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***Campylobacter jejuni* outer membrane vesicle-associated proteolytic activity promotes bacterial invasion by mediating cleavage of intestinal epithelial cell E-cadherin and occludin**

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SUMMARY

Outer membrane vesicles (OMVs) play an important role in the pathogenicity of Gram-negative bacteria. *Campylobacter jejuni* produces OMVs that trigger IL-8, IL-6, hBD-3 and TNF- α responses from T84 intestinal epithelial cells and are cytotoxic to Caco-2 IECs and *Galleria mellonella* larvae. Proteomic analysis of 11168H OMVs identified the presence of three proteases, HtrA, Cj0511 and Cj1365c. In this study, 11168H OMVs were shown to possess proteolytic activity that was reduced by pre-treatment with specific serine protease inhibitors. OMVs isolated from 11168H *htrA*, *Cj0511* or *Cj1365c* mutants possess significantly reduced proteolytic activity. 11168H OMVs are able to cleave both E-cadherin and occludin, but this cleavage is reduced with OMVs pre-treated with serine protease inhibitors and also with OMVs isolated from *htrA* or *Cj1365c* mutants. Co-incubation of T84 monolayers with 11168H OMVs results in a visible reduction in both E-cadherin and occludin. The addition of 11168H OMVs to the co-culture of live 11168H bacteria with T84 cells results in enhanced levels of bacterial adhesion and invasion in a time- and dose-dependent manner. Further investigation of the cleavage of host cell structural proteins by *C. jejuni* OMVs should enhance our understanding of the interactions of this important pathogen with intestinal epithelial cells.

INTRODUCTION

The enteric pathogen *Campylobacter jejuni* is a Gram-negative, microaerophilic, spiral-shaped bacterium that is the leading cause of bacterial food-borne gastroenteritis worldwide (Epps *et al.*, 2013). *C. jejuni* infection is associated with mild diarrhoea to severe inflammatory enteritis, although the mechanisms of pathogenesis are still poorly understood. In most cases, *C. jejuni* infection is self-limiting, but some cases (1:1000 to 1:2000) can develop serious life-threatening complications, such as an acute autoimmune paralysing neuropathy Guillain–Barré syndrome (GBS) (Nyati *et al.*, 2013). *C. jejuni* infection is a multistep process that includes colonisation of the intestinal mucosa, interactions with and invasion of the human intestinal epithelial cells (IECs) (Young *et al.*, 2007). *C. jejuni* has been reported to invade human IECs either via paracellular or transcellular routes (Boehm *et al.*, 2012, Ó Cróinín *et al.*, 2012, Backert *et al.*, 2013). *C. jejuni* involves the breach of the IEC barrier via paracellular invasion and binding to fibronectin located on the baso-lateral surface (Backert *et al.*, 2013). Two adhesins (CadF and FlpA) facilitate the binding of *C. jejuni* to fibronectin (Monteville *et al.*, 2003, Konkel *et al.*, 2010). However other virulence factors involved in the ability of *C. jejuni* to disrupt both tight junction (TJ) and adherens junction (AJ) proteins remain mostly uncharacterised (Boehm *et al.*, 2012).

TJs and AJs comprise two methods of cell-to-cell adhesion that provide different functions. TJ and AJ complexes are associated with the actin cytoskeleton, with formation and maturation of cell-to-cell contacts involving the reorganisation of the actin cytoskeleton (Hartsock *et al.*, 2008, Anderson *et al.*, 2009, Liang *et al.*, 2014, Van Itallie *et al.*, 2014). TJs regulate the paracellular pathway for the movement of ions and solutes in-between cells. The transmembrane protein occludin is an important component of TJs (Cummins, 2012). AJs mediate the maturation and maintenance of cellular contact. The transmembrane protein E-cadherin is an important component of AJs (van Roy *et al.*, 2008, Maitre *et al.*, 2013). Proteases

secreted by the closely related pathogen *Helicobacter pylori* compromise the integrity of AJs, allowing the bacteria access to the baso-lateral layer of gastric epithelial cells (Hoy *et al.*, 2010). Other bacterial pathogens such as *Shigella flexneri*, disrupt the trafficking of cadherins, whereas *Chlamydia* species secrete proteases to exploit Golgi-derived membranes and create a specialised compartment suitable for bacterial survival and growth (Derre, 2015). Studies with enteric pathogens such as enteropathogenic *Escherichia coli*, *Salmonella enterica* and *Shigella flexneri* have revealed that these enteric pathogens breach the IEC TJ and AJ barrier function using type III secretion systems (T3SS) (Simonovic *et al.*, 2000, Boyle *et al.*, 2006). For example, enteropathogenic *E. coli* T3SS effectors disrupt TJ proteins in human IECs (Muza-Moons *et al.*, 2004, Viswanathan *et al.*, 2004). However *C. jejuni* lacks a classical T3SS (Gundogdu *et al.*, 2007). While the flagellar is proposed for the secretion of virulence proteins in a T3SS manner (Konkel *et al.*, 2004), the few known examples of *C. jejuni* invasion antigens have not been shown to be involved in the breaching of the IECs barrier (Novik *et al.*, 2010). Bacterial outer membrane vesicles (OMVs) have emerged as an important secretion mechanism for many Gram-negative pathogens (Bielaszewska *et al.*, 2013, Altindis *et al.*, 2014, Bonnington *et al.*, 2014, Scanlan, 2014). OMVs induce IECs antimicrobial immunity and apoptosis (Manning *et al.*, 2011, Bielaszewska *et al.*, 2013, Kaparakis-Liaskos *et al.*, 2015). *C. jejuni* produces OMVs that orchestrate pro-inflammatory cytokine responses from IECs (Elmi *et al.*, 2012). Cytolethal distending toxin (CDT) is secreted within *C. jejuni* OMVs, which have been shown to be cytotoxic to *Galleria mellonella* wax worm larvae (Lindmark *et al.*, 2009, Elmi *et al.*, 2012, Jang *et al.*, 2014). Although studies have focused on IECs cytokine responses to OMVs, the significance of other outcomes of OMV-host interactions are currently less studied.

Proteases are critical for homeostasis (Clausen *et al.*, 2011). One role of bacterial proteases is interacting with integral and peripheral barrier IECs proteins, resulting in cytotoxicity or

inflammation (Coleman *et al.*, 2013, Sumitomo *et al.*, 2013, Golovkine *et al.*, 2014). Recent studies have shown that proteases from *Bacteroides fragilis* and *Porphyromonas gingivalis* breakdown E-cadherin, the predominant protein in epithelial AJs (Wu *et al.*, 1998, Katz *et al.*, 2000, Remacle *et al.*, 2014). Amongst the virulence proteins identified by proteomic analysis of *C. jejuni* 11168H OMVs were three proteases (Jang *et al.*, 2014). The high temperature requirement protein (HtrA), Cj0511 and Cj1365c all share common features of serine proteases (Gundogdu *et al.*, 2007).

In this study, we demonstrate that *C. jejuni* 11168H OMVs possess proteolytic activity associated with the three proteases HtrA, Cj0511 and Cj1365c. OMV proteolytic activity was reduced by mutation of either *htrA*, *Cj0511* or *Cj1365c* and also by pre-treatment of OMVs with specific serine protease inhibitors. 11168H OMVs directly cleave E-cadherin and occludin *in vitro* and this activity is associated with HtrA and Cj1365c protease activity but not with Cj0511. Co-incubation of T84 monolayers with *C. jejuni* 11168H OMVs results in a visible reduction in both E-cadherin and occludin. The addition of 11168H OMVs to the co-culture of live 11168H bacteria with T84 IECs enhanced levels of bacterial adhesion and invasion in a time- and dose-dependent manner. Collectively, our data supports the hypothesis that OMVs impact on bacteria-IEC interactions by multiple mechanisms.

RESULTS

***C. jejuni* 11168H OMVs possess serine-protease like activity**

The proteolytic activity of 11168H OMVs was quantified using a FITC-labelled casein assay using trypsin as a control (Fig. 1A). 10 µg of 11168H OMVs yielded activity equivalent to approximately that observed with 10 ng/ml of trypsin. The proteolytic activity of 11168H OMVs was not reduced by pre-treatment with the protease inhibitors EDTA (metalloprotease inhibitor), E64 (cysteine protease inhibitor), Pepstatin A (aspartic protease inhibitor) and leupeptin (cysteine protease inhibitor) (Fig. 1B). However pre-treatment with the serine protease inhibitors phenylmethanesulfonyl fluoride (PMSF) and 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) significantly reduced 11168H OMVs proteolytic activity (Fig. 1B) suggesting that 11168H OMVs possess serine protease-like activity. The proteolytic activity of 11168H OMVs was further confirmed by casein and gelatin-substrate zymography (data not shown).

Mutation of *htrA*, *Cj0511* or *Cj1365c* significantly reduces OMVs proteolytic activity

Having established that *C. jejuni* 11168H OMVs possess proteolytic activity, OMVs were isolated from 11168H *htrA*, *Cj0511* and *Cj1365c* mutants and shown to possess a significantly reduced (all $p < 0.001$) proteolytic activity compared to the OMVs isolated from 11168H wild-type strain (Fig. 2). Pre-treatment of OMVs isolated from the *htrA*, *Cj0511* or *Cj1365c* mutants with the specific serine protease inhibitor PMSF significantly reduced ($p < 0.001$, $p < 0.01$ and $p < 0.05$ respectively) the proteolytic activity compared to similarly pre-treated 11168H OMVs. Pre-treatment of OMVs isolated from the *htrA* or *Cj0511* mutants with the specific serine protease inhibitor AEBSF significantly reduced (both $p < 0.001$) the proteolytic activity compared to similarly pre-treated 11168H OMVs. However pre-treatment of OMVs isolated

from the *Cj1365c* mutant with AEBSF did not significantly reduce the proteolytic activity compared to similarly pre-treated 11168H OMVs.

OMVs isolated from *htrA* or *Cj1365c* mutants exhibit reduced cytotoxicity in the *Galleria mellonella* model of infection

C. jejuni 11168H OMVs have been shown to exhibit cytotoxicity in the *G. mellonella* larvae model of infection (Elmi *et al.*, 2012). *G. mellonella* larvae were injected with OMVs (5 µg) isolated from 11168H wild-type strain, *htrA*, *Cj0511* or *Cj1365c* mutants. At 24 h post-infection, OMVs isolated from the *htrA* and *Cj1365c* mutants were significantly less cytotoxic ($p < 0.001$) than 11168H OMVs (Fig. 3). OMVs isolated from the *Cj0511* mutant also showed reduced cytotoxicity but this difference was not significant compared to 11168H OMVs (Fig. 3). No further mortality was observed at either 48 h or 72 h post-infection (data not shown).

OMVs isolated from *htrA*, *Cj0511* or *Cj1365c* mutants do not exhibit reduced cytotoxicity or immunogenicity to T84 IECs

C. jejuni 11168H OMVs have been shown to exhibit cytotoxicity to IECs (Elmi *et al.*, 2012). The cytotoxicity of OMVs on T84 IECs was measured by quantifying the release of cytosolic lactate dehydrogenase (LDH) as a measure of cell damage. OMVs (100 µg) were co-incubated with T84 IECs for 24 h. The cytotoxic effect of OMVs isolated from the *htrA*, *Cj0511* or *Cj1365c* mutants was not statistically different to the cytotoxic effect of OMVs isolated from the 11168H wild-type strain (Fig. 4).

C. jejuni 11168H OMVs have been shown to induce IL-8 from T84 IECs (Elmi *et al.*, 2012). T84 cells were co-incubated with 11168H OMVs (1 µg, 10 µg or 100 µg) for 24 h and the levels of IL-8 secreted were measured by ELISA (Fig. 5). The levels of IL-8 induced by OMVs

isolated from either the *htrA*, *Cj0511* or *Cj1365c* mutants was not statistically different to the levels induced by OMVs isolated from the 11168H wild-type strain.

***C. jejuni* 11168H OMVs cleave E-cadherin and occludin**

C. jejuni invasion via the paracellular route is thought to involve cleavage of E-cadherin and occludin to allow the bacteria to reach the baso-lateral surface and to bind to fibronectin. Given the proteolytic activity associated with 11168H OMVs, the effect of OMVs on E-cadherin and occludin was investigated. Western blot analysis revealed 11168H OMVs cleave recombinant E-cadherin and release one major cleavage product of ~85 kDa (Fig. 6A). Pre-treatment of 11168H OMVs at either 100°C for 20 mins or with the specific serine protease inhibitor PMSF completely inhibited the cleavage of E-cadherin (Fig. 6B). E-cadherin was not cleaved in the presence of PBS alone. Similar results were observed with recombinant occludin (Fig. 6CD). Having established that *C. jejuni* OMVs cleave recombinant E-cadherin and occludin *in vitro*, we investigated whether *C. jejuni* OMVs cleave E-cadherin and occludin in T84 cells. T84 cell monolayers were co-incubated with 11168H OMVs for 3, 6 and 24 h. Immunofluorescence analysis revealed a reduction in both E-cadherin and occludin within monolayers at all three time points, in comparison to untreated monolayers (Fig. 7).

HtrA and Cj1365c contribute to the OMV-induced cleavage of E-cadherin and occludin

To investigate the role of the individual proteases associated with 11168H OMVs in the cleavage of E-cadherin or occludin, OMVs isolated from the *htrA*, *Cj0511* or *Cj1365c* mutants were co-incubated with E-cadherin or occludin. Western blot analysis indicated that OMVs from the *htrA* and *Cj1365c* mutants exhibited reduced cleavage of E-cadherin, whilst OMVs from the *Cj0511* mutant exhibited cleavage of E-cadherin similar to that of 11168H OMVs (Fig. 8A). Western blot analysis of OMVs from the *htrA* and *Cj1365c* mutants also exhibited

reduced cleavage of occludin, whilst OMVs from the *Cj0511* mutant exhibited cleavage of occludin similar to that of 11168H OMVs (Fig. 8B). Taken together, the data indicated that Cj0511 does not target E-cadherin or occludin, in contrast HtrA and Cj1365c may both contribute to dysregulation of IEC barrier function.

OMVs enhance *C. jejuni* 11168H invasion during co-culture with T84 IECs

Having demonstrated that proteolytic activity is associated with *C. jejuni* OMVs, the role of OMVs during *C. jejuni* interactions with and invasion of IECs was investigated by adding 11168H OMVs (1 µg or 10 µg) to the co-culture of live 11168H bacteria with T84 IECs (MOI 100:1). The addition of OMVs had no significant effect on bacterial adhesion at 3 h, however there was a significant increase at both 6 h and 24 h (Fig. 9A). The addition of OMVs had a more marked effect on bacterial invasion, with significant increases observed at all time points with the numbers of intracellular bacteria increasing in an OMVs dose- and time-dependent manner (Fig. 9B).

DISCUSSION

Recent characterisation of OMVs isolated from *C. jejuni* strains has provided important new insights into bacterial interactions with IECs (Lindmark *et al.*, 2009, Elmi *et al.*, 2012). In this study, we have demonstrated that 11168H OMVs contain three proteases, HtrA, Cj0511 and Cj1365c. To date, HtrA is the only *C. jejuni* protease with an identified host substrate, E-cadherin (Boehm *et al.*, 2012). Investigations into host substrates for other *C. jejuni* proteases have not yet been reported. Here, HtrA and Cj1365c proteases associated with *C. jejuni* OMVs were shown to be responsible for the cleavage of the major AJs and TJs proteins E-cadherin and occludin. The proteolytic activity of OMVs appears to play an important role in enhancing levels of *C. jejuni* interactions with and particularly invasion of IECs. The identification of the proteolytic activity associated with *C. jejuni* OMVs contributes to a growing appreciation that proteases play an important role in diverse aspects of bacterial pathogenesis.

C. jejuni 11168H OMVs were shown to possess proteolytic activity with 10 µg OMVs approximately equivalent to 10 ng/ml trypsin. To further investigate the proteolytic activity associated with *C. jejuni* OMVs, the ability of specific protease inhibitors to reduce OMVs proteolytic activity was investigated. Inhibitors of metalloproteases, cysteine proteases and aspartic proteases had no significant effect on OMVs proteolytic activity. In contrast, inhibitors of serine proteases significantly reduced OMVs proteolytic activity. Proteomic analysis has identified at least three proteases associated with *C. jejuni* OMVs (Elmi *et al.*, 2012, Jang *et al.*, 2014). OMVs isolated from *htrA*, *Cj0511* or *Cj1365c* mutants possess significantly reduced proteolytic activity compared to OMVs isolated from the 11168H wild-type strain. The proteolytic activity of OMVs isolated from the *htrA*, *Cj0511* or *Cj1365c* mutants was further reduced by pre-treatment with the specific serine protease inhibitors PMSF or AEBSF. However this reduction was much lower with the *Cj1365c* OMVs compared to either the *htrA* OMVs or *Cj0511* OMVs, suggesting that the inhibitory effect of PMSF or AEBSF was much

less pronounced. *Cj1365c* OMVs would be expected to contain both HtrA and Cj0511 proteases, so why the effect of serine protease inhibitors is less pronounced on OMVs isolated from the *Cj1365c* mutant is unclear. The *Vibrio cholerae* DegP protease has been shown to play an important role in determining the content of OMVs (Altindis *et al.*, 2014), so *Cj1365c* could play a role incorporating HtrA and Cj0511 into OMVs and in the absence of *Cj1365c*, a yet unidentified protease is incorporated into OMVs in preference to HtrA and Cj0511. It is predicted that *Campylobacter* species encode as many as 64 peptidases (Karlyshev *et al.*, 2014). Proteomic analysis of OMVs isolated from the 11168H *Cj1365c* mutant would be a first step to testing this hypothesis. Originally it was considered that HtrA serine proteases play an important role as a bacterial cytoplasmic stress response factor, but more recently *C. jejuni* HtrA has been shown to be actively secreted and to interact with host protein E-cadherin resulting in bacterial paracellular transmigration (Boehm *et al.*, 2012, Hoy *et al.*, 2012). To our knowledge, this study represents the first demonstration that the *C. jejuni* HtrA and *Cj1365c* proteases cleave occludin. The reduced cleavage ability of OMVs isolated from the *htrA* or *Cj1365c* mutants suggests that both proteases can partially compensate for the absence of the other protease. The Cj0511 protease did not appear to contribute to OMV-induced cleavage of E-cadherin or occludin *in vitro*. Cj0511 was recently characterised as a serine protease with a role in bacterial colonisation (Karlyshev *et al.*, 2014). It is possible that Cj0511 plays a role in the degradation of mucin to promote colonisation *in vivo* or as a source of nutrients, whilst the HtrA and *Cj1365c* proteases play roles in bacterial interactions with host cells. Recently, studies have shown *Bacteroidales* species secrete glycoside hydrolases and polysaccharide in OMVs, which allow other species to utilise plant polysaccharides that would otherwise be inaccessible as a source of nutrients (Elhenawy *et al.*, 2014). As such OMVs could play a role in *C. jejuni* competitive and beneficial interactions with host substrates.

The proteolytic activity associated with Cj1365c supports the annotation of Cj1365c as a putative serine protease with a predicted molecular size of 116.3 kDa. Cj1365c also has the characteristic modular organisation of an autotransporter. Bioinformatic analysis predicts that Cj1365c contains an N-terminal signal sequences (1-15) followed by a Peptidase S8 domain (34-346) and a C-terminal autotransporter domain (786-1007) (www.uniprot.org/uniprot/Q0P8P4) (see Figure S1A). Bioinformatic analysis predicts that HtrA contains a Peptidase S46 domain (104-239) (www.uniprot.org/uniprot/Q0P928) and Cj0511 contains a Peptidase S41 domain (208-369) (www.uniprot.org/uniprot/Q0PB04) (see Figure S1A). The predicted active sites for serine protease activity associated with Cj1365c (D43 / H80 / S292), HtrA (H120 / D151 / S225) and Cj0511 (S303 / K328) are indicated in a sequence alignment of the three proteases (Figure S1B). Blast searching of the MEROPS database (<http://merops.sanger.ac.uk/>) has also confirmed Cj1365c has a subtilisin-like protease motif, which shares 30% sequence identity with the extracellular subtilisin from *Serratia marcescens*. *Cj1365c* has been reported to be absent in *C. jejuni* strains isolated from environmental sources, but present in the large majority (88.6%) of clinical isolates, suggesting that *Cj1365c* may be associated with *C. jejuni* host adaptation and virulence (Champion *et al.*, 2005). The fact that the HtrA, Cj0511 and Cj1365c proteases are associated with a serine motif to facilitate substrate recognition supports the hypothesis that *C. jejuni* serine proteases play an important role in virulence. Proteases from several bacterial pathogens have been demonstrated to cleave IEC proteins including E-cadherin (Costa *et al.*, 2013).

Previously we have shown that the cytotoxicity associated with *C. jejuni* OMVs in the *G. mellonella* larvae model was independent of CDT (Elmi *et al.*, 2012). In contrast, this study has shown that both HtrA and Cj1365c play a role in OMVs cytotoxicity to *G. mellonella* larvae. Studies have shown that *C. jejuni* protease mutants exhibit reduced *G. mellonella* cytotoxicity (Champion *et al.*, 2010). However, the fact that OMVs isolated from the *Cj0511*

mutant exhibit a non-significant decrease in cytotoxicity compared to wild-type OMVs suggests that Cj0511 may not play a role in OMVs cytotoxicity to *G. mellonella* larvae. Recombinant proteases from other pathogenic bacteria have been shown to cause destruction of the *G. mellonella* coagulation response and activation of hemolymph melanisation, which results in larval death. For example, *Pseudomonas aeruginosa* protease IV is a key virulence factor that may modulate the host innate immune system and elastase B might contribute to *P. aeruginosa* pathogenesis (Park *et al.*, 2014). *C. jejuni* HtrA has been shown to play a role in acute ulcerative enterocolitis and extra-intestinal immune responses during bacterial infection of gnotobiotic IL-10 deficient mice (Heimesaat *et al.*, 2014b) and *C. jejuni htrA* mutant infection of an infant mouse model system resulted in reduced intestinal and extra-intestinal pro-inflammatory immune responses (Heimesaat *et al.*, 2014a).

The increase in bacterial adhesion and invasion of IECs in the presence of OMVs may be explained in part by the presence of HtrA, Cj0511 and Cj1365c, which may be acting in a concerted fashion, leading to increased accessibility of *C. jejuni* to the sub-cellular space receptors. The ability of *C. jejuni* to adhere to and invade IECs is reduced by mutation of *htrA* (Brondsted *et al.*, 2005, Boehm *et al.*, 2015), *Cj0511* (Jowiya *et al.*, 2013) or *Cj1365c* (data not shown). *C. jejuni* infection of T84 cells results in a time-dependent decrease in transepithelial electrical resistance (TEER) and a redistribution of occludin from an intercellular to an intracellular location (Chen *et al.*, 2006). Previously the cleavage of occludin by *C. jejuni* has been suggested to occur via an as yet unknown mechanism(s) (MacCallum *et al.*, 2005). The *in vitro* time-dependent cleavage of E-cadherin and occludin and the visible reduction in E-cadherin and occludin associated with T84 monolayers after incubation with *C. jejuni* OMVs both support the role of these proteases in the mechanism(s) resulting in increased bacterial adhesion and invasion in the presence of OMVs. This is consistent with the recent report of the

role of HtrA in *C. jejuni* transmigration of IECs, suggesting new outlooks on the mechanisms used by this important enteric pathogen to interact with and invade IECs (Boehm *et al.*, 2012). The data in this study adds to a growing body of evidence that the release of OMVs plays an important role in bacterial pathogenesis. *C. jejuni* OMVs possess proteolytic activity *in vitro*, which can be inhibited by specific serine protease inhibitors. This OMVs proteolytic activity is associated with the HtrA, Cj0511 and Cj1365c proteases. Both the HtrA and Cj1365c proteases are involved in the *C. jejuni* OMV-induced proteolytic cleavage of E-cadherin and occludin. In addition OMVs increased the numbers of live *C. jejuni* interacting with and invading IECs in a time- and dose-dependent manner. Further investigations into the molecular mechanism(s) by which proteases modulate TJs and AJs proteins and IEC barrier function are required. How *C. jejuni* OMV-induced proteolytic cleavage of TJs and AJs proteins is modulated by physiological or pathological stimuli *in vivo* is a novel area of pathogenesis requiring further investigation. A greater understanding of the role that *C. jejuni* OMVs play during bacterial interactions with host cells will allow new insights into how this important pathogen interacts with IECs and causes disease.

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EXPERIMENTAL PROCEDURES

Bacterial strains and culture conditions

The *C. jejuni* wild-type strain used in this study was 11168H, a hypermotile derivative of the sequence strain NCTC11168 that shows higher levels of caecal colonisation in a chick colonisation model (Jones *et al.*, 2004). The *C. jejuni* 11168H *htrA*, *Cj0511* and *Cj1365c* mutants were obtained from the LSHTM *Campylobacter* Resource Facility (<http://crf.lshtm.ac.uk/index.htm>). All mutations were confirmed by PCR screening and by sequencing as described previously (Gundogdu *et al.*, 2011). *C. jejuni* strains were grown either on blood agar plates containing Columbia agar base (Oxoid, UK) supplemented with 7% (v/v) horse blood (TCS Microbiology, UK) and *Campylobacter* Selective Supplement (Oxoid) or in Brucella broth (Oxoid) with shaking at 75 rpm in a microaerobic chamber (Don Whitley Scientific, UK) containing 85% N₂, 10% CO₂ and 5% O₂ at 37°C. Kanamycin (Sigma-Aldrich, UK) was added to blood agar plates or to Brucella broth as required at the concentration of 50 µg/ml. Unless otherwise stated, *C. jejuni* strains were grown on blood agar plates for 24 h prior to use in all subsequent experiments.

Isolation of *C. jejuni* OMVs

C. jejuni OMVs were isolated as described previously (Elmi *et al.*, 2012). *C. jejuni* from a 24 h blood agar plate were resuspended in 1 ml Brucella broth and used to inoculate 100 ml pre-equilibrated Brucella broth to an OD₆₀₀ of 0.1. Cultures were grown for 14 h to mid-log phase then centrifuged at 10,000 x *g* for 15 mins at 4°C. The resulting supernatant was filtered through a 0.22 µm membrane (Millipore, UK) then the filtrate concentrated to 2 ml using an Ultra-4 Centrifugal Filter Unit with a nominal 10 kDa cutoff (Millipore). The concentrated filtrate was ultra-centrifuged at 150,000 x *g* for 3 h at 4°C using a TLA 100.4 rotor (Beckman Instruments, USA). All isolation steps were carried out at 4°C and the resulting OMV pellet

was resuspended in phosphate buffered saline (PBS) and stored at -20°C. OMV samples were plated out on blood agar plates and incubated under both microaerobic and aerobic conditions for 48 h to confirm the absence of viable bacteria. Approximately 200 µg OMVs by protein content were isolated from 100 ml of culture supernatant, determined by BCA assay (Fisher Scientific, UK). Amounts of OMVs used in subsequent experiments are quantified by protein content.

Quantitative determination of OMVs proteolytic activity

The proteolytic activity of OMVs was determined using a Protease Fluorescent Detection Kit (Sigma-Aldrich) using a FITC-labelled casein substrate. OMVs (10 µg in 10 µl) were mixed with 20 µl incubation buffer and 20 µl substrate then incubated at 37°C in the dark for 24 h. The reaction was stopped by adding 200 µl 0.6 N trichloroacetic acid to precipitate any remaining substrate, which was removed by centrifugation at 10,000 x g for 10 mins at 4°C. The supernatant obtained (10 µl) was diluted in 1 ml assay buffer. The digested FITC-casein substrate has absorption / emission maxima at 485 nm / 535 nm, and fluorescence intensity was recorded using a Multi-Mode Microplate reader (Molecular Devices, UK). Bovine trypsin (1 µg/ml) was used as positive control in all experiments.

The effects of different protease inhibitors (EDTA, E64, pepstatin A, leupeptin, PMSF and AEBSF) on proteolytic activity were determined by pre-incubating 10 µg OMVs with 1 µl of each inhibitor (Protease Inhibitor Set, G-Biosciences, U.S.A) in PBS at 37°C for 30 mins before incubation with FITC-labelled casein substrate at 37°C in the dark for 24 h. Appropriate controls (no substrate and no enzyme) were performed.

***Galleria mellonella* larvae model of infection**

G. mellonella larvae (LiveFoods Direct, UK) were kept on wood chips at 16°C. Experiments were performed with slight modifications from the original published methodology (Champion *et al.*, 2010) as described previously (Gundogdu *et al.*, 2011). Briefly, 5 µg of 11168H, *htrA*, *Cj0511* or *Cj1365c* OMVs in a 10 µl volume were injected into the right foremost leg of the *G. mellonella* larvae by micro-injection (Hamilton, Switzerland). For each experiment, 10 *G. mellonella* larvae were injected and experiments were repeated three times where larvae of the same approximate weight were chosen. Controls were both non-injected larvae or larvae injected with 10 µl of sterile PBS. Larvae were incubated at 37°C and survival recorded at 24 h intervals for 72 h.

Cell lines, media and culture conditions

The human T84 colon cancer epithelial cells were obtained from the National Type Culture Collection. T84 cells were maintained at sub-confluence in DMEM / F-12 (Invitrogen, UK) supplemented with 10% (v/v) FCS, 1% (w/v) non-essential amino acids and 1% (w/v) penicillin-streptomycin (Sigma-Aldrich) at 37°C in a 5% CO₂ humidified atmosphere. Cells were split around 80-90% confluence and seeded at 5 x 10⁵ cells per well into 24-well tissue culture plates (Corning Glass Works, Netherlands) using 1 ml volumes of cell culture media per well. Medium was replenished every 2 days. For ELISA experiments, the medium was removed and monolayers washed three times with PBS, then maintained in antibiotic-free medium supplemented with 10% (v/v) serum (Invitrogen) for 24 h before each experiment.

Cytotoxicity detection assay

The CytoTox 96® non-radioactive cytotoxicity assay (Promega, UK) was used to quantify the cell damage induced by co-culture with *C. jejuni* OMVs. Briefly, T84 cells were challenged with 100 µg of 11168H, *htrA*, *Cj0511* or *Cj1365c* OMVs. After co-incubation at 37°C for 24

h, cell supernatants were analysed for the release of lactate dehydrogenase (LDH). Non-challenged cells represented the 0% cytotoxicity negative control. Total lysis of cells following treatment with 1% (v/v) Triton X-100 represented the 100% cytotoxicity positive control.

Enzyme-linked immunosorbent assay (ELISA)

T84 cells were co-cultured with 100 µg, 10 µg or 1 µg of 11168H OMVs or *htrA*, *Cj0511* or *Cj1365c* OMVs for 24 h. The levels of IL-8 secretion were assessed using a commercially available sandwich ELISA kit according to manufacturer's instructions (E-Biosciences, UK). Detection was performed using a Dynex MRX II 96 well plate reader (Dynex, U.S.A) at an absorbance of 450 nm (A_{450}) and analysed using Revelation software (Dynex).

***in vitro* cleavage of E-cadherin and occludin by *C. jejuni* OMVs**

The cleavage of recombinant TJs and AJs proteins (E-cadherin and occludin) by 11168H OMVs or *htrA*, *Cj0511* or *Cj1365c* OMVs was determined as described previously (Baek *et al.*, 2011). Briefly, OMVs (10 µg) were incubated with 1 µg recombinant human E-cadherin or occludin (R&D Systems, UK) in PBS at 37°C for 3 and 16 h. For analysis of E-cadherin or occludin cleavage, reactions were mixed with 2X sample loading buffer (125 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 10% (v/v) 14.3 M β-mercaptoethanol, 10% (v/v) glycerol, 0.006% (w/v) bromophenol blue) and boiled for 10 min. Proteins were separated using NuPAGE® Novex® 12% Bis-Tris protein gels (Invitrogen) then electrotransferred onto a nitrocellulose blotting membrane (GE Healthcare, UK) using the Semi-dry Trans-Blot system (GE Healthcare). Membranes were incubated in a blocking buffer (2% (w/v) skimmed milk (Tesco, UK) in PBS) for 1 h at room temperature. After removal of blocking buffer, membranes were rinsed three times with 0.1% (v/v) Tween-20 in PBS then incubated 1 h at room temperature with primary mouse anti-E-Cadherin or primary rabbit anti-occludin (Abcam, UK) (1:1,000).

Following primary antibody incubation, membranes were washed four times with 0.1% (v/v) Tween-20 in PBS followed by incubation with an infrared fluorescence-conjugated secondary antibody (either goat anti-mouse IR800 or goat anti-rabbit IR680 (Licor Biosciences, UK) prepared in a 1:10,000 dilution of blocking buffer) at room temperature for 1 h. Membranes were scanned and analysed using a Licor Odyssey® (Licor Biosciences).

Immunofluorescence staining of T84 cells

T84 cells were grown on glass coverslips (thickness 0.16 ± 0.005 mm; VWR, UK) in 24-well plates. Cells were incubated with 10 μ g of *C. jejuni* 11168H OMVs. After 3, 6 and 24 h, cells were washed twice with PBS and fixed with 4% (v/v) paraformaldehyde (VWR) in PBS for 20 min at room temperature. After three washes with PBS, cells were permeabilised with 0.1% (v/v) Triton X-100 in PBS at room temperature for 30 min. Cells were washed three times with PBS and blocked with 3% (w/v) bovine serum albumin (BSA) (Sigma-Aldrich) in PBS for 1 h. Following blocking, cells were incubated with anti-E-cadherin (1:400) or anti-occludin (1:400) (Abcam) in blocking buffer for 1 h at room temperature. Cells were washed three times with PBS and incubated with secondary antibodies (anti-mouse IgG Alexa Fluor 488 (1:1000) or anti-rabbit IgG Alexa Fluor 488 (1:1000) (Invitrogen) in blocking buffer for 1 h. After washing, coverslips were mounted with Vectashield with DAPI (Vector Labs, UK). Images were captured on a confocal microscope (Zeiss LSM 510 confocal microscope (Carl Zeiss, Jena, Germany)) then processed in LSM 510 Image Browser. Laser intensity was kept consistent between samples.

Interaction and invasion assays

T84 cell monolayers (~80% confluence in 24-well tissue culture plates) were infected with bacteria at a multiplicity of infection (MOI) of 100:1 for 3, 6 or 24 h as described previously

(Elmi *et al.*, 2012). For interaction experiments, the monolayers were washed three times with PBS then lysed with 0.2% (v/v) Triton X-100. The cell lysates were serially diluted, 200 μ l volumes plated onto blood agar plates and incubated for 72 h before colonies were counted. For invasion experiments, the monolayers were washed three times with PBS, incubated in cell culture medium containing gentamicin (150 μ g/ml) for 2 h at 37°C to kill extracellular bacteria, then treated as for the interaction experiments.

Statistical analysis

All experiments represent at least three biological replicates with each experiment performed in triplicate. All data were analysed using Prism statistical software (Version 6, GraphPad Software, USA). Values were expressed as mean \pm SEM. Variables were compared for significance using Two-Way Analysis of Variance (ANOVA) and the Bonferroni test with one asterisk (*) indicating a *p* value between 0.01 and 0.05, two asterisks (**) indicating a *p* value between 0.001 and 0.01 and three asterisks (***) indicating a *p* value < 0.001.

FIGURE LEGENDS

FIG. 1. *C. jejuni* 11168H OMVs possess proteolytic activity. (A) Quantification of protease activity of *C. jejuni* 11168H OMVs compared to trypsin. Protease activity was detected after incubating FITC-labelled casein substrate with OMVs or trypsin at 37°C in the dark for 24 h. (B) Quantification of protease activity of *C. jejuni* 11168H OMVs in the presence of different protease inhibitors. Protease activity is expressed as the percentage of untreated 11168H OMVs (defined as 100%). *** = $p < 0.001$, *ns* = no significant difference.

FIG. 2. OMVs isolated from *Cj0511*, *Cj1365c* and *htrA* mutants display significantly reduced proteolytic activity compared to 11168H OMVs. Quantification of protease activity of OMVs isolated from *C. jejuni* 11168H wild-type strain, *Cj0511*, *Cj1365c* or *htrA* mutants in the absence or presence of specific serine inhibitors AEBSF or PMSF. Protease activity is expressed as the percentage of untreated 11168H OMVs (defined as 100%). * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

FIG. 3. OMVs isolated from *Cj1365c* and *htrA* mutants display a significant reduced cytotoxicity in *G. mellonella* larvae. *G. mellonella* larvae were injected with a 10 µl inoculum of 11168H OMVs (5 µg) or *Cj0511*, *Cj1365c* and *htrA* OMVs (5 µg) by microinjection in the right foremost leg. Larvae were incubated at 37°C and survival recorded after 24 h. PBS and no injection controls were also performed. For each experiment, 10 *G. mellonella* larvae were infected and experiments were performed in triplicate. *** = $p < 0.001$, *ns* = no significant difference.

FIG. 4. Cytotoxic effect of OMVs isolated from 11168H, *Cj0511*, *Cj1365c* and *htrA* mutants on T84 cells. The cytotoxic effect on the T84 cells was quantified by the release of

cytosolic lactate dehydrogenase (LDH) as a measure of cell damage. Non-challenged T84 cells represented 0% cytotoxicity (Uninfected Cells) and total lysis of T84 cells following treatment with 1% (v/v) Triton X-100 represented 100% cytotoxicity (Positive control).

FIG. 5. Induction of IL-8 from T84 intestinal epithelial cells by OMVs isolated from 11168H, Cj0511, Cj1365c and htrA mutants. T84 IECs responses to 24 h co-incubation with OMVs isolated from *C. jejuni* 11168H wild-type strain, *Cj0511*, *Cj1365c* or *htrA* mutants (100 µg, 10 µg or 1 µg) were assessed. Levels of IL-8 secreted from T84 cells following co-incubation with OMVs were quantified using a human IL-8 ELISA. ** = $p < 0.01$; *** = $p < 0.001$; *ns* = no significant difference.

FIG. 6. *C. jejuni* 11168H OMVs cleaves E-Cadherin and occludin *in vitro*. (A&B) Recombinant E-cadherin was incubated with 10 µg of 11168H OMVs, heat treated OMVs or phenylmethanesulfonyl fluoride (PMSF) treated OMVs for 3 h or 16 h as indicated at 37°C. Samples were separated by SDS-PAGE under reducing conditions and cleavage was detected by Western blotting with anti-E-cadherin antibody. (C&D) Recombinant occludin was incubated with 10 µg of 11168H OMVs, heat treated OMVs or phenylmethanesulfonyl fluoride (PMSF) treated OMVs for 3 h or 16 h as indicated at 37°C. Samples were separated by SDS-PAGE under reducing conditions and cleavage was detected by Western blotting with anti-occludin antibody. The position of molecular mass markers (kDa) is indicated to the left of the blot.

FIG. 7. Co-incubation of *C. jejuni* 11168H OMVs with T84 intestinal epithelial cell monolayers results in a visible reduction in E-cadherin and occludin. T84 IECs were co-incubated with 11168H OMVs (10 µg) for 3 h, 6 h and 24 h. The cells were fixed, permeabilised

and probed with E-cadherin (Top Panel) or occludin (Bottom Panel) antibodies. Untreated T84 cells were included as a control (Left Panels). White arrowheads indicate gap between cells where E-cadherin or occludin is disrupted in T84 IECs co-incubated with OMVs.

FIG. 8. Reduction in cleavage of E-Cadherin or occludin by OMVs isolated from *htrA* and *Cj1365c* mutants. Recombinant E-cadherin (**A**) or occludin (**B**) was incubated with OMVs isolated from *C. jejuni* 11168H wild-type strain, *Cj0511*, *Cj1365c* or *htrA* mutants as indicated. Samples were separated by SDS-PAGE and subjected to Western blot analysis using anti-E-cadherin or anti-occludin antibody. The position of molecular mass markers (kDa) is indicated to the left of the blot.

FIG. 9. Co-incubation of *C. jejuni* 11168H OMVs with intestinal epithelial cells prior to co-culture enhances bacterial invasion. T84 IECs were co-incubated with 11168H OMVs (10 µg or 1 µg) during infection with live *C. jejuni* 11168H (MOI 100:1). T84 cells were either lysed and numbers of interacting bacteria assessed (**A**) or incubated with gentamicin (150 µg/ml) for 2 h to kill extracellular bacteria, then lysed and numbers of intracellular bacteria assessed (**B**). * = $p < 0.05$; *** = $p < 0.001$.

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