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CAMPYLOBACTER JEJUNI SERINE PROTEASE HTRA PLAYS AN IMPORTANT ROLE IN HEAT TOLERANCE, OXYGEN RESISTANCE, HOST CELL ADHESION, INVASION, AND TRANSMIGRATION

Manja Boehm*, Judith Lind, Steffen Backert and Nicole Tegtmeyer

Department of Biology, Division of Microbiology, Friedrich Alexander University Erlangen/Nuremberg, Staudtstr. 5, D-91058 Erlangen, Germany

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Campylobacter jejuni is an important pathogen of foodborne illness. Transmigration across the intestinal epithelial barrier and invasion are considered as primary reasons for tissue damage triggered by *C. jejuni*. Using knockout mutants, it was shown that the serine protease HtrA may be important for stress tolerance and physiology of *C. jejuni*. HtrA is also secreted in the extracellular environment, where it can cleave junctional host cell proteins such as E-cadherin. Aim of the present study was to establish a genetic complementation system in two *C. jejuni* strains in order to introduce the wild-type *htrA* gene *in trans*, test known *htrA* phenotypes, and provide the basis to perform further mutagenesis. We confirm that reexpression of the *htrA* wild-type gene in Δ*htrA* mutants restored the following phenotypes: 1) *C. jejuni* growth at high temperature (44 °C), 2) growth under high oxygen stress conditions, 3) expression of proteolytically active HtrA oligomers, 4) secretion of HtrA into the supernatant, 5) cell attachment and invasion, and 6) transmigration across polarized epithelial cells. These results establish a genetic complementation system for *htrA* in *C. jejuni*, exclude polar effects in the Δ*htrA* mutants, confirm important HtrA properties, and permit the discovery and dissection of new functions.

Keywords: HtrA, secretion, chaperone, flagellum, E-cadherin, molecular pathogenesis, cellular invasion, signaling, transwell, paracellular, virulence, stress response

Abbreviations: BHI, brain heart infusion; CFU, colony forming; FCS, fetal calf serum; MH, Mueller-Hinton; MOI, multiplicity of infection; NaCl, sodium chloride; DPBS, Dulbecco's phosphate buffered saline; TER, transepithelial electrical resistance; OD, optical density; HtrA, high temperature resistant protein A

Introduction

Foodborne and waterborne diseases are responsible for very high rates of morbidity and mortality in humans worldwide. The World Health Organization reported that these diseases kill an estimated number of 2.2 million people annually, most of whom are children [1]. The Gram-negative pathogen *Campylobacter jejuni* is the most common cause of bacterial foodborne infections in developed countries [2, 3]. *C. jejuni* colonizes the intestinal tract of a wide range of wild and domestic birds as well as agriculturally important mammals. Thus, handling and consumption of contaminated poultry meat products or raw milk have been established as the most frequent sources of campylobacteriosis in humans [4]. An infectious dose of a few hundred bacteria is sufficient to establish colonization in the human host. The clinical symptoms of *Campylobacter* infections include diarrhea, abdominal pain, and fever, which in rare cases can lead to the development of more serious complications such as reactive arthritis and Guillain–Barrè and Miller–Fisher syndromes [5, 6]. In contrast to the disease-causing infections in humans, no apparent symptoms are found in colonized poultry or other avian species, indicating a well-adapted commensalism in the avian cecum [2, 7]. In line with these observations, *C. jejuni* exhibits a microaerophilic lifestyle with optimal growth temperature at 37–42 °C.

During transmission, *C. jejuni* is exposed to multiple stress conditions, such as osmotic stress, pH changes, atmospheric oxygen exposure, and temperature fluctuation,

* Corresponding author: Manja Boehm Ph.D.; Friedrich Alexander University Erlangen/Nuremberg, Department of Biology, Division of Microbiology, Staudtstr. 5, D-91058 Erlangen, Germany; Phone: ++49 9131 8528588; Fax: ++49 9131 8528082; E-mail: manja.boehm@fau.de

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requiring a resilient stress response system [7–9]. *C. jejuni* is a typical facultative intracellular pathogen due to its capabilities to adhere to and invade into human gut epithelial cells [2]. However, a main difference between *C. jejuni* infection of humans and poultry is the apparently elevated number of bacteria entering epithelial cells in the human gut [2]. This led to the common view that *C. jejuni* adhesion to and entrance of human intestinal epithelial cells may represent critical steps, which are essential for disease development [10].

The *C. jejuni* flagellar genes and motility are required for invasion of intestinal epithelial cells, and increased mucosal viscosity resulted in enhanced bacterial motility, adherence and invasion [11]. The *C. jejuni* flagellum also serves as a type-III secretion system for the extracellular release of factors that may control bacteria–host interactions [12–15]. These secreted effector proteins include the so-called *Campylobacter* invasion antigens (CiaA-H) and flagellar coexpressed determinants (FedA-D) [13, 16–18]. Moreover, *C. jejuni* binding to host target cells has been reported to include a series of outer membrane adhesins such as PEB1, MOMP, JlpA, CadF, and FlpA [10, 16, 19–21]. In addition, the chaperone domain of serine protease HtrA (high temperature requirement A) contributes to cell binding and invasion, probably by assisting in properly folding of one or more of the above adhesins [22, 23]. A prerequisite of *C. jejuni* host cell invasion is the induction of cytoskeletal rearrangements driven by the small Rho GTPases Cdc42 and Rac1 [24]. These GTPases are activated upon binding and have significant impact during the course of invasion [16, 24]. The signaling cascades stimulating Cdc42 and Rac1 are complex and require CadF, certain receptors (fibronectin, integrin-β_1, PDGF- and EGF-receptor tyrosine kinases), cytosolic kinases (Src, FAK, and PI3-kinase), and guanine exchange factors (Tiam-1, Vav-2, and DOCK180), which mediate cytoskeletal remodelling in host cells followed by bacterial uptake [25, 26].

Recent studies have demonstrated that HtrA is a novel secreted virulence factor of *C. jejuni* [22, 23, 26]. HtrA proteins are widely conserved in many bacteria and represent a group of heat shock induced serine proteases with supplementary chaperone function [27–30]. HtrA proteases commonly consist of a signal peptide, a trypsin-like serine protease module, and 1–2 PDZ domains to facilitate protein–protein interactions [31]. HtrA proteins have significant impact on the survival and virulence properties of multiple pathogens [28, 30, 31]. For example, as compared to wild-type (wt) *C. jejuni*, it was shown that growth of Δ*htrA* mutants is severely diminished at 44 °C and tolerance of the Δ*htrA* mutant against oxygen stress is also significantly reduced [22]. These proteases have been commonly found to act in the periplasm, where they assemble proteolytic active oligomers with crucial function in controlling protein quality [32, 33]. For a long time, it was believed that HtrA family members are strictly operating only intracellularly within the bacteria. However, we have recently unraveled a new feature of HtrA during infection. In *C. jejuni* and its close relative *Helicobacter pylori*, HtrA proteins are actively secreted in the extracellular space, where they can hijack host proteins [26, 34, 35]. Infection experiments *in vitro* indicated that HtrA can open the cell-to-cell junctions in cell monolayers by cleaving-off the extracellular domain of the surface adhesion protein E-cadherin followed by paracellular transmigration of the bacteria [26, 35]. Deletion of the *htrA* gene leads to a de-

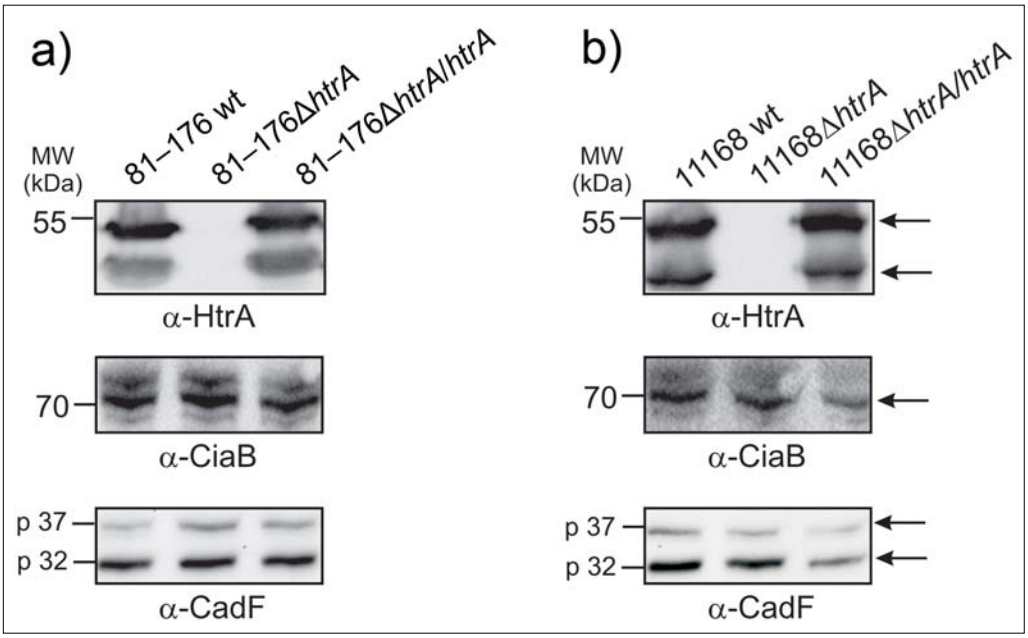

Fig. 1. Western blotting confirms genetic complementation of wild-type (wt) *htrA* gene in *C. jejuni* Δ*htrA* deletion mutants. The wt *htrA* gene with its own promoter was complemented in the Δ*htrA* deletion mutants of *C. jejuni* strains 81–176 (a) and NCTC11168 (b). Bands show presence of HtrA protein expression. As loading controls, the α-CiaB and α-CadF blots confirmed that equal amounts of protein were present in each sample

fect in E-cadherin shedding and impaired transmigration of *C. jejuni* across monolayers of polarized epithelial cells *in vitro* [26]. An insect infection model has shown that the outcome of a *C. jejuni* infection is also affected by HtrA, because fewer *Galleria mellonella* larvae are killed by an *htrA* mutant as compared to the wt strain [36]. In addition, using *C. jejuni* Δ*htrA* deletion mutants, we have shown that HtrA plays a pivotal role in inducing host cell apoptosis and immunopathology in the gut of mice [37, 38]. Goal of the present study was to establish a genetic complementation system in *C. jejuni* to confirm the importance of HtrA in heat tolerance, oxygen stress resistance, epithelial cell adhesion, invasion, and transmigration of the bacteria.

Results

Complementation confirms the importance of HtrA in heat tolerance and oxygen stress resistance by C. jejuni

We have generated several Δ*htrA* deletion mutants in the well-known *C. jejuni* strains 81–176 and NCTC11168 [29, 39]. Next aim was to develop a fast and reliable genetic complementation system of the wt *htrA* gene for further investigation of the importance of this factor both during conventional growth and infection processes. For this purpose, the *htrA* gene of *C. jejuni* NCTC11168 including its own promoter was ligated to a kanamycin resistance gene, and this cassette was inserted in the pseudogene downstream of *Cj*0208, a region which is present in both strains. Correct integration of *htrA* in the *C. jejuni* chromosomes of 81–176 and NCTC11168 was confirmed by PCR and standard sequencing (data not shown). Reexpression of HtrA proteins was verified by Western blotting *(Fig. 1a/b)*. The α-CiaB and α-CadF blots served as loading controls and confirmed that equal amounts of protein were present in each lane.

Next, we investigated the growth of *C. jejuni* strains on Mueller Hinton (MH) agar plates at 37 °C, 42 °C, and 44 °C under microaerobic conditions (~5% O_2) or at 42 °C in the presence of ~18% O_2 *(Fig. 2)*. All strains were growing equally well at 37 °C and 42 °C under microaerobic conditions, but growth of the 81–176Δ*htrA* mutant was severely impaired at 44 °C and tolerance of the mutant to an atmosphere containing 18% O_2 was strongly reduced as compared to the isogenic wt strain *(Fig. 2a–d)*. Genetic complementation of the wt *htrA* gene in Δ*htrA* mutant strain restored *C. jejuni* 81–176 growth in the presence of 5% O_2 at 44 °C *(Fig. 2c)* and growth in the presence of 18% O_2 at 42 °C *(Fig. 2d)*. The same experiments were performed with wt, Δ*htrA* mutant, and complemented *htrA* in strain NCTC11168 with similar results compared to strain 81–176 (data not shown). These experiments demonstrate that *C. jejuni* HtrA plays a crucial role in heat tolerance and oxygen stress resistance of these bacteria.

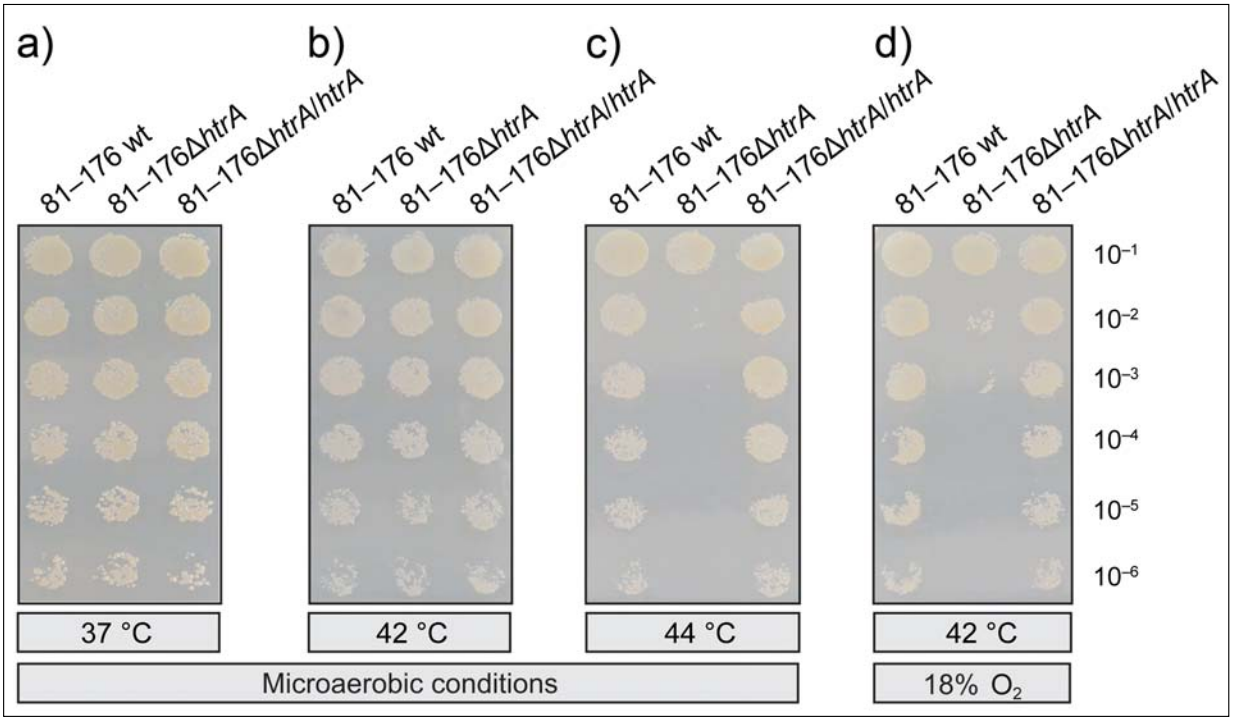

Fig. 2. Genetic complementation confirms the importance of HtrA in heat tolerance and oxygen stress resistance by *C. jejuni.* Serial dilutions of *C. jejuni* strains [81–176 wild-type (wt), 81–176Δ*htrA*, and complemented 81–176Δ*htrA*/*htrA*] were spotted onto MH agar plates as indicated. The plates were incubated for 3 days in jars under microaerobic conditions at 37 °C (a), 42 °C (b), and 44 °C (c) or at 42 °C in the presence of 17–18% O_2 (d). Representative sections of the agar plates from three independent experiments are presented. The results show that reintroduction of wt *htrA* gene in the Δ*htrA* mutant strain restored *C. jejuni* growth under the indicated conditions. Experiments were done in triplicates

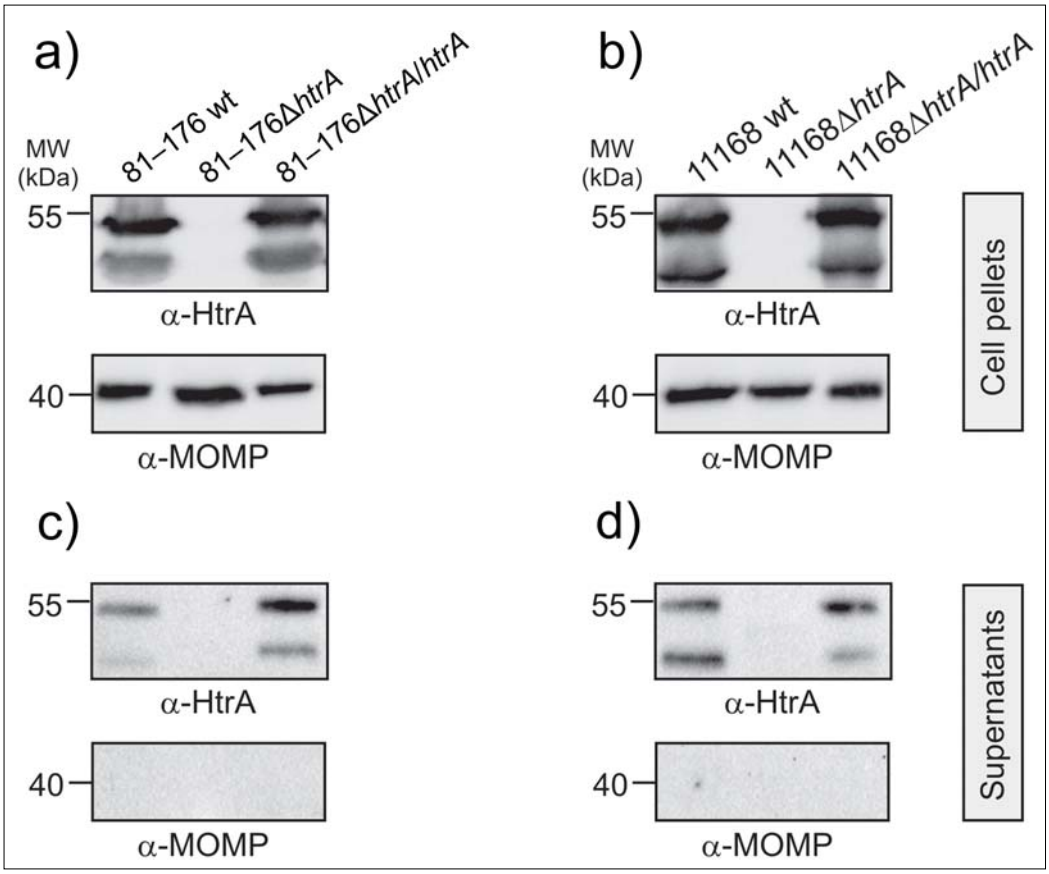

Fig. 3. Western blotting showing presence of HtrA protein in bacterial pellets (top) and culture supernatants (bottom). Wild-type (wt), isogenic Δ*htrA* deletion mutants, and complemented Δ*htrA/htrA* strains of *C. jejuni* 81–176 (a, b) and NCTC11168 (c, d) were grown in BHI medium with 10% FCS for 12 h at 37 °C, and then fractionated. Equal amounts of protein in each bacterial pellet and absence of bacterial lysis in the supernatants were confirmed by probing with α-MOMP antibody

Secretion of HtrA in the extracellular space is restored by the complemented C. jejuni *strains*

To test if HtrA is secreted by the complemented *C. jejuni* variants, all above described strains were grown for 12 h in brain heart infusion (BHI) liquid broth medium containing 10% fetal calf serum (FCS). 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 *(Fig. 3a–d)*. The results show that all wt *C. jejuni* and *htrA* complemented strains exhibit similarly strong HtrA signals in the supernatant fraction while the corresponding Δ*htrA* deletion mutants did not *(Fig. 3b/d)*. As control, the bacterial pellets and supernatants were probed with α-MOMP antibodies. The α-MOMP blots of bacterial cell pellets show strong bands of similar intensity as expected, confirming that equal amounts of protein were present in each sample *(Fig. 3a/c)*, while the supernatants are devoid of MOMP, indicating absence of lysed bacteria and cell debris *(Fig. 3b/d)*. Taken together, these experiments indicate that the complemented strains restored their ability to deliver HtrA proteins in the extracellular environment.

HtrA proteins of the complemented C. jejuni *strains form proteolytically active oligomers*

Next, we aimed to analyze if complemented HtrA can form proteolytically active oligomers. For this purpose, the fractionated samples generated for *Fig. 3* were subjected to casein zymography. Bacterial pellets and sterile filtered culture supernatants were loaded onto a 0.1% casein containing gel and separated under nondenaturating conditions [35]. The results show that HtrA from both wt *C. jejuni* and complemented strains gave rise to active HtrA oligomers with a molecular weight ranging from ~120 kDa to more than 200 kDa in the pellet (*Fig. 4a*, arrows) and supernatant (*Fig. 4b*, arrows). As control, corresponding signals for proteolytically active HtrA were completely absent in both Δ*htrA* deletion mutants as expected *(Fig. 4a/b)*.

Complementation of htrA *restores cell adhesion and invasion properties of* C. jejuni

Recent work has shown that the chaperone domain of HtrA contributes to binding and invasion of epithelial cells by

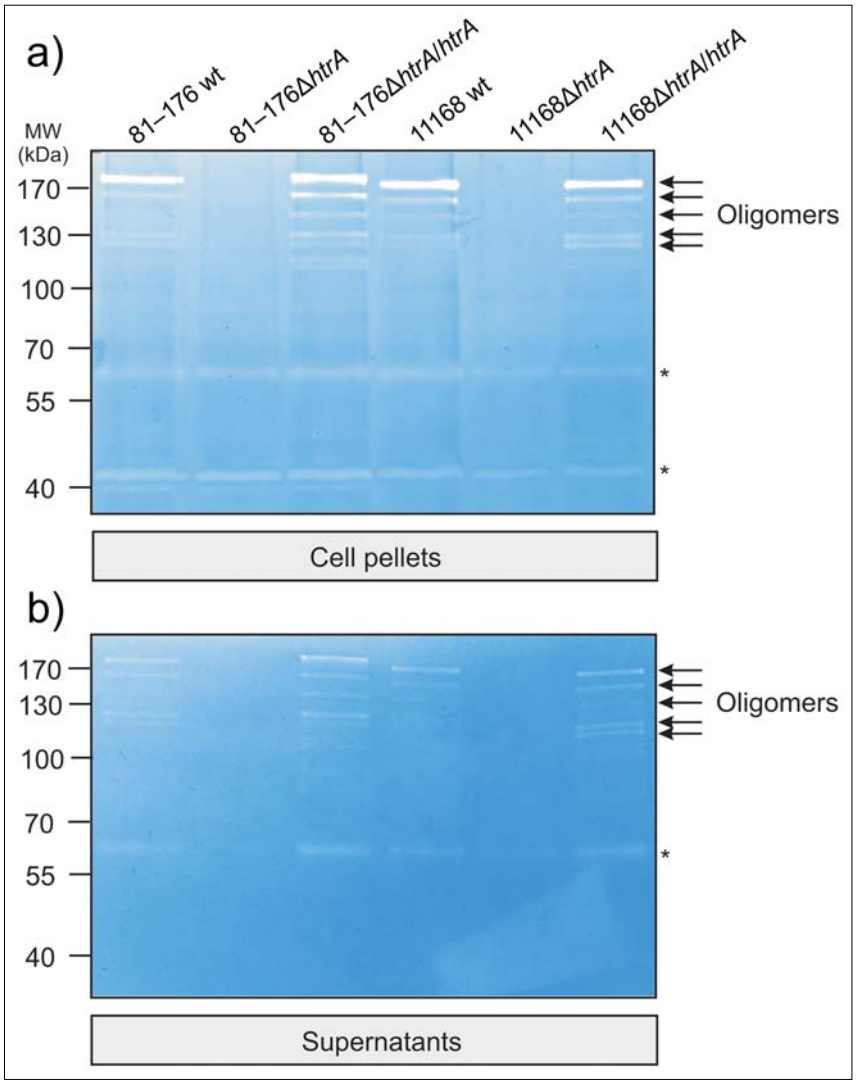

Fig. 4. Secreted and cell-associated HtrA of the complemented *C. jejuni* strains generate proteolytically active oligomers. Wild-type (wt), isogenic Δ*htrA* deletion mutants, and complemented Δ*htrA/htrA* strains of *C. jejuni* 81–176 and NCTC11168 were grown in BHI medium with 10% FCS for 12 h at 37 °C. Bacterial pellets (a) and culture supernatants (b) were prepared and subjected to investigation of protease activity by casein zymography. The position of proteolytically active oligomeric HtrA proteins is indicated with arrows. Asterisks label the position of two other proteolytically active protein bands at ~65 kDa and ~42 kDa, respectively

C. jejuni NCTC11168 [23]. We therefore aimed to test if our *htrA* complemented strains can restore this phenotype. For this purpose, nonpolarized INT-407 intestinal epithelial cells were infected for 6 h with the above strains, followed by gentamicin protection assay and determination of the colony forming units (CFU). The results show that both *C. jejuni* wt and *htrA* complemented strains are able to adhere to INT-407 cells with similar extent, while both Δ*htrA* deletion mutants exhibit a strong reduction in the number of cell-associated bacteria *(Fig. 5a/b)*. In control experiments, 250 μg/mL gentamicin killed all extracellular bacteria (data not shown). In agreement with the cell binding properties of described strains, we found that both wt *C. jejuni* and *htrA* complemented strains are able to invade INT-407 cells, while the two Δ*htrA* deletion mutants show strong deficiency for invasion *(Fig. 6a/b)*. These results further support the view that HtrA is involved in *C. jejuni* adhesion to and invasion of intestinal epithelial cells.

Transmigration of C. jejuni *across polarized epithelial cells is restored by genetic complementation of HtrA*

Finally, we aimed to analyze if the complemented *C. jejuni* strains can restore the transmigration defect of Δ*htrA* mutants across polarized epithelial cells reported recently [26, 39]. To test this important function of HtrA, polarized MKN-28 cells were seeded and differentiated over 14 days in a transwell filter system. The transepithelial electrical resistance (TER) was determined over time and reached values between 140 and 150 Ω/cm^2 in the confluent monolayer, similar to previously reported data [40]. MKN-28 cells were then infected with the *C. jejuni* strains, followed by determination of CFU in the bottom chambers during the time course of 0.5 to 24 h. The results indicate that *C. jejuni* 81–176 wt and the *htrA* complemented strains transmigrated quickly during the first 30 min and increased up to 900,000 CFU over time, while the isogenic

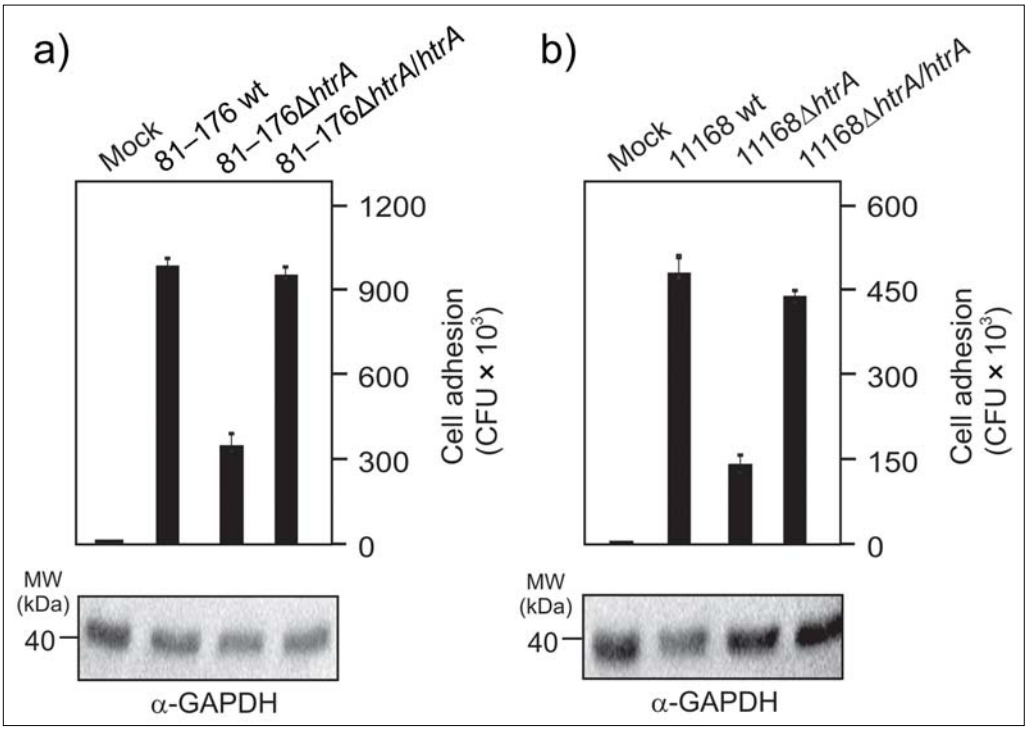

Fig. 5. Genetic complementation of *htrA* restores binding of *C. jejuni* to human intestinal epithelial cells. INT-407 cells infected for 6 h with wild-type (wt), isogenic Δ*htrA* deletion mutants, and complemented Δ*htrA*/*htrA* strains of *C. jejuni* 81–176 (a) or NCTC11168 (b). Cell adhesion of the *C. jejuni* strains was analyzed by gentamicin protection assay. The α-GAPDH blot served as loading control and confirmed that equal amounts of protein are present in each sample. Bars represent averages and standard deviations of three independent experiments

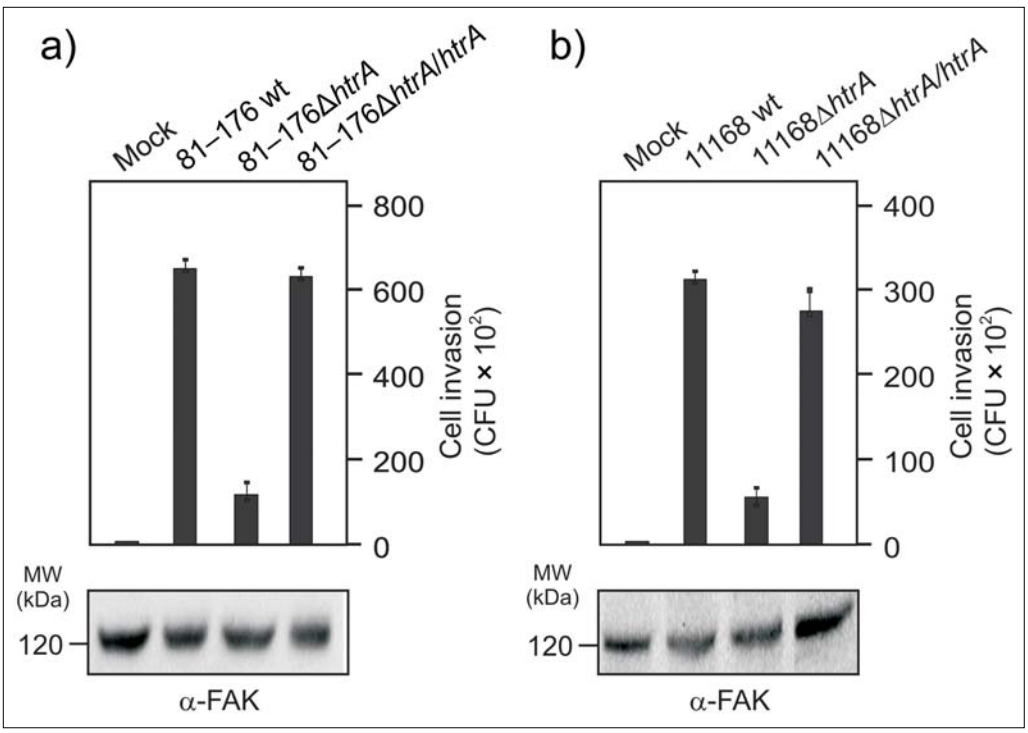

Fig. 6. Genetic complementation of *htrA* restores invasive properties of *C. jejuni* in human intestinal epithelial cells. INT-407 cells were infected for 6 h with wild-type (wt), isogenic Δ*htrA* deletion mutants, and complemented Δ*htrA/htrA* strains of *C. jejuni* 81–176 (a) or NCTC11168 (b). Intracellular *C. jejuni* were quantified by gentamicin protection assay. The α-FAK blot served as loading control and confirmed that equal amounts of protein are present in each sample. Bars represent averages and standard deviations of three independent experiments

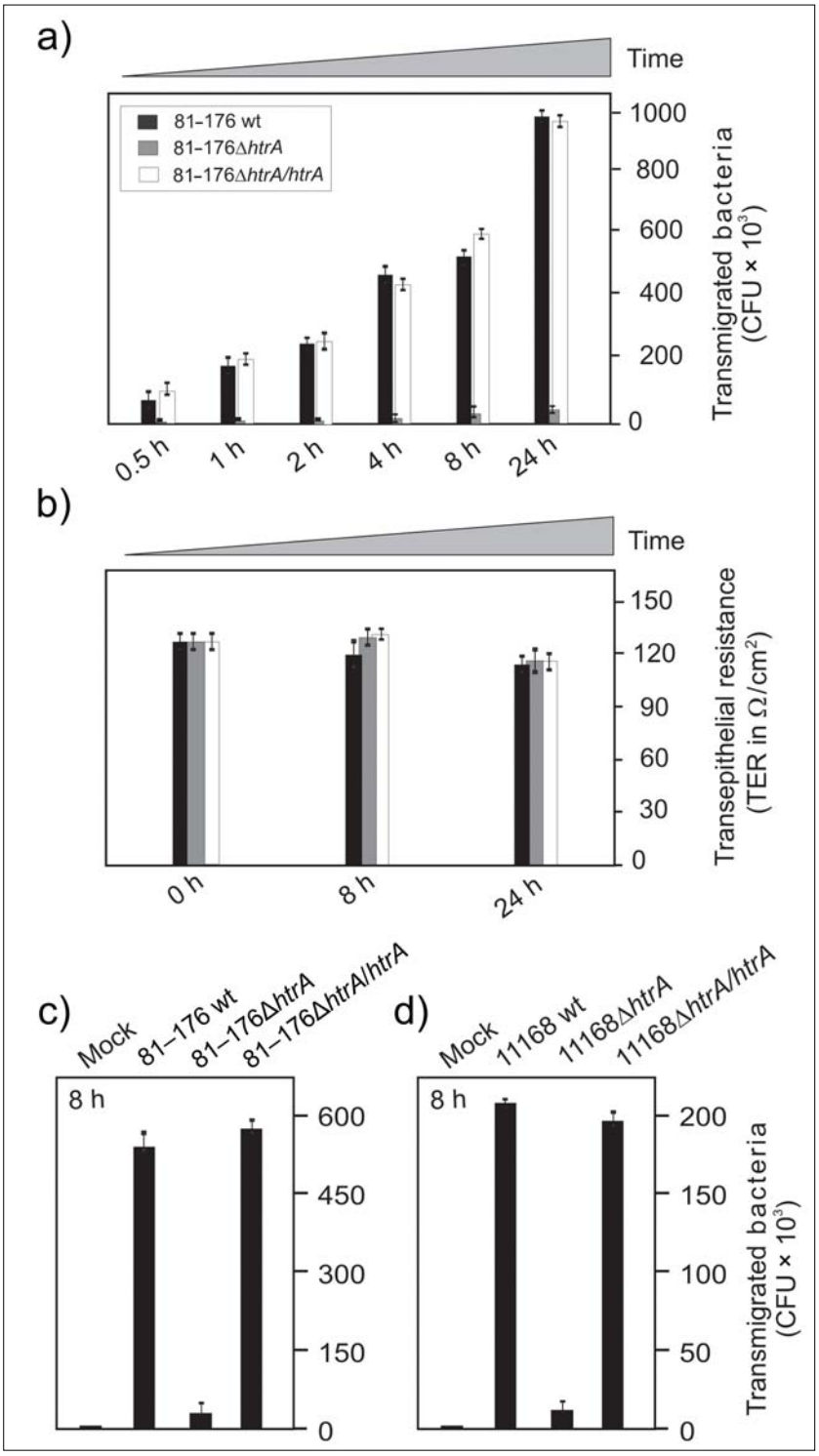

Fig. 7. Transmigration of *C. jejuni* across polarized epithelial cells is restored by genetic complementation of *htrA*. Differentiated MKN-28 epithelial cells were grown in a transwell filter system for 14 days to reach a confluent monolayer. The cells were infected in the apical chamber. A time course of infection with wild-type (wt), isogenic Δ*htrA* deletion mutants, and complemented Δ*htrA/htrA* strains of *C. jejuni* 81–176 (a–c) or NCTC11168 (d) is shown. Transmigrated bacteria were harvested from the bottom chambers, grown on MH agar plates, and CFUs were determined in triplicates (a, c, d). The transepithelial resistance (TER) was measured before and after infection and did not change significantly during the indicated time course (b)

Δ*htrA* deletion mutant did not *(Fig. 7a)*. We also measured the TER values and did not detect significant differences before and after infection *(Fig. 7b*). Similar transmigration rates were obtained when we compared the various strains of 81–176 *(Fig. 7c)* and NTCT111638 *(Fig. 7d*) in parallel experiments. As control, nonpathogenic *Escherichia coli*

Top-10 did not transmigrate under the same conditions as expected (data not shown). These observations further support the view that *C. jejuni* HtrA plays an important role during transmigration of these bacteria across polarized epithelial cells.

Discussion

HtrA is a conserved periplasmic protein that exhibits both protease and chaperone activities with important function in protein quality control in many bacteria [31–33]. In addition, it has been reported that HtrA is crucial for the virulence of numerous bacterial pathogens such as *Yersinia enterocolitica* [41], *Salmonella enterica* [42], *Klebsiella pneumonia* [43], *Shigella flexneri* [44], *Listeria monocytogenes* [45], and *Chlamydia trachomatis* [46]. It is well-known that HtrA is critical for stress tolerance and survival of most bacteria, because it degrades and prevents aggregation of periplasmic proteins that can misfold under stress conditions [31–33], but only a few reports have studied the discrete functions of HtrA protease and chaperone activities during infection [27–30].

Our previous reports suggested that HtrA of two gastrointestinal pathogens, *C. jejuni* and *H. pylori*, can be secreted into the cell culture supernatant, where HtrA plays a major role in crossing polarized epithelial monolayers by these bacteria *in vitro* [26, 34, 35]. In addition, using *C. jejuni* Δ*htrA* deletion mutants, we have shown that HtrA plays a pivotal role in inducing host cell apoptosis and immunopathology during campylobacteriosis in the gut of two mouse models *in vivo* [37, 38]. However, knockout mutagenesis of individual genes in the bacteria can generate polar effects; thus, genetic complementation is necessary. In the present study, we complemented the wt *htrA* gene in two *C. jejuni* Δ*htrA* deletion mutants in order to 1) confirm its important role in heat tolerance, oxygen stress resistance, epithelial cell adhesion, invasion, and transmigration of the bacteria and to 2) establish a tool for further mutagenesis of *htrA* across the entire gene.

During transmission, *C. jejuni* requires significant capabilities to adapt to and survive in various natural environments. In the present study, we first investigated the importance of HtrA in the stress tolerance of *C. jejuni* and demonstrate that it is required both for heat and oxygen tolerance, which may be important for successful survival of the bacteria during the contamination cycle. In agreement with previous studies, we show in the present work that HtrA is required for *C. jejuni* growth at high temperatures. Brøndsted and coworkers proposed that *C. jejuni* HtrA is a functional ortholog of HtrA in *E. coli*, which is involved in the degradation of nonnative proteins in the periplasm [22]. Thus, the temperature sensitivity of the *C. jejuni* Δ*htrA* mutants could be a result of the accumulation of misfolded proteins in this subcellular compartment, similar to observations made in *E. coli* [31–33]. This conclusion is supported by the complementation of the temperature-sensitive phenotype of an *E. coli htrA* mutant with *C. jejuni* wt *htrA* [22]. Remarkably, it was found that deletion of *htrA* in *C. jejuni* leads to overexpression of two cytoplasmic chaperones, DnaK and ClpB [22], that are well-known factors involved in the refolding of heat-denatured proteins and solubilization of larger protein aggregates in *E. coli* by the expenditure of ATP [47, 48]. Questions arise as to whether the accumulation of misfolded proteins in the periplasm increases the level of cytoplasmic chaperones and how exactly the signal is transmitted from periplasm to cytoplasm, which should be investigated in future studies.

In contrast to the growth and survival of bacterial *htrA* mutants of other pathogenic Gram-negative bacteria [41, 49, 50], the *C. jejuni* Δ*htrA* mutants showed reduced oxygen tolerance [22]. Even though *C. jejuni* is a microaerobic organism, it is able to perform aerobic respiration [51], and an increase in oxygen tension could lead to the accumulation of oxidatively damaged proteins in the periplasm that require HtrA for removal [22]. Our experiments confirmed these earlier observations and showed that this phenotype in the *C. jejuni* Δ*htrA* mutant can be restored by wt *htrA* complementation.

In addition, we tested if the *C. jejuni* strains can generate proteolytic active HtrA oligomers. Using casein zymography, we could demonstrate that wt and *htrA* complemented *C. jejuni* formed caseinolytic active oligomers with expected sizes of trimers and higher. These observations are in agreement with reports on HtrA in other bacteria such as *E. coli*, where the HtrA oligomers are highly proteolytic active rather than the monomer [52]. These oligomers were found both in total cell lysates and culture supernatants of *C. jejuni*; however, it remained unknown how HtrA can cross the bacterial outer membrane. HtrAs in Gram-negative bacteria such as *C. jejuni* contain a signal peptide important for Sec-dependent cleavage and transport of the protease across the inner membrane into the periplasm [26, 34, 35]. Commonly, HtrAs exhibit no sequence homology to typical autotransporters, which process themselves by autoproteolysis. This is in line with our observation that the HtrA protease activity is not required for secretion [39]. Thus, HtrA very likely requires the assistance of other bacterial factors for delivery. Candidates are secretion systems called type I–VII (T1SS–T7SS), but various available full genome sequences of *C. jejuni* do not encode orthologs of classical T1SS–T7SS transporters [53–55]. For example, a putative conjugative T4SS has been only found on the pVir plasmid, but pVir is not present in the used strain NCTC11168, and is therefore not involved in HtrA secretion. A few putative T2SS components (encoded by *cj1470* and *cj1474c* genes) have been noted in NCTC11168, which could be involved in protein secretion [53]. However, the genes *cj1343c*, *cj1470c, cj1471c, and cj1474c* were later described to be involved in natural competence and DNA import into *C. jejuni* [56]. Whether these genes may play a role in HtrA secretion remains to be elucidated. Forthcoming experiments should study in detail how HtrA is secreted by *C. jejuni*.

Even though HtrA has been described traditionally as a stress response protein, other findings indicate that HtrA has also specific functions during infection that may be stress-independent. Previous studies have shown that binding of *C. jejuni* to epithelial host cells is highly dependent on HtrA expression [22]. In addition, an *htrA* mutant was detected in a *C. jejuni* transposon library screened for reduced invasion capability [57]. Both studies concluded that particularly the chaperone activity of HtrA may have a significant impact on the interaction of *C. jejuni* and host cells. Lack of HtrA reduced bacterial binding to epithelial cells 5–10 times more [22], as compared to any other known adhesin [16, 25, 58–60], suggesting a pleiotropic effect. Impaired adherence and invasion of the *htrA* mutant cannot be explained by reduced *C. jejuni* motility, since the *htrA* mutation does not affect these parameters [26]. Instead, Brøndsted and coworkers suggested that the reduced adherence of the Δ*htrA* mutants to epithelial cells can be caused by misfolding or improper localization of one or more adhesion factors [22]. One such option is the CadF adhesin for the basolateral receptor fibronectin, which could explain why apical colonization is similarly high between Δ*htrA* mutants and wt *C. jejuni* in the mouse gut *in vivo* [37, 38]. Alternatively, HtrA may influence other processes in the periplasm such as N-linked glycosylation of *C. jejuni* surface proteins like PEB3 and thereby could impact bacterial adherence to epithelial cells [61]. These various options should be investigated in future.

Finally, deletion of the *htrA* gene in two strains resulted in strong deficiency of *C. jejuni* to travel across polarized MKN-28 epithelial cells cultivated in a transwell chamber [26, 35]. We demonstrate here that genetic complementation of *htrA* can also restore the deficiency of Δ*htrA* mutants to transmigrate across polarized epithelial cells. Thus, HtrA is a pivotal factor that may allow *C. jejuni* to cross the intestinal barrier in order to access deeper tissues *in vivo*. In fact, live *C. jejuni* were recovered from other organs in infected animals such as the spleen [62–64], liver [63–65], mesenteric lymph nodes [63], and blood [66]. This suggests that *C. jejuni* exhibits the capability not only to adhere to and enter into enterocytes, but can also travel within the host, pass the intestinal epithelial barrier, enter the lamina propria, and even access other organs of various infected hosts. Thus, the contribution of HtrA in these processes should be studied in more detail.

Taken together, our results demonstrate that various described phenotypic defects of *C. jejuni* Δ*htrA* mutants can be restored by genetic complementation of the wt *htrA* gene. This suggests that we have established a reliable genetic system serving as a tool for further mutagenesis of *htrA* across the entire gene and more in-depth functional studies.

Materials and Methods

E. coli *and* C. jejuni *strains*

The *E. coli* strain Top-10 was purchased from Invitrogen (Darmstadt/Germany) and grown in conventional LB broth medium (10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl). The two *C. jejuni* wt isolates 81–176 and NCTC11168 were used because their genome sequences are available. The corresponding isogenic knockout mutants 11168Δ*htrA* and 81–176Δ*htrA* were previously described [22, 23, 26]. The *C. jejuni* strains were commonly cultured at 37 °C for 48 h on *Campylobacter* blood-free selective agar base containing selective supplement or on MH agar plates (Oxoid, Wesel/Germany). In case of the mutant *C. jejuni* strains, all agar plates were supplemented with 30 µg/ml of kanamycin or 30 µg/ml of chloramphenicol, respectively [67]. *C. jejuni* was grown under microaerobic conditions generated by CampyGen packs in 3.5 l anaerobic jars of Oxoid [68].

Genetic complementation of the htrA *gene in* C. jejuni

The plasmid pCam-148 was kindly provided by Dr. Dennis Linton (University Manchester/UK) and used for genetic complementation of the *htrA* gene in the *C. jejuni* chromosome. In brief, pCam-148 harbors a 2178-bp fragment of *C. jejuni* NCTC11168 chromosomal DNA (position 205,297 to 207,475 containing a pseudogene downstream of Cj0208) cloned into the *Sma*I restriction site of plasmid pUC18. pCam-148 contains a unique *Spe*I restriction site in the *C. jejuni* sequence. This *Spe*I site was used to introduce three additional restriction sites (*Not*I, *Mlu*I, and *Nru*I) by the primer annealing approach. Afterwards, a 1694-bp fragment of the *htrA* gene of *C. jejuni* NCTC11168 was amplified by PCR, including the 200-bp upstream and 75-bp downstream sequences (with promotor and terminator). The resulting PCR product (with flanking *Not*I and *Mlu*I sites, respectively) was introduced into the pSB-249 vector, and then further subcloned into the *Not*I and *Mlu*I sites of pCam-148. As a selection marker, we used the 795-bp *aph* gene with its own promoter permitting kanamycin resistance (from plasmid pRYSK12, kindly provided by Dr. Sabine Kienesberger, University Graz/Austria). This kanamycin resistance cassette was cloned into the *Mlu*I and *Nru*I sites of pCam-148 adjacent to the *htrA* gene, resulting in the final *htrA* complementation construct. This plasmid was then transformed into the *C. jejuni* NCTC11168Δ*htrA* and 81–176Δ*htrA* deletion mutants, respectively (named NCTC11168Δ*htrA/htrA* or 81–176Δ*htrA*/*htrA*). Correct integration of *htrA* in the *C. jejuni* chromosomes was approved by PCR, and expression of HtrA proteins was confirmed by Western blotting.

Growth of C. jejuni *under stress conditions on MH agar plates*

Wt *C. jejuni*, Δ*htrA* mutants, and corresponding Δ*htrA/htrA* complemented strains were grown for 48 h on MH agar plates at 37 °C under microaerobic conditions as described above. All bacterial strains were suspended in BHI medium. The optical density was determined by mea-

suring the suspension at λ = 600 nm (OD_{600}) in a UV/Vis spectrometer Lambda 2 (Perkin Elmer, Waltham/USA) and adjusted to 0.1. Serial dilutions (10^{-1} through 10^{-6}) were prepared, and 10 µL volumes were spotted onto MH agar plates. These plates were incubated for 3 days either under microaerobic conditions at 37 °C, 42 °C or 44 °C, or at 42 °C in atmosphere containing ~18% O_2 produced by the candle jar procedure as described [22]. Experiments were repeated at least three times.

HtrA secretion assays

Wt *C. jejuni* and mutant strains were suspended in BHI medium supplemented with 10% FCS. The optical density was determined and adjusted to OD_{600} = 0.2. To allow HtrA secretion in the culture supernatant, the bacteria were incubated for 12 h shaking at 160 rpm. The cell pellets and the supernatants were separated by centrifugation at 4000 rpm. The supernatants were then passed through 0.21 µm sterile filters (Sigma-Aldrich, Taufkirchen/Germany) to remove remnant bacterial cells. Absence of live bacteria in the supernatant was verified by the lack of CFU after 3 days of incubation on MH agar plates. The obtained bacterial pellets and supernatants were then analyzed by Western blotting and casein zymography as described below.

Infection of INT-407 cells

INT-407 human embryonic intestinal epithelial cells, a typical nonpolarized cell line, was obtained from the American Type Culture Collection (ATCC CCL-6). The cells were grown at 37 °C in 5% CO_2 using six-well plates with MEM medium containing Earle's salts and L-Glutamine (Gibco, Darmstadt/Germany) and Pen/Strep antibiotics cocktail (Invitrogen). After reaching confluency of ~70%, cells were washed twice with Dulbecco's phosphate buffered saline (DPBS, Sigma-Aldrich) and replaced by fresh antibiotics-free medium 12 h before infection. For infection, *C. jejuni* was harvested from MH agar plates, suspended in BHI medium, and the number of bacteria was determined through optical density measurement at OD_{600}. The INT-407 cells were infected using a multiplicity of infection (MOI) of 100.

Gentamicin protection assay

After infection, the INT-407 cells were washed three times with 1 ml of prewarmed MEM medium per well to remove nonbound *C. jejuni*. To determine the CFU corresponding to intracellular bacteria, the cells were treated for 2 h with 250 µg/ml gentamicin (Sigma-Aldrich), washed three times with medium, and then incubated with 1 ml of 0.1% (*w*/*v*) saponin (Sigma-Aldrich) in DPBS at 37 °C for 15 min to lyse the cells. Afterwards, the treated cells were suspended thoroughly, diluted, and plated on MH agar plates. To determine the total CFU of cell-associated bacteria, the infected cell monolayers were incubated with 1 ml of 0.1% (*w*/*v*) saponin in DPBS for 15 min at 37 °C without prior treatment with gentamicin. The resulting suspensions were diluted and plated on MH agar plates as described above. For each strain, the level of bacterial adhesion and uptake was determined by calculating the number of CFU as described [69]. All experiments were performed in triplicates.

Infection of MKN-28 cells in a transwell filter system

The human polarized epithelial cell line MKN-28 was obtained from the Japanese Collection of Research Biosources (JCRB0253). MKN-28 cells are cultured at 37 °C in 5% CO_2 using RPMI-1640 medium containing L-Glutamine, 25 mM HEPES, and Pen/Strep antibiotics cocktail (Invitrogen). To study bacterial transmigration, the cells were grown for 14 days in a transwell filter system with pore size of 3.0 µm (Corning B.V. Lifescience, Schiphol/Netherlands) placed in 12-well plates. As control, the TER values were measured every 2 days followed by medium change as described [26]. Twelve hours before infection, the growth medium was removed, cells were washed with DPBS, and fresh medium without antibiotics was added. Infections were carried out with the different *C. jejuni* strains using a MOI of 50 for the indicated periods of time (30 min to 24 h). At each given time point, 10 µL aliquots from the basolateral chamber of the transwell were taken and incubated on MH agar plates for 3 days under microaerobic conditions. The number of transmigrated bacteria was determined by counting the CFU in triplicates.

Casein zymography

Bacterial lysates, culture supernatants, or recombinant HtrA were separated under nonreducing conditions in gels containing casein. Subsequently, gels were renatured in 2.5% Triton-X-100 and equilibrated in developing buffer [26–35]. Caseinolytic activity was visualized by staining with 0.5% Coomassie Blue R250.

Antibodies and Western blotting

Two polyclonal antibodies, α-FAK and α-GAPDH, were purchased from Santa Cruz (Heidelberg/Germany). In addition, we generated polyclonal antibodies raised against conserved peptides corresponding to the following amino acid (aa) residues in four *C. jejuni* proteins: HtrA (aa 288–301: QGDTKKAYKNQEGA), CadF (aa 293–306: QDNPRSSNDTKEGR), CiaB (aa 597–610: C-EIDNSGEFERYKKK), and MOMP (aa 400–413: C-NLDQGVNTNESADH). For this purpose, all four peptides were synthesized with an additional amino-termi-

nal cysteine residue (Biosynthan, Berlin/Germany), used for covalent conjugation to the *Limulus polyphemus* hemocyanin carrier protein [70]. Two rabbits each were immunized using standard protocols of Biogenes GmbH (Berlin/Germany). The resulting antisera were affinity-purified, and the specificity against the HtrA, CadF, CiaB, and MOMP proteins was confirmed by SDS–PAGE and Western blotting. Horseradish peroxidase-conjugated antirabbit polyvalent sheep immunoglobulin was used as secondary antibody (DAKO, Glostrup/Denmark). Blots were developed with ECL Plus Western blot reagents (GE Healthcare, Munich/Germany) as described [71, 72].

Statistics

All data were evaluated using Student *t*-test with SigmaStat 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 +/− signs represent standard deviation.

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Competing interests

The authors have declared that no competing interests exist.

Author contributions

Conceived and designed the experiments: M.B., N.T.
Performed the experiments: M.B., N.T., J.L.
Analyzed the data: M.B., N.T., J.L., S.B.
Wrote the paper: M.B., S.B., N.T.

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