The bile salt sodium taurocholate induces *Campylobacter jejuni* outer membrane vesicle production and increases OMV-associated proteolytic activity.

Abdi Elmi[1], Amber Dorey[1], Eleanor Watson[2], Heena Jagatia[1], Neil F. Inglis[2], Ozan Gundogdu[1], Mona Bajaj-Elliott[3], Brendan W. Wren[1], David G.E. Smith[4] & Nick Dorrell[1]*

[1] Faculty of Infectious & Tropical Diseases, London School of Hygiene & Tropical Medicine, Keppel Street, London, WC1E 7HT, UK.

[2] Moredun Research Institute, Pentlands Science Park, Penicuik, EH26 0PZ, UK.

[3] Infection, Immunity, Inflammation and Physiological Medicine, UCL Institute of Child Health, 30 Guilford Street, London, WC1N 1EH, UK.

[4] School of Engineering & Physical Sciences, Heriot-Watt University, Edinburgh, EH14 4AS, UK.

*Corresponding author:
Mailing address: Faculty of Infectious & Tropical Diseases, London School of Hygiene & Tropical Medicine, Keppel Street, London, WC1E 7HT, United Kingdom.
Tel: +44 (0)20 7927 2838.
E-mail: nick.dorrell@lshtm.ac.uk

Running title: Sodium taurocholate induces *Campylobacter jejuni* OMVs.

SUMMARY

Campylobacter jejuni, the leading cause of bacterial acute gastroenteritis worldwide, secretes an arsenal of virulence-associated proteins within outer membrane vesicles (OMVs). *C. jejuni* OMVs contain three serine proteases (HtrA, Cj0511 and Cj1365c) which cleave the intestinal epithelial cell (IEC) tight and adherens junction proteins occludin and E-cadherin, promoting enhanced *C. jejuni* adhesion to and invasion of IECs. *C. jejuni* OMVs also induce IECs innate immune responses. The bile salt sodium taurocholate (ST) is sensed as a host signal to co-ordinate the activation of virulence-associated genes in the enteric pathogen *Vibrio cholerae.* In this study, the effect of ST on *C. jejuni* OMVs was investigated. Physiological concentrations of ST do not have an inhibitory effect on *C. jejuni* growth until the early stationary phase. Co-culture of *C. jejuni* with 0.1% or 0.2% (w/v) ST stimulates OMV production, increasing both lipid and protein concentrations. *C. jejuni* ST-OMVs possess increased proteolytic activity and exhibit a different protein profile compared to OMVs isolated in the absence of ST. ST-OMVs exhibit enhanced cytotoxicity and immunogenicity to T84 IECs and enhanced killing of *Galleria mellonella* larvae. ST increases the level of mRNA transcripts of the OMVs-associated serine protease genes and the *cdtABC* operon that encodes the cytolethal distending toxin. Co-culture with ST significantly enhances the OMVs-induced cleavage of E-cadherin and occludin. *C. jejuni* OMVs also cleave the major endoplasmic reticulum (ER) chaperone protein BiP/GRP78 and this activity is associated with the Cj1365c protease. This data suggests that *C. jejuni* responds to the presence of physiological concentrations of the bile salt ST which increases OMV production and the synthesis of virulence-associated factors that are secreted within the OMVs. We propose that these events contribute to pathogenesis.

INTRODUCTION

Campylobacter jejuni is a motile Gram-negative bacterium regarded as the leading cause of bacterial food-borne gastroenteritis worldwide (Kaakoush *et al.*, 2015, Skarp *et al.*, 2016). *C. jejuni* possesses multiple virulence factors, such as an *O*-linked glycosylated flagella (Wassenaar *et al.*, 1991, Mertins *et al.*, 2013), a polysaccharide capsule and lipo-oligosaccharide (LOS) (Bacon *et al.*, 2001, Maue *et al.*, 2013), multiple adhesins (Monteville *et al.*, 2003), a cytolethal distending toxin (CDT) (Johnson *et al.*, 1988, Whitehouse *et al.*, 1998, Lara-Tejero *et al.*, 2001) and multiple proteases (Brondsted *et al.*, 2005, Karlyshev *et al.*, 2014, Elmi *et al.*, 2016).

A defining feature of bacterial pathogens is the ability to sense host metabolites and precisely co-ordinate the expression of multiple virulence factors (Finlay *et al.*, 1997, Fang *et al.*, 2016a, Fang *et al.*, 2016b, Olive *et al.*, 2016). Bile is a well-characterised host metabolite, biosynthesised from cholesterol by hepatocytes in the liver (Joyce *et al.*, 2016, Vitek *et al.*, 2016). Bile is excreted from the gall bladder into the small intestine, particularly in the duodenum, jejunum and proximal ileum. The physiological concentration of bile in the intestine can be as high as 20 mM in the duodenum, then progressively decreasing to 10 mM in the jejunum, reaching as little as 4 mM in the ileum (Hofmann *et al.*, 2008, Maldonado-Valderrama *et al.*, 2011). In the intestine, bile emulsifies dietary fats and facilitates absorption of lipid nutrients. In addition, owing to the heterogeneous mixture of bile salts, bilirubin, phospholipids, cholesterol and enzymes, bile also has detergent-like toxicity and functions as a potent antimicrobial agent, solubilising membrane lipids including the dissociation of bacterial membrane proteins (Hofmann *et al.*, 1967, Begley *et al.*, 2005, Garidel *et al.*, 2007, Boyer, 2013). For many enteric pathogens including *Escherichia coli* O157:H7*, Vibrio cholerae, Shigella flexneri* and *Vibrio parahaemolyticus*, bile acts as a signal to modulate global gene expression of virulence factors (Alam *et al.*, 2010, Gotoh *et al.*, 2010, Faherty *et* *al.*, 2012, Hamner *et al.*, 2013, Bachmann *et al.*, 2015, Sanchez *et al.*, 2016, Sistrunk *et al.*, 2016). The conjugated bile salt sodium taurocholate (ST) has been shown to act as a host signal for the *V. cholerae* 7th pandemic O1 El Tor biotype strain N16961 (Yang *et al.*, 2013), inducing intermolecular di-sulphide bond formation in the transmembrane transcription activator TcpP (Yang *et al.*, 2013). More recently, the mechanisms by which *V. parahaemolyticus* senses bile salts via the type III secretion system 2 (T3SS2) regulators VtrA and VtrC have been identified (Li *et al.*, 2016). Analysis of the crystal structure of the periplasmic domains of the VtrA/VtrC heterodimer revealed that VtrA and VtrC form a protein complex on the surface of the outer membrane creating a barrel-like structure that binds to bile salts and induces *V. parahaemolyticus* to secrete toxins (Li *et al.*, 2016).

As with the other *Proteobacteria* pathogens *Haemophilus ducreyi*, *Aggregatibacter actinomycetemcomitans* and *Shigella dysenteriae*, *C. jejuni* possesses CDT which interferes with normal cell cycle progression, resulting in G_2 arrest (Pickett *et al.*, 1996, Whitehouse *et al.*, 1998, Purdy *et al.*, 2000). CDT (encoded by the *cdtABC* operon) is the only identified toxin in *C. jejuni*, yet the role that host metabolites such as bile salts play in inducing CDT secretion is unclear. Like many other enteric pathogens, *C. jejuni* encounters bile salts during human infection (Drion *et al.*, 1988, Raphael *et al.*, 2005). The bile salt sodium deoxycholate induces differential expression of *C. jejuni* virulence genes, notably increasing the expression of *Campylobacter* Invasion Antigen (Cia) genes, leading to enhanced invasion of IECs by *C. jejuni* (Malik-Kale *et al.*, 2008). However the effects of the taurocholate-conjugated hydrophilic bile salts such as ST on *C. jejuni* physiology or the role of ST as a host cellular signal for *C. jejuni* have not been previously investigated.

Outer membrane vesicles (OMVs) are nano-sized structures (50 –250nm in diameter) that are released by all Gram-negative bacteria (Kulkarni *et al.*, 2014, Schwechheimer *et al.*, 2015). OMVs are enriched with an active molecular cargo of virulence factors such as adhesins, invasins, toxins, proteases, antigenic proteins and non-protein antigens such as lipo-polysaccharide (LPS). OMVs are associated with a multi-faceted role in bacterial pathogenesis (Schertzer *et al.*, 2013, Kaparakis-Liaskos *et al.*, 2015, Schwechheimer *et al.*, 2015, Vanaja *et al.*, 2016). So far only limited studies on the role of stress response pathways on bacteria vesiculation have been reported (McBroom *et al.*, 2007, Macdonald *et al.*, 2013, Schwechheimer *et al.*, 2013a, Schwechheimer *et al.*, 2013b, Schwechheimer *et al.*, 2014). Whilst these studies have contributed to the understanding of the role of OMVs in bacterial pathophysiology, there are probably many roles of OMVs in bacterial pathogenesis that are still poorly defined. These questions are particularly relevant for understanding *C. jejuni* pathogenesis that in comparison to other enteric pathogens, is poorly understood.

C. jejuni produces OMVs enriched with an active arsenal of virulence factors (Lindmark *et al.*, 2009, Elmi *et al.*, 2012). More recently we have highlighted the role of three serine proteases (HtrA, Cj0511 and Cj1365c) secreted in *C. jejuni* OMVs which cleave the intestinal epithelial cell (IEC) tight and adherens junction proteins occludin and E-cadherin, promoting enhanced *C. jejuni* adhesion to and invasion of IECs (Elmi *et al.*, 2016). The role of *C. jejuni* OMVs in pathogenesis and the emerging role of bile salts such as ST in inducing bacterial virulence led to the investigation of the effect of ST on *C. jejuni* OMVs production. ST stimulates *C. jejuni* 11168H OMVs production, increasing both the protein concentration and lipid content of OMVs. ST enhances the proteolytic activity associated with *C. jejuni* OMVs, leading to enhanced OMVs cytotoxicity and immunogenicity to IECs. ST increases the level of mRNA transcripts of the OMVs-associated serine protease genes and also the *cdtABC* operon. Collectively, our data demonstrates that *C. jejuni* responds to physiological concentrations of the bile salt ST, increasing OMVs production and the synthesis of virulence-associated factors that are secreted within the OMVs.

RESULTS

Co-culture with sodium taurocholate increases both the protein concentration and lipid content of *C. jejuni* OMVs

As bile salts have bactericidal properties (Begley *et al.*, 2005, Merritt *et al.*, 2009), the effect of ST on the growth of *C. jejuni* was investigated. No significant difference in growth rate as measured by OD_{600} readings (Figure 1A) or CFU counts (Figure 1B) was observed when *C. jejuni* 11168H was cultured in Brucella broth in the presence of 0.1% or 0.2% (w/v) ST compared to bacteria cultured in Brucella broth alone. To investigate whether ST had any damaging effects on *C. jejuni* membranes, LIVE/DEAD BacLight staining was performed using microplate fluorescence and confocal microscopic analysis of *C. jejuni* 11168H after co-culture in Brucella broth for 12 hours in the presence of 0.1% or 0.2% (w/v) ST. No reduction in the numbers of live *C. jejuni* cells was observed in the presence of ST (Figure 1C), indicating that the viability of *C. jejuni* cells were not affected by either of these concentrations of ST. Confocal microscopic analysis of *C. jejuni* 11168H also revealed the membrane integrity of the bacterial cells was not affected (Figure 1D).

OMVs isolated from *C. jejuni* 11168H cultured in the presence of 0.1% or 0.2% (w/v) ST (ST-OMVs) had significantly higher protein concentrations than OMVs isolated from the same volume of *C. jejuni* 11168H cultured in the absence of ST (Figure 2A). In addition, the lipid content of *C. jejuni* ST-OMVs was also increased compared to OMVs isolated from *C. jejuni* 11168H cultured in the absence of ST (Figure 2B). To establish the role of ST in enhancing *C. jejuni* OMV formation, the amount of Keto-deoxy-d-manno-8-octanoic acid (Kdo) associated with OMVs was assessed. Kdo is a characteristic constituent of Gram-negative bacterial LPS and LOS (Brade *et al.*, 1984). The amount of Kdo associated with OMVs isolated from *C. jejuni* 11168H cultured in the presence of 0.1% or 0.2% (w/v) ST was significantly increased (Figure 2C). To extend these findings, OMVs were separated on SDS-PAGE gel and stained with silver stain to visualise LOS. The presence of 0.1% or 0.2% (w/v) ST resulted in enhanced staining of OMVs-associated LOS compared OMVs isolated in the absence of ST (Figure 2D).

Proteomic analysis indicates an increase in the number of proteins associated with *C. jejuni* ST-OMVs

To further investigate the significance of ST on *C. jejuni* OMV production, the proteome of OMVs isolated from *C. jejuni* 11168H grown in the presence of 0.2% (w/v) ST was investigated. A total of 185 proteins were identified (Table S1), 131 of which were also identified in our earlier proteomic analysis of *C. jejuni* 11168H OMVs (Elmi *et al.*, 2012). The proportions of proteins from each cluster of orthologous groups (COG) categorization are shown in Figure 3. Of the 34 new proteins identified as specific to ST-OMVs, one of the *Campylobacter* invasion antigens CiaI (Cj1450) (Buelow *et al.*, 2011) was identified as well as the Cj1365c protease and the bile salt response protein Cj0561c. Cj1614 and Cj0755, which are involved in the binding, uptake or export of trace metals, and the 2-oxoglutarate-acceptor oxidoreductase subunit OorABC were also identified along with Cj0628, a putative autotransporter protein that functions as an adhesin with a role in colonisation (Ashgar *et al.*, 2007).

Co-culture with sodium taurocholate significantly enhances OMVs proteolytic activity

C. jejuni 11168H OMVs contain biologically active serine proteases that interact with IEC proteins to enhance *C. jejuni* adherence to and invasion of IECs (Elmi *et al.*, 2012, Elmi *et al.*, 2016). *C. jejuni* 11168H ST-OMVs exhibited a statistically significant enhanced proteolytic activity compared to OMVs isolated from *C. jejuni* 11168H cultured in the absence of ST (Figure 4).

ST-OMVs exhibit enhanced cytotoxicity and immunogenicity to T84 intestinal epithelial cells

Given ST-OMVs exhibit enhanced proteolytic activity, the cytotoxicity of *C. jejuni* OMVs and ST-OMVs co-incubated with T84 IECs for 24 hours was investigated. Increased levels of cytosolic lactate dehydrogenase (LDH) after co-incubation of T84 IECs with ST-OMVs was observed compared to co-incubation with OMVs (Figure 5A). The induction of interleukin-8 (IL-8) from T84 cells by OMVs and ST-OMVs was quantified using ELISA (enzyme-linked immunosorbent assay). ST-OMVs significantly enhanced induction of IL-8 compared to co-incubation with OMVs (Figure 5B). The effect of ST on the OMV-induced up-regulation of IL-8 was also investigated. ST enhanced the mRNA transcription of IL-8 in T84 IECs (Figure 5C).

ST-OMVs exhibit enhanced cytotoxicity in the *Galleria mellonella* model of infection

To investigate the effect of ST on *C. jejuni* OMVs *in vivo*, 11168H ST-OMVs and OMVs were injected into *G. mellonella* larvae. Injection with ST-OMVs resulted in an increase in killing of larvae compared to injection with OMVs (Figure 6). To investigate whether ST had any toxicity towards *G. mellonella* larvae, ST alone was also injected. No killing was observed in the *G. mellonella* larvae injected with 10 µl of either 0.1% or 0.2% (w/v) ST.

Co-culture with sodium taurocholate results in differential expression of genes encoding OMV-associated CDT and serine proteases

The effects of ST on the relative expression of genes involved in *C. jejuni* cytotoxicity (*cdtABC* operon) and proteolytic activity (*htrA*, *Cj0511* and *Cj1365c*) were investigated. Total RNA was isolated from *C. jejuni* 11168H cultured in the absence of ST or in the presence of 0.1% or 0.2% (w/v) ST. Expression of both the *cdtABC* operon (Figure 7A) and the serine protease genes (Figure 7B) was significantly enhanced relative to the level of the housekeeping DNA gyrase gene *gyrA* (Ritz *et al.*, 2009). In agreement with the proteolytic and cytotoxicity data, these data imply that ST enhances the expression of *C. jejuni* 11168H virulence-associated genes to facilitate enhanced proteolytic activity, cytotoxicity and immunogenicity.

Co-culture with sodium taurocholate significantly enhances the OMV-induced cleavage of occludin and E-cadherin *in vitro*

C. jejuni OMVs cleave the major tight junction (TJ) and adheren junctions (AJ) proteins occludin and E-cadherin (Elmi *et al.*, 2016). Recombinant occludin and E-cadherin were co-incubated with OMVs and ST-OMVs. Incubation with ST-OMVs resulted in an increase in the cleaved form of both occludin and E-cadherin compared to incubation with OMVs (Figure 8AB).

C. jejuni 11168H OMVs cleave the major endoplasmic reticulum (ER) chaperone protein BiP/GRP78

The Cj1365c protease possesses a subtilase domain. The amino acid sequences of the subtilase domain share 20% identity with the A subunit (SubA) of the subtilase cytotoxin SubAB of Shiga toxigenic *Escherichia coli* (STEC). STEC SubA mediates an unusual toxicity of the $SubAB_5$ toxin with an extreme substrate specificity towards ER chaperone GRP78/BiP (Paton *et al.*, 2006). Despite the low level of sequence identity, the position of the key residues in the catalytic serine active site of Cj1365c at position 274 (numbering corresponds to the protein without signal sequence) is conserved in STEC SubA, suggesting that GRP78/BiP may serve as a substrate for the Cj1365c protease. The activity of OMVs isolated from *C. jejuni* in the presence or absence of ST to cleave recombinant GRP78/BiP was therefore investigated. *C. jejuni* OMVs were able cleave GRP78/BiP and this cleavage was enhanced with ST-OMVs

(Figure 9A). OMVs isolated from the 11168H wild-type strain and from the three protease mutants (*htrA*, *Cj0511* and *Cj1365c*) were co-incubated with recombinant GRP78/BiP. Only OMVs isolated from the *Cj1365c* mutant exhibited reduced ability to cleave GRP78/BiP (Figure 9B).

DISCUSSION

C. jejuni OMVs are cytotoxic, immunogenic and proteolytic (Elmi *et al.*, 2016). However the effect of host metabolites such as bile salts on *C. jejuni* OMVs secretion, content and function has not been studied. Recently genetic, biochemical and transcriptome analyses have been used to reveal the role of bile salts in inducing the pathogenesis of different enteric bacteria (Joyce *et al.*, 2016, Sistrunk *et al.*, 2016). Bile salts mediate reciprocal host-pathogen crosstalk (Gotoh *et al.*, 2010, Chaand *et al.*, 2013) and there is increasing evidence supporting the role of bile salts in regulating bacterial virulence gene expression (Yang *et al.*, 2013, Li *et al.*, 2016, Xue *et al.*, 2016). ST plays an important role in inducing *V. cholerae* virulence gene expression (Xue *et al.*, 2016), However the effect of ST on C. *jejuni* has not been investigated. In this study, we have characterised the role of ST in modulating *C. jejuni* 11168H OMVs secretion, content and function. Both the protein and lipid concentration of 11168H OMVs were increased when *C. jejuni* was cultured in the presence of physiologically relevant concentrations of ST (0.1% and 0.2% w/v ST). Changes in OMV secretion, content and function under various growth conditions have also been observed in a number of other bacterial pathogens (Tashiro *et al.*, 2010, Choi *et al.*, 2014, Metruccio *et al.*, 2016).

Proteomic analysis of *C. jejuni* 11168H ST-OMVs identified all 131 proteins previously identified associated with 11168H OMVs (Elmi *et al.*, 2012), but also a further 34 proteins, including proteins expected to be involved *C. jejuni* response to bile salts such as Cj0561c for efficient colonisation (Guo *et al.*, 2008). The presence of CiaI (Cj1450) was of note as this is the first time that one of the Cia proteins has been identified associated with OMVs. Previously the secretion mechanism of CiaI via the flagellum was shown to be independent of host cell contact (Barrero-Tobon *et al.*, 2014). During colonisation of the human intestine, *C. jejuni* will be exposed to a number of different bile salts. The bile salt sodium deoxycholate (SD) increases the expression of *cia* genes (Malik-Kale *et al.*, 2008), however the effect of ST on *cia* gene expression has not been investigated. It is possible that a combination of SD and ST would increase the number of Cia proteins that could be identified associated with *C. jejuni* OMVs. Secretion of Cia proteins within OMVs would provide a new mechanism for the delivery of these virulence determinants into host cells.

The three proteases (HtrA, Cj0511 or Cj1365c) associated with OMVs are conserved in *C. jejuni* and inactivation of each has been linked to the attenuation of *C. jejuni* physiology, virulence and/or immunogenicity (Brondsted *et al.*, 2005, Karlyshev *et al.*, 2014, Jowiya *et al.*, 2015). The enhanced proteolytic activity of 11168H ST-OMVs indirectly indicates a more pathogenic *C. jejuni* response during colonisation of a host after the bacteria senses bile salts. The cytotoxic and immunogenic properties of OMVs are well characterised (Kaparakis-Liaskos *et al.*, 2015, Pathirana *et al.*, 2016). The enhanced cytotoxicity and immunogenicity of ST-OMVs may be associated with the increase in concentrations of proteases and CDT secreted in ST-OMVs, based on the qPCR analysis which showed that ST significantly up-regulates expression of *htrA*, *Cj0511*, *Cj1365* and the *cdtABC* operon. This supports an emerging consensus that bile salts in the intestine play important roles in modulating bacterial gene expression. The enhanced expression of *cdtA* is consistent with the dynamics of operon expression with a recent study reporting that there is a linear relationship between transcription distance and gene expression in operons (Lim *et al.*, 2011). This observation also indicates the importance of CdtA and CdtC which are involved in delivery of CdtB which possesses type I deoxyribonuclease-like activity that mediates IECs DNA damage, triggering the response of the cell cycle checkpoint and results in G2 arrest (Lara-Tejero *et al.*, 2001). Internalisation of CdtB is also positively correlated with CdtA and CdtC as purified CdtB alone has no effect on IECs, but when combined with CdtA and CdtC, IECs exhibited cell cycle arrest in the G2/M phase (Elwell *et al.*, 2001, Lara-Tejero *et al.*, 2001). However in contrast to *Vibrio* species, the mechanism(s) of ST interactions with *C. jejuni* remains unclear. *C. jejuni* lacks orthologues of

V. parahaemolyticus VtrA and VtrC that create a barrel-like structure that can bind to bile salts and trigger the production of cholera toxin. ST might cause changes in *C. jejuni* outer membrane proteins or alter levels and/or activities of yet unidentified *C. jejuni* proteins that could regulate synthesis and protein folding machinery or post-translational modifications. *C. jejuni* has orthologues of the Maintenance of lipid asymmetry (Mla) pathway proteins which retrograde phospholipid transport from the OM back to the IM and are involved in bacterial OMVs biogenesis in *V. cholerae* and *Haemophilus influenzae* (Roier et al., 2016a, Roier et al., 2016b). However further studies are required to identify the precise mechanism of *C. jejuni* ST-induced OMV biosynthesis.

ST-OMVs enhanced the cleavage of both occludin and E-cadherin and this was more pronounced for occludin than for E-cadherin. One explanation is that *C. jejuni* may preferentially bind and cleave apically located tight junction proteins such as occludin that function to seal the paracellular passage that is crucial for IEC integrity. *C. jejuni* also invades IECs via an actin filament-mediated mechanism and actin filaments interface with tight junction proteins (Biswas *et al.*, 2003). In addition, this result reinforces previous findings that correlated significant decrease in transepithelial electrical resistance (TEER) with redistribution and dephosphorylation of occludin instead of E-cadherin (Chen *et al.*, 2006). Loss of occludin in IECs infected with *C. jejuni* also led to significant alteration in tight junction transmembrane proteins (MacCallum *et al.*, 2005). Similar studies in *Escherichia coli* (EPEC) and *Salmonella enterica serovar Typhimurium* also showed a pathogenic mechanism associated with occludin-specific redistribution (Sakakibara *et al.*, 1997, McNamara *et al.*, 2001, Bertelsen *et al.*, 2004). It appears that a number of virulence-associated serine proteases secreted by different enteric pathogens preferentially alter the expression and distribution of occludin, indicating that occludin might be an important IEC target that shapes the bacterial-host interplay (Guttman *et al.*, 2008, Awad *et al.*, 2017, Eichner *et al.*, 2017). Future studies should focus on understanding the precise mechanisms of OMV-associated serine proteases that enteric pathogens including *C. jejuni* use to interact with and cleave/modify occludin.

The ER chaperone GRP78/BiP protein is highly conserved and essential for the survival of eukaryotic cells. GRP78/BiP also maintains the permeability barrier of the ER membrane and plays a crucial role in the unfolded-protein response (UPR) as the ER stress-signalling master regulator. For the first time *C. jejuni* OMVs have also been shown to cleave the recombinant ER chaperone GRP78/BiP, a key cellular target for AB_5 toxins (Paton *et al.*, 2006, Beddoe *et al.*, 2010, Paton *et al.*, 2010). This activity was only reduced in OMVs isolated from a *Cj1365c* mutant, suggesting that it is this protease responsible for the cleavage of GRP78/BiP. Cj1365c is a multi-domain protein, containing an autotransporter domain as well as a subtilisin-like serine domain. Serine proteases belonging to the family of subtilisin-like proteases are secreted by a wide variety of bacterial pathogens and have been shown to cleave host proteins including immunoglobulins, complement compounds and proteins of the extracellular matrix (Male, 1979, Juarez *et al.*, 1999, Mortensen *et al.*, 2011). Considering previous studies have suggested that the expression of CDT is not directly linked to *C. jejuni* disease severity (Abuoun *et al.*, 2005, Mortensen *et al.*, 2011), this observation may also highlight the importance of the Cj1365c protease in *C. jejuni* pathogenesis. The Cj1365c protease has previously been suggested to be one of the *C. jejuni* virulence factors associated with the development of bloody diarrhoea, independent of pVir plasmid (Louwen *et al.*, 2006). The novel AB_5 toxin that cleaves GRP78/BiP also occurs in the loss of enterocyte effacement negative STEC O113:H21 outbreak strain (Newton *et al.*, 2009).

In summary, ST is important not only for the up-regulation of the genes encoding HtrA, Cj0511, Cj1365c and CDT *in vitro*, but also for the modulation of *C. jejuni* OMV secretion, content and function. Our findings and other recent reports also highlight the importance of ST in inducing bacterial virulence. Enhanced *C. jejuni* vesiculisation induced by ST can also be linked to enhanced cytotoxicity to both IECs and *Galleria mellonella* larvae as well as enhanced immunogenicity and proteolytic activity. This data suggests that *C. jejuni* responds to the presence of the bile salt ST by increasing OMV production and changing the protein content of OMVs to enhance pathogenesis.

Acknowledgements

We would like to acknowledge Neveda Naz and Emma Taylor for technical assistance.

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). *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% N_2, 10% CO_2 and 5% O_2 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 and Quantification 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 OD_{600} of 0.1. Cultures were grown for 12 h ($OD_{600} \approx 1.2$) then centrifuged at 4,000 x *g* for 30 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 performed at 4°C and the resulting OMVs pellet was resuspended in phosphate buffered saline (PBS) and stored at -20°C. OMVs samples were plated out on blood agar plates and incubated under both microaerobic and aerobic conditions for 72 h to confirm the absence of viable bacteria. The protein concentration of the OMVs was quantified using a bicinchoninic-acid assay (BCA Protein Assay; Thermo Fisher Scientific, UK) using BSA as the protein standard. 10 µg of each OMVs sample, based on protein concentration, was analysed using SDS-PAGE and stained using Silver stain Reagent (Thermo Fisher Scientific). The LOS profiles of the OMVs were examined using standard methods (Naito *et al.*, 2010). The OMVs samples were separated on 12% (w/v) sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gels and then visualised using silver staining.

Further quantification of OMVs

Further OMV quantification was performed as described previously (Lee *et al.*, 1999) by measuring keto-deoxy-d-manno-8-octanoic acid (Kdo) content. Keto-deoxy-d-manno-8-octanoic acid from *E. coli* LPS (Sigma-Aldrich) was used as the standard. For the quantification of the lipid content of OMVs, the fluorescent lipophilic dye FM4-64 (Thermo Fisher Scientific) was used to a final concentration of 5 µg/ml (McBroom *et al.*, 2006). OMVs alone and the FM4-64 probe alone were used as negative controls.

Mass spectrometry analysis

Proteins from three separate *C. jejuni* OMVs preparations were resolved on SDS-PAGE gels then submitted to the Moredun Proteomics Facility for analysis. Each gel lane was excised and sliced horizontally from top to bottom to yield equal gel slices 2.5 mm deep. Each of the resulting gel slices was then subjected to standard in-gel destaining, reduction, alkylation and trypsinolysis procedures (Shevchenko *et al.*, 1996). Digests were transferred to low-protein-binding HPLC sample vials immediately prior to liquid chromatography-electrospray ionisation-tandem mass spectrometry (LC-ESI-MS/MS) analysis. Liquid chromatography was performed using a Dionex Ultimate 3000 nano-HPLC system (Thermo Fisher Scientific) comprising a WPS-3000 well-plate micro auto sampler, a FLM-3000 flow manager and column compartment, a UVD-3000 UV detector, a LPG-3600 dual-gradient micropump and a SRD-3600 solvent rack controlled by Chromeleon™ chromatography software (www.thermoscientific.com/dionex). A micro-pump flow rate of 246 µl/min was used in combination with a cap-flow splitter cartridge, affording a $^{1}/_{82}$ flow split and a final flow rate of 3 µl/min through a 5 cm x 200 µm ID monolithic reversed phase column (Thermo Fisher Scientific) maintained at 50°C. Samples of 4 µl were applied to the column by direct injection. Peptides were eluted by the application of a 15 min linear gradient from 8-45% solvent B (80% acetonitrile (Rathburn Chemicals, UK), 0.1% v/v formic acid) and directed through a 3 nl UV detector flow cell. LC was interfaced directly with a 3-D high capacity ion trap mass spectrometer (amaZon-ETD, Bruker Daltonics, Germany) via a low-volume (50 µl/min maximum) stainless steel nebuliser (Agilent Technologies, UK / G1946-20260) and ESI. Parameters for tandem MS analysis were based on those described previously (Batycka *et al.*, 2006).

Database mining

Deconvoluted MS/MS data in mgf (Mascot Generic Format) format was imported into ProteinScape™ V3.1 (Bruker Daltonics) proteomics data analysis software for downstream mining of databases utilising the Mascot™ V2.4.1 (Matrix Science, UK) search algorithm. The protein content of each individual gel slice was established using the “Protein Search” feature of ProteinScape™, whilst separate compilations of the proteins contained in all gel slices for each sample were produced using the “Protein Extractor” feature of the software. Mascot search parameters were set in accordance with published guidelines (Taylor *et al.*, 2005) using fixed (carbamidomethyl “C”) and variable (oxidation “M” and deamidation “N,Q”) modifications along with peptide (MS) and secondary fragmentation (MS/MS) tolerance values of 0.5 Da and allowing for a single ^{13}C isotope. Molecular weight search (MOWSE) scores attained for individual protein identifications were inspected manually and considered significant only if a) two peptides were matched for each protein, with each matched peptide containing an unbroken "*b*" or "*y*" ion series represented by of a minimum of four contiguous amino acid residues or b) one peptide was matched for each protein, containing an unbroken "*b*" or "*y*" ion series represented by of a minimum of eight contiguous amino acid residues.

Files were searched with MASCOT software against *C. jejuni* NCTC 11168 protein databases derived from genomic sequence available at NCBI (Genbank, AL111168) (http://www.ncbi.nlm.nih.gov/).

The NCTC 11168 annotated genome sequence indicates Clusters of Orthologous Group (COG) assignments (Tatusov *et al.*, 1997) which were used to predict functional classification. Additionally the Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1990), InterProScan (http://www.ebi.ac.uk/interpro/search/sequence-search) and The Koyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg/) (Kanehisa *et al.*, 2000) were used to investigate protein function.

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 as described previously (Elmi *et al.*, 2016). OMVs (10 µg in 10 µl, based on protein concentration) from *C. jejuni* cultured in Brucella broth in the presence or absence of 0.1% or 0.2% (w/v) ST 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.

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% (v/v) non-essential amino acids and 1% (v/v) penicillin-streptomycin (Sigma-Aldrich) at 37°C in a 5% CO_2 humidified atmosphere. Cells were split around 80-90% confluence and seeded at 5 x 10^6 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 T84 cells with OMVs isolated from *C. jejuni* cultured in Brucella broth in the presence or absence of 0.1% or 0.2% (w/v) ST. Briefly, T84 cells were challenged with 100 μg OMVs based on protein concentration. 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) for IL-8 quantitation

T84 cells were co-cultured with 100 µg or 10 µg of OMVs based on protein concentration isolated from *C. jejuni* cultured in Brucella broth in the presence or absence of 0.1% or 0.2% (w/v) ST 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).

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 OMVs based on protein concentration isolated from *C. jejuni* cultured in Brucella broth in the presence or absence of 0.1% or 0.2% (w/v) ST, 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 using larvae of the same approximate weight. 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.

Quantitative real-time polymerase chain reaction (qRT-PCR).

For measurements of gene expression, total RNA from either *C. jejuni* cultured in Brucella broth in the presence or absence of 0.1% or 0.2% (w/v) ST or T84 cells was extracted with Qiagen RNAeasy mini kit (Life Technologies). DNA contamination was removed with DNA-free treatment (Ambion). The purified RNA was quantified using a Nanodrop machine

(NanoDrop Technologies, UK). Complementary DNA (cDNA) was synthesised with a Superscript III first-strand synthesis system (Life Technologies) according to the manufacturer's protocol. Briefly, 2 µg total RNA was used for the reverse-transcription reaction mixture (Fisher Scientific) using either random hexamers or oligo(dT) primers. Quantitative real-time PCR reactions were performed in triplicate using SYBR green master mix (Applied Biosystems) with ABI7500 machine (Applied Biosystems). Relative gene expression comparisons were performed using ΔΔCT method (Schmittgen *et al.*, 2008), (where CT is threshold cycle) normalising the mean cycle threshold of each gene to the *gyrA* gene, which is considered a stably expressed housekeeping gene (Ritz *et al.*, 2009). Primer efficiencies were tested with genomic DNA dilution series and primer sequences are listed in Table 1.

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

The cleavage of recombinant TJ or AJ proteins (occludin or E-cadherin respectively) by OMVs was determined as described previously (Baek *et al.*, 2011, Elmi *et al.*, 2016). Briefly, OMVs (10 µg based on protein concentration) from *C. jejuni* cultured in Brucella broth in the presence or absence of 0.1% or 0.2% (w/v) ST were incubated with 1 µg recombinant human occludin or E-cadherin (R&D Systems, UK) in PBS at 37°C for 16 h. For analysis of occludin or E-cadherin 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 12% (w/v) Bis/Tris precast gels (Invitrogen) then transferred to nitrocellulose using an iBlot gel transfer device (Life Technologies). 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 rabbit anti-occludin or primary mouse anti-E-Cadherin or (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).

in vitro **cleavage of GRP78/BiP**

C. jejuni OMVs cleavage of GRP78/BiP was performed as previously described (Nagasawa *et al.*, 2014). Briefly, 20 μg of OMVs based on protein concentration were incubated with PBS buffer with GRP78/BiP (Fisher Scientific) in a final volume of 25 μl at 37°C for 16 h. The reactions were stopped by addition of 2X sample loading buffer. The samples were analysed by SDS-PAGE and western blot as described above using GRP78/BiP primary antibody.

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 ± 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.

TABLES

Table 1. qRT-PCR primers used in this study.

Gene name	Sequence 5’-3’	Melting Temp (°C)	Source
Cj1365cF	GGTGTAGCCGATGATGCTTT	55.4	This study
Cj1365cR	GCCCCATAAGCCACTCCATA	56	This study
Cj0511F	TGGTGGAAGTGCTAGTGCAA	55.7	This study
Cj0511R	GGTTTTACACCCACTGCTTG	54.2	This study
Cj1228cF	TGATTGATGGTTTGAGTTTGAGAA	52.9	This study
Cj1228cR	TTCACTTTGTCCAACACCTATG	53	This study
cdtAF	TAGCGGTGCTGATTTAGTACCT	55.6	This study
cdtAR	CATCGCCAAATCCTTTGCTATCG	56.6	This study
cdtBF	GAACAGCCACTCCAACAGGACG	60.3	This study
cdtBR	CGATTAGCTCCTACATCAACGCGA	58.6	This study
cdtCF	GCCTTTGCAACTCCTACTGGAGAT	56.9	This study
cdtCR	GCTCCAAAGGTTCCATCTTCTAAG	55.5	This study
gryAF	CCCACTTGCTAAAGTGCGTGA	54.6	This study
gyrAR	CCTTACCACCTCTGCTTTGC	55.5	This study

FIGURE LEGENDS

Figure 1. ST does not affect the growth or outer membrane integrity of *C. jejuni* 11168H wild-type strain.

Growth curves of *C. jejuni* 11168H cultured in Brucella broth without ST or Brucella broth supplemented with 0.1% (w/v) ST or 0.2% ST (w/v) quantified by **(A)** OD_{600} or **(B)** Colony Forming Units (CFU). **(C)** Quantitative analysis of *C. jejuni* 11168H stained with LIVE/DEAD BacLight for the purpose of evaluating outer membrane integrity phenotype. **(D)** Representative fluorescence confocal images showing the relative live/dead cells of *C. jejuni* 11168H cultured without ST or with 0.1% (w/v) or 0.2% (w/v) ST. Cells were stained with LIVE/DEAD BacLight (Life Technologies) (green = viable cells, red = dead cells). Scale bar, 10 μm.

Figure 2. ST increases the protein concentration of OMVs from *C. jejuni* 11168H wild-type strain.

Quantification of the increase in OMVs isolated from *C. jejuni* 11168H in Brucella broth supplemented with 0.1% (w/v) or 0.2% (w/v) ST compared to without ST. The OD_{600} of ST treated 11168H cultures were normalised to the OD_{600} of untreated 11168H cultures for each OMV isolation. **(A)** BCA assay to determine protein concentration of OMVs. *, $P < 0.05$; ****, $P < 0.0001$. **(B)** Relative fold increase of OMVs determined using FM4-64 dye. *, $P < 0.05$; ****, $P < 0.0001$. **(C)** Relative fold increase of OMVs determined using 3-deoxy-d-*manno*-octulosonic acid (Kdo). ****, $P < 0.0001$. **(D)** LOS levels associated with OMVs as indicated by silver staining following separation by SDS-PAGE.

Figure 3. Proteomic analysis of ST-OMVs.

Major protein functional categories (COGs) identified in *C. jejuni* 11168H ST-OMVs following proteomic analysis.

Figure 4. Increased proteolytic activity of ST-OMVs.

Quantification of proteolytic activity of OMVs isolated from 11168H cultured without ST or with 0.1% (w/v) or 0.2% (w/v) ST. FITC labelled casein was incubated with OMVs for 24 hr and OMV proteolytic activity assessed. **, $P < 0.01$; ****, $P < 0.0001$.

Figure 5. Increased cytotoxic activity and immunogenicity of ST-OMVs.

(A) Cytotoxic effect of OMVs isolated from 11168H in Brucella broth without ST or supplemented with 0.1% (w/v) or 0.2% (w/v) ST on T84 IECs after 24 hr co-incubation. The cytotoxic effect on the T84 cells was measured by quantifying 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). ***, $P < 0.001$. **(B)** Immunogenic effect of OMVs isolated from 11168H in Brucella broth without ST or supplemented with 0.1% (w/v) or 0.2% (w/v) ST on T84 IECs after 24 hr co-incubation. Levels of IL-8 secreted during *C. jejuni* OMV interactions with T84 cells were quantified using a human IL-8 ELISA. **(C)** Relative transcript levels of IL-8 in T84 cells co-incubated with OMVs from 11168H cultured without ST or supplemented with 0.1% (w/v) or 0.2% (w/v) ST. Relative transcript levels of GAPDH was used as an internal control.

Figure 6. ST enhances the cytotoxicity of *C. jejuni* OMVs in the *Galleria mellonella* infection model.

G. mellonella larvae were injected with a 10 µl inoculum of OMVs (5 µg) from 11168H cultured in either Brucella broth without ST or supplemented with 0.1% (w/v) or 0.2% (w/v) ST. Larvae were incubated at 37°C, with survival and appearance recorded every 24 h. PBS and no-injection controls were used. For each experiment, 10 *G. mellonella* larvae were infected and experiments were repeated in triplicate. *, $P < 0.05$.

Figure 7. qRT-PCR analysis of transcription in *C. jejuni* 11168H wild-type strain co-incubated with ST.

Quantitative real-time-PCR (qRT-PCR) analysis was performed using *cdtA*, *cdtB*, *cdtC*, *Cj0511*, *Cj1365c* and *htrA* transcript-specific primers. *gyrA* mRNA was used as an internal control. **(A)** The relative expression of *cdtA*, *cdtB*, *cdtC*. **(B)** The relative expression of *Cj0511*, *Cj1365c* and *htrA*. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Figure 8. Increased cleavage by ST-OMVs of Occludin and E-cadherin.

Recombinant Occludin or E-Cadherin were incubated with 10 µg of OMVs isolated from 11168H cultured in Brucella broth without ST or supplemented with 0.1% (w/v) or 0.2% (w/v) ST. Reaction mixtures were stopped and aliquots separated by SDS-PAGE and immunoblotted with antibodies against **(A)** Occludin or **(B)** E-Cadherin. Arrows indicate putative cleaved band. The negative control with phosphate buffered saline showed no detectable cleavage product.

Figure 9. Increased cleavage by ST-OMVs of GRP78/BiP.

(A) Recombinant GRP78 was incubated with 20 µg of OMVs isolated from 11168H cultured in Brucella broth without ST or supplemented with 0.1% (w/v) or 0.2% (w/v) ST. Reaction mixtures were stopped, aliquots separated by SDS-PAGE and immunoblotted with a GRP78 antibody. Arrows indicate putative cleaved band. The negative control with phosphate buffered saline showed no detectable cleavage product. **(B)** Recombinant GRP78 was incubated with 20 µg of OMVs isolated from either *htrA*, *Cj0511* or *Cj1365c* mutants cultured in Brucella broth and compared to 11168H OMVs as above.

REFERENCES

Abuoun, M., Manning, G., Cawthraw, S.A., Ridley, A., Ahmed, I.H., Wassenaar, T.M. and Newell, D.G. (2005). Cytolethal distending toxin (CDT)-negative Campylobacter jejuni strains and anti-CDT neutralizing antibodies are induced during human infection but not during colonization in chickens. *Infect Immun* **73,** 3053-3062.

Alam, A., Tam, V., Hamilton, E. and Dziejman, M. (2010). vttRA and vttRB Encode ToxR family proteins that mediate bile-induced expression of type three secretion system genes in a non-O1/non-O139 Vibrio cholerae strain. *Infect Immun* **78,** 2554-2570.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. (1990). Basic local alignment search tool. *J Mol Biol* **215,** 403-410.

Ashgar, S.S., Oldfield, N.J., Wooldridge, K.G., Jones, M.A., Irving, G.J., Turner, D.P. and Ala'Aldeen, D.A. (2007). CapA, an autotransporter protein of Campylobacter jejuni, mediates association with human epithelial cells and colonization of the chicken gut. *J Bacteriol* **189,** 1856-1865.

Awad, W.A., Hess, C. and Hess, M. (2017). Enteric Pathogens and Their Toxin-Induced Disruption of the Intestinal Barrier through Alteration of Tight Junctions in Chickens. *Toxins (Basel)* **9**.

Bachmann, V., Kostiuk, B., Unterweger, D., Diaz-Satizabal, L., Ogg, S. and Pukatzki, S. (2015). Bile Salts Modulate the Mucin-Activated Type VI Secretion System of Pandemic Vibrio cholerae. *PLoS Negl Trop Dis* **9,** e0004031.

Bacon, D.J., Szymanski, C.M., Burr, D.H., Silver, R.P., Alm, R.A. and Guerry, P. (2001). A phase-variable capsule is involved in virulence of Campylobacter jejuni 81-176. *Mol Microbiol* **40,** 769-777.

Baek, K.T., Vegge, C.S. and Brondsted, L. (2011). HtrA chaperone activity contributes to host cell binding in *Campylobacter jejuni*. *Gut Pathog* **3,** 13.

Barrero-Tobon, A.M. and Hendrixson, D.R. (2014). Flagellar biosynthesis exerts temporal regulation of secretion of specific Campylobacter jejuni colonization and virulence determinants. *Mol Microbiol* **93,** 957-974.

Batycka, M., Inglis, N.F., Cook, K., Adam, A., Fraser-Pitt, D., Smith, D.G*., et al.* (2006). Ultra-fast tandem mass spectrometry scanning combined with monolithic column liquid chromatography increases throughput in proteomic analysis. *Rapid Commun Mass Spectrom* **20,** 2074-2080.

Beddoe, T., Paton, A.W., Le Nours, J., Rossjohn, J. and Paton, J.C. (2010). Structure, biological functions and applications of the AB5 toxins. *Trends Biochem Sci* **35,** 411-418.

Begley, M., Gahan, C.G. and Hill, C. (2005). The interaction between bacteria and bile. *FEMS Microbiol Rev* **29,** 625-651.

Bertelsen, L.S., Paesold, G., Marcus, S.L., Finlay, B.B., Eckmann, L. and Barrett, K.E. (2004). Modulation of chloride secretory responses and barrier function of intestinal epithelial cells by the Salmonella effector protein SigD. *Am J Physiol Cell Physiol* **287,** C939-948.

Biswas, D., Itoh, K. and Sasakawa, C. (2003). Role of microfilaments and microtubules in the invasion of INT-407 cells by Campylobacter jejuni. *Microbiol Immunol* **47,** 469-473.

Boyer, J.L. (2013). Bile formation and secretion. *Compr Physiol* **3,** 1035-1078.

Brade, H. and Rietschel, E.T. (1984). Alpha-2----4-interlinked 3-deoxy-D-manno-octulosonic acid disaccharide. A common constituent of enterobacterial lipopolysaccharides. *Eur J Biochem* **145,** 231-236.

Brondsted, L., Andersen, M.T., Parker, M., Jorgensen, K. and Ingmer, H. (2005). The HtrA protease of Campylobacter jejuni is required for heat and oxygen tolerance and for optimal interaction with human epithelial cells. *Appl Environ Microbiol* **71,** 3205-3212.

Buelow, D.R., Christensen, J.E., Neal-McKinney, J.M. and Konkel, M.E. (2011). Campylobacter jejuni survival within human epithelial cells is enhanced by the secreted protein CiaI. *Mol Microbiol* **80,** 1296-1312.

Chaand, M. and Dziejman, M. (2013). Vibrio cholerae VttR(A) and VttR(B) regulatory influences extend beyond the type 3 secretion system genomic island. *J Bacteriol* **195,** 2424-2436.

Champion, O.L., Karlyshev, A.V., Senior, N.J., Woodward, M., La Ragione, R., Howard, S.L*., et al.* (2010). Insect infection model for *Campylobacter jejuni* reveals that *O*-methyl phosphoramidate has insecticidal activity. *J Infect Dis* **201,** 776-782.

Chen, M.L., Ge, Z., Fox, J.G. and Schauer, D.B. (2006). Disruption of tight junctions and induction of proinflammatory cytokine responses in colonic epithelial cells by Campylobacter jejuni. *Infect Immun* **74,** 6581-6589.

Choi, C.W., Park, E.C., Yun, S.H., Lee, S.Y., Lee, Y.G., Hong, Y*., et al.* (2014). Proteomic characterization of the outer membrane vesicle of Pseudomonas putida KT2440. *J Proteome Res* **13,** 4298-4309.

Drion, S., Wahlen, C. and Taziaux, P. (1988). Isolation of Campylobacter jejuni from the bile of a cholecystic patient. *J Clin Microbiol* **26,** 2193-2194.

Eichner, M., Protze, J., Piontek, A., Krause, G. and Piontek, J. (2017). Targeting and alteration of tight junctions by bacteria and their virulence factors such as Clostridium perfringens enterotoxin. *Pflugers Arch* **469,** 77-90.

Elmi, A., Nasher, F., Jagatia, H., Gundogdu, O., Bajaj-Elliott, M., Wren, B. and Dorrell, N. (2016). Campylobacter jejuni outer membrane vesicle-associated proteolytic activity promotes bacterial invasion by mediating cleavage of intestinal epithelial cell E-cadherin and occludin. *Cell Microbiol* **18,** 561-572.

Elmi, A., Watson, E., Sandu, P., Gundogdu, O., Mills, D.C., Inglis, N.F*., et al.* (2012). Campylobacter jejuni outer membrane vesicles play an important role in bacterial interactions with human intestinal epithelial cells. *Infect Immun* **80,** 4089-4098.

Elwell, C., Chao, K., Patel, K. and Dreyfus, L. (2001). Escherichia coli CdtB mediates cytolethal distending toxin cell cycle arrest. *Infect Immun* **69,** 3418-3422.

Faherty, C.S., Redman, J.C., Rasko, D.A., Barry, E.M. and Nataro, J.P. (2012). Shigella flexneri effectors OspE1 and OspE2 mediate induced adherence to the colonic epithelium following bile salts exposure. *Mol Microbiol* **85,** 107-121.

Fang, F.C., Frawley, E.R., Tapscott, T. and Vazquez-Torres, A. (2016a). Bacterial Stress Responses during Host Infection. *Cell Host Microbe* **20,** 133-143.

Fang, F.C., Frawley, E.R., Tapscott, T. and Vazquez-Torres, A. (2016b). Discrimination and Integration of Stress Signals by Pathogenic Bacteria. *Cell Host Microbe* **20,** 144-153.

Finlay, B.B. and Cossart, P. (1997). Exploitation of mammalian host cell functions by bacterial pathogens. *Science* **276,** 718-725.

Garidel, P., Hildebrand, A., Knauf, K. and Blume, A. (2007). Membranolytic activity of bile salts: influence of biological membrane properties and composition. *Molecules* **12,** 2292-2326.

Gotoh, K., Kodama, T., Hiyoshi, H., Izutsu, K., Park, K.S., Dryselius, R*., et al.* (2010). Bile acid-induced virulence gene expression of Vibrio parahaemolyticus reveals a novel therapeutic potential for bile acid sequestrants. *PLoS One* **5,** e13365.

Gundogdu, O., Mills, D.C., Elmi, A., Martin, M.J., Wren, B.W. and Dorrell, N. (2011). The *Campylobacter jejuni* transcriptional regulator Cj1556 plays a role in the oxidative and aerobic stress response and is important for bacterial survival *in vivo*. *J Bacteriol* **193,** 4238-4249.

Guo, B., Wang, Y., Shi, F., Barton, Y.W., Plummer, P., Reynolds, D.L.*, et al.* (2008). CmeR functions as a pleiotropic regulator and is required for optimal colonization of Campylobacter jejuni in vivo. *J Bacteriol* **190,** 1879-1890.

Guttman, J.A. and Finlay, B.B. (2008). Subcellular alterations that lead to diarrhea during bacterial pathogenesis. *Trends Microbiol* **16,** 535-542.

Hamner, S., McInnerney, K., Williamson, K., Franklin, M.J. and Ford, T.E. (2013). Bile salts affect expression of Escherichia coli O157:H7 genes for virulence and iron acquisition, and promote growth under iron limiting conditions. *PLoS One* **8,** e74647.

Hofmann, A.F. and Hagey, L.R. (2008). Bile acids: chemistry, pathochemistry, biology, pathobiology, and therapeutics. *Cell Mol Life Sci* **65,** 2461-2483.

Hofmann, A.F. and Small, D.M. (1967). Detergent properties of bile salts: correlation with physiological function. *Annu Rev Med* **18,** 333-376.

Johnson, W.M. and Lior, H. (1988). A new heat-labile cytolethal distending toxin (CLDT) produced by Campylobacter spp. *Microb Pathog* **4,** 115-126.

Jones, M.A., Marston, K.L., Woodall, C.A., Maskell, D.J., Linton, D., Karlyshev, A.V.*, et al.* (2004). Adaptation of *Campylobacter jejuni* NCTC11168 to high-level colonization of the avian gastrointestinal tract. *Infection and Immunity* **72,** 3769-3776.

Jowiya, W., Brunner, K., Abouelhadid, S., Hussain, H.A., Nair, S.P., Sadiq, S.*, et al.* (2015). Pancreatic amylase is an environmental signal for regulation of biofilm formation and host interaction in Campylobacter jejuni. *Infect Immun* **83,** 4884-4895.

Joyce, S.A. and Gahan, C.G. (2016). Bile Acid Modifications at the Microbe-Host Interface: Potential for Nutraceutical and Pharmaceutical Interventions in Host Health. *Annu Rev Food Sci Technol* **7,** 313-333.

Juarez, Z.E. and Stinson, M.W. (1999). An extracellular protease of Streptococcus gordonii hydrolyzes type IV collagen and collagen analogues. *Infect Immun* **67,** 271-278.

Kaakoush, N.O., Castano-Rodriguez, N., Mitchell, H.M. and Man, S.M. (2015). Global Epidemiology of Campylobacter Infection. *Clin Microbiol Rev* **28,** 687-720.

Kanehisa, M. and Goto, S. (2000). KEGG: kyoto encyclopedia of genes and genomes. *Nucleic Acids Res* **28,** 27-30.

Kaparakis-Liaskos, M. and Ferrero, R.L. (2015). Immune modulation by bacterial outer membrane vesicles. *Nat Rev Immunol* **15,** 375-387.

Karlyshev, A.V., Thacker, G., Jones, M.A., Clements, M.O. and Wren, B.W. (2014). Campylobacter jejuni gene cj0511 encodes a serine peptidase essential for colonisation. *FEBS Open Bio* **4,** 468-472.

Kulkarni, H.M. and Jagannadham, M.V. (2014). Biogenesis and multifaceted roles of outer membrane vesicles from Gram-negative bacteria. *Microbiology* **160,** 2109-2121.

Lara-Tejero, M. and Galan, J.E. (2001). CdtA, CdtB, and CdtC form a tripartite complex that is required for cytolethal distending toxin activity. *Infect Immun* **69,** 4358-4365.

Lee, C.H. and Tsai, C.M. (1999). Quantification of bacterial lipopolysaccharides by the purpald assay: measuring formaldehyde generated from 2-keto-3-deoxyoctonate and heptose at the inner core by periodate oxidation. *Anal Biochem* **267,** 161-168.

Li, P., Rivera-Cancel, G., Kinch, L.N., Salomon, D., Tomchick, D.R., Grishin, N.V. and Orth, K. (2016). Bile salt receptor complex activates a pathogenic type III secretion system. *Elife* **5**.

Lim, H.N., Lee, Y. and Hussein, R. (2011). Fundamental relationship between operon organization and gene expression. *Proc Natl Acad Sci U S A* **108,** 10626-10631.

Lindmark, B., Rompikuntal, P.K., Vaitkevicius, K., Song, T., Mizunoe, Y., Uhlin, B.E.*, et al.* (2009). Outer membrane vesicle-mediated release of cytolethal distending toxin (CDT) from Campylobacter jejuni. *BMC Microbiol* **9,** 220.

Louwen, R.P., van Belkum, A., Wagenaar, J.A., Doorduyn, Y., Achterberg, R. and Endtz, H.P. (2006). Lack of association between the presence of the pVir plasmid and bloody diarrhea in Campylobacter jejuni enteritis. *J Clin Microbiol* **44,** 1867-1868.

MacCallum, A., Hardy, S.P. and Everest, P.H. (2005). Campylobacter jejuni inhibits the absorptive transport functions of Caco-2 cells and disrupts cellular tight junctions. *Microbiology* **151,** 2451-2458.

Macdonald, I.A. and Kuehn, M.J. (2013). Stress-induced outer membrane vesicle production by Pseudomonas aeruginosa. *J Bacteriol* **195,** 2971-2981.

Maldonado-Valderrama, J., Wilde, P., Macierzanka, A. and Mackie, A. (2011). The role of bile salts in digestion. *Adv Colloid Interface Sci* **165,** 36-46.

Male, C.J. (1979). Immunoglobulin A1 protease production by Haemophilus influenzae and Streptococcus pneumoniae. *Infect Immun* **26,** 254-261.

Malik-Kale, P., Parker, C.T. and Konkel, M.E. (2008). Culture of Campylobacter jejuni with sodium deoxycholate induces virulence gene expression. *J Bacteriol* **190,** 2286-2297.

Maue, A.C., Mohawk, K.L., Giles, D.K., Poly, F., Ewing, C.P., Jiao, Y.*, et al.* (2013). The polysaccharide capsule of Campylobacter jejuni modulates the host immune response. *Infect Immun* **81,** 665-672.

McBroom, A.J., Johnson, A.P., Vemulapalli, S. and Kuehn, M.J. (2006). Outer membrane vesicle production by Escherichia coli is independent of membrane instability. *J Bacteriol* **188,** 5385-5392.

McBroom, A.J. and Kuehn, M.J. (2007). Release of outer membrane vesicles by Gram-negative bacteria is a novel envelope stress response. *Mol Microbiol* **63,** 545-558.

McNamara, B.P., Koutsouris, A., O'Connell, C.B., Nougayrede, J.P., Donnenberg, M.S. and Hecht, G. (2001). Translocated EspF protein from enteropathogenic Escherichia coli disrupts host intestinal barrier function. *J Clin Invest* **107,** 621-629.

Merritt, M.E. and Donaldson, J.R. (2009). Effect of bile salts on the DNA and membrane integrity of enteric bacteria. *J Med Microbiol* **58,** 1533-1541.

Mertins, S., Allan, B.J., Townsend, H.G., Koster, W. and Potter, A.A. (2013). Role of motAB in adherence and internalization in polarized Caco-2 cells and in cecal colonization of Campylobacter jejuni. *Avian Dis* **57,** 116-122.

Metruccio, M.M., Evans, D.J., Gabriel, M.M., Kadurugamuwa, J.L. and Fleiszig, S.M. (2016). Pseudomonas aeruginosa Outer Membrane Vesicles Triggered by Human Mucosal Fluid and Lysozyme Can Prime Host Tissue Surfaces for Bacterial Adhesion. *Front Microbiol* **7,** 871.

Monteville, M.R., Yoon, J.E. and Konkel, M.E. (2003). Maximal adherence and invasion of INT 407 cells by Campylobacter jejuni requires the CadF outer-membrane protein and microfilament reorganization. *Microbiology* **149,** 153-165.

Mortensen, N.P., Schiellerup, P., Boisen, N., Klein, B.M., Locht, H., Abuoun, M.*, et al.* (2011). The role of Campylobacter jejuni cytolethal distending toxin in gastroenteritis: toxin detection, antibody production, and clinical outcome. *APMIS* **119,** 626-634.

Nagasawa, S., Ogura, K., Tsutsuki, H., Saitoh, H., Moss, J., Iwase, H.*, et al.* (2014). Uptake of Shiga-toxigenic Escherichia coli SubAB by HeLa cells requires an actin- and lipid raft-dependent pathway. *Cell Microbiol* **16,** 1582-1601.

Naito, M., Frirdich, E., Fields, J.A., Pryjma, M., Li, J., Cameron, A.*, et al.* (2010). Effects of sequential Campylobacter jejuni 81-176 lipooligosaccharide core truncations on biofilm formation, stress survival, and pathogenesis. *J Bacteriol* **192,** 2182-2192.

Newton, H.J., Sloan, J., Bulach, D.M., Seemann, T., Allison, C.C., Tauschek, M.*, et al.* (2009). Shiga toxin-producing Escherichia coli strains negative for locus of enterocyte effacement. *Emerg Infect Dis* **15,** 372-380.

Olive, A.J. and Sassetti, C.M. (2016). Metabolic crosstalk between host and pathogen: sensing, adapting and competing. *Nat Rev Microbiol* **14,** 221-234.

Pathirana, R.D. and Kaparakis-Liaskos, M. (2016). Bacterial membrane vesicles: Biogenesis, immune regulation and pathogenesis. *Cell Microbiol* **18,** 1518-1524.

Paton, A.W., Beddoe, T., Thorpe, C.M., Whisstock, J.C., Wilce, M.C., Rossjohn, J.*, et al.* (2006). AB5 subtilase cytotoxin inactivates the endoplasmic reticulum chaperone BiP. *Nature* **443,** 548-552.

Paton, A.W. and Paton, J.C. (2010). Escherichia coli Subtilase Cytotoxin. *Toxins (Basel)* **2,** 215-228.

Pickett, C.L., Pesci, E.C., Cottle, D.L., Russell, G., Erdem, A.N. and Zeytin, H. (1996). Prevalence of cytolethal distending toxin production in Campylobacter jejuni and relatedness of Campylobacter sp. cdtB gene. *Infect Immun* **64,** 2070-2078.

Purdy, D., Buswell, C.M., Hodgson, A.E., McAlpine, K., Henderson, I. and Leach, S.A. (2000). Characterisation of cytolethal distending toxin (CDT) mutants of Campylobacter jejuni. *J Med Microbiol* **49,** 473-479.

Raphael, B.H., Pereira, S., Flom, G.A., Zhang, Q., Ketley, J.M. and Konkel, M.E. (2005). The Campylobacter jejuni response regulator, CbrR, modulates sodium deoxycholate resistance and chicken colonization. *J Bacteriol* **187,** 3662-3670.

Ritz, M., Garenaux, A., Berge, M. and Federighi, M. (2009). Determination of rpoA as the most suitable internal control to study stress response in C. jejuni by RT-qPCR and application to oxidative stress. *J Microbiol Methods* **76,** 196-200.

Roier, S., Zingl, F.G., Cakar, F., Durakovic, S., Kohl, P., Eichmann, T.O.*, et al.* (2016a). A novel mechanism for the biogenesis of outer membrane vesicles in Gram-negative bacteria. *Nat Commun* **7,** 10515.

Roier, S., Zingl, F.G., Cakar, F. and Schild, S. (2016b). Bacterial outer membrane vesicle biogenesis: a new mechanism and its implications. *Microb Cell* **3,** 257-259.

Sakakibara, A., Furuse, M., Saitou, M., Ando-Akatsuka, Y. and Tsukita, S. (1997). Possible involvement of phosphorylation of occludin in tight junction formation. *J Cell Biol* **137,** 1393-1401.

Sanchez, L.M., Cheng, A.T., Warner, C.J., Townsley, L., Peach, K.C., Navarro, G.*, et al.* (2016). Biofilm Formation and Detachment in Gram-Negative Pathogens Is Modulated by Select Bile Acids. *PLoS One* **11,** e0149603.

Schertzer, J.W. and Whiteley, M. (2013). Bacterial outer membrane vesicles in trafficking, communication and the host-pathogen interaction. *J Mol Microbiol Biotechnol* **23,** 118-130.

Schmittgen, T.D. and Livak, K.J. (2008). Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc* **3,** 1101-1108.

Schwechheimer, C. and Kuehn, M.J. (2013a). Synthetic effect between envelope stress and lack of outer membrane vesicle production in Escherichia coli. *J Bacteriol* **195,** 4161-4173.

Schwechheimer, C. and Kuehn, M.J. (2015). Outer-membrane vesicles from Gram-negative bacteria: biogenesis and functions. *Nat Rev Microbiol* **13,** 605-619.

Schwechheimer, C., Kulp, A. and Kuehn, M.J. (2014). Modulation of bacterial outer membrane vesicle production by envelope structure and content. *BMC Microbiol* **14,** 324.

Schwechheimer, C., Sullivan, C.J. and Kuehn, M.J. (2013b). Envelope control of outer membrane vesicle production in Gram-negative bacteria. *Biochemistry* **52,** 3031-3040.

Shevchenko, A., Wilm, M., Vorm, O. and Mann, M. (1996). Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Anal Chem* **68,** 850-858.

Sistrunk, J.R., Nickerson, K.P., Chanin, R.B., Rasko, D.A. and Faherty, C.S. (2016). Survival of the Fittest: How Bacterial Pathogens Utilize Bile To Enhance Infection. *Clin Microbiol Rev* **29,** 819-836.

Skarp, C.P., Hanninen, M.L. and Rautelin, H.I. (2016). Campylobacteriosis: the role of poultry meat. *Clin Microbiol Infect* **22,** 103-109.

Tashiro, Y., Ichikawa, S., Shimizu, M., Toyofuku, M., Takaya, N., Nakajima-Kambe, T.*, et al.* (2010). Variation of physiochemical properties and cell association activity of membrane vesicles with growth phase in Pseudomonas aeruginosa. *Appl Environ Microbiol* **76,** 3732-3739.

Tatusov, R.L., Koonin, E.V. and Lipman, D.J. (1997). A genomic perspective on protein families. *Science* **278,** 631-637.

Taylor, G.K. and Goodlett, D.R. (2005). Rules governing protein identification by mass spectrometry. *Rapid Commun Mass Spectrom* **19,** 3420.

Vanaja, S.K., Russo, A.J., Behl, B., Banerjee, I., Yankova, M., Deshmukh, S.D. and Rathinam, V.A. (2016). Bacterial Outer Membrane Vesicles Mediate Cytosolic Localization of LPS and Caspase-11 Activation. *Cell* **165,** 1106-1119.

Vitek, L. and Haluzik, M. (2016). The role of bile acids in metabolic regulation. *J Endocrinol* **228,** R85-96.

Wassenaar, T.M., Bleumink-Pluym, N.M. and van der Zeijst, B.A. (1991). Inactivation of Campylobacter jejuni flagellin genes by homologous recombination demonstrates that flaA but not flaB is required for invasion. *EMBO J* **10,** 2055-2061.

Whitehouse, C.A., Balbo, P.B., Pesci, E.C., Cottle, D.L., Mirabito, P.M. and Pickett, C.L. (1998). Campylobacter jejuni cytolethal distending toxin causes a G2-phase cell cycle block. *Infect Immun* **66,** 1934-1940.

Xue, Y., Tu, F., Shi, M., Wu, C.Q., Ren, G., Wang, X.*, et al.* (2016). Redox pathway sensing bile salts activates virulence gene expression in Vibrio cholerae. *Mol Microbiol.*

Yang, M., Liu, Z., Hughes, C., Stern, A.M., Wang, H., Zhong, Z.*, et al.* (2013). Bile salt-induced intermolecular disulfide bond formation activates Vibrio cholerae virulence. *Proc Natl Acad Sci U S A* **110,** 2348-2353.