Extracellular vesicles in the pathogenesis of *Campylobacter jejuni*

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Abstract

Bacteria in genus *Campylobacter* are the leading cause of foodborne infections worldwide. Here we describe the roles of extracellular vesicles in the pathogenesis of these bacteria and current knowledge of vesicle biogenesis. We also discuss the advantages of this alternative secretion pathway for bacterial virulence.

Keywords

Extracellular vesicles; Secretion system; Virulence factors; Gram-negative bacteria; *Campylobacter*.

1. Introduction

Campylobacter is a genus of spirally curved, Gram-negative, non-fermentative, microaerophilic, and non-spore-forming zoonotic bacteria. The majority of *Campylobacter* species are characterized by their rapid, corkscrew-like motility, which is mediated by an unsheathed, polar flagellum at one or both ends. Since the recognition of the genus *Campylobacter* in 1991, its taxonomy has undergone numerous modifications. According to the most-recent taxonomic structure, there are currently 50 species of *Campylobacter* and 16 subspecies yet to be described [1], although the classification scheme of the WHO recognizes only 17 species and 6 subspecies [2]. Of these, the four thermophilic species *Campylobacter jejuni*, *Campylobacter coli*, *Campylobacter lari*, and *Campylobacter upsaliensis* are those most commonly associated with human infection.

Campylobacters are the most common cause of foodborne bacterial gastroenteritis worldwide, with 1.5 million cases estimated annually in the United States [3] and 137,107 cases reported in Europe in 2022 (UK non-included) [4]. The overall economic burden of campylobacteriosis was estimated at about €3 billion in the EU [5], and US$ 4 billion in the US annually [6]. More than 90% of human campylobacterosis cases are caused by *C. jejuni* and *C. coli*, with the vast majority (over 80%) due to *C. jejuni* [7]. Unlike *Salmonella* or *Listeria*, *Campylobacter* spp. are not able to multiply within foods and are not associated with large outbreaks. More than 90% of cases of human campylobacteriosis are sporadic.

Identification of campylobacters is challenging using traditional microbiological culturing techniques. These bacteria are considered to be fastidious microorganisms because they can neither ferment nor oxidize carbohydrates. Their optimum growth temperature is between 37–42°C, but their culturing requires special medium and conditions such as the addition of blood to a culture broth and mandatory microaerobic environments [8]. The

biochemical/phenotypical characterization of campylobacters is also difficult because they can change their distinctive "S"-shape into a coccoid form. Given these challenges, the increased incidence of illnesses associated with these bacteria, and the inadequacies of current therapies, there is an urgent need for the development of new strategies for control and treatment. Such strategies will draw inspiration from studies of *Campylobacter*'s ability to invade, replicate within the host, and survive in the face of stressors, research that is rapidly improving our understanding of its dissemination, ability to escape from host immune defenses, and adaptation to different environments.

In this review, we first introduce the main virulence factors involved in various steps of infection by *Campylobacter*, and then present a synthesis of current knowledge concerning the role of bacterial extracellular vesicles (BEVs) in its pathogenesis. We discuss the importance of BEVs in this bacterium's lifestyle, dissemination, and infection process, particularly given the absence of other genes for prototypical secretion systems in the genome of *Campylobacter*.

2. Campylobacteriosis and virulence of campylobacters

Campylobacters, like other bacteria, have developed complex systems for exporting effectors and virulence factors in order to adapt to adverse conditions or colonize new habitats. When exposed to an unfavorable environment, campylobacters produce virulence factors that can also act as antimicrobial resistance factors or niche factors at various steps along the food chain or during infection. In addition, in the presence of oxygen or under antibiotic pressure, *C. jejuni* may enter a "persister state" [9], a non-growing state in which reduction/dysregulation in metabolic processes enables bacterial cells to survive lethal stress conditions. The metabolic burst in the persister phenotypic variant has been proposed to

result from a reduction in membrane potential due to remodeling of the electron transport chain [9], which prevents hyperpolarization of the inner membrane and curbs intracellular alkalization. In the persister state, cells of *C. jejuni* demonstrate reduced respiration and nutrient transport and considerable modulation of gene expression; however, when environmental conditions become favorable again, these bacteria may return to their original phenotype and cause infection [9].

This zoonosis is transmitted through consumption of under-cooked poultry meat and meat products (up to 80% of cases), as well as raw milk, shellfish, vegetables, and contact with animals [10]. The majority of cases of campylobacteriosis are reported during the summer season, likely associated with outdoor barbecues [11]. However, campylobacters can also survive in water, and infection may occur following contact with contaminated water during recreational activities. Clinical symptoms of illness range from self-limiting diarrhea to severe inflammatory and bloody diarrhea, sometimes associated with systemic issues. Most of the time, symptoms follow an incubation period of 2 to 5 days and are limited to gastroenteritis with diarrhea, fever, abdominal cramps, and vomiting lasting from 2 to 10 days [7]. The infective dose in a human host is low, around 500–800 bacteria [12]. Even if the majority of *C. jejuni* infections remain uncomplicated, this pathogen is the main infectious agent identified in peripheral neuropathies such as Guillain-Barré syndrome [13] or Miller-Fisher syndrome, which can lead to death in 2–12% of patients, depending on their age. Finally, campylobacteriosis may result, in rare cases, in intestinal complications (colitis, appendicitis, irritable bowel syndrome, colorectal cancer, Barrett's esophagus) or systemic infections (endocarditis, pneumonia, neonatal sepsis). The true incidence of campylobacteriosis is estimated to be under-reported by a factor of 10, mainly due to underdiagnosis, misdiagnosis or improper sample collection and testing [14].

The clinical presentation of *Campylobacter* infections is influenced by a range of virulence factors used by the bacterium to target host cells, adhere to mucus, invade epithelial cells, and cause cell damage. To date, the pathogenesis of *C. jejuni* remains poorly understood due to the complexity of the genetic mechanisms involved and a lack of appropriate animal models for research. However, multiple virulence factors expressed by this bacterium are implicated in crucial steps of pathogenesis, as illustrated in Fig. 1.

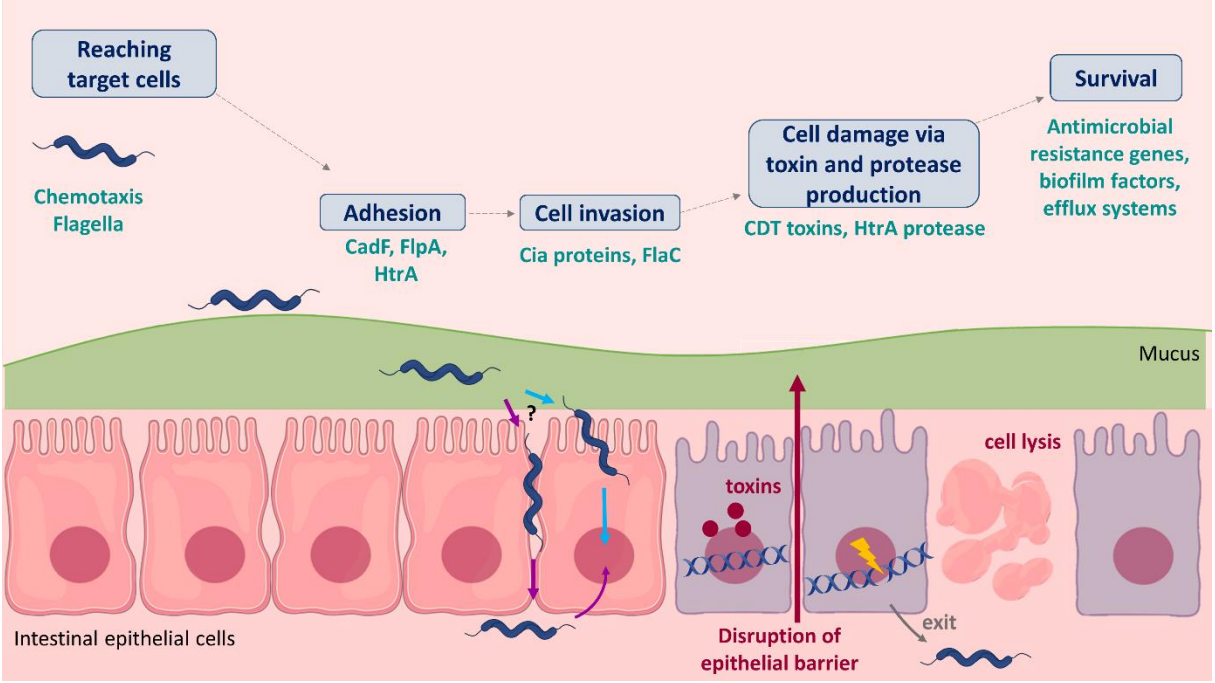


Figure 1. The process of infection by the intestinal pathogen *Campylobacter jejuni* and the virulence factors implicated. The infectious process of *C. jejuni* begins when the bacteria reach the intestinal epithelial cells of the host and then adhere to and invade those cells. Two models are presented for *C. jejuni*'s migration through the epithelium (violet / sky blue). Once internalized, *C. jejuni* produces CDT toxins (cytolethal distending toxins) and proteases, which provoke cell death and inflammation. Then, the bacteria may survive or be cleared from the host. Virulence factors are noted in green.

2.1. Reaching target host cells

For enteric pathogens, a key step of infection is colonization of the intestinal epithelial cells. This process requires the ability to penetrate the mucus layer covering epithelial cells in order to reach the target cells and then internalize. *Campylobacter* species are naturally able to swim very rapidly in viscous environments due to their helical shape and amphitrichous flagella [15]. Indeed, *C. jejuni* has been documented to travel at a higher velocity in mucus than most other bacteria (55 to 100 $\mu m.s^{-1}$) [16].

Given the crucial role of the flagellum in the colonization process, the flagellar proteins are considered to act as bacterial virulence factors. In addition to motility, the flagellum is involved in biofilm formation, adhesion, internalization, and protein secretion [17,18]. The basic structure of a bacterial flagellum consists of an extracellular filament connected via a hook to a basal body formed of rings and a rod. The basal body complex comprises the MS ring (FliF protein), the C ring rotor (FliG, FliM, FliN, FliY), the P-ring in the peptidoglycan layer and the L-ring in the outer membrane (Flg I and FlgH, respectively, described in other Gram-negative bacteria), the rod (FlgF and FlgG), and a motor (MotA, MotB) responsible for the rotation force. This assemblage anchors the flagellum to the bacterial envelope with the help of a hook (FlgE) and surrounds the type III secretion system (T3SS) localized in the inner membrane. The flagellar T3SS is composed of the proteins FlhA, FlhB, FliO, FliP, FliQ, and FliR, and exports distal flagellar fragments through the membranes to construct the extracellular filament, formed by the two glycosylated flagellins FlaA and FlaB [19]. The monomers at the distal end of the filament are covered with the flagellar capping protein FliD [20].

A bacterium's route and directional changes during swimming are determined by the rotational direction of the flagellum, which alternates between clockwise and counter-clockwise depending on extracellular signals. The rotational direction of the flagellum is usually coupled to chemosensory receptors, enabling the bacterium to reach a favorable

environment [21]. Bacterial movement driven by gradients of oxygen, nutrients, or temperature—called chemotaxis—may play an important role in both the commensal and pathogenic lifestyles of *Campylobacter*. Various extracellular signals are detected by methyl-accepting chemotaxis proteins (MCPs), also called transducer-like proteins (Tlps). These proteins are classified into three groups (A, B, and C) depending on their structure. Novel Tlp proteins were recently identified (Tlp14-25), in addition to the 13 Tlps previously described [22–24]. Once the external stimuli bind to Tlps, a signal is relayed to chemotaxis proteins (Che) in the cytoplasm. These proteins initiate a signal transduction cascade resulting in directional rotation of the flagellum. To date, 6 Che proteins have been described in *C. jejuni* [23]. Notably, the loss of CheY creates bacteria that are non-motile and non-invasive. For *C. jejuni*, chemotaxis is as essential as motility for reaching intestinal cells and colonizing the gut of chickens and mammals [25].

2.2. Adhesion and invasion of host cells: two models for transepithelial migration of C. jejuni

After reaching the gut, *C. jejuni* must cross the mucus layer and adhere to intestinal epithelial cells in order to invade them. A series of studies in human biopsies have shown that *C. jejuni* is able to cross the epithelial barrier and enter underlying tissues, organs, and the bloodstream [26]. However, the mechanism of this transmigration through the intestinal epithelium is still debated and not well understood. To explain how *C. jejuni* is able to cross polarized epithelial cells, two transmigration routes have been hypothesized: (i) the transcellular route, in which bacteria cross the epithelial barrier through adhesion and cell entry at the apical pole and exit at the basal pole [27], and (ii) the paracellular route, in which pathogens open cell junctions using proteases like HtrA [28] and make their way between cells by adhering to basal

extracellular matrix components like fibronectin before finally invading a cell at the basal pole (Fig. 2). The adhesion and invasion steps of pathogenesis may therefore occur either at the apical pole or at the basal one, depending on the route taken by *C. jejuni*. Currently, studies have been converging towards the paracellular pathway, considering the importance of fibronectin-binding and HtrA-mediated adherens junctions cleavage for the internalization of *C. jejuni* [29,30].

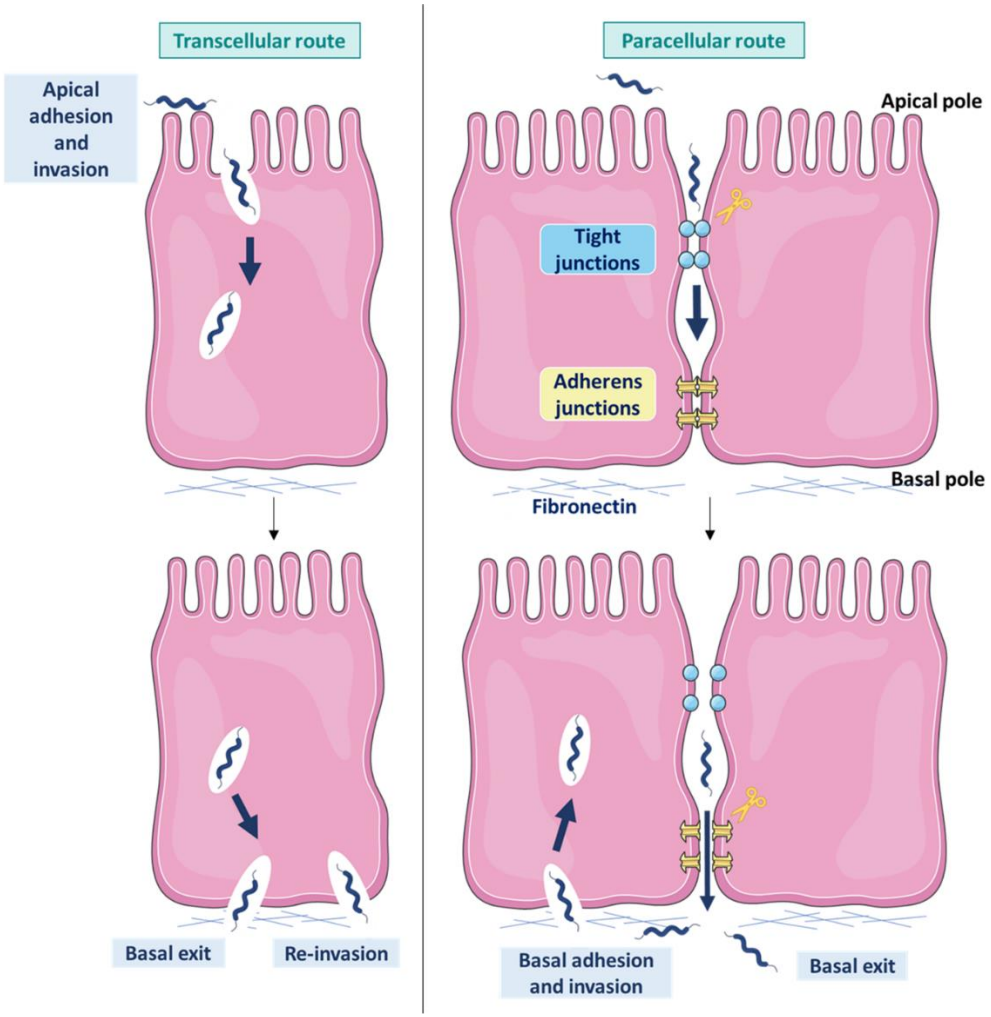


Figure 2. The two models hypothesized for the epithelial transmigration of *Campylobacter jejuni* during infection. In the transcellular route, bacteria adhere to and invade the apical pole of epithelial cells and exit by the basal pole. In the paracellular route, bacteria cleave cell junctions to cross the epithelial barrier between two cells, and once at the basal pole, adhere to and invade the cell.

2.2.1. Adhesion

As for many bacteria, adhesion of *C. jejuni* is a multifactorial process. Several binding factors have been implicated in the successful interaction with host cells and the development of disease. Depending on the transmigration route taken by the bacterium, adhesion may occur either at the apical pole (adhesion to mucus or directly to epithelial cells) or at the basal one (adhesion to extracellular matrix components like fibronectin).

Certain adhesion factors found in *C. jejuni* have been shown to promote basal adhesion to fibronectin, a large and ubiquitous glycoprotein that is the main component of the extracellular matrix of mammalian cells. Binding to this protein is the primary step of attachment to tissue surfaces for numerous pathogens. Of the adhesins that have been found to bind to fibronectin, three are of particular interest: the major outer membrane protein, MOMP [31], and the outer membrane proteins CadF (*Campylobacter* spp. adhesion to fibronectin) and FlpA (fibronectin-like protein A). Studies of *cadF* and *flpA* mutants have revealed impairments in binding to epithelial cells, colonization of the chicken gut, and the establishment of severe disease in germ-free mice [32,33]. These proteins promote adhesion via their fibronectin-binding sites, which consist of a four-amino-acid motif (Phe-Arg-Leu-Ser) for CadF and a nine-amino-acid motif (Trp-Arg-Pro-His-Pro-Asp-Phe-Arg-Val) for FlpA [34].

Other proteins enhance the adhesive properties of bacteria via their chaperone activity, assisting in the folding of outer membrane proteins and thus in modifications to the bacterial surface. The best example is the serine protease HtrA, a highly conserved periplasmic protein displaying both protease and chaperone activity. A loss of HtrA chaperone activity leads to a strong reduction in bacterial binding to epithelial cells, more so than the loss of any other known adhesin [35].

Some proteins have been identified as adhesins but the mechanism by which they bind epithelial cells is still unknown. An example of this is Peb1, the first protein identified in *C. jejuni* as being involved in bacterial adhesion to HeLa cells and the colonization of mice [36]. Another example is CapA, an autotransporter protein of the outer membrane. CapA is not conserved among all strains of *C. jejuni*, but when it is present this surface-exposed protein plays a role in binding to epithelial cells and is required for efficient colonization of chickens [37]. Some flagellar proteins seem to hold a role also in adhesion, such as FlaC which binds HEp-2 cells, by an unknown mechanism [38]. Finally, mutation of the *comB3* gene (encoded in the *C. jejuni* resistance plasmid pVir) was found to result in decreased adhesion and invasion of INT-407 cells, [39].

The adhesion process also appears to depend on certain non-protein components, such as polysaccharides or plasmids. *C. jejuni* can produce lipooligosaccharides (LOS) and highly variable capsular polysaccharides but not lipopolysaccharides (LPS). It is through these polysaccharides that the *C. jejuni* capsule participates in the processes of adherence and invasion. For example, strains with mutations in three LOS synthesis genes (*wlaRG*, *wlaTB*, and *wlaTC*) all displayed reduced adhesion to chicken embryo fibroblasts [40]. Interestingly, the similarity between the LOS of *C. jejuni* and neuronal gangliosides is thought to explain the link between *C. jejuni* and Guillain-Barré Syndrome.

2.2.2. Cell invasion

In 2001, Rivera-Amill *et al*. demonstrated that certain proteins—designated Cia for *Campylobacter* spp. invasion antigens—are secreted during co-cultivation of *C. jejuni* and intestinal cells [41]. More precisely, they identified a temporal association between the secretion of Cia proteins and the invasion of cells by *C. jejuni*: Cia proteins are secreted after

30 minutes of co-culturing, at the precise moment when a rapid increase in the internalization of bacteria is observed. Because the genome of *C. jejuni* lacks genes associated with a classical type III secretion system, the flagellar export apparatus was identified as the likely mechanism of Cia secretion [41]. Further investigations into CiaB suggested that this protein may be essential for cell invasion, as three mutants with insertional disruptions of the *ciaB* gene exhibited a significant reduction in internalization in host cells [42]. Interestingly, Cia protein synthesis was found to be enhanced in the presence of deoxycholate bile acid, but Cia secretion was unchanged. This finding suggests that production of Cia proteins may occur early in the colonization process, but secretion is only initiated once the bacterium has adhered to its long-term colonization site [41].

In addition to their roles in adhesion, some flagellins and flagellar components also contribute to epithelial cell invasion by *C. jejuni*. In studies of mutant strains, Grant *et al*. demonstrated that flagella are important for *C. jejuni*'s internalization in epithelial cells [18]. Notably, the protein FlaC, which, like Cia proteins, is secreted by the flagellar apparatus, has been shown to be required for invasion [38]. Similarly, the Δ*motAB* mutant strain, lacking the genes encoding the flagellar motor, demonstrated a significant decrease in cell invasion capacity [43].

Finally, some lysophospholipids contained in the membrane of *C. jejuni* are also considered to be novel virulence factors. Short lysophosphatidylethanolamines (lysoPE) were reported to permeabilize host cell membranes and thus cause cell damage via oxidative stress [44].

2.3. Toxin production and pro-inflammatory factors

After *C. jejuni* interacts with epithelial cells, it releases a holotoxin – cytolethal distending toxin (CDT) – that may be responsible for the cytopathic effects of *C. jejuni* infection. CDT belongs to a family of bacterial toxins that affect the epithelial cell layer and interrupt the cell division process, leading to cell death [45]. This toxin is produced by a wide range of pathogenic Gram-negative bacteria including *Escherichia coli*, *Helicobacter* spp., and other species of *Campylobacter*. The CDT of *C. jejuni* belongs to the AB family of toxins, which feature an active subunit (CdtB) and two binding subunits (CdtA and CdtC) encoded by the *cdtA*, *cdtB*, and *cdtC* genes located in the same operon [46].

The subunits CdtA and CdtC are thought to enable the binding of CDT to epithelial cells by attaching themselves to the cholesterol-rich lipid rafts of the cell membrane, thus facilitating the entry of CdtB into the cell cytoplasm. CdtB is now known to be the toxic component, as a microinjection or transfection of only this subunit into host cells causes the same cell cycle arrest observed with the complete CDT toxin. Once in the cell, CdtB reaches the cell nucleus and causes DNA damage (double-strand breaks). CdtB is thought to act as a deoxyribonuclease I, as it shares structural similarity with DNAse I–like proteins [47]. Host cells react to this DNA damage by initiating a regulatory cascade that includes phosphorylation of the histone protein H2AX, leading to the recruitment of Rad50, a DNA-repair protein for double-strand breaks [48]. This process blocks the cell cycle at the G2/M interphase to allow for DNA repair. If the damage is too extensive, repair fails and the cell cycle arrest leads to cellular distention, senescence, and finally cell death [49].

The CDT toxin has been reported to provoke autophagy and apoptosis in vitro and inflammation in vivo, along with cell senescence. A recent study in colonic epithelial cells highlighted CDT-induced pyroptosis, a process of pro-inflammatory programmed cell death characterized by the emergence of large bubbles from the plasma membrane, which leads to

cell membrane rupture and eventually the release of cell content. This form of programmed cell death was originally known to be triggered by pro-inflammatory caspases but it now appears that it can also be mediated by virulence factors [50]. Specifically, Gu *et al*. demonstrated the induction of gasdermin E–mediated pyroptosis in response to the CDT of *C. jejuni*. After CDT exposure, there was a considerable elevation in ROS levels in epithelial cells, initiating the caspase-9/caspase-3/gasdermin E pyroptosis pathway [50]. In addition to this toxin-induced inflammation, some virulence factors cause damage to the intestine by triggering inflammatory responses. For example, the lipoprotein JlpA activates NF-KB and p38 MAP kinase pathways in Hep-2 cells [51].

2.4. Survival

During commensal carriage in food animals, *C. jejuni* must cope with various stresses related to oxygen levels, desiccation, disinfectants, or temperature shocks. In these environments, *C. jejuni* may also encounter bile salts, antibiotics, and defenses of the host immune system. Resistance to many drugs and bile salts is often mediated by the interaction between two *Campylobacter* multidrug efflux pumps (CME), encoded by the *cmeABC* operon and the *cmeDEF* operon [52–54]. To protect itself from disinfection protocols, dessication, and high oxygen levels, *C. jejuni* is able to form biofilms [55]. In addition, this bacterium is able to enter a viable-but-not-culturable (VBNC) state in response to osmotic and temperature shocks, pH modification, or nutrient starvation [56]. A final form of defense are capsular polysaccharides (CPS) that coat *C. jejuni* cells, which are involved in cell wall maintenance but have also been implicated in evasion from the host immune system [57].

3. Extracellular vesicles in Gram-negative bacteria and *C. jejuni*

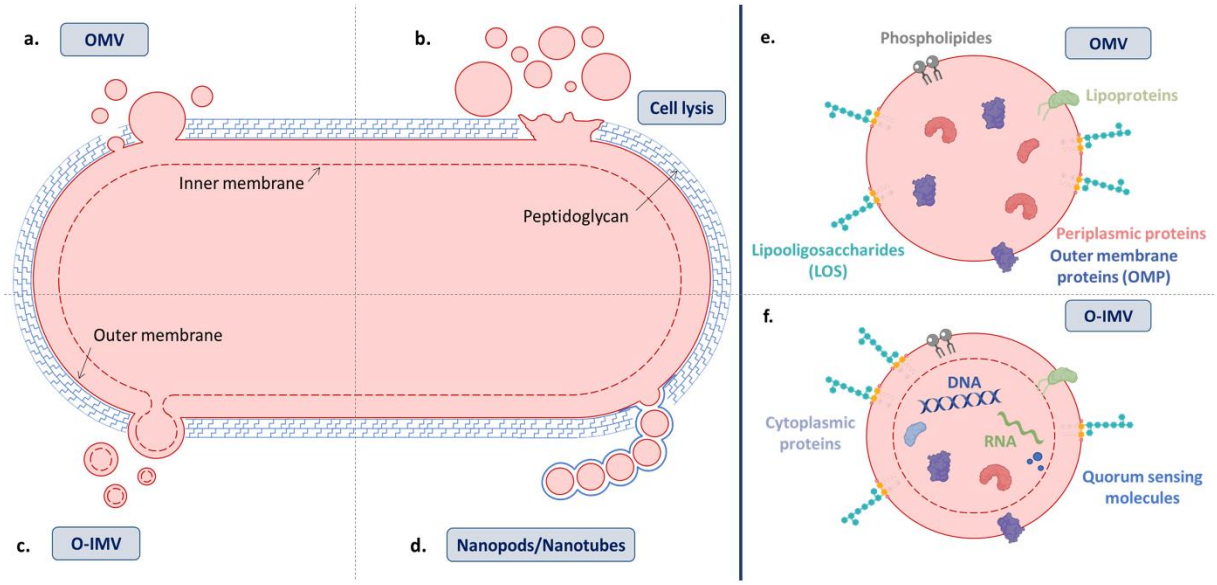
3.1. Secretion of extracellular vesicles in Gram-negative bacteria

Bacteria constitutively release extracellular vesicles, termed bacterial extracellular vesicles (BEVs), which vary in their composition, size, content, and architecture depending on the biogenesis route, growth phase, and environmental conditions present (e.g., pH, temperature, nutrient content, ionic force, antibiotic pressure, or oxygen stress). The liberation of membrane vesicles from Gram-negative bacteria is thought to be an alternative secretion system that facilitates both interbacterial and bacterial-host cargo transfer. Indeed, vesicles from Gram-negative bacteria were shown to integrate into the membranes of foreign bacteria, both Gram-positive and Gram-negative [58]. Vesicles may also adhere to or integrate into eukaryotic cells, promoting adherence of the parent bacterium to host cells. There is an increasing body of evidence that BEVs are the cause of numerous pathologies, through either bacterial-bacterial or bacterial-host interactions [59,60]. Nanosized BEVs are released in both favorable and unfavorable physiological conditions as a mechanism for cell-free intracellular communication.

Through their roles in bacterial short- and long-distance communication, stress response, detoxification, lateral gene transfer, and intracellular competition, BEVs allow bacteria to interact rapidly and adapt to their environments, and thus to cause various pathologies [61]. In particular, BEVs have a direct impact on pathogenesis by delivering toxins and other virulence factors to the host during infection [62]. Indeed, in *C. jejuni*, the well-characterized toxin CDT is secreted in its active form via BEVs [63]. Bacterial vesicles may internalize into epithelial or immune cells or fuse with eukaryotic membranes to release their cargo inside the cytoplasm. In addition to toxin delivery, BEVs enable long-distance delivery of virulence factors such as adhesins, colonization factors, outer membrane proteins, LPSs, flagellins, and proteases [64]. BEVs are particularly suitable for delivering insoluble proteins and proteins lacking signal peptides. Additionally, by densely packaging virulence factors, BEVs

311 increase their stability and concentration until delivery to the target. They also provide a
312 protective barrier, safeguarding their contents against degradation by host proteases or
313 nucleases, or low/high concentrations of salt or cations. Compared to classical secretion
314 systems that export individual virulence factors, BEVs may enhance pathogenesis through the
315 coordinated delivery of multiple, densely packed factors.

316 BEVs can be easily observed in bacterial preparations by electron microscopy. They
317 may be formed from cellular debris during cell lysis (Fig. 3a), but can also be generated through
318 bacterial metabolic activities. The generation of extracellular vesicles under non-lytic
319 conditions is beneficial for bacteria by eliminating misfolded proteins and relieving membrane
320 stress. In this process, the outer membrane swells to form outer membrane vesicles (OMVs)
321 that are then released in the medium (Fig. 3b). The resulting liberated spherical particles,
322 ranging in diameter from 10 to 500 nm, consist mainly of periplasmic proteins and envelope
323 components such as phospholipids, lipoproteins, LPSs, lipooligosaccharides (LOS), and outer
324 membrane proteins (Fig. 3e). Moreover, Gram-negative bacteria may produce double-
325 membrane bilayer vesicles via the protrusion of both the outer and cytoplasmic membrane
326 (Fig. 3c). These so-called outer-inner membrane vesicles (O-IMVs) are believed to carry
327 bacterial cytoplasmic content, e.g., proteins, ATP, nucleic acids, effectors, and quorum-
328 sensing molecules (Fig. 3c and f) [65]. Several Gram-negative bacteria have also been shown
329 to liberate nanosized filaments attached to OMVs [59,66]. When a nanofilament forms as a
330 continuous tubular structure that resembles multiple OMVs, it is called a nanotube (Fig. 3d);
331 when the nanofilament is instead attached to a single OMV, it is called a nanopod. Nanotubes
332 and nanopods may bridge different cells together and facilitate their communication via
333 OMVs.



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335 **Figure 3. Diverse mechanisms of bacterial extracellular vesicle (BEV) secretion and content**
336 **of the resulting vesicles.** (**a**) OMVs can be formed by a bleb of the outer membrane or (**b**)
337 during cell lysis. (**c**) BEVs may also be formed by a protrusion of both the inner and outer
338 membranes, forming O-IMVs. (**d**) BEVs can be secreted in a nanofilament containing one
339 vesicle (nanopods) or several (nanotubes). (**e-f**) These different mechanisms result in
340 differences in the contents of secreted vesicles.

341

342 *C. jejuni* cells, which are approximately 1–4 µm long, can liberate vesicles of diameters
343 reaching 300–500 nm (Fig. 4). The biogenesis and liberation of BEVs of this size represent a
344 huge loss of both membrane and energy. This strongly suggests that BEVs have vital roles in
345 the maintenance of normal bacterial physiological activities. Campylobacters have been
346 observed to produce BEVs both in the presence and absence of stress. To date, though, no
347 model has been proposed for BEV biogenesis in *Campylobacter*, and the mechanisms
348 described in other Gram-negative bacteria appear to be species-specific. For instance,
349 *Pseudomonas aeruginosa*, which has a highly fluid membrane, can produce BEVs only when
350 outer membrane fluidity is reduced, which occurs in response to the interaction between the
351 quorum-sensing pseudomonas quinolone signal (PQS) molecule and lipid A protein [67]. Once

the outer membrane is stabilized, the membrane curvature enables formation of BEVs. Another mechanism, proposed for *Salmonella*, involves modifications in the connections between the cell wall and the outer membrane that allow blebbing [68]. This mechanism is supported by the observation that BEVs are released with high frequency at cell division sites. Nevertheless, this model cannot be considered universal because BEVs can also be released from other cell sites. Finally, bacteria whose flagellum is sheathed with LPS, such as *Vibrio fischeri* or *Vibrio cholerae*, have been shown to release BEVs via flagellar rotation [69].

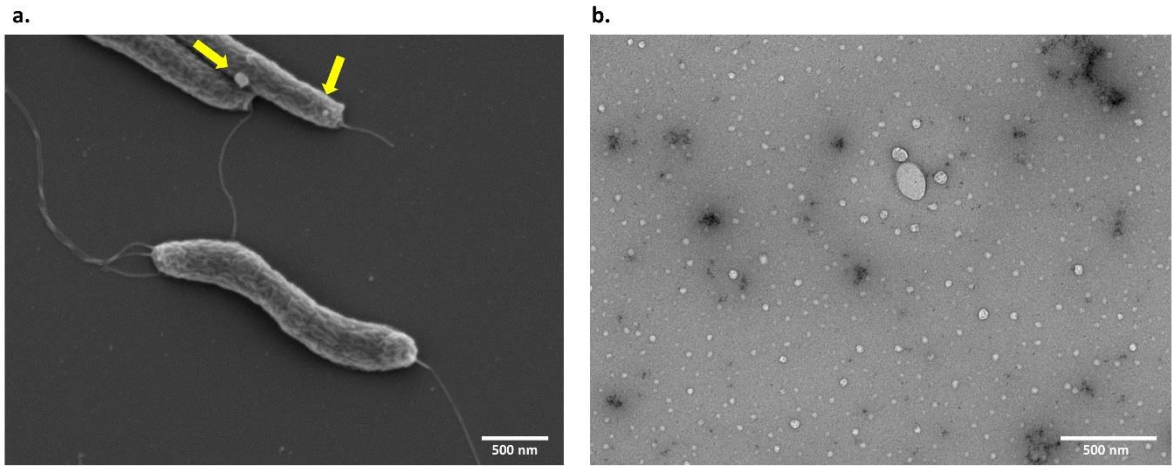

Figure 4. Scanning electron microscopy image of *Campylobacter jejuni* and its purified extracellular vesicles. (**a**) *C. jejuni* on a BHI plate. Its S-shaped morphology, polar flagella, and extracellular vesicles (yellow arrows) can be observed. (**b**) Extracellular vesicles secreted by *C. jejuni* are heterogenous in size, ranging in diameter from around 20 nm to 300 nm. Scale bars = 500 nm.

One model that may be more generally applicable is that proposed by Roier *et al.* based on the maintenance of lipid asymmetry (MLA) pathway [70]. This pathway is highly conserved in Gram-negative bacteria and is involved in the retrograde trafficking of phospholipids from the outer leaflet of the outer membrane to the inner membrane. When this retrograde trafficking is blocked or down-regulated, the accumulation of phospholipids in the outer leaflet of the outer membrane leads to an asymmetric expansion and the formation of BEVs.

The role of this pathway in BEV production in *C. jejuni* was confirmed by Davies *et al*. [71]. Using the bile salt sodium taurocholate as a potential regulator, they observed that *C. jejuni* regulated BEV production through the MLA pathway without compromising membrane stability. In the presence of sodium taurocholate, BEV production was enhanced in the wild-type strain but not in an *mlaA* mutant that lacked the outer membrane component of the MLA pathway. This work suggests that *C. jejuni* is able to produce BEVs of different sizes, numbers, and cargos depending on signals from the host gut, and moreover, that such environmental signals may be responsible for triggering different mechanisms for BEV biogenesis.

3.2. Implication of BEVs in C. jejuni virulence

In contrast to archetypal Gram-negative bacteria, which have six secretion systems for exporting molecules (Type I to Type VI, T1SS-T6SS), genetic analyses of *Campylobacter* isolates suggest that this bacterium does not possess type I, type IV or type V secretion system and only a few genes encoding protein components of type II secretion system have been identified. Thus, *C. jejuni* may rely only on Type III and VI secretion systems to export biological macromolecules [72]. Unlike the majority of Gram-negative pathogenic bacteria that possess a dedicated T3SS (e. g. *Salmonella*), *C. jejuni* has no dedicated T3SS. Instead, its flagellum holds a dual role of motility and protein export [72]. It transports not only flagellar proteins such as flagellin and allied proteins, but also releases some non-flagellar virulence factors such as the invasion antigen Cia proteins and FlaC, which plays a role in cell adhesion and invasion [38]. Concerning the type IV secretion system, the 54,065 genomes of *C. jejuni* and *C. coli* from the PubMLST database were screened and only 7 genes associated with T4SS were identified, with the majority encoded in plasmids pVir and pTet [72]. This result suggests that most of *Campylobacter* isolates do not produce a T4SS. Finally, in 2012, a study showed that some *C.*

jejuni isolates encode a T6SS involved in bile salt survival, adhesion to host cells and invasion [73]. More recent studies tried to evaluate the percentage of *C. jejuni* isolates encoding a functional T6SS but the results vary a lot (between 27 to 74% of clinical isolates were positive). [72]. A more detailed analysis is needed to elucidate whether the T6SS is functional in *C. jejuni*.

Due to the absence of some prototypical secretion systems (type I, II, V and often type IV), campylobacters must use alternative mechanisms to secrete virulence factors in host cells and so exert their pathogenic effects. Like other Gram-negative bacteria, campylobacters seem to rely on extracellular vesicles to transport virulence factors. As mucosal pathogens, campylobacters use BEVs to coordinate the secretion of major virulence factors (active proteins, nucleic acids, metabolites, peptidoglycan fragments) and to deliver them in the cytoplasm of host cells rather than in the surrounding medium. Indeed, it has been demonstrated that *C. jejuni* uses BEVs to deliver toxins [63] and certain proteins that promote adhesion and invasion of host cells [74]. BEVs carrying CDT have been shown to directly damage cellular DNA and induce apoptosis in a range of mammalian cell lines [75]. Recently, it was shown that CDT is mainly located in EVs and induces epithelial cell distension, even in absence of surface proteins. Interestingly, this study elucidated partially the mechanism of EV uptake in *C. jejuni* by showing that EVs binds preferentially to complex glycans of host cells (e.g. Lewis antigens, blood group A antigens, gangliosides) [76]. Furthermore, the addition of isolated BEVs was found to improve the adhesion and internalization of *C. jejuni* to intestinal epithelial cells, confirming the importance of BEVs in establishing infection [77,78].

A few studies have attempted to characterize the proteinaceous cargo of the BEVs secreted by *C. jejuni* under different conditions. Vesicles have been reported to contain numerous and diverse proteins described or predicted as virulence factors with a role in the pathogenesis of *C. jejuni* (Table 1). First among these are the flagellar proteins (flagellins FlaA,

FlaB, FlaC, and FlaD, and flagellar hook proteins FlaE, FlgE, FlgK, FlgL, FlgP, and FliD), which have been found in BEVs secreted by different strains of *C. jejuni* (81-716 and 11168) and at different growing temperatures (37°C and 42°C)[79–82]. As mentioned earlier, these proteins, which form components of the flagellum, are considered virulence factors of pathogenic bacteria because of the importance of the flagellum in motility, adhesion, and stimulation of the host immune system [83–85]. Many different adhesins have also been found in vesicles, notably the fibronectin binding proteins FlpA and CadF, the lipoproteins RlpA and JlpA, and the capsule polysaccharide export protein KpsD [74,79–81]. In addition, vesicles have been found to contain three outer membrane proteins identified as virulence factors: the Omp18 protein, implicated in adhesion and immune system stimulation; the ChuA heme receptor protein, involved in adhesion and invasion of host cells; and the major outer membrane protein (MOMP) PorA, which plays a role in the adhesion of *C. jejuni* to intestinal cells [81]. An analysis of the content of vesicles from strain 81-176 detected a chemoreceptor protein, CheV [80], which is involved in chemotaxis, driving the directional changes during swimming to allow bacteria to reach target cells, and is thus implicated in the first step of pathogenesis. Vesicles secreted by *C. jejuni* strain 81-176 were also found to contain the molecular chaperone GroEL, which has multiple roles in pathogenesis, namely adhesion, invasion, and immune system stimulation. BEVs from strains 81-187 and 11168 have been reported to contain several antigens, for example the well-known Peb antigens (Peb1A, 2, 3 and 4), which play roles in adhesion, invasion, and survival, and CjaA and CjaC, which promote inflammation [62],[65-67]. The immunogenic CjaA is loosely connected with the inner membrane, but it can be transported to the cell surface and is present in EVs [86].

The serine protease HtrA—responsible for the disruption of tight and adherens junctions during invasion—was identified in the BEV content of strains 81-176, 11168, and 11168H. In

addition, studies highlighted the proteolytic activity of *C. jejuni* BEVs and identified several proteases implicated in this process. Elmi *et al*. showed that the proteases HtrA and Cj1365c contained in the *C. jejuni* BEVs contributed to the cleavage of E-cadherin and occludin, resulting in the disruption of adherens and tight junctions, respectively [78]. This cleavage of tight and adherens junctions induced by BEVs was shown to be enhanced when bile salt sodium taurocholate was added to the medium [87].

Interestingly, vesicles also contained some components of efflux pumps, like CmeA and CmeC, which have been implicated in antibiotic resistance and survival [80,81]. Finally, several studies have reported the presence of the CDT subunits CdtA, CdtB, and CdtC in vesicles secreted by different strains of *C. jejuni*. In brief, proteomic analyses of BEV content have confirmed that numerous virulence factors, representing all steps of *C. jejuni* pathogenesis, are packed into these vesicles [63,74].

<table>
<tr><th>Virulence factor</th><th>Type</th><th>Role</th><th>Step of pathogenesis concerned</th><th>Reference</th></tr>
<tr><td>FlaA and FlaB</td><td>Flagellins</td><td rowspan="6">Corkscrew motility, immune stimulating</td><td rowspan="6">Reaching target cells
Adhesion
Invasion</td><td>[79–81]</td></tr>
<tr><td>FlaC</td><td>Flagellins</td><td>[81]</td></tr>
<tr><td>FlaD</td><td>Putative flagellin</td><td>[79]</td></tr>
<tr><td>FlaE and FlgP</td><td rowspan="2">Flagellar hook proteins</td><td>[81]</td></tr>
<tr><td>FlgL, FlgE and FlgC</td><td>[81,82]</td></tr>
<tr><td>FliD and FlgK</td><td>Flagellar hook proteins</td><td>[79]</td></tr>
<tr><td>CadF</td><td rowspan="2">Fibronectin binding protein</td><td>Adhesin</td><td>Adhesion</td><td>[74,79–81]</td></tr>
<tr><td>FlpA</td><td>Adhesin</td><td>Adhesion</td><td>[74]</td></tr>
<tr><td>RlpA</td><td>Lipoprotein</td><td>Cell wall biogenesis</td><td>Adhesion</td><td>[81]</td></tr>
<tr><td>JlpA</td><td>Surface-exposed lipoprotein</td><td>Adhesin</td><td>Adhesion</td><td>[80]</td></tr>
<tr><td>Omp18</td><td>Outer membrane protein</td><td>Immune system stimulation</td><td>Adhesion
Cell damage (via inflammation)</td><td>[81]</td></tr>
</table>

PorA	MOMP	Ion transport (porin) and adhesion to fibronectin	Adhesion and cell damage (porin)	[79,81]
KpsD	Capsule polysaccharide export protein	Capsule biosynthesis	Adhesion	[79,81]
ChuA	Outer membrane haem receptor protein	TonB-dependent heme receptor	Adhesion Invasion	[80,81]
GroEL	Molecular chaperone	Adhesion Immune system stimulation	Adhesion Cell damage (via inflammation)	[81]
CheV	Chemoreceptor protein	Chemotaxis	Reaching target cells	[80]
CjaA, CjaC	*C. jejuni* antigens	Immune system stimulation	Cell damage (via inflammation)	[79–81]
Peb1A, 2, 3	Major antigen protein	Biofilm formation, motility, host cell invasion	Adhesion Invasion Survival	[80,81]
Peb4				[74,81]
HtrA	Serine proteases and chaperone	Folding of adhesins Disruption of tight and adherens junctions	Adhesion Invasion	[74,79,80]
CmeA and CmeC	Efflux pump subunits	Resistance to broad range of antimicrobial	Survival	[80,81]
CdtA, CdtB and CdtC	Cytolethal distending toxin subunits	DNAse like protein toxin making double DNA break in host cells	Cell damage (via DNA damage)	[63,74,81]

Table 1. Virulence factors of *Campylobacter jejuni* found in the proteinaceous cargo of bacterial extracellular vesicles (BEVs) by proteomic studies and the contribution of these factors to the pathogenic process. MOMP = major outer membrane protein

Recently, Khan *et al*. demonstrated that BEVs of *C. jejuni* with different cargos and morphological attributes induced different, biologically relevant host responses [88]. Using FRET and fluorescence dye dequenching assays, they showed *C. jejuni* BEVs are fusogenic and specialized for transporting macromolecules. They reported that host cell–specific differences in BEV uptake from the extracellular medium were mediated by heterogeneity in vesicles caused by underlying differences in the membrane phospholipids acquired from the source bacteria and the abundance of surface proteins. Furthermore, when human and avian cells

were compared as host models, the uptake of different OMVs was observed to vary preferentially among different target cells. However, the intracellular accumulation of BEVs was concentration-dependent in both cell lines. Using pharmacological inhibitors, this study pointed that although the dynamin-dependent pathway was predominant in BEV uptake for both cell types, the actin filament-dependent micropinocytosis was involved in BEV internalization in avian cells [88]. All together, these data suggest that better characterization of individual BEVs may shed light on their complex roles in bacterial infections.

With respect to BEV characterization, it is interesting to note that *C. fetus* releases BEVs with a distinctive S-layer on the surface [89]. This S-layer is a bidimensional crystalline structure formed by various surface-layer proteins, which mediates the evasion of the immune response and is involved in cell adhesion. Moreover, the S-layer serves as a means of protection from external stress for the bacterium. BEVs coated with an S-layer are probably more resistant to adverse environmental conditions, which may prolong the life of the released vesicles. It remains to be seen whether other *Campylobacter* species also produce BEVs enveloped with an S-layer.

4. Conclusion

Despite the large amount of energy resources required, the non-lytic production of BEVs throughout the life of a bacterium is evolutionarily conserved in both Gram-negative and Gram-positive species. In Gram-negative bacteria, BEVs can be released from different cellular locations and, due to the protection provided by the lipid bilayer, enable the transportation of various molecules in their active forms. BEVs are involved in many cellular functions, including mediating bacterial adaptation to various environments, facilitating intercellular communication, delivering virulence factors, or transferring DNA.

In *C. jejuni,* BEVs are associated with the maintenance of virulence, stress response, improvements in cell adherence, colonization in different hosts, and responses to signals from the host gut and microbiota. The lipid bilayer protects transported molecules from host proteases and immune cells and delivers molecules directly to or near their target. Additionally, vesicles can play a significant role in bacterial communication by transporting molecules between cells.

Although many studies have reported on the biogenesis and function of bacterial extracellular vesicles, the formation and role of BEVs in campylobacters merit further investigation. Depending on the environmental signals present, temperature, or the identity of the host (avian or human cells), *C. jejuni* releases BEVs of various sizes, charges, and cargos, but the reasons and mechanisms underlying these differences remain unclear. New analytical methods are needed to distinguish the variety of regulatory roles these structures play during pathogenesis. In particular, imaging techniques that enable dynamic monitoring are sorely needed for investigations of the biogenesis and fate of BEVs. Additionally, further research is required into the mechanisms by which BEV content and function adapt to the external environment of the bacterium; such knowledge will help to elucidate the specific functions of BEVs in pathogenesis. Understanding BEVs biogenesis, cargo and uptake into host cells can also be crucial for the development of BEVs-based vaccines to prevent *C. jejuni* infections in humans, as has been tested for chickens [90]. Based on proteomic studies, it is clear that BEVs transport a high diversity of virulence factors with roles in all steps of infection, plainly demonstrating that BEV secretion is a key mechanism for *C. jejuni* virulence. Identification of the factors that influence the production and composition of BEVs in the gut will be key in improving our understanding of both *Campylobacter* physiology and the functioning of holobionts.

Declaration of competing interest

The authors have no conflicts of interest to declare.

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