Amino-Terminal Processing of Helicobacter pylori Serine Protease HtrA: Role in Oligomerization and Activity Regulation

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The HtrA family of serine proteases is found in most bacteria, and plays an essential role in the virulence of the gastric pathogen Helicobacter pylori. Secreted H. pylori HtrA (HtrAHp) cleaves various junctional proteins such as E-cadherin disrupting the epithelial barrier, which is crucial for bacterial transmigration across the polarized epithelium. Recent studies indicated the presence of two characteristic HtrAHp forms of 55 and 52 kDa (termed p55 and p52, respectively), in worldwide strains. In addition, p55 and p52 were produced by recombinant HtrAHp, indicating auto-cleavage. However, the cleavage sites and their functional importance are yet unclear. Here, we determined the amino-terminal ends of p55 and p52 by Edman sequencing. Two proteolytic cleavage sites were identified (H46/D47 and K50/D51). Remarkably, the cleavage site sequences are conserved in HtrAHp from worldwide isolates, but not in other Gram-negative pathogens, suggesting a highly specific assignment in H. pylori. We analyzed the role of the amino-terminal cleavage sites on activity, secretion and function of HtrAHp. Three-dimensional modeling suggested a trimeric structure and a role of amino-terminal processing in oligomerization and regulation of proteolytic activity of HtrAHp. Furthermore, point and deletion mutants of these processing sites were generated in the recently reported Campylobacter jejuni ΔhtrA/htrAHp genetic complementation system and the minimal sequence requirements for processing were determined. Polarized Caco-2 epithelial cells were infected with these strains and analyzed by immunofluorescence microscopy. The results indicated that HtrAHp processing strongly affected the ability of the protease to disrupt the E-cadherin-based cell-to-cell junctions. Casein zymography confirmed that the amino-terminal region is required for maintaining the proteolytic activity of HtrAHp. Furthermore, we demonstrated that this cleavage influences the secretion of HtrAHp in the extracellular space as an important prerequisite for its virulence activity. Taken together, our data demonstrate that amino-terminal cleavage of HtrAHp is conserved in this pathogen and affects oligomerization and thus, secretion and regulatory activities, suggesting an important role in the pathogenesis of H. pylori.

Keywords: C. jejuni, H. pylori, HtrA, secretion, chaperone, E-cadherin, molecular pathogenesis, virulence
INTRODUCTION

Infection by the Gram-negative pathogen *Helicobacter pylori* can cause chronic, mostly asymptomatic, gastritis, whereas more severe gastric diseases including adenocarcinoma, mucosa-associated lymphoid tissue (MALT) lymphoma and peptic ulceration occur less often (Polk and Peek, 2010). In fact, about half of the human world population carries these bacteria, and the majority of patients are colonized persistently by *H. pylori* (Pachathundikandi et al., 2016; Smolka and Schubert, 2017). The interplay between several bacterial, host and environmental factors is crucial for the virulence of the pathogen, largely influencing the clinical outcome of an infection (Servetas et al., 2016; Figueiredo et al., 2017; Gobert and Wilson, 2017; Shimizu et al., 2017). Besides the two well-known bacterial pathogenicity factors, the vacuolating toxin (VacA) associated with cellular vacuolation, apoptosis or immune cell inhibition, the cytotoxin-associated genes pathogenicity island (cagPAI), which encodes a type IV secretion system (T4SS) for the delivery of the CagA protein across the bacterial membrane into the host cell, additional *H. pylori* determinants are important for virulence (Backert and Clyne, 2011; Bridge and Merrell, 2013; Naumann et al., 2017). The FlaA protein, which is involved in flagella-mediated motility, the urease being essential for neutralizing the acidic pH in the human stomach and inflammasome activation, as well as various adhesins are important determinants for the virulence of *H. pylori* (Salama et al., 2013; Koch et al., 2015; Pachathundikandi et al., 2015; Javaheri et al., 2016; Bugaytssova et al., 2017). Furthermore, the serine protease high-temperature requirement A protein of *H. pylori* (HtrAHp) was recently identified as a novel secreted virulence factor, which can target various host cell surface proteins (Hoy et al., 2010, 2012; Backert et al., 2016; Schmidt et al., 2016a,b; Tegtmeyer et al., 2016, 2017).

In many bacterial pathogens, HtrA proteases are widely conserved and play an important role in the virulence and survival of microbes under stress conditions (Hoy et al., 2013; Backert et al., 2017; Posselt et al., 2017; Skorko-Glonek et al., 2017). In addition to the protease function, HtrA acts as a chaperone being responsible for protein quality control and degradation of misfolded proteins in the periplasm (Kim and Kim, 2005; Hoy et al., 2013), suggesting that HtrA is highly active under harsh conditions (Hoy et al., 2013). In Gram-negative bacteria, HtrA proteases are actively transported in the periplasmic space and form proteolytic active oligomers (Singh et al., 2011; Skorko-Glonek et al., 2017). However, the mature form of HtrA in *E. coli* (HtrAEc; 48 kDa) produces two 43 kDa polypeptides by partial auto-cleavage. This process seems to be positively stimulated under reducing conditions by substrates or peptides resulting from degraded HtrA products (Skorko-Glonek et al., 2003; Jomaa et al., 2009). Recent studies have shown that HtrA from the gastrointestinal pathogens *H. pylori* and *Campylobacter jejuni* can be actively secreted into the extracellular space (Löwer et al., 2008; Hoy et al., 2010, 2012; Boehm et al., 2012; Abfalter et al., 2016; Schmid et al., 2016b; Wessler and Backert, 2018). For *H. pylori* and *C. jejuni* it was demonstrated that secreted and recombinant HtrA can cleave various host cell proteins such as the ectodomain of the cell-to-cell adhesion protein E-cadherin (Hoy et al., 2010, 2012; Boehm et al., 2012; Schmidt et al., 2016b). In addition, HtrAHp cleaves fibronectin (Hoy et al., 2010) and the tight junction proteins occludin and claudin-8 (Tegtmeyer et al., 2017). In case of E-cadherin, cleaving-off the extracellular domain by HtrA can open the cell-to-cell junctions in the host cell monolayer and, thus, promote paracellular transmigration of both bacterial pathogens (Boehm et al., 2012; Hoy et al., 2012). By infecting polarized Caco-2 or MKN-28 cells, respectively, we were able to show that overexpression of HtrAHp in *H. pylori* led to an increased damage of cell-to-cell junctions and significant decreased TER-values over time (Harrer et al., 2017). In addition, the transepithelial migration by *H. pylori* was elevated as a result of increased HtrA expression (Harrer et al., 2017). Moreover, a crucial role of HtrA in the immunopathology and induction of host cell apoptosis in the gut was demonstrated by infecting mice with *C. jejuni* (Heimesaat et al., 2014a,b). For *H. pylori* it was shown that an elevated expression of HtrA is associated with an increased proteolytic activity that led to a positive regulation of T4SS-dependent translocation and phosphorylation of CagA in epithelial cells (Harrer et al., 2017). Furthermore, the generation of htrA knockout mutants for about hundred worldwide *H. pylori* isolates failed so far, suggesting an outstanding importance of HtrAHp for yet unknown cellular processes of *H. pylori* (Salama et al., 2004; Hoy et al., 2010; Tegtmeyer et al., 2016). In line with these observations, we have demonstrated that specific pharmacological inhibition of the HtrA protease activity killed *H. pylori* effectively, whereas the growth of other pathogens such as *Salmonella* or *Shigella* was not affected (Tegtmeyer et al., 2016).

Generally, HtrA proteins are composed of an amino-terminal signal peptide, which is followed by the trypsin-like protease domain and finally one or two carboxy-terminal PDZ domains (Gottseman et al., 1997; Frees et al., 2013). Three HtrA homologs, DegS, DegQ, and DegP, are expressed by *E. coli* representing the best-characterized HtrA model systems (Singh et al., 2011; Skorko-Glonek et al., 2013). While DegP works as an ATP-independent chaperone protease, DegS is characterized as a regulatory protease (Bass et al., 1996; Ruiz and Silhavy, 2005; Jiang et al., 2008). However, DegQ functions as a pH-related protein and chaperone, which is involved in protein quality control. Thus, this protease plays a very important role under acid stress conditions (Bai et al., 2011; Sawa et al., 2011; Malet et al., 2012). We have shown that the htrA gene is present in more than one thousand *H. pylori* strains from Europe, Asia, North and South America as well as Australia, and the HtrAHp protein sequence is conserved within these isolates (Tegtmeyer et al., 2016). Analysis of bacterial lysates by Western blotting indicated that HtrAHp is expressed as a double-band with molecular weights of 52 kDa (p52) and 55 kDa (p55), respectively. In addition, recombinant HtrAHp showed this characteristic double-band, suggesting processing of the protein (Tegtmeyer et al., 2016). Here, we aimed to investigate the function of HtrAHp processing and to identify the involved protease. Even though active secretion of HtrAHp in the extracellular space might be conserved among various *H. pylori* isolates, the underlying
mechanism is currently not understood (Tegtmeier et al., 2016). Thus, we also investigated the role of HtrA<sup>Hp</sup> processing during protein secretion and proteolytic activity regulation.

### MATERIALS AND METHODS

**H. pylori, E. coli, and C. jejuni Growth**

*Helicobacter pylori* strains 35A, 26695, and P12 were used for Edman sequencing. Generally, *H. pylori* was grown at 37°C for 48–72 h on GC agar (Oxoid, Wesel/Germany) plates supplemented with 10% donor horse serum (Biochrom AG, Berlin/Germany), 1% vitamin mix, 10 μg/mL vancomycin, 1 μg/mL nystatin, and 5 μg/mL trimethoprim (Kumar Pachathundikandi et al., 2011; Wiedemann et al., 2012). The *C. jejuni ΔhtrA/htrA<sup>Hp</sup>* complementation system was applied to mutagenize the amino-terminal cleavage sites of HtrA<sup>Hp</sup> (Boehm et al., 2017). The corresponding isogenic knockout mutant *C. jejuni* 81–176ΔhtrA was characterized earlier (Brendsted et al., 2005; Baek et al., 2011; Boehm et al., 2012). Commonly, the *C. jejuni* strains were grown at 37°C for 48–72 h on Campylobacter blood-free selective agar base containing selective supplement. If required for mutants, 20 μg/mL of chloramphenicol and/or 50 μg/mL kanamycin were added. All antibiotics were obtained from Sigma-Aldrich (St. Louis, MO/United States). Both *C. jejuni* and *H. pylori* were cultured under microaerobic conditions produced by CampyGen packs in 2.5 L anaerobic jars (Oxoid) (Patel et al., 2013; Tenguria et al., 2014). Chemically competent One Shot™ TOP-10 *E. coli* (Invitrogen, Darmstadt/Germany) were grown in LB broth medium consisting of 10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl.

**Amino-Terminal Sequencing of *H. pylori* HtrA**

Edman sequencing of proteins was performed on blotted proteins on a PVDF membrane using the ABI Procise 494 sequencer. HtrA fragments were eluted and amino-terminal sequencing done by Alphalyse A/S (Odense/Denmark).

**Mutagenesis of *H. pylori* htrA and Genetic Complementation in *C. jejuni***

For expression of the amino-terminal tagged GST-fusion protein, the pGEX-6P-1 plasmid encoding the HtrA<sup>Hp</sup> of strain 26695 (without signal peptide) was applied in this study (Lower et al., 2008). This construct was a kind gift from Silja Wessler (University of Salzburg/Austria) and used as template for the generation of amino-terminal deleted variants of *H. pylori* 26695 HtrA. The mutagenesis PCR was performed using Platinum<sup>™</sup> Taq DNA Polymerase High Fidelity (High Fidelity buffer, Thermo Fisher Scientific, Darmstadt/Germany) with 400 ng template DNA following the manufacturer’s instructions. Primer pairs J/L or K/L were used for the construction of ΔN1 or ΔN2 mutants, respectively (Table 1). For amplification, an initial denaturation for 2 min at 94°C, followed by 30 cycles consisting of denaturation at 94°C for 30 s, annealing at 63°C for 30 s, and elongation at 68°C for 90 s was performed. Then the PCR products (flanked by EcoRI/BamHI restriction sites) were digested by the corresponding enzymes (NEB, Frankfurt/Main/Germany), ligated into pGEX-6P-1 using T4 DNA Ligase (Promega, Mannheim/Germany) and transformed in One Shot™ TOP-10 *E. coli* strain following manufacturer’s protocol. To prove the correct integration of the amino-terminal deletions, the plasmids were sequenced by GATC Biotech (Konstanz/Germany). The constructs were used for protein overexpression in *E. coli* BL21.

**HtrA Secretion Assay**

For secretion of HtrA proteins into the supernatant, *C. jejuni* wt, isogenic ΔhtrA deletion mutant and ΔhtrA/htrA<sup>Hp</sup> variants were suspended in BHI medium (Oxoid) supplemented with the mutant-specific antibiotics or without, respectively. For monitoring the secretion of HtrA, the bacteria were grown starting at OD<sub>600nm</sub> = 0.3 and shaking at 160 rpm. The culture reached an OD<sub>600nm</sub> ≥ 0.8 and 0.9, the cellular and secreted proteins were separated by centrifugation at 1,500 × g for 10 min. The supernatants were again centrifuged for 15 min at 17,000 × g to remove cell debris and sterile filtered (Brisslert et al., 2005; Boehm et al., 2012). Bacterial pellets (cellular proteins) and supernatants (secreted proteins) were used for Western Blotting.
or casein zymography, respectively. All secretion assays were done at least in triplicates.

**Antibodies**

Polyclonal antibodies against *C. jejuni* HtrA and CiaB were characterized previously (Boehm et al., 2015). Moreover, we generated a polyclonal antibody against HtrA<sub>Hp</sub> using the recombinant protein as antigen. Two rabbits each were immunized using standard protocols by BioGenes GmbH (Berlin/Germany). Immunization was carried out in accordance with German Tierschutzgesetz and Tierschutz-Versuchsverordnung as implementation of the EU directive 2010/63/EU. The protocol was registered and approved by Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei Mecklenburg-Vorpommern (LALLF M-V, Rostock/Germany). All sera were affinity-purified (BioGenes GmbH). The specificity against HtrA<sub>Hp</sub> and cross-reactivity with *C. jejuni* HtrA (HtrA<sup>G</sup>) were verified by Western Blotting (Boehm et al., 2017). For detection of GST, the goat polyclonal antibody against GST was applied (GE Healthcare, Freiburg/Germany).

**Overexpression of HtrA in E. coli**

For the overexpression of the GST-tagged *H. pylori* 26695 HtrA variants, the constructs (see mutagenesis of *H. pylori* htrA) have been transformed into *E. coli* BL21 (NEB). Briefly, the overexpression was induced by 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) as described (Löwer et al., 2008). After 4 h induction, the bacteria were harvested at 1,500 g for 15 min. The protein overexpression was analyzed by standard Coomassie staining and Western Blotting.

**SDS–PAGE and Western Blotting**

Bacterial cell pellets were adjusted to equal amounts by adding 1× SDS buffer consisting of 62.6 mM Tris–HCl, 2% sodium dodecyl sulfate, 0.01% bromophenol blue, 5% glycerin and reducing agent (Thermo Fisher Scientific), while the supernatants were mixed with 4× SDS buffer. Prior to the separation by SDS–PAGE on 10% polyacrylamide gels, the protein samples were boiled for 10 min at 94°C (Moose et al., 2001). The separated proteins were then analyzed by staining with Coomassie Brilliant Blue R-250 (Bio-Rad, Munich/Germany). For Western Blotting, the proteins were blotted onto PVDF membranes (Immobilon-P, Merck Millipore, Darmstadt/Germany) and blocked in TBS-T buffer (pH 7.4, 0.2 M Tris, 1.4 M sodium chloride and 1% Tween-20) containing 5% milk powder for 1 h at room temperature or at 4°C overnight (Boehm et al., 2011). After addition of the primary antibodies for 2 h at room temperature, horseradish peroxidase-conjugates anti-rabbit polyclonal sheep immunoglobulin was applied as secondary antibody (Zhang et al., 2015). Antibody detection was performed using 1.41 mM luminol in 0.1 Tris–HCl (pH 6.8) supplemented with 0.61 mM p-coumaric acid solved in DMSO and 0.02% hydrogen peroxide. Unless otherwise indicated, all chemicals were obtained from Carl Roth (Karlsruhe/Germany).

**Casein Zymography**

Bacterial cell pellets were adjusted to equal amounts by adding phosphate buffered saline and separated in polyacrylamide gels supplemented with 0.1% casein. The gels were renatured in 2.5% Triton-X-100 and equilibrated in developing buffer as described previously (Hoy et al., 2010, 2012). To maintain proteolytic cleavage of casein, the gels were incubated in developing buffer at 37°C overnight. Finally, the caseinolytic activity was visualized using 0.5% Coomassie Brilliant Blue R-250 (Bio-Rad).

**Quantification of Signals in Western Blotting**

To investigate the protein expression, band signals on immunoblots were quantified using the Image Lab software (Bio-Rad). The control band was set as 1 and differences are shown as relative amounts of secreted HtrA<sub>Hp</sub>. For statistical comparison, Mann–Whitney test using GraphPad Prism 5.01 Software (La Jolla, CA/United States) was performed. Statistical significance was defined as *p < 0.05.

**Immunofluorescence Staining**

Polarized Caco-2 cells (ATCC HTB-37) obtained from human colon adenocarcinoma were cultured in RPMI-1640 medium (Invitrogen, Karlsruhe/Germany). The cells were seeded at a concentration of 1.0 × 10<sup>3</sup> cells in 12-well plates and grown for 72 h. For infection, *C. jejuni* isolates were harvested in BHI broth and incubated at 37°C overnight (Boehm et al., 2011). After addition of the primary antibodies for 2 h at room temperature, horseradish peroxidase-conjugates anti-rabbit polyclonal sheep immunoglobulin was applied as secondary antibody (Zhang et al., 2015). Antibody detection was performed using 1.41 mM luminol in 0.1 Tris–HCl (pH 6.8) supplemented with 0.61 mM p-coumaric acid solved in DMSO and 0.02% hydrogen peroxide. Unless otherwise indicated, all chemicals were obtained from Carl Roth (Karlsruhe/Germany).

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**TABLE 1 | Primer sequences used for mutagenesis PCR.**

| Primer | Number | Mutation | Sequence (5′-3′) |
|--------|--------|----------|-----------------|
| A      | 822F2  | H46A/D47A| TAC GCC GCT TCT ATT AAG GAT TCG A |
|        | 822R   | H46A/D47A| AGA ATA GAT CGT ATC TTT AG |
| C      | 823F2  | K50A/D51A| ATT GGC GCT TGG ATT AAA GCG GTG |
|        | 823R   | K50A/D51A| GCT AGA TAA GAA TGG TGC TAA GA |
| E      | 924F   | H46A/D47A/K50A/D51A | ATT GGC GCT TGG ATT AAA GCG GTG GTG |
| F      | 924R   | H46A/D47A/K50A/D51A | AGA AGC GGC GTA AGA ATA GAT GCT ATG C |
| G      | 939F1  | ΔN2      | GAT TAA AGC GGT GAT GAA TAT CAC TAC TG |
|        | 939R   | ΔN2      | GAT GCA GCA ATT AAA GCA CTT GCT AAA C |
| J      | 935F   | pGEX_ΔN1 | CCG CTG GCA TCC TCT ATT AAG GAC GC |
| K      | 935F1  | pGEX_ΔN2 | CCG CTG GGA TCC TCT ATT AAG GGC GTG |
| L      | 935R   | pGEX_ΔN1/2| CQA CCC GGG AAT TGT CAT TTC ACC AAA ATG |
medium and the bacterial numbers were determined as optical density (OD) at 600 nm using a spectrophotometer (Eppendorf, Hamburg/Germany). Infections were performed at a multiplicity of infection (MOI) of 50. Twenty four hours post-infection, the cells were fixed and subjected to immunofluorescence staining as described (Harrer et al., 2017). Antibodies against the extracellular domain of E-cadherin (CD324; BD Biosciences, San Jose, CA/United States) and C. jejuni (Dako, Glostrup/Denmark) were applied. As secondary antibodies, TRITC (tetramethylrhodamine isothiocyanate)-conjugated goat anti-rabbit and FITC (fluorescein isothiocyanate)-conjugated goat anti-mouse (Thermo Fisher Scientific, Darmstadt/Germany) were used. The specimens were analyzed by the Leica DM4000B fluorescence microscope with different lasers (Leica Microsystems, Wetzlar/Germany). The images were obtained by LAS AF computer software (Leica Microsystems, Wetzlar/Germany) and optimized in contrast and brightness using Image J-win64 version 2.0. For each condition, representative images are shown.

Three-Dimensional Modeling of HtrA
The structure of trimeric HtrA<sup>Hp</sup> was modeled using the homologous structure DegS from <i>E. coli</i> (PDB: 4RQY; de Regt et al., 2015). The homologous region (37% sequence identity; 67% sequence similarity) covers the amino acid residues 36–369 of HtrA<sup>Hp</sup>, with the exception of the residues 69–93 that have no equivalent in the template structure. Therefore, modeling was restricted to amino acid residues 36–68 and 94–369. Modeller 9.16 (Webb and Sali, 2014) was used for modeling and RasMol for structure analysis and visualization (Sayle and Milner-White, 1995). The quality of the model was assessed using RAMPAGE (Lovell et al., 2003) and WHATCHECK (Hooft et al., 1996). The resulting model exhibited a good backbone geometry (98% of all residues in the favored regions of the Ramachandran Plot) and no steric clashes larger 0.35 Å were observed.

RESULTS

Identifying Amino-Terminal Cleavage Sites in HtrA of <i>H. pylori</i>
Analysis of HtrA<sup>Hp</sup> protein expression in various <i>H. pylori</i> strains revealed that the monomer formed a double-band under reducing conditions, called p55 and p52 (Tegtmeier et al., 2016). To identify the cleavage sites in HtrA<sup>Hp</sup>, protein samples of <i>H. pylori</i> strains 35A, 26695, and P12 were subjected to Edman sequencing. Besides the predicted signal peptide, we identified additional amino-terminal cleavage sites between the amino acids at positions H46/D47 or K50/D51, respectively, giving rise to the p55 and p52 HtrA<sup>Hp</sup> forms (Figure 1A). The characteristic HtrA<sup>Hp</sup> double-band was also seen after purification of recombinant HtrA<sup>Hp</sup> from <i>E. coli</i> (Tegtmeier et al., 2016), suggesting auto-processing of the protease.

Next, we investigated the conservation of these amino-terminal cleavage sites in HtrA<sup>Hp</sup> from <i>H. pylori</i> isolates collected in various geographical areas of the world. For this purpose, HtrA<sup>Hp</sup> protein sequences of several <i>H. pylori</i> strains with African, European, Asian, American, and Australian origin were aligned using Clustal Omega (McWilliam et al., 2013). We found that the processing sites between the amino acid positions H46/D47 and K50/D51 are highly conserved within all investigated <i>H. pylori</i> isolates (Figure 1B). Moreover, multiple sequence alignment of HtrA<sup>Hp</sup> with HtrA protein sequence from several other Gram-negative gastrointestinal pathogens revealed that the identified cleavage sites are present in <i>H. pylori</i> and <i>C. jejuni</i>, but not in other Gram-negative pathogens including Escherichia, Salmonella, Shigella, Yersinia, and Vibrio species (Figure 1C). These results led us to propose that the highly conserved amino-terminal processing sites of HtrA<sup>Hp</sup> might have an important function in <i>H. pylori</i>.

Amino-Terminal Cleavage Affects the Secretion of HtrA<sup>Hp</sup>
To investigate a potential regulatory role of the amino-terminal auto-processing sites on HtrA<sup>Hp</sup> secretion, we next studied the involved sequences using the SignalP and SecretomeP databases, predicting a possible secretion based on the signal peptide (Signal P) or a non-classical secretion (SecretomeP), respectively ( Bendtsen et al., 2005; Petersen et al., 2011). Using the modified amino acid sequence of HtrA<sup>Hp</sup>, we obtained that mutation of the amino-terminal cleavage sites has no effect on the protein secretion (Table 2).

HtrA<sup>Hp</sup> is an essential gene in <i>H. pylori</i> and cannot be mutagenized (Salama et al., 2004; Tegtmeier et al., 2016). We therefore applied the recently established genetic complementation system in <i>C. jejuni</i>, where we can express the htrA gene of <i>H. pylori</i> strain G27 (<i>C. jejuni</i>ΔhtrA/htrA<sup>Hp</sup>) to investigate the importance of the above identified amino-terminal cleavage sites on HtrA<sup>Hp</sup> secretion experimentally (Boehm et al., 2017). For this purpose, various point and deletion mutations of the amino-terminal cleavage sites were generated (Supplementary Figure 1A). The mutated htrA<sup>Hp</sup> gene variants were then transformed into the ΔhtrA deletion mutant of <i>C. jejuni</i> strain 81–176. Correct integration of modified htrA<sup>Hp</sup> in the <i>C. jejuni</i> 81–176 chromosome was confirmed by PCR and standard sequencing (data not shown). Expression of the modified HtrA<sup>Hp</sup> proteins in <i>C. jejuni</i> was verified by immunoblotting.

To investigate if the amino-terminal cleavage sites have an effect on the protein secretion, the above-described HtrA<sup>Hp</sup> variants were grown in BHI liquid broth medium. Secreted and cellular proteins were analyzed by immunoblotting using an antibody against HtrA<sup>Hp</sup> (Figures 2A,B). All corresponding mutants showed a strong and similar expression of HtrA<sup>Hp</sup>, while the ΔhtrA deletion mutant and the <i>C. jejuni</i> 81–176 wild-type (wt) did not (Figure 2A). Hence, for the ΔhtrA mutant no secretion was noted, whereas the ΔhtrA/htrA<sup>Hp</sup> revealed profound HtrA<sup>Hp</sup> secretion as expected (Figure 2B). However, the wt complementant and variants with only one mutated amino-terminal cleavage site (HtrA<sup>Hp</sup> H46A/D47A or HtrA<sup>Hp</sup> K50A/D51A) exhibited strong HtrA<sup>Hp</sup> secretion levels, while mutation of both cleavage sites resulted in decreased HtrA<sup>Hp</sup> secretion (Figure 2B). An HtrA<sup>Hp</sup> variant missing the entire
The p55 and p52 forms of HtrA\textsubscript{Hp} are generated by amino-terminal cleavage and are conserved in worldwide \textit{H. pylori} strains. (A) The domain structures of full-length (FL), p55 and p52 HtrAs are shown. The amino-terminal auto-processing sites, which lead to the generation of p55 and p52 forms, were identified by Edman sequencing in HtrA\textsubscript{Hp} of \textit{H. pylori} strains 35A, 26695, and P12. In addition to the cleaved signal peptide (gray box), cleavage sites between H46/D47 and K50/D51 (highlighted with red and scissors), respectively, were determined. (B) The amino-terminal sequences of worldwide \textit{H. pylori} strains were aligned using Clustal Omega, showing that the cleavage sites H46/D47 and K50/D51 are highly conserved (red boxes). The exact cleavage positions are marked by arrows. (C) Furthermore, the amino-terminal protein sequence of HtrA\textsubscript{Hp} was aligned to that of HtrAs from other Gram-negative gastrointestinal pathogens, revealing that the above cleavage sites (red boxes) are unique for \textit{H. pylori} and \textit{C. jejuni}. However, for the HtrA homologs from \textit{S. enterica}, \textit{E. coli}, and \textit{S. flexneri} other amino-terminal auto-processing sites at C69/Q70 and Q82/K83 were found (marked with blue).
The HtrA
Hp
amino-terminus including both cleavage sites (HtrA
Hp
ΔN2) revealed only residual secretion levels, suggesting that the amino-terminus plays a role in the secretion, but not in the general expression of HtrA
Hp
(Figure 2B, asterisk). As control, probing of the cellular and secreted protein fractions with an antibody against HtrA
Cj
revealed strong expression and secretion for the C. jejuni 81–176 wt, but not for ΔhtrA and the ΔhtrA/htrA
Hp
mutants as presumed (Figures 2A,B). Moreover, using an antibody against FlaA, a protein known to be not secreted, we were able to show that the supernatant was free of cell debris and cellular proteins, thus excluding artificial lysis of the bacteria (Figure 2B). As FlaA is expressed in the cellular fraction of all samples equally well, we showed that similar amounts of protein were present (Figure 2A). As another control, detecting the Campylobacter invasion antigen B (CiaB) as a well-known secreted protein (O Cróinín and Backert, 2012) approved that equal amounts of secreted proteins were present, and thus, the differences in HtrA
Hp
secretion resulted from mutating the HtrA
Hp
amino-terminal cleavage sites (Figure 2B).

To quantify HtrA
Hp
protein secretion levels upon deleting the HtrA
Hp
amino-terminus, the relative amounts of secreted HtrA
Hp
were determined. Quantification of secreted HtrA
Hp
levels revealed significant lower levels for HtrA
Hp
ΔN2 compared to the ΔhtrA/htrA
Hp
wt complementant (Figure 2C), underlying the importance of the amino-terminus for HtrA
Hp
delivery into the extracellular environment.

### TABLE 2 | Amino-terminal auto-processing sites of HtrA
Hp
appear to have no effect on protein secretion.

| SignalP-value* (Signal peptide based secretion) | SecP-value* (Non-classical secretion) |
|-----------------------------------------------|--------------------------------------|
| HtrA
Hp
H46A                                      | 0.644                                 |
| HtrA
Hp
D47A                                      | 0.644                                 |
| HtrA
Hp
K50A                                      | 0.644                                 |
| HtrA
Hp
D51A                                      | 0.644                                 |
| HtrA
Hp
H46A/D47A                                 | 0.645                                 |
| HtrA
Hp
K50A/D51A                                 | 0.645                                 |
| HtrA
Hp
H46A/D47A/K50A/D51A                       | 0.645                                 |
| HtrA
Hp
ΔN1                                      | 0.623                                 |
| HtrA
Hp
ΔN2                                      | 0.659                                 |

Signal P and SecretomeP databases were used to predict a possible effect of the amino-terminal cleavage sites of HtrA
Hp
on the protein secretion. As template the modified amino acid sequence of HtrA from H. pylori G27 was applied. *SignalP-value above 0.57 indicates a possible protein secretion. *SecP-value above 0.50 indicates a possible protein secretion.

### Amino-Terminal Cleavage Is Involved in Regulating the Proteolytic Activity of HtrA
Hp

Next, we aimed to investigate if amino-terminal cleavage affects the proteolytic activity of HtrA
Hp
. To test this idea, cellular proteins of the above-described HtrA
Hp
variants expressed in the C. jejuni 81–176 ΔhtrA/htrA
Hp
complementation system were subjected to casein zymography. First of all, in

### FIGURE 2 | Helicobacter pylori HtrA auto-processing sites might affect HtrA
Hp
secretion in C. jejuni strain 81–176. Point and deletion mutations of the HtrA
Hp
amino-terminal cleavage sites (compare Supplementary Figure 1A) were generated and expressed in the ΔhtrA mutant of C. jejuni strain 81–176. In addition to the amino-terminal mutants, ΔhtrA, ΔhtrA/htrA
Hp
wt complementant and C. jejuni 81–176 wt were grown in BH1 liquid broth medium. (A) The cellular proteins were subjected to immunoblotting analyzing HtrA
Hp
. All samples revealed the expression of the HtrA
Hp
monomer as p55 or p52 forms (indicated by arrows). Detection of HtrA
Cj
and FlaA expression served as control. (B) In addition, secreted proteins were immunoblotted for the presence of HtrA
Hp
, and as control for HtrA
Cj
, FlaA, and CiaB. The HtrA
Hp
p55 or p52 forms (indicated by arrows) were found in the wt complemented and point mutant strains, while the secretion of HtrA
Hp
ΔN2 was strongly decreased (asterisk). (C) The band intensities of secreted HtrA
Hp
were quantified densitometrically and the relative amount of secreted protein is given. Significant differences were analyzed using Mann–Whitney test (*p < 0.05). All secretion assays were performed in triplicates.
C. jejuni only the p55 HtrA\textsubscript{Hp} form showed proteolytic in-gel activity, while the p52 form did not (Supplementary Figure 2). However, C. jejuni 81–176 wt showed strong caseinolytic active HtRA trimers (arrow). Moreover, for the cellular proteins of HtrA\textsubscript{Hp}ΔN2 no proteolytic activity was found and the H46A/D47A/K50A/D51A mutant was strongly reduced (yellow asterisk), while the other HtrA\textsubscript{Hp} variants carrying single point mutations in the amino-terminal cleavage sites, the wt complementant and C. jejuni wt showed strong caseinolytic active HtRA monomers. As control, no activity was detected for the Δhtra deletion mutant as expected (Supplementary Figure 2).

Together, these data suggest a role of the HtrA\textsubscript{Hp} amino-terminus in regulating/maintaining the proteolytic activity of this enzyme.

To investigate the importance of the amino-terminus on the proteolytic activity of HtrA\textsubscript{Hp} in more detail, wt HtrA\textsubscript{Hp}, ΔN1 or ΔN2 were expressed as GST-tagged variants in E. coli strain BL21 (Supplementary Figure 1B). E. coli BL21 were induced for 4 h with IPTG and the resulting bacterial lysates were subjected to Coomassie staining, confirming that equal amounts of protein were present (Figure 3A). HtrA\textsubscript{Hp} wt missing only the signal peptide (ASP) revealed a strong overexpression of the fusion protein (p55 HtrA\textsubscript{Hp} with GST-tag of ∼70 kDa) as detected by Coomassie-staining (Figure 3A, black asterisk). Moreover, a slight expression of the HtrA\textsubscript{Hp} monomer (55 kDa) without GST-tag was found (Figure 3A, arrow). Similar expression patterns were observed for the amino-terminal deletion variants of HtrA\textsubscript{Hp}, ΔN1, and ΔN2. These modified variants have only a slightly lower molecular weight compared to the wt HtrA\textsubscript{Hp} (ASP) resulting from the loss of the amino-terminus (Figure 3A, yellow asterisks). In addition, bacterial lysates were subjected to immunoblotting using antibodies against the HtrA\textsubscript{Hp} protein or GST, respectively (Figure 3B). Detection of HtrA\textsubscript{Hp} confirmed the strong overexpression of the p55 HtrA\textsubscript{Hp} monomer, with or without GST-tag (Figure 3B, arrows). Additionally, similar expression patterns were obtained for the p52 forms (Figure 3B, red asterisks). As control, E. coli BL21 without a plasmid exhibited no expression of HtrA\textsubscript{Hp} or GST-tagged protein, respectively (Figures 3A,B). Together, these findings confirmed the successful overexpression of HtrA\textsubscript{Hp} variants in E. coli BL21, useful for further analysis of HtRA activity.

As next, the above generated E. coli BL21 lysates were subjected to casein zymography to investigate an effect of the shortened amino-terminus of HtrA\textsubscript{Hp} on the proteolytic activity. Wt HtrA\textsubscript{Hp} (ASP) showed strong caseinolytic bands at about 70 and 55 kDa resulting from the fusion protein of HtrA\textsubscript{Hp} with GST-tag or the HtrA\textsubscript{Hp} protein without tag, respectively (Figure 3C, arrows). Reduced proteolytic activity was also detected for the corresponding wt p52 HtrA\textsubscript{Hp} monomers, with and without GST-tag (Figure 3C, red asterisks). In addition, caseinolytic active bands at about 210 and 165 kDa were found, corresponding to the HtrA\textsubscript{Hp} ASP trimer with or without GST tag, respectively (Figure 3C, arrows). Importantly, both HtrA\textsubscript{Hp} variants missing the amino-terminus (ΔN1 and ΔN2) revealed no caseinolytic activity, albeit the proteins are strongly expressed (Figure 3C, yellow asterisks). These experiments demonstrate that deletion of the amino-terminus resulted in abrogation of HtrA\textsubscript{Hp} proteolytic activity.

**Modifications Within the Amino-Terminus of HtrA\textsubscript{Hp} Affect Disruption of Cell-to-Cell Junctions**

As changes within the amino-terminus interfere with the proteolytic activity of HtrA\textsubscript{Hp}, we aimed to study the outcome during infection. For this purpose, confluent polarized Caco-2 cells were infected with the above-characterized C. jejuni 81–176Δhtra/htrA\textsubscript{Hp} variants and corresponding control strains for 24 h. Subsequently, the cells were fixed and subjected to immunofluorescence staining using antibodies against the adherens junction protein E-cadherin and C. jejuni. We could confirm that the signals of the C. jejuni bacteria being attached to the host cells were equally high between the infected samples, while the mock control cells showed no bacteria as expected (red, Figure 4). Moreover, while the uninfected mock control cells exhibited the typical E-cadherin signals between the neighboring cells in the monolayer, infection with C. jejuni wt and the Δhtra/htrA\textsubscript{Hp} complementant led to a significant disruption of the E-cadherin staining in a HtrA-dependent fashion (green, Figures 4A–D). Individual cells showing downregulated or mislocated E-cadherin signals are marked with blue and yellow arrowheads, respectively. Infection with strains expressing HtrA\textsubscript{Hp} carrying mutations in the single cleavage sites H46A/D47A or K50A/D51A, respectively, also resulted in the downregulation and mislocalization of E-cadherin signals, but less pronounced (Figures 4E,F). In contrast, the E-cadherin patterns were still widely intact during infection with the HtrA\textsubscript{Hp} double mutant H46A/D47A/K50A/D51A or HtRa/HtrA\textsubscript{Hp} ΔN2 deletion variant, respectively, suggesting an important role of amino-terminal HtrA\textsubscript{Hp} cleavage at both sites on protease activity, and thus, on damaging cell-to-cell junctions (Figures 4G,H).

**Modeling the Structure of the Trimeric HtRA**

Finally, we aimed to investigate the potential importance of HtRA auto-processing by structural modeling. The model of HtrA\textsubscript{Hp} revealed a trimer as known from other HtRA proteins in Gram-negative bacteria. Interestingly, this trimer is stabilized via interactions of the amino-terminal arm (residues 36–45), which protrudes from the protease domain and interacts with the adjacent subunits, thereby interlocking the conformation (Figures 5A,B). The intermolecular interactions of this arm are conserved among different members of the HtRA family of proteases including DegP, DegQ, and DegS. We selected DegS as a modeling template because it exhibits the highest local sequence similarity to HtrA\textsubscript{Hp} in the amino-terminal region. The model also revealed that the H46/D47 cleavage site is located almost exactly between the N-terminal arm and the globular part of the enzyme. Thus, a cleavage at this position will remove the N-terminal arm, which is responsible for the majority of the inter-subunit interactions (Figure 5C). The lack of these interactions will drastically reduce trimer...
FIGURE 3 | Importance of auto-processing for oligomerization and caseinolytic activity of HtrA<sup>Hp</sup> expressed in E. coli. The amino-terminus of HtrA<sup>Hp</sup> was mutagenized by generating deletion variants and expressed as GST-tagged variants in E. coli BL21 (compare Supplementary Figure 1B). E. coli BL21 expressing GST-tagged HtrA<sup>Hp</sup> without the signal peptide (∆SP) and empty vector E. coli BL21 were used as controls. (A) After induction of protein expression by IPTG, the protein lysates were subjected to Coomassie staining. Overexpression of the HtrA<sup>Hp</sup> p55 monomers and the GST-tagged variants was observed (arrows). The amino-terminal deletion variants (∆N1 and ∆N2, yellow asterisks) exhibit a lower molecular weight compared to HtrA<sup>Hp</sup>1 SP (black asterisk). (B) The bacterial lysates were subjected to immunoblotting against HtrA<sup>Hp</sup> and GST. Overexpression of the GST-tagged HtrA<sup>Hp</sup> p55 variants was confirmed by detecting the HtrA<sup>Hp</sup> or GST protein, respectively (arrows). In addition, immunoblotting against HtrA<sup>Hp</sup> showed the presence of HtrA<sup>Hp</sup> monomers without GST-tag (arrow). The p52 form is migrating slightly below p55 (red asterisks). (C) Finally, the bacterial lysates were analyzed by casein zymography. For wt HtrA<sup>Hp</sup> ∆SP, a strong caseinolytic activity of the HtrA<sup>Hp</sup> monomers and its GST-tagged variants was observed (arrows). The p52 form is migrating slightly below p55 (red asterisks). Moreover, caseinolytic activity for the HtrA<sup>Hp</sup> trimer and its GST-tagged variant was shown (arrows). The ∆N1 and ∆N2 variants revealed no proteolytic activity (yellow asterisks). All assays were done in triplicates.
FIGURE 4 | Continued
FIGURE 4 | Amino-terminal auto-processing of HtrA\(^{Hp}\) influences the disruption of cell-to-cell junctions during infection with C. jejuni. Polarized Caco-2 cells were (A) left uninfected (mock) or infected for 24 h with (B) C. jejuni 81–176 wt, (C) \(\Delta\)htrA or (D) \(\Delta\)htrA/htrA\(^{Hp}\) wt. (E–H) Infection with C. jejuni 81–176 expressing point and deletion mutants of HtrA\(^{Hp}\) amino-terminal auto-processing sites (compare Supplementary Figure 1A). The cells were subjected to immunofluorescence staining detecting E-cadherin (green) and C. jejuni (red). Arrowheads mark cells showing significantly downregulated (blue) or locally disrupted (yellow) E-cadherin signals. The nuclei were stained with DAPI (blue). The scale bar corresponds to 10 \(\mu\)m.
stability and is expected to result in the dissociation of the subunits.

**DISCUSSION**

The serine protease HtrA is a conserved periplasmic protein and has an important impact on the virulence and survival of various Gram-negative pathogens (Ingmer and Brøndsted, 2009; Frees et al., 2013; Skorko-Glonek et al., 2013). Inactivation of the htrA gene and generation of ΔhtrA knockout mutants have been already described in various bacteria, suggesting that this gene is not essential in the majority of pathogens (Humphreys et al., 1999; Pedersen et al., 2001; Cortés et al., 2002; Purdy et al., 2002; Wilson et al., 2006; Hoy et al., 2010; Gloeckl et al., 2013; Heimesaat et al., 2014a,b), while the inactivation of htrA in a large number of worldwide H. pylori isolates failed (Salama et al., 2004; Tegtmeyer et al., 2016). In addition, recent studies have shown that pharmacological inhibition of the HtrAHp activity leads to killing of H. pylori, but did not affect the growth of Salmonella or Shigella species, underlying the essentiality of the gene in H. pylori (Tegtmeyer et al., 2016). Here, we discovered and characterized amino-terminal auto-processing events of HtrAHp. Our data demonstrate that cleavage of HtrAHp appears at specific and conserved sites in worldwide strains, and significantly reduced the stability of trimer formation, and thus affect oligomerization, secretion and regulatory activities of the protease with an important role in the pathogenesis of H. pylori.

**Amino-Terminal Cleavage of HtrAHp and Effects on Trimerization**

Analyses of more than one thousand worldwide H. pylori strains demonstrated that HtrAHp is conserved among these isolates and that the mature full-length HtrAHp proteins are expressed as a p55 and p52 double-band (Tegtmeyer et al., 2016). We proposed a role of HtrAHp auto-processing for its proteolytic activity and extracellular transport. Using Edman sequencing we identified two amino-terminal cleavage sites at positions H46/D47 or K50/D51 and their cleavage gives rise to the p55 and p52 HtrAHp protein forms. Moreover, we could demonstrate that these cleavage sites are highly conserved among H. pylori strains originating from different countries worldwide. Interestingly, these sites have been also found in HtrA of the close relative C. jejuni, but not in other Gram-negative bacteria. By comparison, for the HtrA homolog DegP from E. coli auto-cleavage was also identified, after amino acid position C69 or Q82, respectively (Skórko-Glonek et al., 1995), which is different from the cleavage we observed in HtrAHp. Interestingly, HtrA proteins from bacteria and higher organisms can form proteolytically active trimers, and trimerization sequences were reported. For example, it was proposed that the multimerization of E. coli DegP occurs via the interactions between Q-linker sequences, a less conserved region in the protease domain (Kim and Kim, 2005). In addition, several mammalian HtrA proteins exhibit the trimerization motif QYNFIA, which is conserved and located at the amino-terminus (Nam et al., 2006). Using deletion variants of this motif in the mitochondrial serine protease HtrA2, trimerization was abrogated (Li et al., 2002). Especially, a phenylalanine residue (F149) in this motif is crucial for HtrA homotrimer formation in mammals (Nam et al., 2006). Regarding the trimerization of HtrAHp, our structural model suggested that an amino-terminal arm, comprising the amino acid residues 36–45, plays an important role in mediating the interaction between individual HtrAHp monomers, and thus, is able to influence the stability of the trimer. These findings lead us to suggest that the auto-processing sites identified in H. pylori have an essential regulatory role and might be crucial for the pathogenesis of H. pylori and eventually for C. jejuni, which deserves further investigation in prospective studies.

**Mutational Effects on HtrA Secretion and Proteolytic Activity**

To reconsider the impact of the amino-terminal cleavage sites, we aimed to investigate the effect on secretion and proteolytic activity in bacteria. Research on the functions of HtrAHp is
complicated because the gene is essential in \textit{H. pylori} and \textit{ΔhtrA} knockout mutants are not available (Salama et al., 2004; Tegtmeyer et al., 2016, 2017). Thus, we decided to apply the recently established genetic complementation system of \textit{htrA} from \textit{H. pylori} strain G27 in \textit{C. jejuni} 81–176 (Boehm et al., 2017). It is well known that HtrA proteins in Gram-negative bacteria including \textit{H. pylori} contain a signal peptide being important for the Sec-dependent transport of the protein across the inner membrane into the periplasm (Claussen et al., 2002; Ingmer and Brondsted, 2009; Frees et al., 2013; Skorko-Glonek et al., 2013), while its transport across the outer membrane remains unclear. To investigate the proposed roles of the H46/D47 or K50/D51 cleavage sites, we constructed point mutations in both cleavage sites, either separately (H46A/D47A and K50A/D51A) or together (H46A/D47A/K50A/D51A). In addition, we deleted the amino-terminus of HtrA including both cleavage sites (ΔN2) and expressed the HtrA\textsuperscript{Hp} mutants in \textit{C. jejuniΔhtrA} deletion variant. We confirmed equal expression of HtrA\textsuperscript{Hp} variants, while the secretion was significantly downregulated by the H46A/D47A or K50A/D51A mutants, respectively, leading to structural changes within the trimer, resulting in a disturbed HtrA\textsuperscript{Hp} secretion. Moreover, we investigated the role of amino-terminal auto-cleavage on the caseinolytic activity of HtrA\textsuperscript{Hp}, which is also conserved among hundred worldwide \textit{H. pylori} isolates (Tegtmeyer et al., 2016). Our studies showed that mutation of the amino-terminus including both cleavage sites (ΔN2) resulted in the loss of HtrA activity in the \textit{C. jejuni} complementation system. To further characterize if the reduction of activity resulted from loss of the entire amino-terminus, we created HtrA\textsuperscript{Hp} variants without the amino-terminus including cleavage site H46/D47 (ΔN1) or H46/D47 and K50/D51 (ΔN2), respectively, and expressed the HtrA\textsuperscript{Hp} variants heterologously in \textit{E. coli} BL21. As observed in \textit{C. jejuni}, HtrA\textsuperscript{Hp}ΔN2 showed no proteolytic activity when expressed in \textit{E. coli} BL21. In addition, no caseinolytic activity was detected for the ΔN1 mutant. This might be an effect of a disturbed interaction between the subunits within the HtrA trimer. HtrA\textsuperscript{Hp} shows no sequence homology to typical auto-transporters, which process themselves by auto-proteolysis (Boehm et al., 2013), indicating that the auto-cleavage sites might not affect the secretion, but can modulate the inter-trimer interactions. Caseinolytic active oligomers with sizes of 13 and higher were found in multiple tested \textit{H. pylori} isolates from different origins, both in total cell lysates and culture supernatant (Tegtmeyer et al., 2016). In addition, studies on HtrA in \textit{E. coli} have shown that the oligomers are highly proteolytic active compared to the monomer (Krojer et al., 2008). In line with these observations, deletion of the trimerization motif in human HtrA2 showed that the formation of the homotrimer is essential for precise function of the enzyme including its proteolytic activity (Li et al., 2002; Nam et al., 2006). Recently, it was experimentally also shown that trimerization plays a fundamental role for the activation of human HtrA1 by an allosteric mechanism (Cabrera et al., 2017). Further, computational studies of DegS suggest that disassembly of a DegS trimer inhibits proteolytic activity (Lu et al., 2016). Therefore, an altered interaction within the trimers resulting from the loss of the amino-terminus could lead to destabilization of the trimer and thus, to the lack of the proteolytic activity of HtrA\textsuperscript{Hp}.

**Importance of Amino-Terminal HtrA Sequences to Disrupt E-cadherin in Caco-2 Cells**

Previous studies revealed that binding of the bacteria to the epithelial host cells depends on the HtrA expression (Brondsted et al., 2005). Furthermore, secreted HtrA\textsuperscript{Hp} can cleave the ectodomain of the adherens junction protein E-cadherin in polarized gastric epithelial cell models \textit{in vitro} and \textit{in vivo} (Weydig et al., 2007; Schirrmeister et al., 2009; Hoy et al., 2010, 2012; Boehm et al., 2012; Tegtmeyer et al., 2016). Infecting polarized epithelial cells with our generated HtrA\textsuperscript{Hp} variants carrying mutations in the amino-terminal cleavage sites showed a lower disruption of the E-cadherin pattern compared to the wt HtrA complementant. This underlines the proposal, that disturbed trimer interaction caused by the missing or modified amino-terminus, respectively, led to an altered proteolytic activity of HtrA\textsuperscript{Hp}. Thus, the HtrA-dependent disruption of E-cadherin based cell-to-cell junctions is affected. Besides E-cadherin, HtrA\textsuperscript{Hp} can act directly on the tight junction proteins occludin and claudin-8 (Tegtmeyer et al., 2017). Consequently, an effect of the HtrA\textsuperscript{Hp} amino-terminal processing on the cleavage of occludin and claudin-8 should be further investigated in prospective studies. Our data suggest that the conserved amino-terminal cleavage sites H46/D47 and K50/D51 are important for the auto-processing of HtrA\textsuperscript{Hp} and seem to be involved in oligomerization and hence, in regulating activity and secretion of the novel virulence factor HtrA. In line, amino-terminal cleavage being important for trimer stabilization seems to influence the disruption of the epithelial cell barrier by affecting HtrA-dependent E-cadherin cleavage.

**CONCLUSION**

Here, we presented for the first time that the p55 and p52 forms of HtrA\textsuperscript{Hp} result from amino-terminal cleavage of the protein between amino acid positions H46/D47 or K50/D51, respectively. Moreover, these amino-terminal cleavage sites are conserved within \textit{H. pylori} and \textit{C. jejuni}. Three-dimensional modeling of HtrA showed that the amino-terminus of HtrA\textsuperscript{Hp} might be essential for oligomerization of HtrA monomers and stabilization of the trimer. Point and deletion mutants of these identified amino-terminal processing sites were constructed to investigate effects on protein secretion or activity using Western Blotting or Casein zymography, respectively. Interestingly, loss of the entire amino-terminus including these cleavage sites could lead to strong structural changes within the trimer being deleterious for the activity. Lack of certain interactions within the trimer caused by mutagenesis of the amino-terminal cleavage sites might also led to destabilization of the trimer and a lower enzymatic efficiency and protein transport. In addition, infection of Caco-2 cells confirmed that the loss of entire amino-terminus led to...
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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.00642/full#supplementary-material

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AUTHOR CONTRIBUTIONS

NT and SB conceptualized the study. NA, NT, and HS performed the experiments and generated the data. NT, SB, NA, HS, and JS-G analyzed and interpreted the data. SB and NA wrote the paper. All authors revised and agreed on the manuscript.

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