

Function of Serine Protease HtrA in the Lifecycle of the Foodborne Pathogen *Campylobacter jejuni*

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Campylobacter jejuni is a major food-borne zoonotic pathogen, responsible for a large proportion of bacterial gastroenteritis cases, as well as Guillain-Barré and Miller-Fisher syndromes. During infection, tissue damage is mainly caused by bacteria invading epithelial cells and traversing the intestinal barrier. *C. jejuni* is able to enter the lamina propria and the bloodstream and may move into other organs, such as spleen, liver, or mesenteric lymph nodes. However, the involved molecular mechanisms are not fully understood. *C. jejuni* can transmigrate effectively across polarized intestinal epithelial cells mainly by the paracellular route using the serine protease high-temperature requirement A (HtrA). However, it appears that HtrA has a dual function, as it also acts as a chaperone, interacting with denatured or misfolded periplasmic proteins under stress conditions. Here, we review recent progress on the role of HtrA in *C. jejuni* pathogenesis. HtrA can be transported into the extracellular space and cleaves cell-to-cell junction factors, such as E-cadherin and probably others, disrupting the epithelial barrier and enabling paracellular transmigration of the bacteria. The secretion of HtrA is a newly discovered strategy also utilized by other pathogens. Thus, secreted HtrA proteases represent highly attractive targets for anti-bacterial treatment and may provide a suitable candidate for vaccine development.

Keywords: HtrA, *C. jejuni* proteases, E-cadherin, fibronectin, occludin, integrins, LOS, molecular pathogenesis, cellular invasion, signaling, TER, outer membrane vesicles (OMVs), tight junction, adherens junction, virulence

Introduction: The Lifestyle of *C. jejuni*

Foodborne illnesses are a major public health problem that have proven to be difficult to resolve, despite multiple and focussed efforts over the past 25 years. Diarrhoeal diseases are responsible for high rates of morbidity and mortality in developing nations, and are also frequent in industrialized countries [1–3]. It has been estimated that bacterial and viral infections of the gastrointestinal tract together kill approximately 2.2 million people annually worldwide, which has a large negative impact on public health and presents a serious burden to public health services [4–6]. Meanwhile, in a number of countries, *Salmonella* infections have decreased in the past decade, the decrease in infections caused by *Campylobacter* species has been less or absent, so that in some countries these infections are now more frequent than infections by *Salmonella*, pathogenic *Escherichia*, or other bacterial enteropathogens [7–9].

Campylobacter jejuni is a zoonotic pathogen [10]. These highly motile, spiral-shaped Gram-negative bacteria require microaerobic conditions and a temperature between 37 °C and 42 °C for growth. These conditions allow these bacteria to asymptotically colonize the gastrointestinal tract of a wide range of wild and domestic birds and animals. The natural niche of *C. jejuni* is the avian intestine, and it is commonly present in poultry [11]. Consumption of contaminated (animal-derived) foods is a major means of transmission to humans, and food-borne campylobacteriosis cases account for 400–500 million cases annually [12], corresponding to estimated 33 million Disability Adjusted Life Years

(DALYs) [3]. However, not all infections result in disease, and it is estimated that the majority of humans encounters with *C. jejuni* remain without symptoms [13], although prolonged asymptomatic colonisation is uncommon in developed countries. When disease occurs, it presents with symptoms ranging from mild, non-inflammatory, self-limiting diarrhea to severe inflammatory bloody diarrhea that may require hospitalization [14–17]. *C. jejuni* infection can also lead to potentially more severe sequelae, such as the Miller-Fisher or Guillain-Barré syndromes [10, 18, 19].

Multiple efforts to unravel the molecular mechanisms of *C. jejuni* virulence have identified a number of bacterial factors that have a role in the pathogenesis of this organism, but their exact activities and interplay with host factors are still poorly understood. In order to cause infection, *C. jejuni* enters the human gut via the oral route, where it can colonize the mucus layer of the small intestine or attach to intestinal epithelial cells [20]. Quorum sensing plays an important role in *C. jejuni* colonization as it can regulate the expression of multiple genes involved in virulence, toxin production, motility, chemotaxis, and biofilm formation, thus contributing to environmental adaptation [21]. Bacterial adhesion to and invasion into epithelial gastrointestinal cells are considered as a primary cause of tissue damage in humans [22]. In order to adhere to cells, the mucus layer has to be crossed, for which the highly motile bacteria are well equipped. Bacterial adhesion is then enabled by specific adhesins, which are exposed on the bacterial surface and bind to host cell receptors. Currently, at least seven different types of proteins have all been proposed to act as bacterial adhesins: *Campylobacter* adhesion to fibronectin (CadF) [23, 24], major outer membrane protein (MOMP) [25], periplasmic binding protein (PEB1) [26], P95 [27], *jejuni* lipoprotein A (JlpA) [28, 29],

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Campylobacter autotransporter protein A (CapA) [30], and fibronectin-like protein A (FlpA) [31, 32]. The contribution of each of these factors to pathogenesis is under much debate.

Following adhesion to epithelial cells, many strains of *C. jejuni* are able to enter cells, though in vitro determined levels of invasion can be lower than those obtained with strongly invasive organisms, such as *Salmonella enterica* or enteroinvasive *Escherichia coli* strains [33]. Using in vitro models of invasion, it was observed that non-motile *C. jejuni* mutants invade less efficiently [34], and since motility was shown to be required for invasion and translocation through cells [35], it is possible that the bacteria use motion force to propel themselves into the cells by means of their flagella. Alternatively, the flagellum may serve as a delivery device for virulence factors, which trigger host cell entry, as has been suggested for *Campylobacter* invasion antigens C [36] and D [37] (CiaC and CiaD). In order to reach deeper tissues, *C. jejuni* may not only pass the epithelial barrier by cellular invasion and escape, but also by paracellular translocation across tight junctions. The bacteria are surprisingly fast in doing so, at least in confluent cell layers in vitro [35]. The secretion of bacterial factors into host cells has been proposed as a further contribution to virulence [39], although classical type-III or type-IV secretion systems (T3SSs or T4SSs) are absent. Secreted effector proteins include *Campylobacter* invasion antigens (Cia) [40] and flagellar co-expressed determinant (Fed) proteins [41, 42]. Amongst the secreted proteins with a function in virulence of *C. jejuni*, the serine protease high-temperature requirement A (HtrA) can be added, which is involved in stress response, as well as in virulence [43].

Bacterial proteins need to be properly folded to maintain their function, but an array of stresses can cause denaturation and protein aggregation, which are deleterious to the cell. These malfunctioning proteins and their aggregates need to be removed, for which a cell uses specialized proteases. The serine proteases of the HtrA family degrade denatured proteins and in that way limit damage produced by a variety of stresses [44]. Their name reminds of their protective role in temperature stress, as they were first discovered in *E. coli* being responsible for a high temperature requirement phenotype [45]. A protease known as DegP in *E. coli* [46] turned out to be identical to HtrA, so that this bifunctional protein is now known by both names. Subsequently, DegQ and DegS were discovered in *E. coli*, which were similar in structure and function to DegP/HtrA [47]. A sequence alignment of DegP and DegQ revealed an amino acid identity of 60%. The main difference is the lack of 20 amino acids in the N-terminally positioned LA-Loop of DegQ. The LA-Loop for his part is important for the stabilization of the inactive hexameric conformation of DegP. It has been postulated that DegQ is able to compensate the lack of DegP [48]. In turn, other studies showed that DegP, DegQ, and DegS mutants might have the same phenotype, suggesting they have different roles. Additionally, the re-expression of DegQ or DegS did not replace function of DegP in a knockout mutant [47, 49]. Different studies indicate that DegP and DegQ have different roles in the bacterial periplasm and could not give the answer whether they share overlapping functions [50]. *Campylobacter jejuni* only contains one HtrA-type protein, which is a DegQ homolog, and although that name is used in *Campylobacter* genome annotations, we will refer to it as HtrA [38, 51] here. All known HtrA proteins combine a proteolytic functional domain with one or two peptide-binding domains displaying ATP-independent chaperone activity [44, 52]. As will be discussed in this review, the HtrA homolog of *C. jejuni* enhances adhesion, cellular invasion, and bacterial transmigration via the paracellular route and is also involved in stress tolerance. As such, it plays a pivoting role in the life cycle of this organism.

General Structure and Function of Bacterial HtrA Proteins

Tolerance to oxidative and heat stress is of key importance for every organism and includes the degradation of intracellular irreversibly misfolded proteins [53, 54]. Among others, HtrA proteases play an important role in these stress responses and can be found in both prokaryotes and eukaryotes including humans [44, 52, 55, 56]. HtrA undergoes oligomerization and conformational changes when encountering denatured proteins, which switches on its proteolytic activity. These oligomerization and configuration processes have been studied in detail for HtrA homologs from a number of organisms, such as *Thermotoga maritima* [57], *E. coli* [58, 59], *Legionella pneumophila* [60] and *Helicobacter pylori* [61]. Bacterial HtrAs are predominantly localized in the periplasm and *de novo* protein typically contains an N-terminal signal peptide that must be removed during transport across the inner membrane before the protein becomes active. Thus, the key role of HtrA may be to protect the cell against denatured proteins in the periplasm, whereas other proteases are responsible for protein maintenance in the cytosol.

The protease domain of HtrA contains a His–Asp–Ser catalytic triad (HDS), and this domain is followed by one or two PDZ (protein–protein interaction) domains, depending on the species and HtrA variant [62–64] (Figure 1A). Of the three *E. coli* HtrA homologues, most detailed insights are available for DegP, and since there are no fundamental differences with DegQ, we briefly review DegP here. It was shown that in the periplasm, DegP polymerizes into a hexamer (DegP₆) that is enzymatically inactive. In this configuration, the proteolytic active site is hidden in a pouch within the hexamer structure, as reviewed more extensively elsewhere [65]. In the configuration of DegP₆, the PDZ1 domain can be reached only by unfolded proteins. Binding of such denatured proteins to PDZ1 of DegP₆ results in destabilization of the hexamer by rearrangements of the PDZ1 and PDZ2 domains. This facilitates the assembly of higher order oligomers DegP₁₂ and DegP₂₄, where the PDZ1 domain is now fixed to interact with a sensory loop of the protease domain, called L3. The loop rearranges and causes a remodelling of the active domain into its active state. PDZ1, the proteolytic site, and L3 are now able to bind and degrade misfolded periplasmic proteins or to protect proteins from being damaged [58, 65, 66].

Subtle conformational shifts result in different functions of HtrA homologues in different organisms. Thus, whereas *E. coli* HtrA/DegP is involved in heat stress, HtrA of Gram-positive *Lactococcus lactis* is required for the tolerance to a variety of environmental stress conditions: HtrA-deficient mutants exhibit increased sensitivity to heat, ethanol, puromycin, and sodium chloride [67]. Similar data were obtained for Firmicute *Listeria monocytogenes*, with regard to heat, puromycin-induced, and additionally, oxidative stress, resulting in a reduced ability for biofilm formation when its HtrA gene was mutated [68].

Recent studies revealed a direct or indirect participation of HtrA homologues in virulence of a variety of bacterial pathogens. In *Clostridium difficile*, inactivation of an HtrA homolog increased virulence in a hamster model, resulting from upregulation of toxin production and reduced spore formation, so in this organism, HtrA seems to down-regulate virulence [69]. In most other pathogens, HtrA enhances virulence, e.g., in *Streptococcus pneumoniae*, inactivation of HtrA resulted in attenuation in a mouse model for pneumonia [70]. In *H. pylori*, HtrA is required for paracellular translocation across a confluent polarized cell layer, enabling the bacteria to deliver their virulence factor cytotoxin-associated gene A (CagA) to the basolateral membrane of the host cells, even though infection initiates at the apical side [51, 71–74]. For this, the serine protease activity of *H. pylori* HtrA cleaves proteins of tight junctions like occludin, claudin-8, and the major adherens junction protein E-cadherin of epithelial cells, allowing

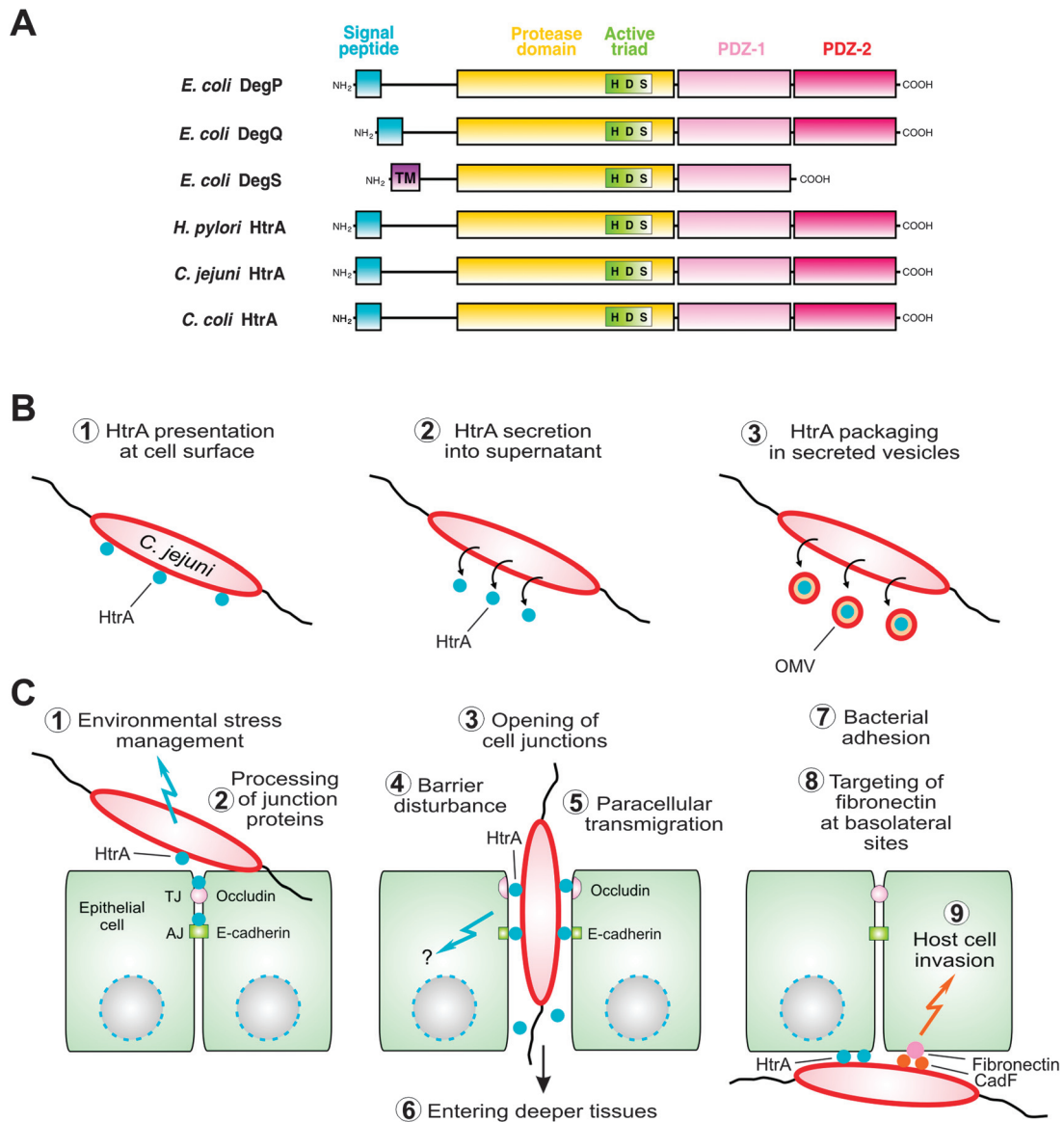


Figure 1. Domain architecture of HtrA, secretion routes of the protease, and involvement in virulence properties by *C. jejuni*. (A) Representation of the domain structure of characteristic HtrA family proteases. The protease domain is shown in yellow, PDZ-1 and PDZ-2 domains in red, and the three conserved amino acid residues (histidine, aspartate, and serine; HDS) at the active centre in green. A signal peptide is highlighted in light blue squares and a transmembrane (TM) domain with magenta. (B) Proposed secretion routes of *C. jejuni* HtrA into the extracellular space are diverse and complex. As shown in this scheme, HtrA can be presented at the bacterial cell surface (1), delivered in the supernatant as soluble protein (2), or shed in the form of outer membrane vesicles (OMVs) (3). (C) HtrA proteases are involved in *C. jejuni* stress management (1), as well as cleavage of host cell factors such as E-cadherin in the adherens junctions (AJs) and possibly other factors, such as occludin in the tight junctions (TJs) (2), followed by opening of cell-to-cell junctions (3). Cleavage of junctional proteins leads to disruption of the epithelial barrier (4), paving the way for bacteria to transigrate across the cell monolayer by a paracellular route (5) and to enter deeper tissues (6). In addition, HtrA is also involved in bacterial adhesion by a yet unknown mechanism (7), basolateral targeting of fibronectin by CadF (8), and CadF-dependent invasion of host target cells (9).

the pathogen to transigrate to the basolateral side, where it can inject the effector protein CagA in an integrin-dependent fashion [51, 71–76]. The second role of HtrA of *H. pylori* is to assist in secretion of oncogene CagA into host cells by means of a T4SS [75–77]. Cleavage of E-cadherin by HtrA homologues is not exclusive for *H. pylori*, as it has also been demonstrated for enteropathogenic *E. coli* strains and *Shigella flexneri*, but not for the urogenital pathogen *Neisseria gonorrhoeae* [51]. It is now evident that HtrA proteins are virulence factors for a number of pathogens [54, 78, 79], to which *C. jejuni* can be added, as will be discussed in the next sections.

Role of *C. jejuni* HtrA in Regulating Intracellular Stress Responses

A homolog of the *htrA* gene is present in every completely sequenced *C. jejuni* and *C. coli* genome. The HtrA proteins of

C. jejuni and *C. coli* are highly similar, and their closest phylogenetic neighbours are HtrA of *H. pylori* and DegQ of *E. coli* [38, 51] (Figure 1A). During the transfer between individual hosts, *C. jejuni* has to survive several stressors, including heat and oxidative stress. High temperatures and atmospheric oxygen induce protein damage that needs to be avoided or repaired. Studies revealed that *C. jejuni* HtrA is required for the tolerance to heat stress. HtrA is not essential for growth at the temperature range that is normal for this thermophile, as a knockout mutant lacking HtrA was able to grow similarly to wild-type at temperatures between 37 °C and 42 °C. However, already at 44 °C, the number of colonies of an HtrA deletion mutant was decreased 10^{-4} -fold compared to its isogenic wild-type [80, 81]. The protective effect of HtrA against temperature stress is due to its chaperone function, as a mutation inactivating the proteolytic function of the protein (by replacing the catalytically active serine residue in the active center by alanine, S197A) did not have an

effect on stress tolerance [82]. Similar results were obtained using the antibiotic puromycin, which increases the intracellular and periplasmic amount of misfolded proteins. When bacteria lacking HtrA were treated with puromycin, smaller and fewer colonies were formed than was observed for wild-type [80]. Proteome analysis of the $\Delta htrA$ strain used in that study revealed an increase of ClpB and DnaK at 42 °C or higher that was dependent of HtrA; the expression levels of those stress-response factors changed little at 37 °C. The cytoplasmic chaperones ClpB and DnaK are involved in the refolding and disaggregation of denatured proteins [83, 84] and can be used as indicators for increased intracellular stress, since they are activated by the presence of unfolded proteins. The described observations lead to the suggestion that *C. jejuni* attempts to compensate for the missing HtrA by increasing the expression of DnaK and ClpB [80]. Only the inactivation of the HtrA-proteolytic domain, as was demonstrated with a mutant in the S197A point mutant, had the same upregulating effect on the expression of ClpB and DnaK in response to high temperatures as complete deletion of *htrA* [82]. This shows a requirement of the functional HtrA protease domain to upregulate ClpB and DnaK chaperones that can deal with cytosolic denatured proteins. Besides tolerance to high temperatures, *C. jejuni* HtrA is also important for the bacteria to survive oxidative stress. A $\Delta htrA$ mutant showed a decreased viability under high oxygen exposure (18% O₂ at temperatures up to 42°C) [80, 82]. Contrary to this, the sensitivity to oxidative stress inducers such as paraquat, cumene hydroperoxide, and H₂O₂ was not affected by *htrA* deletion [80]. Interestingly, the proteolytic activity of HtrA is essential for bacterial growth during simultaneous heat and oxidative stress: the S197A mutant could not grow at all during this double stress exposure [38, 82]. These observations identify *C. jejuni* HtrA as a key player in stress tolerance, increasing the bacteria's survivability under unfavourable conditions that it typically encounters during its lifecycle.

Secretion of *C. jejuni* HtrA into the Extracellular Space

HtrA of *C. jejuni* is not only a periplasmic stress-response protein, but it is also secreted into the extracellular medium [38, 51]. Surface exposition and secretion of HtrA were shown to be highly efficient in two different model strains, *C. jejuni* strain 81–176 and strain NCTC11168 [85] (Figure 1B). This secretion is independent of the protease activity of HtrA, as was demonstrated with the S197A mutation introduced in NCTC11168 [85]. This observation excludes the possibility of autosecretion of HtrA via a type I secretion system employed by certain hemolysins of other bacteria [86].

For a number of secreted effector proteins of *C. jejuni*, flagella have been shown to be involved in their extracellular transport, as mentioned above for the Cia and Fed proteins [34, 41, 87]. Although *C. jejuni* does not contain the genes required to build a classical T3SS, there are striking structural similarities between flagella and T3SSs, both in their architecture and in their bio-assembly [88]. Flagellar filaments are more flexible and are attached to the body with a rotating motor, while T3SS form more rigid filaments that do not rotate, but bacterial flagella can act as an injectosome to deliver effectors inside a target cell, just like T3SS do [88, 89]. Early observations had already described that the secretion of *C. jejuni* Cia proteins is induced during incubation with human intestinal epithelial cells, and this process is contact-dependent [90]. Proteins CiaC and CiaD may be secreted in a similar manner [36, 37]. Secretion of Cia proteins was further shown to be induced by the presence of foetal calf serum (FCS) in the culture medium of *C. jejuni* [87]. In view of these observations, it was assessed if the secretion of HtrA was dependent on flagella and whether it was induced by the presence of foetal calf serum. It turned out that a flagella-deficient $\Delta flaA/flaB$ and other

flagellar gene mutants were still able to secrete HtrA; thus, its secretion into the extracellular space is not dependent on flagella [85]. The absence of CiaB secretion was noted for the tested mutants, confirming the observations of Konkel and colleagues [40]. The same study further identified that the secretion of HtrA was induced by the presence of foetal calf serum [85].

Various bacterial pathogens utilize shedding of outer membrane vesicles (OMVs) for the delivery of virulence factors into host cells [91], and it shows that HtrA is present in purified OMVs from *H. pylori* [92]. It became more likely that this could also be the case for *C. jejuni*, with the observation that OMVs play a role in interaction between *C. jejuni* and intestinal epithelial cells [93] (Figure 1B). This study presented a proteome analysis of *C. jejuni* OMVs, revealing a number of proteins important for pathogenesis and host immune response [93]. Indeed, it was subsequently shown that OMVs of *C. jejuni* strain 11168H contain HtrA, together with two other proteases encoded by Cj0511 and Cj1365c [94]. The observation that these OMVs displayed proteolytic activity that could be reduced by serine protease inhibitors further provides evidence for their role in transporting active HtrA [94]. Future studies are needed to study the molecular mechanism(s) of secretion in more detail and to identify and study additional *C. jejuni* factors driving this process.

HtrA Substrates and Paracellular Transmigration of *C. jejuni* In Vitro

HtrA has been also shown to play a role during various interactions of *C. jejuni* with host cells (Figure 1C). Polarized epithelial cell models are particularly valuable for the study of *C. jejuni* activities on cell barrier permeability, transepithelial electrical resistance (TER), mode of transmigration, and cell invasion [38, 95, 96]. Electron microscopy was applied to compare the interaction of *C. jejuni* with non-polarised INT-407 cells with polarized Caco-2 cells, showing that the efficiency of *C. jejuni* invasion of Caco-2 cells was two- to threefold less, as compared to INT-407 cells [96]. Additional studies showed that *C. jejuni* entered the intercellular space between two neighboring cells [93, 94]. It was therefore suggested that *C. jejuni* can translocate across polarized Caco-2 cell monolayers, both by passing through and between cells (Figure 1C). Remarkably, *C. jejuni* triggered not only its own translocation but also that of non-pathogenic *E. coli* [97]. Several research groups supported the paracellular pathway as a major transmigration route [35, 38, 98], whereas other groups favoured the transcellular trail [97, 99, 100]. It was further shown that various *C. jejuni* strains mutated in the *htrA* gene could no longer transmigrate across polarized cells, although the flagellar motility of the mutants was not affected [39]. The reduced transmigration properties, as well as the impaired growth at elevated temperature (44 °C) and under high oxygen conditions, could be restored by *trans* expression of the *htrA* gene [81], being in line with expectations expressed two years earlier [43]. In analogy to the catalytic activity of *H. pylori* HtrA, it has been shown that secreted *C. jejuni* HtrA can degrade extracellular host proteins, such as the cell surface adhesion protein and tumour-suppressor E-cadherin [38, 50, 51, 85, 101]. In particular, *C. jejuni* HtrA cleaves off the extracellular NTF (N-terminal fragment) domain of E-cadherin, thus leading to the destruction of adherence junctions and subsequent downregulation of barrier function, which enables the bacteria to transmigrate across polarized MKN-28 epithelial cells via paracellular movement [38].

In accordance to HtrA function in transmigration, expression of the protease-deficient S197A mutant leads to significantly reduced transmigration of *C. jejuni* across polarized epithelial cells [85]. Further investigations showed that the TER values are not dramatically reduced during *C. jejuni*

infection, although this is typically the case with other pathogens, such as *Shigella flexneri*, *Salmonella typhimurium*, *Listeria monocytogenes*, or *Neisseria gonorrhoeae*. This indicates that permanent opening of the cell-to-cell junctions may not occur as a result of *C. jejuni* HtrA enzymatic activity, and therefore, there is no loss of host cell integrity during transmigration [38]. During the last years, several studies confirmed early observations that factors other than HtrA may also play an important role during the transmigration of *C. jejuni* across polarized epithelial cells [35]. It seems that flagella, the flagella-associated bacterial motility, and the Cia proteins are all crucial for the transmigration process [35, 38, 102]. Further indications for the paracellular route came from another study, which showed that an $\Delta cadF$ mutant, who is deficient in fibronectin binding, also transmigrated as effectively as the *C. jejuni* wild-type strain F38011 [98]. However, another report counteracted with the observations above by showing that a LOS-deficient $\Delta cstII$ mutant in *C. jejuni* GB11 featured a strong imperfection in invasion and transmigration; therefore, this study favoured the transcellular route [100]. At the moment, there is no conclusive explanation for these different observations. Thus, more studies are required to confirm the exact mechanism of *C. jejuni* transmigration, whereby the possibility of strain-specific differences should also be considered.

Importance of HtrA in Host Cell Adhesion and Invasion by *C. jejuni* In Vitro

A hallmark of *C. jejuni* pathogenesis is its ability to adhere to epithelial cells. The major adhesin is the 37 kDa CadF protein [25, 103–106]. CadF allows the bacteria to bind to host fibronectin, a component of the extracellular matrix, present in the basolateral but not apical surface of polarized epithelial cells [23]. Thus, *C. jejuni* employs a major basolateral receptor for attaching to, as well as entering into, epithelial cells. Bacterial binding to host target cells decreased up to 10 times when HtrA was deleted [107], an effect that appears more pronounced compared to those of the inactivation of other proposed adhesins, such as FlpA, CapA, or JlpA [28–32]. In line with these observations, *C. jejuni* HtrA does not cleave fibronectin, unlike its counterpart in *H. pylori* [38]. Thus, fibronectin is resistant to the proteolytic activity of *C. jejuni* HtrA, underlining the importance of fibronectin for adhesion and invasion of *C. jejuni*. However, host cell attachment was not strictly dependent on the proteolytic activity of HtrA, as S197A mutants were still able to attach at significant levels to host cells. This suggests that the chaperone part of HtrA is mostly responsible for enhanced adherence capacity of *C. jejuni* [107]. Not only adherence to, but also invasion into epithelial cells is affected when HtrA is inactivated [38, 81, 107]. The reduced cell adherence and invasion could also be restored by *trans* expression of HtrA, in the same way like the transmigration properties of the used strains [81]. Taken together, both the chaperone and protease activity of HtrA are required for optimal adherence and internalization of *C. jejuni* to epithelial host cells. In general, HtrA has been viewed as a stress response protein, but it seems that HtrA has also specific functions during the infection process that may be stress-independent [107, 108]. This observation is in line with an increasing notion that the chaperone activity of HtrA may be involved in folding of adhesins and other virulence factors and thus could contribute in this way to host cell binding and invasion of *C. jejuni*. Alternatively, *C. jejuni* HtrA could also bind directly to host cell surface protein(s) to act as an adhesion itself, but there are as yet no such indications in the literature [108].

Function of HtrA during *C. jejuni* Infection of Mice In Vivo

Most studies to unravel the virulence mechanisms of *C. jejuni* are restricted to in vitro models, which is why experimental

in vivo data elucidating the molecular mechanisms of this pathogen are scarce. One of the reasons is that conventional mice display a pronounced colonization resistance phenotype against *C. jejuni*, which is caused by the host-specific composition of their intestinal microbiota [109]. However, recently it was revealed that this commensal polymicrobial barrier can be overcome by complete eradication of the murine gut microbiota [109–112]. Remarkably, mice in which the depleted microbiota had been reconstituted with human gut microbiota following faecal microbiota transplantation could be stably infected with *C. jejuni* [109]. Furthermore, *C. jejuni* infected mice displayed immunopathological key features of human campylobacteriosis that were dependent on toll-like receptor 4 (TLR-4) signalling through *C. jejuni* lipooligosaccharide (LOS) [109]. Subsequently, two mice models for *Campylobacter* infection study were developed: conventional infant wild-type mice could be infected with *C. jejuni* that resulted in self-limiting disease [113, 114], whereas secondary abiotic IL-10^{-/-} knockout mice in which the murine gut microbiota was eradicated following broad-spectrum antibiotic treatment resulted in lethal disease upon peroral *C. jejuni* wild-type infection [115]. The latter provided an excellent model to study the role of HtrA during murine infection by *C. jejuni*.

It turned out that HtrA has a significant effect on the outcome of infection in secondary abiotic IL-10^{-/-} mice. Six days following infection with either *C. jejuni* wild-type or a $\Delta htrA$ mutant strain, both groups of animals harboured comparable loads of either strain alongside the gastrointestinal tract, but the mice infected with the $\Delta htrA$ mutant presented with notably less severe clinical symptoms [116]. Their intestinal as well as extra-intestinal (i.e., hepatic and renal) symptoms, including systemic immunopathological sequelae of infection, were far less severe compared to wild-type infected control mice that were prone to death [116].

These intriguing results were further supported using the conventional infant mice model, which mimics human clinically manifesting acute infection less accurately. Upon being perorally infected, immediately after weaning (i.e., at the age of 3 weeks *post partum*), conventional infant mice exhibited acute enterocolitis that resolves within two weeks, followed by an asymptomatic carrier status with overt *C. jejuni*-induced intestinal and extra-intestinal immune responses [113, 114]. One week following *C. jejuni* $\Delta htrA$ strain infection, these infant mice exhibited comparable intestinal bacterial loads, but they presented with less distinct colonic epithelial apoptosis as compared to *C. jejuni* wild-type-strain-infected counterparts; the latter was accompanied by lower pro-inflammatory cytokine levels and less expression of the matrix-degrading enzyme matrix metalloproteinase-2 (MMP-2) in the large intestine [116]. Conversely, colonic numbers of proliferating cells were increased in mice infected with *C. jejuni* $\Delta htrA$ as compared to those infected with wild-type bacteria, thereby counteracting *C. jejuni*-induced cell damage [116]. Remarkably, the better outcome of infected mice due to *htrA* gene deficiency was not restricted to the intestinal tract, but could also be observed in extra-intestinal locations as well, as indicated by less pronounced inflammatory changes in the liver, kidneys, and lung of $\Delta htrA$ versus parental-strain-infected infant mice [116]. These in vivo results clearly support the view that HtrA is a virulence factor of *C. jejuni*, as it is required for the penetration and spread into deeper tissue, thereby eliciting pro-inflammatory cytokines and local host cell apoptosis.

Other Proteases Identified in *C. jejuni*

While *C. jejuni* only encodes one homolog of HtrA, the bacteria produce other proteases that may also have fundamental functions in gastrointestinal colonization and infection. The specific lifestyle and amino acid-dependent metabolism of *C. jejuni* are

similar to those of commensal gut bacteria, but differ significantly from those of other gastrointestinal pathogens. In contrast to the most frequent enteritis-inducing bacteria in the Enterobacteriaceae family, which use sugar substrates to fuel their requirements for energy and carbohydrate structures, *C. jejuni* depends on the catabolization of oligopeptides and amino acids released by proteases from proteins derived from diet, commensal microbiota, and the host [117]. Thus, proteases play an exceptional role in feeding *C. jejuni* with essential amino acids, which therefore become of extraordinary importance to maintain the virulence properties. Because of the key role of proteases in the establishment and multiplication in the gastrointestinal lumen of humans and other warm-blooded vertebrates, earlier scientific investigations have focused on the use of a single amino acid insertion into the highly conserved FtsH protease homolog of *C. jejuni*, which bears a specific molecular signature present in the Campylobacterales subgroup of the epsilon proteobacteria [118]. Recently, more than 45 peptidase-related proteins were identified by in silico analysis of the *C. jejuni* NCTC11168 genome [119]. A more detailed experimental analysis revealed that the Cj0511 serine peptidase, which was shown to be present in *C. jejuni* OMVs in conjunction with the HtrA and Cj1365c proteases [94], is essential for chicken colonization. The findings that OMVs produced in the presence of the bile salt taurocholic acid exerted elevated proteolytic activity and were found to be required for *C. jejuni* invasion and transmigration in vitro underlined the functions of the secreted proteases in *C. jejuni* virulence and intestinal pathogenesis [120]. Taken together, all these findings provided the first evidence that the other proteases of *C. jejuni* might represent excellent targets for antibacterial therapy and the protection of livestock from *C. jejuni* colonization. This was further confirmed by the observations that *C. jejuni* mutants deficient in the zinc metalloendopeptidase PepF, the putative family C26 endopeptidase CJJ81176_1416, or the carboxyl-terminal protease CtpA resulted in a severe reduction in the ability of *C. jejuni* to colonize the intestines of mice [121]. Finally, a recent investigation on feed additives in chicken revealed significantly higher numbers of *C. jejuni* in broiler chickens which received a protease enriched diet [122].

Concluding Remarks

Campylobacter jejuni is one of the most important zoonotic pathogens and leading cause of bacterial acute gastroenteritis worldwide and of potentially more severe diseases, such as reactive arthritis or Guillain-Barré syndrome. The pathogen exhibits varying pathogenicity properties and secretes a variety of virulence-associated proteins [1–4, 16, 17, 33, 123–125]. Crossing the intestinal epithelial barrier of the host and cellular invasion by *C. jejuni* are described as the primary reasons of gut tissue damage in humans. The serine protease HtrA of *C. jejuni* is efficiently secreted into the culture supernatant, although the exact mechanism of the secretion remains to be elucidated. In addition to its function to increase the stress resistance, HtrA has been identified as a novel secreted virulence factor. By means of its proteolytic activity, the protein opens cell-to-cell junctions by cleaving E-cadherin and probably other host factors such as occludin (Figure 1C). This enables *C. jejuni* to enter underlying tissues, such as the lamina propria, from where it can invade the bloodstream and possibly reach distinct organs, such as the spleen, liver, or mesenteric lymph nodes. Various models exist to explain how the pathogen can trigger its own transmigration across polarized intestinal epithelial cells in vitro. We favour the paracellular transmigration mechanism, involving bacterial factors, such as flagellum and serine protease HtrA.

The ongoing spread of antibiotic resistances has become an increasing concern, both for gastrointestinal and other bacterial

pathogens. The development of novel antibiotic substances has dramatically reduced, and drug resistances are becoming a priority in human medicine worldwide. Facing this new hazard, alternative treatments are urgently required. The discovery of HtrA as a novel class of bacterial virulence factors, including *C. jejuni* and other pathogens, suggests that secreted HtrA represents a very attractive new anti-bacterial target [53, 54, 78, 79, 126–129].

One option could be the specific inhibition of extracellular HtrA by compounds that do not need to enter the bacteria in order to be effective. This strategy has the advantage that such compounds would not affect colonization per se, limiting the selection pressure for resistance, either in the pathogen or in endogenous commensals. While many of the current antibiotics also affect commensal bacteria, the search for pathogen-selective HtrA inhibitors represents a very encouraging drug discovery strategy. Taken together, targeting of HtrA protease may represent an inspiring new option to elaborate new therapies for gastrointestinal diseases, but this requires more research. Thus, future studies on HtrA proteases will become a rewarding topic in the field.

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Authors' Contributions

All authors contributed to the writing of this review.

Conflicts of Interest

The authors declare that they have no conflict of interest.

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