EXTRACELLULAR SECRETION OF PROTEASE HtrA FROM *CAMPYLOBACTER JEJUNI* IS HIGHLY EFFICIENT AND INDEPENDENT OF ITS PROTEASE ACTIVITY AND FLAGELLUM

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The serine protease HtrA of *C. jejuni* has been identified as a novel secreted virulence factor which opens cell-to-cell junctions by cleaving E-cadherin. Efficient *C. jejuni* transmigration across polarized human epithelial cells requires the intact flagellum and HtrA; however, the mechanism of HtrA secretion into the supernatant is unknown. Here we show that HtrA secretion is highly efficient and does not require its proteolytic activity because the protease-inactive S197A mutant is secreted like wild-type HtrA. In addition, the flagellar mutants $\Delta flaA/B$, $\Delta fliI$, $\Delta flgH$, $\Delta flhA$, $\Delta flhB$, and $\Delta flgS$ were also able to secrete HtrA in high amounts, while they were strongly attenuated in secreting the well-known invasion antigen CiaB. We also tested several culture media and cell lines of different origin such as human, mouse, hamster, dog, and chicken for their ability to influence HtrA secretion. Interestingly, HtrA was effectively secreted in the presence of most but not all cell lines and media, albeit at different levels, but secretion was significantly higher when fetal calf serum (FCS) was added. These results demonstrate that HtrA secretion by *Campylobacter* proceeds independent of HtrA's protease activity, the flagellum and origin of cell lines, but can be strongly enhanced by molecular compound(s) present in FCS.

Keywords: HtrA, secretion, flagellum, E-cadherin, molecular pathogenesis, cellular invasion, signaling, virulence

Abbreviations: BHI, brain heart infusion; CCM, cell culture medium; CFU, colony forming; CiaB, *Campylobacter* invasion antigen B; FCS, fetal calf serum; MH, Mueller–Hinton; MOI, multiplicity of infection; NaCl, sodium chloride; PBS, phosphate buffered saline; T3SS, type III secretion system

Introduction

Pathogenic food-borne bacteria represent one of the leading causes of morbidity and mortality in humans. Reports by the World Health Organization WHO indicate that the human population suffers from ~4.5 billion cases of gastroenteritis per year, causing about 1.8 million deaths worldwide [1]. Various *Campylobacter* species, and in particular *C. jejuni*, represent the leading cause of enteric bacterial infections and acute diarrheal disease worldwide [2–4]. Dif-

ferent *Campylobacters* are classical zoonotic pathogens, living predominantly as commensals in the gastrointestinal tract of a wide range of wild and domestic birds as well as mammals, including most, if not all, agriculturally important animals. Thus, contaminated food products represent the major source of *Campylobacter* infections in humans [5]. Disease outcome in humans varies from mild, non-inflammatory, self-limiting diarrhea to severe, inflammatory, bloody diarrhea lasting for several weeks [3, 5]. In addition, *C. jejuni* is associated with the development

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of the reactive arthritis and peripheral neuropathies, the Miller–Fisher and Guillain–Barré syndromes in a minority of infected persons [6, 7]. The availability of numerous fully completed genome sequences of different *C. jejuni* isolates has identified a pathogen which exhibits a high degree of strain-to-strain variation. This natural heterogeneity has made studies on the virulence properties particularly challenging. An important characteristic of different foodborne pathogens (including *C. jejuni*) is their ability to bind to and enter human gut epithelial cells causing intestinal tissue damage. One of the key differences between infection of humans and chickens by *C. jejuni* is the apparently higher number of bacteria invading epithelial cells in the human host [3]. This suggests that both bacterial adherence to and entrance of intestinal epithelial cells may be critical steps that are essential for disease development [8]. Early studies indicated that the *C. jejuni* flagellar genes and motility are required for invasion of epithelial cells *in vitro*, and increased mucosal viscosity resulted in enhanced motility, adherence and invasion [9]. A series of papers reported on the extracellular release of *C. jejuni* proteins into the medium in the presence of fetal calf serum (FCS) or upon host cell contact, which was abrogated when certain flagellar genes were mutated. This led some groups to propose that the *C. jejuni* flagellum may act as a classical type III secretion system (T3SS) for the extracellular transport or injection of proteins, which then influence bacterial–host interactions [10–13]. The first identified secreted factor depending on the flagellum was the *Campylobacter* invasion antigen B (CiaB), a 73-kDa protein with weak homology to T3SS effectors of other pathogens [11]. This paper showed that, while *ciaB* mutants exhibited no reduction in adherence to non-polarized INT-407 cells, a significant reduction of intracellular bacteria was apparent. However, the exact function of CiaB is still not clear. In addition, *C. jejuni* adherence to cells has been shown to involve a variety of different outer membrane adhesins, including JlpA, PEB1 and the fibronectin binding proteins CadF and FlpA [14–17]. *Campylobacter jejuni* invasion of INT-407 and other cell lines has been observed to induce rearrangements of the host cytoskeleton by small Rho GTPases, which are directly linked to bacterial uptake [18]. Various lines of evidence indicated that the GTPase members, Rac1 and Cdc42, but not RhoA, are activated during infection and play a significant role in the invasion process [17, 18]. The detailed signaling pathways leading to the activation of Rac1 and Cdc42 involve CadF targeting of fibronectin and require certain receptors (integrins, EGF and PDGF receptors), kinases (FAK, Src, and PI3-kinase) and guanine exchange factors (DOCK180, Vav2, and Tiam-1). Studies using knockout cell lines, scanning electron microscopy, GTPase, and other signaling assays have shown that *C. jejuni* triggers the CadF → fibronectin → integrin-β1 → FAK/Src → EGFR/PDGFR → PI3-kinase → Vav2 and CadF → fibronectin → integrin-β1 → FAK → DOCK180/Tiam-1 signaling cascades to activate Cdc42 and Rac1, respectively, stimulating cytoskeletal rearrangements and bacterial uptake [18, 19].

We have recently shown that the serine protease HtrA (high temperature requirement A) is a novel virulence factor of *C. jejuni* [20–23]. HtrA family proteins constitute a group of conserved heat shock induced serine proteases with additional chaperone function, which were identified to influence the adhesion and invasion properties of different bacterial pathogens [24, 25]. Many bacterial HtrAs are localized in the periplasm, where they are involved in various aspects of intracellular protein quality control [26, 27]. The group of HtrA proteins typically consists of a signal peptide, a trypsin-like serine protease domain and one or two PDZ domains for protein–protein interactions. The protease domain consists of an active site, called catalytic triad, which includes the central serine residue at position 197 [28]. It was generally believed for a long time that HtrAs are strictly acting intracellularly within the bacteria. Very recently, however, we have discovered a completely new feature of HtrAs during infection with *C. jejuni* (and *H. pylori*). We surprisingly found that HtrA can be actively secreted into the extracellular environment, where it can cleave the cell surface adhesion protein and tumor-suppressor E-cadherin [22, 23, 29]. Infection studies with *C. jejuni* and *in vitro* cleavage assays showed that HtrA can cleave-off the 90-kDa extracellular NTF domain of E-cadherin to open the cell-to-cell junctions [22, 23]. Deletion of the *htrA* gene or expression of a protease-deficient S197A point mutant in *C. jejuni* lead to defects in E-cadherin shedding and transmigration of the bacteria across polarized human MKN-28 epithelial cells, but did not lead to loss of the flagella or reduced bacterial motility [23].

However, the secretion mechanism of HtrA is completely unknown. The aim of the present study was therefore to investigate different culture conditions, host cell lines, media, and the role of HtrA's protease activity and flagellar genes for HtrA secretion by *C. jejuni*.

Results

Secretion of HtrA is highly efficient in wild-type C. jejuni and isogenic strain expressing the protease-inactive S197A mutant

The secretion route of HtrA in bacteria is unknown. In Gram-negative bacteria, including *C. jejuni*, HtrAs contain a signal sequence important for Sec-dependent processing and delivery of the protease over the bacterial inner membrane into the periplasm [22, 23]. However, how HtrAs can cross the bacterial outer membrane is yet completely unclear. We therefore aimed to characterize requirements for the HtrA secretion process in *C. jejuni*. For this purpose, we utilized two *C. jejuni* wild-type (wt) strains, 81-176 and NCTC11168, its isogenic $\Delta htrA$ deletion mutants and the protease-inactive NCTC11168 *htrA*-S197A mutant. First, we confirmed the presence or the absence of HtrA protease activities by casein zymography (Fig. 1A).

Fig. 1. Secretion of *C. jejuni* HtrA into the culture supernatant is independent of its protease activity. (A) Total cell lysates of *C. jejuni* wild-type (wt) strain 81-176, 81-176ΔhtrA, NCTC11168 wt and NCTC11168 htrAS197A were investigated for protease activities by casein zymography. The position of proteolytically active multimeric HtrA proteins is indicated with red arrows. The asterisk indicates the protease-inactive HtrA multimer formed by the S197A point mutant. Equal amounts of protein per sample was confirmed by immunoblotting using an α-MOMP antibody as control. (B) HtrA secretion assay in BHI medium with 10% FCS. Wt *C. jejuni* NCTC11168 and isogenic ΔhtrA deletion and htrAS197A point mutants were grown for 12 hours at 37 °C. Bacterial supernatants and cell pellets were filtered and prepared as described in *Materials and methods*. The presence of secreted HtrA proteins in the supernatant was investigated by immunoblotting using the α-HtrA antibody and as control with α-MOMP antibody. To ensure equal amounts of bacteria in each sample, the blot for bacterial pellets was stripped and reprobed with α-CiaB and α-MOMP antibodies

The position of proteolytically active multimeric HtrA proteins in the two lanes with wt bacteria is indicated with red arrows. The asterisk indicates the protease-inactive HtrA multimer formed by the S197A point mutant. Equal amounts of bacteria per sample were confirmed by immunoblotting using α-MOMP antibodies (*Fig. 1A*), recognizing the 45-kDa major outer membrane protein of *C. jejuni* [30].

HtrA secretion assays were established recently [22, 23]. To test if the protease activity of HtrA is required for its secretion into the extracellular space, wt NCTC11168 and NCTC11168 htrAS197A, and ΔhtrA mutants were grown in BHI medium with 10% FCS for 12 hours. Bacterial supernatants and cell pellets were prepared, and the presence of secreted HtrA proteins in the supernatant was investigated by immunoblotting using α-HtrA antibodies. The results show that wt *C. jejuni* and the htrAS197A point mutant exhibited similarly strong HtrA signals in the supernatant fraction, while the ΔhtrA deletion mutant did not as expected (*Fig. 1B*). As control, the supernatants were also probed with α-MOMP antibodies, exhibiting no signals at all. This indicates that the supernatants are devoid of lysed bacteria and cell debris. In contrast, the α-MOMP and α-CiaB blots of bacterial cell pellets showed strong bands of similar intensity, confirming that equal amounts of protein were present in each sample (*Fig. 1B*). Taken together, these experiments indicate that the protease-inactive HtrA S197A mutant can be delivered into the culture supernatant like wt HtrA, thus the translocation process is independent of HtrA's protease activity.

Secretion of HtrA into the culture supernatant is independent of C. jejuni flagellar genes

A tempting hypothesis was that HtrA maybe secreted into the extracellular milieu using the flagellum as it has been shown for CiaB and other *C. jejuni* factors [10–13]. To test this idea, *C. jejuni* wt strain 81-176 and its isogenic flagellar mutants ΔflaA/B, ΔfliI, ΔflgH, ΔflhA, ΔflhB, and ΔflgS [32–34] were subjected to HtrA secretion assays as described above. The blots of supernatant fractions showed that all flagellar mutants exhibited similar strong HtrA signals as compared to wt *C. jejuni* (*Fig. 2A*). This was confirmed by densitometric measurements of HtrA band intensities, revealing the relative amounts of secreted HtrA per strain. The signal corresponding to secreted HtrA in the isogenic ΔflaA/B was even slightly stronger than the signal for wt *C. jejuni* (*Fig. 2B*). These results let us conclude that secretion of HtrA into the extracellular environment proceeds independently of the intact flagellum. To further verify that our assays work properly, the supernatant blot was stripped and reprobed with α-CiaB antibodies to monitor CiaB secretion in the same experiment. The results showed strong signals for secreted CiaB in wt *C. jejuni* and ΔhtrA control, but 5–10 times weaker signals were obtained with the various flagellar mutants (*Fig. 2A*) as confirmed by densitometric measurements (*Fig. 2B*). The corresponding bacterial pellets

Fig. 2. Secretion of *C. jejuni* HtrA into the culture supernatant is independent of 19 flagellar genes. (A) *C. jejuni* wild-type (wt) strain 81-176 and its isogenic Δ htrA and flagellar mutants Δ fliA/B, Δ fliL, Δ fliH, Δ fliA, Δ fliB and Δ fliS were grown in MH medium with 10% FCS for 12 hours at 37 °C. Bacterial supernatants and cell pellets were filtered and prepared as described in *Materials and methods*. The presence of secreted HtrA and CiaB proteins in the supernatant was investigated by immunoblotting using α -HtrA and α -CiaB antibodies. (B) Densitometric measurements of HtrA and CiaB band intensities of panel A revealed the relative amounts of secreted HtrA and CiaB proteins, respectively. The strongest band in each blot corresponds to 100%. (C) To ensure equal amounts of bacteria in each sample, the corresponding bacterial pellets were probed with α -HtrA and α -*C. jejuni* antibodies

revealed similar protein amounts per sample, indicating that differences seen in the amounts of CiaB were not due to different quantities of bacteria per sample (Fig. 2C). This confirms observations that secretion of CiaB, but not HtrA, is mediated by flagellar genes. As control, we have also tested various flagellar mutants of *H. pylori*, which also secrete HtrA in high amounts with slight but no significant differences (Suppl. Fig. 1A/B).

Secretion of HtrA in the presence of several cell lines and different culture media

As next, we aimed to investigate if HtrA secretion by *C. jejuni* is influenced by the presence of different culture media, cell lines of different mammalian origin and FCS. For this purpose, different cell lines of human, dog, mouse, hamster, or chicken origin (Table 1) were grown in cell culture medium. After reaching ~70% confluency, the medium was replaced by fresh cell culture medium, MH or BHI medium in the presence or absence of 10% FCS. *Campylobacter jejuni* wt strains 81-176 or NCTC11168, respectively, were added, and HtrA secretion into the cell culture supernatant was investigated after co-incubation. The results for 81-176 showed different signal intensities of secreted HtrA in the supernatant fraction depending on the cell line and medium type (Fig. 3). This suggests that the amount of secreted HtrA clearly depends on environmental signals. A clear tendency revealed in most, but not all, cases that addition of FCS enhanced the amount of extracellular HtrA. In BHI medium plus FCS, we detected the strongest HtrA signals, irrespective of the cell line and origin. Basic levels of secreted HtrA were similar between cell lines of human, dog, mouse, hamster, and chicken cells. Interestingly,

Fig. 3. Secretion of HtrA in the presence of several cell lines and different culture media. (A) Different indicated cell lines of human, dog, mouse, hamster or chicken origin were grown in cell culture medium (CCM, summarized in Table 1). After reaching ~70% confluency, the medium was replaced by fresh CCM, MH or BHI medium in the presence or absence of 10% FCS as indicated. Cells were then infected for 6 hours with *C. jejuni* wild-type (wt) strain 81-176. Cell culture supernatants were sterile filtered to remove traces of bacteria and cell pellets were harvested by centrifugation. HtrA secretion into the supernatant was investigated using the α -HtrA antibody. Quantitation data are summarized in Table 2. (B) To ensure equal amounts of bacteria in each sample, the corresponding bacterial pellets were probed with α -HtrA antibodies

almost no HtrA signals were seen in cell culture or MH medium with the human cell line 23132/87. By comparison, the amount of secreted HtrA in cell culture medium was also low with human cell lines MKN-7, MKN-74, and T-84 but very strong in the presence of BHI and FCS (Fig. 3). The overall quantitation data for HtrA secretion are summarized in Table 2. Similar results for HtrA secretion were seen with strain NCTC11168, albeit the intensity of HtrA signals were mostly one to two times lower as compared to strain 81-176 (data not shown). Thus, HtrA of *C. jejuni* is effectively secreted into the cell culture supernatant and facilitated by extracellular signals with different secretion properties.

Discussion

The mucosa in the human intestine forms a tight barrier, which protects against host cell invasion by commensals, non-pathogenic microbes residing in the intestinal lumen. However, various enteric pathogens, including *Salmonella*, *Listeria*, *Shigella*, and *Campylobacter*, can translocate across polarized epithelial cells via the paracellular route or transcellular route, respectively. Previous studies have shown that *C. jejuni* can transmigrate across Caco-2 and other polarized cell monolayers without a concomitant loss in transepithelial electrical resistance, indicating that *C. jejuni* can pass polarized epithelial cells whose integ-

Table 1. Mammalian cell lines and chicken primary cells used in this study

Cell line	Source (catalogue #)	Origin	Origin, patient (age, year)	Cell culture medium ^a
MKN-7	JCRB (1025)	Human stomach	Adenocarcinoma, male (39)	RPMI 1640 (Gibco), 2 mM L-glutamine, 25 mM HEPES, 10%FCS (Gibco)
MKN-74	JCRB (0255)	Human stomach	Liver metastasis from stomach cancer, male (37)	RPMI 1640 (Gibco), 2 mM L-glutamine, 25 mM HEPES, 10%FCS (Gibco)
23123/87	DSMZ (ACC-201)	Human stomach	Primary gastric adenocarcinoma, male (72)	RPMI 1640 (Gibco), 2 mM L-glutamine, 25 mM HEPES, 10%FCS (Gibco)
Caco-2	ATCC (CRL-2102)	Human colon	Colorectal adenocarcinoma, male (72)	DMEM (Gibco), 4.5 g/L D-glucose, 4 mM L-glutamine, 110 mg/L sodium pyruvate, 10%FCS (Gibco)
T84	ATCC (CCL-248)	Human colon	Colorectal carcinoma, derived from metastatic site: lung, male (72)	DMEM (Gibco), 4.5 g/L D-glucose, 4 mM L-glutamine, 110 mg/L sodium pyruvate, 10%FCS (Gibco)
MDCK	ATCC (CCL-34)	Canine kidney	Cocker spaniel, female, adult (unknown)	DMEM (Gibco), 4.5 g/L D-glucose, 4 mM L-glutamine, 110 mg/L sodium pyruvate, 10%FCS (Gibco)
NIH3T3	ATCC (CRL-1658)	Mouse embryonic fibroblasts	Mouse embryo (unknown)	DMEM (Gibco), 4.5 g/L D-glucose, 4 mM L-glutamine, 110 mg/L sodium pyruvate, 10%FCS (Gibco)
CHO-K1	ATCC (CCL-61)	Chinese hamster ovary	Hamster, female (unknown)	DMEM (Gibco), 4.5 g/L D-glucose, 4 mM L-glutamine, 110 mg/L sodium pyruvate, 10%FCS (Gibco)
PCC cells	Primary cells	Chicken cecum	Leghorn chicken (3 weeks)	Quantum 286 (PAA), 5% chick serum (Sigma-Aldrich), 0.5 % chick embryo extract (MP Biomedicals)

Abbreviations: ATCC, American Type Culture Collection (www.atcc.org); DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (www.dsmz.de); JCRB, Japanese Collection of Research Biosources (<http://cellbank.nibio.go.jp/>); PCC, primary chicken cecal.

^a DMEM, Dulbecco's modified Eagle's medium; Quantum 286, complete medium for epithelial cells; RPMI 1640, Roswell, Park Memorial Institute 1640 medium

Table 2. Secretion properties of *C. jejuni* 81-176 HtrA into various culture media in the presence of different cell lines^a

Cell line	CCM	CCM + FCS	MH	MH + FCS	BHI	BHI + FCS
MKN-7	+	++	++	+++	++++	++++
MKN-74	+	+++	++	++++	++++	++++
23123/87	–	+	–	++	+	++++
Caco-2	++++	+++	+	++	+++	++++
T84	++	+++	+	++++	+++	++++
MDCK	++++	+++	++++	+++	++++	++++
NIH3T3	++++	++++	++	+++	+++	++++
CHO-K1	++	+++	++	+++	++	++
PCC cells	+++	++++	++	++++	+++	++++

Abbreviations: CCM, cell culture medium (for details see Table 1); MH, Mueller Hinton; BHI, Brain heart infusion; FCS, fetal calf serum; PCC, primary chicken cecal.

^a Intensity of HtrA signals in supernatant after 6 h of culturing. Signals correspond to relative amounts of secreted HtrA: +++++, very strong; +++ strong; ++ moderate; + weak; – no or very weak

ity, however, remains intact [35–38]. We are interested in characterizing bacterial and host factors involved in *C. jejuni* transmigration. Our previous data suggested that HtrA from *C. jejuni*, a well-known periplasmic serine protease and chaperone, can be secreted into the cell culture supernatant and was shown to play a major role in crossing polarized epithelial monolayers [22, 23]. In particular, we found that deletion of *htrA* or replacing wt HtrA by a protease-inactive S197A mutant exhibited a strong transmigration defect, indicating that HtrA's protease activity indeed plays a role in this process. We could further show that recombinant *C. jejuni* HtrA can cleave E-cadherin, a major adherent junctional protein, into several subfragments *in vitro* and during infection *in vivo*. Thus, HtrA-mediated cleavage of E-cadherin (and probably other junctional proteins) may be involved in *C. jejuni* transmigration.

The process of HtrA secretion into the medium by Gram-negative bacteria is completely unclear. In this study, we aimed to pinpoint conditions of HtrA secretion by *C. jejuni*. We could show that HtrA secretion is a highly efficient process and can be easily detected in non-concentrated culture supernatants by Western blotting using α -HtrA antibodies. Interestingly, HtrA secretion does not require its proteolytic activity because the protease-resistant S197A mutant can be secreted into the supernatant similar to wild-type HtrA. Rivera-Amill and Konkel [39] have previously shown that secretion of *C. jejuni* Cia proteins such as CiaB is induced upon incubation with human intestinal cells, thus is contact dependent. Cia secretion can be also induced upon incubating *C. jejuni* in medium supplemented with FCS [39]. To investigate the effective conditions for HtrA secretion, we tested several culture media (MH, BHI, DMEM, and RPMI-1640) and cell lines of different origin such as human (MKN-7, MKN-74, 23132/87, Caco-2, and T84), mouse (NIH3T3), hamster (CHO-K1), dog (MDCK), and chicken (primary intestinal cells) for their ability to influence HtrA release. Interestingly, HtrA was effectively secreted into the culture supernatant in the presence of most but not all cell lines and media, albeit at different levels, but secretion was significantly higher (about twofold to fivefold) when FCS was added. The synthesis and secretion of the Cia proteins were not affected by heat-treatment of FCS, indicating that the stimulating molecule in serum is heat stable [39]. The actual stimulatory molecule is still unknown but was not unique to FCS as sera from other sources including human, pig, sheep, goat, rabbit, mouse, and chicken also induced the synthesis and release of the Cia proteins [39]. These findings collectively indicate that the secretion of the Cia and HtrA proteins can be induced in the presence of various host cells or in a cell-free system by incubating *C. jejuni* in serum-supplemented tissue culture medium.

The secretion of Cia proteins has been shown to require a functional flagellar export system which acts as a type III secretion system [8, 40]. The above similarity between CiaB and HtrA secretion capabilities with respect to enhancement by FCS let us therefore suggest that HtrA

may follow the same secretion route as compared to CiaB. We thus tested if the flagellar mutants $\Delta flaA/B$, $\DeltafliI$, $\Delta flgH$, $\Delta flhA$, $\Delta flhB$, and $\Delta flgS$ are defective for secretion of both HtrA and CiaB. Unexpectedly, we found that while all flagellar mutants were strongly attenuated in secreting CiaB, HtrA secretion remained widely unaffected under the same conditions. Similar results were obtained for various flagellar mutants in *H. pylori*, which also did not affect HtrA secretion. This suggests that HtrA secretion is clearly independent of the flagellum, and in *C. jejuni* is therefore different as compared to the pathway described for CiaB. In contrast to CiaB, HtrAs in Gram-negative bacteria, such as *C. jejuni* and *H. pylori*, contain a signal sequence important for Sec-dependent processing and delivery of the protease over the bacterial inner membrane into the periplasm [22, 23, 29]. However, how HtrAs can cross the bacterial outer membrane is not known. Commonly, HtrAs do not exhibit homology to classical autotransporters which process themselves by autoprolysis, which is in agreement with our observation that HtrAs protease activity is not required for secretion.

Thus, HtrA delivery very likely needs additional bacterial factor(s) for the secretion process as known from other secretory systems of types I–VII. However, various genome sequencing reports have shown that most classical types I–VII transporter systems are missing in *C. jejuni* [41, 42, 43]. For example, a type IV secretion system has been found on the plasmid pVir, but pVir is not present in the used strain NCTC11168, and is therefore not involved in HtrA secretion. A few genes in NCTC11168 (*cj1470* and *cj1474c*) have been noted as putative type II secretion components which could be involved in protein secretion [41]. However, the genes *cj1343c*, *cj1470c*, *cj1471c*, and *cj1474c* were then described to have a role in natural competence and DNA import into *C. jejuni* [44]. If these genes are involved in HtrA secretion remains to be investigated. Another possibility is the extracellular transport of HtrA via outer membrane vesicles (OMVs), which are continuously shed from the bacteria. We have indeed evidence that HtrA is present in purified OMVs from *H. pylori* [45], but if this also the case for *C. jejuni* is unknown. Future studies should investigate systematically how HtrA is secreted into the extracellular space by Gram-negative bacteria.

Materials and methods

Bacterial strains

The wild-type *C. jejuni* strains NCTC11168 and 81-176 were used in this study. The isogenic mutants 81-176 $\Delta htrA$, 11168 $\Delta htrA$ and 11168 $\Delta htrA$ S197A were recently described [20–23]. The flagellar mutants $\Delta flaA/B$, $\DeltafliI$, $\Delta flgH$, $\Delta flhA$, $\Delta flhB$, and $\Delta flgS$ in strain 81-176 were all generated in the strain 81-176 background with introduced streptomycin resistance marker rpsLSm [32]. The mutants $\Delta flgS$ (81-176 rpsLSm flgS::cat-rpsL) and $\Delta flhA$ (81-176 rpsLSm flhA::cat-rpsL) [33] and $\DeltafliI$ (81-176

rpsLSM flhI::cat-rpsL) [34] were previously described. To generate $\Delta flhB$ mutant, strain 81-176 rpsLSm was electroporated with plasmid pSNJ360, a suicide vector that contains flhB::cat-rspL [34]. Transformants were recovered on Mueller–Hinton (MH) agar containing chloramphenicol. Colony PCR was used to verify replacement of flhB with flhB::cat-rpsL on the chromosome and one transformant designated clone SNJ407 was saved. The 81-176 rpsLSm flgH::cat-rpsL mutant was generated as described [46]. Transformants were recovered on MH agar containing chloramphenicol.

Colony PCR was used to verify replacement of flgH gene with flgH::cat-rpsL on the chromosome and one transformant clone designated ALU103 was saved [46]. All *C. jejuni* strains were grown on *Campylobacter* blood-free selective Agar Base (Oxoid) containing *Campylobacter* growth supplement (Oxoid) or on MH agar at 37 °C under microaerobic conditions (generated by CampyGen gas packs, Oxoid) for 48 hours. In case of the *C. jejuni* deletion mutant, agar plates were amended with 30 µg/ml or chloramphenicol. The *H. pylori* strain ATCC53405 and its flagellar mutants were generated and cultivated as described [47, 48].

HtrA secretion assays

Bacterial wild-type and mutant strains were grown in BHI broth medium supplemented with 10% FCS for 12 hours starting with an OD_{600 nm} ~ 0.2. The supernatant and the cell pellets were separated by centrifugation at 4000 rpm, and the supernatant was further purified from remaining bacterial cells by passage through a 0.21 µm sterile filter (Sigma-Aldrich). The resulting bacterial pellets and supernatants were analyzed by immunoblotting and casein zymography as described below. Absence of live bacteria in the supernatant was confirmed by incubation on agar plates showing no growth. Quantitation of α -HtrA signals on Western blots was performed by densitometric measurement of band intensities in the blots using the Lumi-Imager F1 (Roche).

Casein zymography

Bacterial lysates, culture supernatants or recombinant HtrA were separated in casein containing gels under non-reducing conditions. Subsequently, gels were renatured in 2.5% Triton-X-100 and equilibrated in developing buffer [49]. Caseinolytic activity was visualized by staining with 0.5% Coomassie Blue R250.

Host cell lines and infection studies

The origin of the used cell lines and medium conditions is given in Table 1. For the infection studies, about 4×10^5 cells were seeded in 12-well tissue culture plates and

grown for 2–3 days. After reaching about 70–80% confluency, cells were washed two times with PBS. The culture medium was then replaced with fresh medium without antibiotics and starved for 12 hours before infection. Bacteria were suspended in culture medium, added to the cells at a multiplicity of infection (MOI) of 100 and co-incubated with host cells for the indicated periods of time per experiment.

Preparation and cultivation of primary chicken cecal (PCC) cells

Three-week-old white Leghorn chicks hatched from specific pathogen-free eggs (Lohmann Tierzucht GmbH, Germany) were dissected, the ceca separated and transferred into sterile ice-cold PBS with gentamicin (150 µg/ml). The intestines were slit open and thoroughly washed with the same buffer to remove all intestinal contents. Using a pair of sterile scissors, the tissue was minced into extremely fine pieces. The pieces were placed on a dry, collagen from chicken type II coated surface (100 µg/ml, Sigma-Aldrich) to allow them to adhere for up to 30 min. Finally Quantum 286 (complete medium for epithelial cells, PAA Germany) supplemented with 5% chick serum (Sigma-Aldrich), 0.5% chick embryo extract (MP Biomedicals, Germany) and 150 µg/ml gentamicin was added carefully. The cells were cultivated for 2 days in a humidified environment at 37 °C with 5% CO₂. Subsequently, the medium was replaced with fresh culture medium but with reduced concentration of gentamicin (50 µg/ml). When the cultures reached confluence, the cells were sub-cultivated using accutase (PAA, Germany). Tissue pieces were removed by filtration of the cell suspension through a cell strainer. After the second passage, the cells were frozen and stored in the gas phase of liquid nitrogen. The PCC cells could be cultured without loss of viability or morphological organization up to 10 passages.

SDS-PAGE and Western blotting

After infection, the cell culture supernatants were harvested and filtered as described above. Cell pellets were lysed [29], and proteins were separated by SDS-PAGE. The polyclonal rabbit α -HtrA antibody [21, 31], polyclonal rabbit anti-Campylobacter (MyBiosource) were used as described. The α -CiaB antibody was raised against a conserved peptide corresponding to the following conserved amino acid (aa) residues 597–610: C-EIDNSGE-FERYKKK. The α -MOMP antibody was raised against a conserved peptide corresponding to aa residues 400–413: C-NLDQGVNTNESADH. Both peptides were conjugated to *Limulus polyphemus* hemocyanin carrier protein and two rabbits each were immunized according to standard protocols by Biogenes GmbH (Berlin, Germany). The resulting antiserum was affinity-purified and the specificity against the CiaB protein was confirmed by Western

blotting. Horseradish peroxidase-conjugated anti-rabbit polyvalent sheep immunoglobulin was used as secondary antibody (DAKO Denmark A/S, DK-2600 Glostrup, Denmark). Blots were developed with ECL Plus Western blot reagents (GE Healthcare, UK limited Amersham Place, UK) as described [50].

Statistical analysis

All data were evaluated using Student *t*-test with Sigma-Stat statistical software (version 2.0). Statistical significance was defined by $P \leq 0.05$ (*) and $P \leq 0.005$ (**). All error bars shown in figures and those quoted following the $\pm$ signs represent standard deviations.

Competing interests

The authors have declared that no competing interests exist.

Author contributions

Conceived and designed the experiments: MB, NT, SW
 Performed the experiments: MB, NT, IH, BH
 Analyzed the data: MB, NT, IH, BH, SW
 Contributed reagents/materials/analysis tools: DH, LB, TGS, TH, SW, IH
 Wrote the paper: NT

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Supplement

Suppl. Fig. 1. Secretion of *H. pylori* HtrA into the culture supernatant is independent of flagellar genes. (A) *H. pylori* wild-type (wt) strain ATCC43504 and its isogenic flagellar mutants *fliK*, *flhX*, *flhB*, *flhA*, *flhA77* and *flhA454* were grown in BHI medium with 10% FCS for 12 hours at 37 °C. Bacterial supernatants and cell pellets were filtered and prepared as described for *C. jejuni* in *Materials and methods*. The presence of secreted HtrA proteins in the supernatant and pellet was investigated by immunoblotting using α -HtrA antibodies. (B) Densitometric measurements of HtrA band intensities of panel A revealed the relative amounts of secreted HtrA proteins, respectively. The strongest band in each blot corresponds to 100%.