido black-stained proteins, with bovine serum albumin (BSA) as the standard. Carbonylated proteins were derivatized with 2,4-dinitrophenylhydrazine (DNPH), and equal amounts of protein were separated by SDS-PAGE. DNPH-derivatized proteins were detected by immunoblotting with anti-2,4-dinitrophenol (anti-DNP) antibody using an OxyBlot protein oxidation detection kit (Chemicon).

Northern blotting. *C. jejuni* cells were grown under microaerobic conditions to an OD_{600} of 0.4 or 0.7. RNA extraction was performed essentially as described previously (42). Briefly, 1 volume of cells was added to 2 volumes of RNAprotect bacterial reagent (Qiagen), and the mixture was incubated for 5 min at room temperature before the cells were pelleted at 8,000 × *g* for 2 min at 0°C. Subsequently, cells were lysed in Tris-EDTA (TE) buffer containing 1 mg ml^{-1} lysozyme at room temperature for 10 min, and RNA was extracted using an RNeasy kit (Qiagen). Equal amounts of RNA (3 to 5 μg) were denatured at 75°C for 10 min and separated in a 1% agarose gel in 10 mM $NaPO_4$ buffer as described by Pelle and Murphy (50). RNA was blotted onto a positively charged nylon membrane (Hybond-N; GE Healthcare) and subsequently fixed to the membrane by UV light exposure. A gel-purified PCR fragment internal to the *htrA* gene was used as a probe and labeled with [^{32}P]dCTP using Ready-to-Go labeling beads (GE Healthcare), and unincorporated nucleotides were removed using ProbeQuant G-50 microcolumns (GE Healthcare). Probes were hybridized to the membrane overnight at 65°C in 0.5 M $NaPO_4$ (pH 7.2), 7% (wt/vol) SDS after 1 h of prehybridization under the same conditions. To remove unspecifically bound probe, membranes were washed twice in 20 mM $NaPO_4$ (pH 7.2), 1% (wt/vol) SDS. The washed membrane was used to expose a phosphorimage screen at room temperature, and the screen was scanned in a phosphorimager (Cyclone Plus; PerkinElmer).

RESULTS

***C. jejuni* HtrA has both protease and chaperone activities.** A bioinformatic analysis suggested that HtrA of *C. jejuni* is a bifunctional protein possessing protease as well as chaperone activity, since the protein contains the conserved active-site residues characteristic of serine proteases (His_{92}-Asp_{123}-Ser_{197}) as well as two PDZ domains. To explore this predicted dual functionality of HtrA, we constructed a protease-negative mutant protein ($HtrA_{S197A}$) by replacing the serine in the active proteolytic site with alanine. His-tagged $HtrA_{S197A}$ or $HtrA_{wt}$ protein was subsequently expressed in *E. coli* and purified. The protease activity of HtrA was demonstrated by degradation by $HtrA_{wt}$ of β-casein (Fig. 1A), a substrate commonly used to investigate proteolytic activity of HtrA homologs (26, 36, 63). $HtrA_{S197A}$, on the other hand, was unable to degrade this substrate (Fig. 1A), showing that HtrA of *C. jejuni* is a protease, whose proteolytic activity depends on serine 197. Furthermore, the protease activity was temperature dependent (Fig. 1B). The chaperone activity was measured as the ability of purified $HtrA_{S197A}$ to prevent aggregation of denatured lysozyme at various temperatures. Lysozyme aggregation was determined by light scattering and by quantification of large insoluble aggregates, and we found that $HtrA_{S197A}$ decreased the rate of lysozyme aggregation in a dose-dependent manner at all tested temperatures within the growth range of *C. jejuni* (Fig. 2 and data not shown). The ability of $HtrA_{S197A}$ to prevent aggregation was slightly reduced at higher temperatures, especially at 44°C, which may be explained by a lower intrinsic chaperone activity of HtrA at this temperature. In conclusion, these results show that HtrA of *C. jejuni* has both protease and chaperone activities.

Function of HtrA chaperone and protease activities in heat and oxygen tolerance of *C. jejuni*. We previously used a Δ*htrA* mutant to show that *C. jejuni* exposed to high temperature (44°C) or near-aerobic conditions (18% O_2 at 42°C) requires HtrA to form colonies (6). To investigate the contribution of the HtrA chaperone and protease activities to stress tolerance of *C. jejuni*, we constructed a *C. jejuni htrA*(*S197A*) mutant by replacing the wild-type *htrA* allele with a mutated *htrA*(*S197A*) allele. Thus, the *C. jejuni htrA*(*S197A*) mutant lacks HtrA protease activity but retains HtrA chaperone activity. We com-

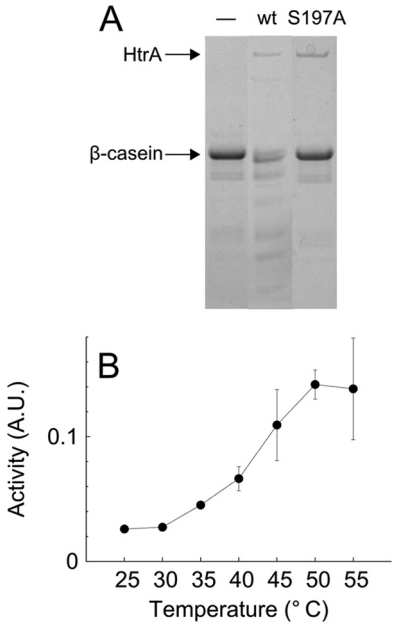

FIG. 1. Protease activity of HtrA *in vitro*. Wild-type HtrA and $HtrA_{S197A}$ were expressed from plasmids pKB1012 and pKB1014, respectively, in *E. coli* and purified. (A) Degradation of β-casein at 37°C in the absence (−) or presence of wild-type HtrA or $HtrA_{S197A}$. (B) Degradation of BODIPY-labeled β-casein at different temperatures in the presence of wild-type HtrA. The activity is the degradation rate measured fluorimetrically at 511 nm in triplicate. The averages from three independent measurements are shown. Error bars indicate the standard deviations. A.U., arbitrary units.

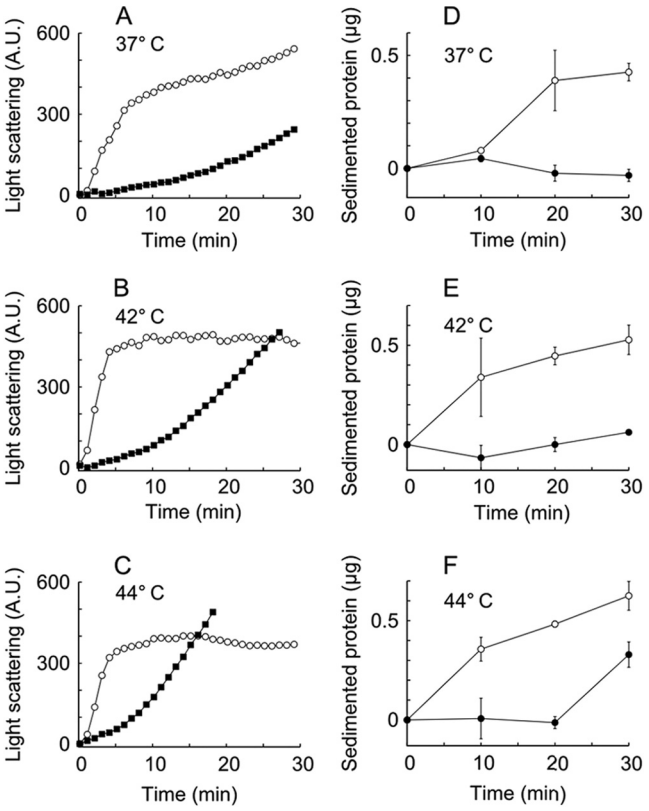

FIG. 2. Chaperone activity of HtrA *in vitro*. $HtrA_{S197A}$ and lysozyme were mixed in a molar ratio of 1:2, and DTT was added at time zero. (A to C) Aggregation of denatured lysozyme in the presence (filled squares) or absence (open circles) of $HtrA_{S197A}$, measured by light scattering at 300 nm. (D to F) Aggregation of denatured lysozyme in the presence (filled circles) or absence (open circles) of $HtrA_{S197A}$, measured by sedimentation of insoluble aggregates at 21,000 × *g* for 10 min. Sedimented protein was quantified as described in Materials and Methods. The values shown are averages from two individual experiments, normalized to the value at time zero. Error bars indicate the standard deviations.

pared the heat and oxygen tolerance of the *htrA*(*S197A*) mutant with that of the wild type and the Δ*htrA* mutant and found that protease activity of HtrA was not required for *C. jejuni* to form colonies either at high temperature (44°C) (Fig. 3A) or at high oxygen tension (18% at 42°C) (Fig. 3B). In contrast, the protease activity of HtrA was required for growth of *C. jejuni* if the two stress conditions were combined (18% O_2 at 44°C), under which condition the *htrA*(*S197A*) mutant failed to form colonies (Fig. 3C). These findings show that HtrA chaperone activity is sufficient to allow growth under mild stress, whereas the protease activity of HtrA is required for growth only when the stress is severe. In addition, the requirement for HtrA at high temperature could be eliminated completely by significantly reducing the oxygen tension, as HtrA was not required for colony formation in a 1% O_2 atmosphere at 44°C (Fig. 3D). Thus, the requirement of HtrA for growth of *C. jejuni* at high temperature depends on the amount of oxygen in the atmosphere.

HtrA is required for tolerance to oxidative stress. To determine if the increased oxygen sensitivity of the *htrA* mutants was mediated by reactive oxygen species (ROS), we tested growth of the wild type and the Δ*htrA* and *htrA*(*S197A*) mutants on solid medium containing ferrous sulfate, sodium bisulfate, and pyruvate (FBP). These compounds act as scavengers that destroy H_2O_2 and O_2^- generated in the growth medium (20, 67). In the presence of these scavengers, the *htrA*(*S197A*) mutant could form colonies at 44°C in an 18% O_2 atmosphere, while the Δ*htrA* mutant failed to form colonies under this condition (Fig. 3E). In contrast, both the Δ*htrA* and *htrA*(*S197A*) mutants were able to form colonies when the temperature was lowered to 42°C (data not shown). Therefore, the increased oxygen sensitivity of *C. jejuni* lacking HtrA must be mediated, at least in part, by exogenous H_2O_2 or O_2^-. To more directly investigate the sensitivity of the *htrA* mutants to oxidative stress induced by reactive oxygen species, cumene hydroperoxide or the intracellular O_2^- generator paraquat (15) was added to the growth medium, and the formation of colonies under microaerobic conditions was investigated. In the presence of these compounds, the ability of Δ*htrA* cells and *htrA*(*S197A*) cells to form colonies was reduced compared to that of the wild type in a temperature-dependent manner (Fig. 3F and G). In agreement with this, a disc diffusion assay with H_2O_2, cumene hydroperoxide, and paraquat showed that the Δ*htrA* and *htrA*(*S197A*) mutants were significantly more sensitive to reactive oxygen species than the wild type and that the difference was more pronounced at 42°C than at 37°C (Table 1). In conclusion, these data show that HtrA plays an essential role in protecting *C. jejuni* against reactive oxygen species.

The role of HtrA in oxidative stress tolerance of *C. jejuni* suggests that HtrA degrades, binds, or refolds oxidatively dam-

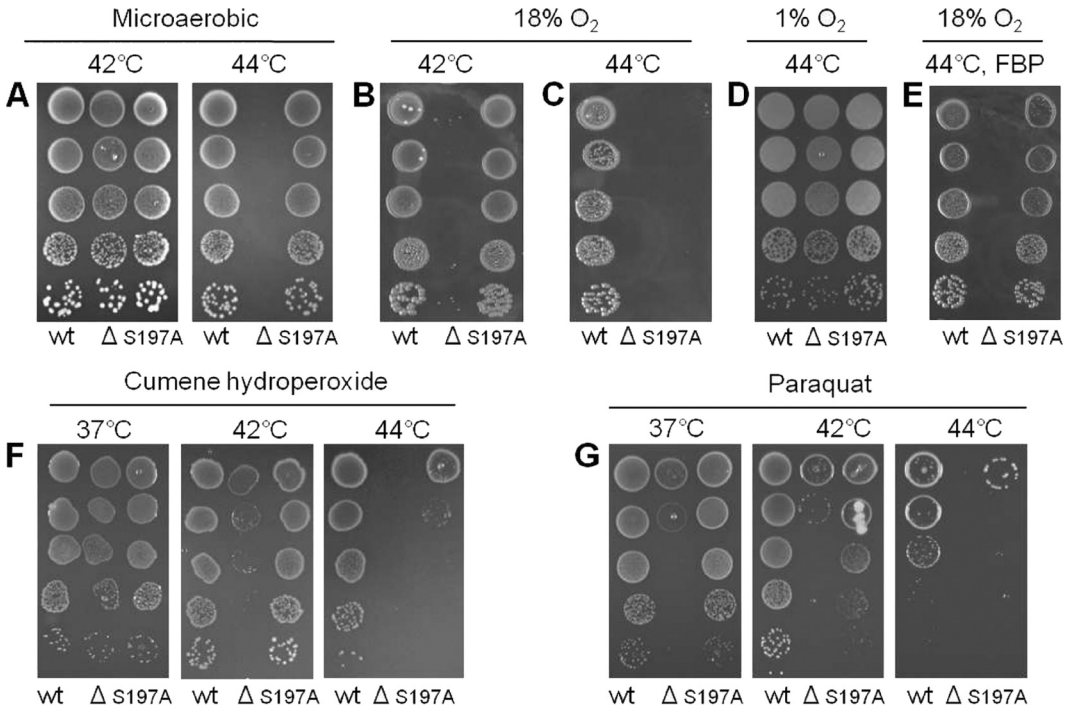

FIG. 3. Effect of temperature and oxidative stress on growth of *C. jejuni htrA* mutants on solid medium. Serial dilutions (10^{-1} to 10^{-5}) of *C. jejuni* NCTC11168 (wild type; wt), LB1281 (Δ*htrA* mutant; Δ), and KB1025 [*htrA*(*S197A*) mutant; S197A] at an OD_{600} of 0.1 were spotted in 10-μl volumes on base II, 5% blood agar plates. The plates were incubated under microaerobic conditions (A, F, and G), in an 18% O_2 atmosphere (candle jar) (B, C, and E), or in a 1% O_2 atmosphere (D). Ferrous sulfate, sodium bisulfate, and pyruvate (FBP), cumene hydroperoxide, or paraquat was added as indicated.

aged proteins in the periplasm. To investigate whether the lack of HtrA affects the level of oxidatively damaged proteins, we assayed protein carbonylation in the wild type and the Δ*htrA* and *htrA*(*S197A*) mutants. Carbonylation is an irreversible modification of proteins exposed to oxidative stress (45), and the level of protein carbonylation can be used as an indicator of the degree of oxidative protein damage. We found no overall increase in the level of carbonylated proteins from Δ*htrA* or *htrA*(*S197A*) cells compared to that from the wild type under microaerobic growth conditions (data not shown) or upon exposure to aerobic growth conditions (Fig. 4). However, since HtrA is a periplasmic protein and since envelope proteins constitute only a small fraction of all cellular protein, a difference in level of protein carbonylation is difficult to detect in whole-cell extracts. Therefore, we performed the analysis of carbonylation with glycine extracts, which contain periplasmic and surface-located proteins (49). Interestingly, we consistently observed a different band pattern with these proteins from the Δ*htrA* mutant than with those from the wild type and the *htrA*(*S197A*) mutant (Fig. 4); however, the specific band differences varied slightly between repeated experiments. These results indicate that HtrA does not lower the level of carbonylated proteins but rather degrades some carbonylated proteins while protecting others in a protease-independent manner.

The protease activity of HtrA is important for protein homeostasis under optimal growth conditions. In the absence of HtrA, the cytoplasmic heat shock chaperones ClpB and DnaK are upregulated in *C. jejuni* (6), suggesting that increased amounts of ClpB and DnaK in *htrA* mutants can be used as an indicator of disturbed protein homeostasis in the cell envelope. The amounts of ClpB and DnaK were increased only slightly in

TABLE 1. Effects of oxidative stress agents on wild-type and *htrA* mutant strains, as measured by disc diffusion assays

Temp (°C)	Oxidative stress agent	Mean diam (mm) of inhibition zone ± SD for[a]:			No. of samples
		Wild type	Δ*htrA* mutant	*htrA*(*S197A*) mutant	
37	H_2O_2	68 ± 5.3	81 ± 7.1**	79 ± 11*	7
	Cumene hydroperoxide	76 ± 6.9	88 ± 10*	88 ± 8.2*	7
	Paraquat	58 ± 4.6	70 ± 2.2**	66 ± 5.2	4
42	H_2O_2	59 ± 5.9	77 ± 5.7***	72 ± 7.0**	7
	Cumene hydroperoxide	73 ± 6.6	91 ± 6.9***	91 ± 11**	7
	Paraquat	50 ± 2.4	64 ± 1.4***	63 ± 2.0***	4

[a] Asterisks indicate significant differences from results for the wild type, based on Student's *t* test, as follows. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

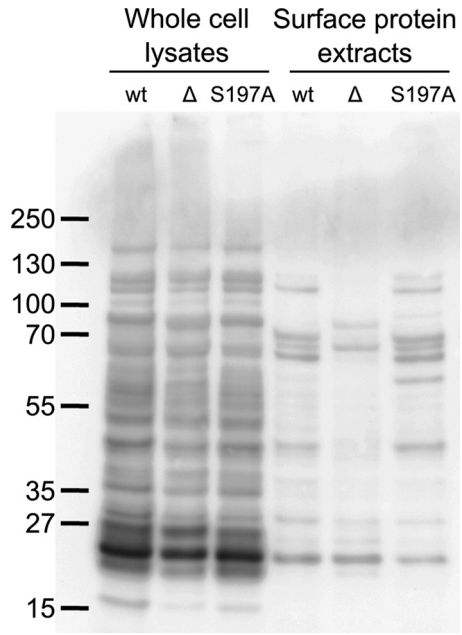

FIG. 4. Protein carbonylation. *C. jejuni* NCTC11168 (wt), LB1281 (Δ*htrA* mutant; Δ), and KB1025 [*htrA*(*S197A*) mutant; S197A] were grown microaerobically overnight at 42°C to stationary phase and then exposed to vigorous shaking for 1 h. Carbonylation of proteins in whole-cell lysates and surface protein extracts was determined as described in Materials and Methods. Equal amounts of protein were loaded in each lane for the lysates and surface extracts. The positions of molecular size standards are indicated on the left (in kilodaltons).

the *htrA*(*S197A*) mutant compared to those in the wild type at 37°C, whereas at 42°C the amounts of ClpB and DnaK in the *htrA*(*S197A*) mutant approached the amounts observed in Δ*htrA* cells (Fig. 5). This indicates that the chaperone activity of HtrA is sufficient to prevent any accumulation of misfolded substrates that may lead to increased amounts of ClpB and DnaK at 37°C. However, at 42°C, misfolded HtrA substrates must be degraded by HtrA to prevent upregulation of ClpB and DnaK. Furthermore, ClpB and DnaK were upregulated in the Δ*htrA* mutant even at low oxidative stress (1% O_2 or added FBP) (data not shown). Thus, a lack of HtrA caused increased levels of ClpB and DnaK, regardless of whether there was oxidative stress. Exposure to 18% O_2 or ROS at 42°C led to an even greater increase in the amounts of ClpB and DnaK in the *htrA*(*S197A*) mutant (data not shown), indicating an increased requirement for protease activity in the presence of oxidative

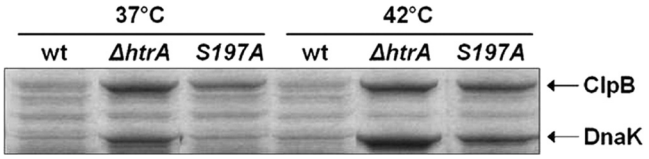

FIG. 5. Effect of lack of HtrA protease activity on induction of cytoplasmic heat shock response. *C. jejuni* NCTC11168 (wt), LB1281 (Δ*htrA* mutant; Δ), and KB1025 [*htrA*(*S197A*) mutant; S197A] were grown in BHI broth at the indicated temperatures in a microaerobic atmosphere. Extracted proteins were separated by SDS-PAGE and stained with Coomassie brilliant blue. ClpB and DnaK were identified previously (6).

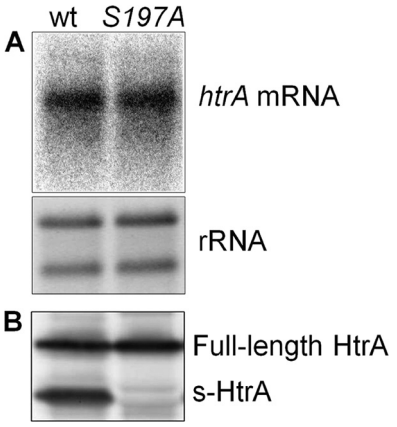

FIG. 6. Effect of HtrA protease activity on HtrA content and *htrA* expression. (A) *htrA* mRNA in exponential-phase-growing *C. jejuni* NCTC11168 (wt) and KB1025 [*htrA*(*S197A*) mutant; S197A] cultures, detected by Northern blotting with an *htrA*-specific DNA probe. (B) Full-length and short HtrA (s-HtrA) in exponential-phase-growing *C. jejuni* NCTC11168 (wt) and KB1025 [*htrA*(*S197A*) mutant; S197A], detected by immunoblotting with an HtrA antibody.

stress. Protein levels of the Δ*htrA* mutant could not be examined at 42°C or under oxidative stress, due to a lack of growth under these conditions (Fig. 3B, F, and G). In conclusion, with ClpB and DnaK levels used as indicators of disturbed protein homeostasis in the cell envelope, the results show that HtrA is important to maintain protein homeostasis in the entire growth interval of *C. jejuni* and that the chaperone activity alone is sufficient to fulfill this role at low temperature.

HtrA protease activity stimulates HtrA production and oligomer disassembly. Because a lack of HtrA protease activity results in the upregulation of ClpB and DnaK at 42°C (Fig. 5), we hypothesized that *htrA* expression may also be upregulated under this condition. We analyzed *htrA* expression in the *htrA*(*S197A*) mutant grown microaerobically at 42°C and found that the amounts of *htrA* mRNA were identical in the wild type and the *htrA*(*S197A*) mutant (Fig. 6A). Thus, *C. jejuni* does not compensate for a loss of HtrA protease activity by increasing the transcription of *htrA*. To examine HtrA protein levels, a polyclonal antibody against HtrA was prepared, and protein extracts from wild-type and *htrA*(*S197A*) cells were analyzed by immunoblotting. Two bands were detected in the wild type by the HtrA antibody: one band corresponding in size to the HtrA monomer (full-length HtrA) and one band corresponding in size to a shorter fragment of HtrA (s-HtrA) (Fig. 6B). Both bands were identified as HtrA by mass spectrometry of the purified wild-type HtrA (data not shown). In contrast, only the full-length HtrA band was detected in protein extracts from *htrA*(*S197A*) cells (Fig. 6B), and no bands were detected in the Δ*htrA* mutant (data not shown). Furthermore, when purified wild-type HtrA was incubated alone at either 37°C or 45°C, a steady increase in the amount of the short HtrA fragment was observed over the course of 3 h (data not shown). These observations imply that the short HtrA fragment is an autocleavage product, a feature also observed for the *E. coli* HtrA ortholog (27, 62). The amounts of full-length HtrA in the wild type and the *htrA*(*S197A*) mutant were identical, as measured by densitometry; however, the wild type contained a substantial

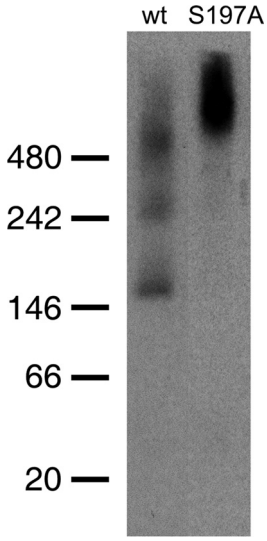

FIG. 7. Visualization of HtrA oligomers in exponential-phase-growing *C. jejuni* NCTC11168 (wt) and KB1025 [*htrA*(*S197A*); S197A] by blue native PAGE followed by immunoblotting with HtrA antibody. The positions of molecular size standards (NativeMark; Invitrogen) are indicated on the left (in kilodaltons).

amount of s-HtrA (1.2-fold the amount of full-length HtrA) (Fig. 6B). Since $HtrA_{S197A}$ and s-HtrA were equally stable for several cell generations (10 h [data not shown]), this indicates that, overall, more HtrA is produced in the wild type than in the *htrA*(*S197A*) mutant.

Purified HtrA from *E. coli* organizes into large 12- and 24-mers upon binding of substrate and reverts to a hexameric state after substrate degradation is complete (32). We analyzed native protein extracts from the wild type and the *htrA*(*S197A*) mutant by immunoblotting to investigate whether such HtrA oligomers were present in *C. jejuni* (Fig. 7). HtrA from the wild type was found as oligomers corresponding in size to trimers, hexamers, and 12-mers, while HtrA from the *htrA*(*S197A*) mutant was found only as larger oligomers. This result shows that the HtrA oligomerization observed *in vitro* also takes place *in vivo* and that disassembly of the large oligomers depends on HtrA protease activity.

DISCUSSION

C. jejuni encodes the periplasmic chaperones HtrA and SurA for folding of periplasmic and outer membrane proteins. HtrA is a conserved protein that contains both protease and chaperone activities, and we previously showed that HtrA is essential for growth of *C. jejuni* in a near-aerobic environment and for prevention of accumulation of misfolded proteins caused by heat (6). In this study, we examined the *in vitro* proteolytic and chaperone activities of HtrA and furthermore explored how these activities contribute to *C. jejuni* stress tolerance *in vivo* by analysis of a defined *htrA* mutant that lacks proteolytic activity.

We show that purified HtrA of *C. jejuni* possesses both serine protease and molecular chaperone activities in the entire temperature growth range of *C. jejuni*. Consistent with findings in *E. coli* (63), the protease activity of *C. jejuni* HtrA is more efficient at high temperature, while the chaperone activity of $HtrA_{S197A}$ is slightly reduced at 44°C. Interestingly, we found that chaperone activity alone is sufficient for growth of *C. jejuni* at high temperature, while *E. coli* and *Salmonella* require plasmid-mediated overexpression of protease-negative HtrA to suppress the temperature-sensitive phenotype of an *htrA* deletion mutant (34, 61), suggesting that the chaperone activity of *C. jejuni* is more efficient. Interestingly, only when the temperature and oxygen stresses are combined is protease activity essential for growth of *C. jejuni.* This observation suggests that severe stress increases the rate of protein damage to a point at which chaperone activity is not sufficient to prevent accumulation of toxic protein aggregates and that, therefore, proteolytic activity is needed to remove the damaged proteins from the HtrA oligomer. However, the observation that the chaperone activity is slightly less efficient in preventing aggregation at 44°C *in vitro* may also explain the requirement for protease activity during severe stress. It is noteworthy that HtrA protease activity is required for growth of *C. jejuni* only under rather extreme conditions, as 44°C is only 1°C below the upper temperature limit for growth of the wild type (16, 59) and 18% oxygen is far from the microaerobic environment ideal for *C. jejuni* (5). Consequently, HtrA chaperone activity may be sufficient to support growth of *C. jejuni* in the gastrointestinal tract of both avian and human hosts, due to the temperatures (42°C and 37°C, respectively) and the low oxygen concentration found in these environments (17).

Interestingly, our analyses of the requirement of HtrA for stress tolerance of *C. jejuni* showed that heat sensitivity of the *htrA* mutant depends on the level of oxidative stress, demonstrating that protein damage caused by increased temperature *per se* does not harm the bacterium enough to require HtrA for degradation or repair. While loss of *htrA* has been shown to cause sensitivity to high temperature in all examined bacteria (9, 11, 35, 41, 48), conditional heat sensitivity has not previously been reported, but revisiting the phenotypes of these mutants will reveal whether oxidative-stress-dependent thermosensitivity is a general feature of *htrA* mutants.

The data presented here show that HtrA plays a central role in protecting *C. jejuni* during oxidative stress, since an *htrA* mutant has increased sensitivity to oxygen, H_2O_2, cumene hydroperoxide, and paraquat. Brøndsted et al. previously reported no difference in sensitivity between the wild type and the Δ*htrA* mutant in a similar disc diffusion assay at 37°C (6). However, the present data show that the sensitivity to these oxidants is most pronounced at 42°C, which may explain the discrepancy between the results. Reactive oxygen species can be generated by oxidation of growth medium constituents (7, 19) or by cellular respiration (65). The oxygen-sensitive phenotypes of the *htrA* mutants are mediated partly by H_2O_2 or O_2^- generated in the growth medium. However, H_2O_2 or O_2^- scavengers fail to rescue growth of the Δ*htrA* mutant at 44°C and 18% O_2, either because they cannot quench H_2O_2 and O_2^- completely or because HtrA also protects against reactive oxygen species arising from cellular respiration. The latter explanation seems likely, since *C. jejuni* encodes a cytochrome bc_1 complex (47), which is a possible source of O_2^- in the periplasm (43), and a formate dehydrogenase, which generates periplasmic H_2O_2 (13, 19). HtrA may act in the oxidative stress defense of *C. jejuni* by degradation or refolding of oxidatively

damaged proteins or, alternatively, by assisting the folding of periplasmic proteins that are part of the oxidative stress defense, such as the cytochrome *c* peroxidases (4, 47). Indeed, analysis of carbonylated surface proteins indicates that degradation of oxidatively damaged proteins by HtrA is limited, since the levels and the band patterns of carbonylated proteins were almost identical in the wild type and the *htrA*(*S197A*) mutant. In contrast, several carbonylated protein bands were absent in the Δ*htrA* mutant, indicating that HtrA may capture oxidatively damaged proteins and thereby protect them from degradation by other proteases. A similar mechanism has been demonstrated *in vitro* with *E. coli* HtrA, which is able to protect a substrate from degradation by an added protease (32). Our data suggest that this role of HtrA is physiologically important *in vivo*, and we propose that HtrA may be responsible for rescuing oxidatively damaged proteins in the periplasm.

Our data suggest that both the chaperone and protease activities of HtrA are essential for maintaining periplasmic protein homeostasis in *C. jejuni* even under optimal growth conditions, which is indicated by increased amounts of DnaK and ClpB in the *htrA* mutants. Thus, ClpB and DnaK are upregulated in response to even low rates of protein misfolding occurring in the *htrA*(*S197A*) mutant at 42°C, while the growth defect is observed only under stress conditions that increase the rate of protein misfolding. In *E. coli* and *Shigella flexneri*, the HtrA chaperone is important for folding of outer membrane proteins and ensures their safe transit across the periplasmic space (32, 51). A similar function in *C. jejuni* would explain the need for HtrA chaperone activity under all growth conditions; however, so far we have not been able to determine if such a function applies to HtrA in *C. jejuni.* The proteolytic HtrA activity affects the structural arrangement of HtrA oligomers found in *C. jejuni*, as the wild type and the *htrA*(*S197A*) mutant contain large HtrA oligomers while the smaller and presumably resting oligomers are present only in the wild type. Consistent with this finding, the turnover of the active *E. coli* HtrA multimers into the resting hexameric conformation takes place after a substrate has been degraded (32). These findings suggest that proteolytically inactive HtrA may become occupied with unfoldable proteins. We therefore propose that the role of HtrA-mediated proteolysis under nonstress conditions in *C. jejuni* is simply to empty the HtrA cavity of unfoldable proteins, which otherwise hinder the proper folding of periplasmic proteins or the transfer of outer membrane proteins across the periplasmic space.

The proteolytic activity of HtrA also mediates autodegradation, which was observed *in vivo* under all tested conditions (37 to 44°C, 1% to 18% O_2), as well as *in vitro*, with purified wild-type HtrA. In contrast, HtrA autodegradation occurs only under reducing conditions in *E. coli*, most likely because *E. coli* HtrA is stabilized by a disulfide bridge in the main regulatory loop (62). However, *C. jejuni* HtrA contains no disulfide bridge, supporting our observation that this protein is more prone to autodegradation. Our results furthermore indicate that the proteolytic activity may be important for regulation of HtrA expression, since, overall, more HtrA is produced in the wild type than in the *htrA*(*S197A*) mutant. However, the increased HtrA production is not controlled at the transcriptional level, as the amounts of *htrA* mRNA are similar in the wild type and the *htrA*(*S197A*) mutant. Expression studies using microarrays have shown that transcription of *htrA* in *C. jejuni* is induced moderately by heat (64) but downregulated in a low-oxygen environment (68). However, it is not known how the synthesis of HtrA is controlled in *C. jejuni*, as the genome does not encode homologs of the σ^E sensing system for extracytoplasmic stress, which regulates periplasmic chaperones in *E. coli* and *Salmonella* (6, 52). In particular, *C. jejuni* does not encode the HtrA paralog DegS, which cleaves an inner membrane protein during stress and initiates a signal cascade resulting in increased *htrA* expression in *E. coli*. It is tempting to speculate that, in the absence of a DegS-like protein, *C. jejuni* employs another mechanism to sense periplasmic stress that combines the regulatory proteolysis and the quality control proteolysis in the same protein. Future work will show whether *C. jejuni* HtrA is involved in such a regulatory mechanism.

C. jejuni is dependent on the HtrA and SurA chaperones to fold outer membrane proteins and maintain periplasmic protein homeostasis important for tolerating stress, since it lacks homologs of the Skp and FkpA chaperones (47) found in other Gram-negative bacteria (2, 60). While SurA does not possess protease activity, the chaperone activities of SurA and HtrA may functionally overlap in *C. jejuni*, as HtrA is upregulated in a *surA* mutant (3). SurA facilitates outer membrane protein biogenesis in *E. coli* (60), and consistent with this function, the outer membrane profile of *C. jejuni* is altered by a *surA* mutation (54). However, HtrA may have a more dominant role than SurA in maintaining periplasmic protein homeostasis, since a lack of SurA does not result in upregulation of DnaK and ClpB (53). Thus, both HtrA and SurA chaperone activities may be involved in folding of envelope proteins in *C. jejuni*, but the mechanism that determines the substrate specificity is so far unknown. However, HtrA may specifically be involved in degradation and folding of heat-damaged proteins, since expression data indicate that only HtrA is important for heat tolerance of *C. jejuni*, as *surA* is downregulated at high temperature (53), while *htrA* is upregulated (64). Thus, in summary, we here present data showing that the HtrA chaperone activity alone is sufficient to protect against either heat or oxidative stress in *C. jejuni*, while the protease activity of HtrA is essential only under severe stress conditions. Finally, other chaperones display additional enzymatic activity (40, 56), and for HtrA, the proteolytic activity may very well be crucial for the overall performance of HtrA as a chaperone.

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