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Comprehensive proteomic profiling of outer membrane vesicles from *Campylobacter jejuni*

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Abstract

Gram-negative bacteria constitutively release outer membrane vesicles (OMVs) during cell growth that play significant roles in bacterial survival, virulence and pathogenesis. In this study, comprehensive proteomic analysis of OMVs from a human gastrointestinal pathogen *Campylobacter jejuni* NCTC11168 was performed using high-resolution mass spectrometry. The OMVs of *C. jejuni* NCTC11168 were isolated from culture supernatants then characterized using electron microscopy and dynamic light scattering revealing spherical OMVs of an average diameter of 50 nm. We then identified 134 vesicular proteins using high-resolution LTQ-Orbitrap mass spectrometry. Subsequent functional analysis of the genes revealed the relationships of the vesicular proteins. Furthermore, known N-glycoproteins were identified from the list of the vesicular proteome, implying the potential role of the OMVs as a delivery means for biologically relevant bacterial glycoproteins. These results enabled us to elucidate the overall proteome profile of pathogenic bacterium *C. jejuni* and to speculate on the function of OMVs in bacterial infections and communication.

Keywords

C. jejuni NCTC11168; Functional annotation; Outer membrane vesicle; Signal peptide; Virulence factor

Introduction

Gram-negative bacteria secrete outer membrane vesicles (OMVs) that consist of lipopolysaccharides (LPS), outer membrane proteins, periplasmic proteins, outer membrane lipids, and virulence factors [1, 2]. In pathogens, OMVs are released extracellularly often attaching to host cells through adhesive molecules then transferring compounds that may modulate the host immune response [3]. They have been used for vaccine development [4]

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or for delivery of foreign proteins such as beta-lactamase [5]. Antigenic OMVs from *Neisseria meningitidis* were used to develop an administrative medicine to combat the microbial infection by boosting host immune response [6, 7]. Thus, bacterial OMVs have drawn much attention due both to their biological functions and potential use.

The human food-borne pathogen *Campylobacter jejuni* is the major bacterial causative agent of food poisoning [8] and infections can lead to Guillain-Barré syndrome (GBS), a disorder affecting the peripheral nervous system [9]. Currently, diverse approaches using genomics and proteomics techniques are being applied to better understand the pathogenesis and physiology of this medically important organism [10–13]. In particular, various proteins (i.e. outer membrane proteins) in *C. jejuni* have been investigated for the immunogenic properties and potential for use in vaccine development [14–16].

Due to the lack of a clear understanding for the biological roles of OMVs, the last decade has seen proteomic analysis of OMVs from a variety of pathogenic bacterial species such as *Escherichia coli*, *Helicobacter pylori*, *Legionella pneumophila*, *Neisseria meningitidis*, *Pseudomonas aeruginosa* etc [17–23]. The vesicular proteome of *C. jejuni*, however, has yet to be sufficiently investigated, despite the likelihood of their role in pathogenesis and virulence. Currently, the OMVs of *C. jejuni* have only been examined as the delivery mechanism for certain toxins [24].

In this study, we investigated the proteome profile of OMVs from the clinically isolated *C. jejuni* NCTC11168 using high-resolution LTQ-Orbitrap mass spectrometry (FT MS). Purified OMVs from bacterial culture supernatants were characterized and prepared for MS analysis. The results of this analysis showed a diverse set of enzymes and known virulence factors were present in the OMVs and may play a meaningful role during host-cell interaction.

Material and methods

Purification of OMVs from bacterial culture supernatants

C. jejuni NCTC11168 (ATCC #700819), originally isolated from a patient, was grown in brain heart infusion (BHI) media under microaerobic conditions (1% O_2, 10% CO_2, 10% H_2 and balanced with N_2) at 37°C. A 10 mL overnight culture was inoculated to 1L of media that was allowed to grow 18 h to mid-log phase. OMVs were purified from culture supernatants, as described previously with some modifications [17]. Briefly, the cells were pelleted using centrifugation at 6,000×g for 20 min at 4°C twice. The supernatants were filtered with 0.22 μm filter device to remove remaining cells. The resultant was centrifuged at 30,000×g for 40 min at 4°C to remove cell debris, large cell bulges or vesicle aggregates, and finally centrifuged with 150,000×g for 3 h at 4°C. The pellets were resuspended with 1.6 mL of 10% OptiPrep iodixanol solution (Sigma-Aldrich)/PBS, then gently overlaid onto the top of 40% (1.9 mL) and 50% (3 mL) of OptiPrep/PBS solutions. The OMVs were isolated from the protein-rich fractions with ultracentrifugation at 150,000×g for 2 h at 4°C, then dissolved with PBS buffer and stored at −80°C prior to use.

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Transmission electron microscopy (TEM)

Isolated OMVs were ten-fold diluted with deionized water and 3 μL of this suspension was added to glow discharged, 200-mesh formvar-carbon coated grids (Pella Inc.), then the samples were stained with 1% uranyl acetate for negative staining. Samples were applied for 45s, blotted with filter paper and air dried. Digital images were recorded at 67,000× magnification, on a Tecnai T12 electron microscope (FEI Company, Hillsboro, OR) operating at 120 keV acceleration and equipped with a Gatan Ultrascan 2×2K CCD camera.

Dynamic light scattering (DLS)

The size distribution of the OMVs was determined using a DynaPro NanoStar light scattering instrument (Wyatt Technology, Santa Barbara, CA). DLS data were collected from 100 μL of PBS-dissolved OMVs by ten 5 s acquisitions at 25°C with a laser at a wavelength of 659 nm.

Western Blot analysis

Subcellular fractions of *C. jejuni* were analyzed by western blotting with soybean agglutinin lectin (SBA, Vector Laboratories Inc, CA). Briefly, the fractionated samples were separated on 12% acrylamide gels, transferred to polyvinylidene fluoride (PVDF) membranes by Trans-Blot Turbo transfer system (Bio-Rad Laboratories, Inc., Hercules, CA), and probed with fluorescein-conjugated SBA lectins (50 nM in PBS) after blocking with 2.5% (w/v) bovine serum albumin in PBS for 1 h at room temperature. After washing unbound lectins, the membranes were scanned with Storm 860 Molecular Imager (Molecular Dynamics) at excitation and emission wavelengths of 450 nm and 520 nm for detection.

Sample preparation for MS analysis

Fifty micrograms of total proteins in OMV sample were digested with trypsin (Promega, Madison, WI) on Amicon ultra centrifugal filter units (30K molecular weight cutoff, Millipore, Billerica, MA) based on a method (FASP; filter-aided sample preparation) developed by Manza et al. [25] and modified by Wisniewski et al. [26]. The OMV and whole lysate (WL) were first dissolved in 1% (w/v) SDS and 0.1 M DTT in 100 mM Tris-HCl (pH 7.6) and incubated at 95°C for 5 min, respectively. Alkylation and digestion were performed according to a published protocol [21]. Desalting of digested peptides was performed on Empore High-Performance extraction disk cartridges C18-SD (3M, St. Paul, MN) as described previously [26]. The final peptide samples were dried and reconstituted with 0.1% (v/v) formic acid/water for MS analysis.

NanoLC-MS/MS analysis

Mass spectrometric experiments were performed using a nanoLC LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a nano-electrospray ion source and an Easy II nanoLC, as described previously [27] with some modifications. A 5 μL sample (1 μg of total proteins) was loaded on a self-packed 5 μm Reprosil C18AQ (Dr. Maisch GmbH, Ammerbuch-Entringen, Germany) column (75 μm × 150 mm). The mobile phases consisted of acetonitrile/formic acid/water (2/0.2/98, v/v/v) for A and acetonitrile/formic acid/water (80/20/0.2, v/v/v) for B. The FASP processed samples

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were analyzed on a 150 min gradient from 2 to 30% B. The LTQ-Orbitrap was operated in data-dependent acquisition mode to automatically alternate between a full scan (m/z 300–1700) in the Orbitrap and CID MS/MS scans in the linear ion trap. The ten most intense peptide ions were isolated for fragmentation. Helium was used as collision gas for CID. Normalized collision energy was 35% and activation time was 30 ms. Unless otherwise noted, three replicate measurements were made at each MS setting. Data acquisition was controlled by Xcalibur 2.0.7 and Tune 2.4 software (Thermo Fisher Scientific).

Database searching

Raw files were processed and searched using MaxQuant (version 1.2.2.5). Spectra were searched against the total predicted *C. jejuni* sequences from UNIPROT (1624 sequences, downloaded on 04/12/09) and common contaminants (262 sequences). The spectra were also searched against an equal number of decoy sequences to estimate the false discovery rate (FDR), similar to as described previously [28]. The specified enzyme was trypsin with up to two missed cleavages. Oxidation of methionine (+15.9949) and acetylation of protein N-terminus (+42.0106) were specified as variable modifications and carbamidomethylation of cysteine (+57.0215) was specified as a fixed modification. All other parameters were the default, including a fragment ion tolerance of 0.5 Da and a maximum precursor ion tolerance of 6 ppm after recalibration. Identifications were filtered to curate a dataset with less than 1% FDR at both the peptide and protein level.

Bioinformatic analyses

Prediction of a signal peptide for the general secretory (Sec) or the twin-arginine translocation (Tat) pathway of the vesicular proteins was performed by PRED-TAT software (http://www.compgen.org/tools/PRED-TAT) [29] and the predicted Tat motif was obtained from TATFIND 1.4 server (http://signalfind.org/tatfind.html) [30]. Additional prediction of the signal peptide for vesicular proteins was done using the SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP) [31] and using PrediSi software (http://www.predisi.de) [32]. A consensus motif for N-glycosylation (D/E-X_1-N-X_2-S/T; X_1 and X_2 can be any amino acids, except proline) was searched using an in-house algorithm.

Results

Isolation and characterization of OMVs from C. jejuni NCTC11168

OMVs, secreted by *C. jejuni* into the extracellular milieu, were prepared from culture supernatants by serial centrifugation to remove cells and larger vesicles. The isolated OMVs were fixed and examined by transmission electron microscopy (TEM). The electron micrographs revealed that the OMVs of *C. jejuni* are spherical in shape with a generally uniform size distribution (Fig. 1A). This method dehydrates the vesicles resulting in an average diameter of 50 nm. Some of the vesicles have collapsed revealing the bilayer (Fig. 1A, white arrow).

Dynamic light scattering (DLS) was used to get an accurate measure of hydrated OMVs. This method directly measures particle size confirming, the homogeneous size distribution seen by TEM. Hydrated, the mean hydrodynamic radius was 77.5 ± 4.6 nm (Fig. 1B).

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OMVs prepared in the laboratory may not reflect the natural population of OMVs but the nano-sized OMVs are considered to biologically most relevant due to their ability to access a variety of environments within the host.

Proteome profiling of OMVs using MS

Along with purifying the OMVs, the periplasm and outer membrane were fractionated. These three sample pools were separated by SDS-PAGE and visualized by Coomassie staining (Fig. 1C). Comparing the bands on the gel, OMVs appeared to possess both outer membrane and periplasmic proteins; however, there were distinct differences indicating that the OMVs were unique from both. Soybean agglutinin lectin (SBA)-reactive bands were also observed from the vesicular proteins in Western Blot analysis, supporting the likely existence of glycoproteins in the membrane vesicles (Fig. 1D). It should be pointed out that the intensity of the positive bands from the OMVs was relatively lower than those from other samples, indicating that only a small fraction of glycoproteins are present in OMV when compared to whole cell lysates. OMV proteins were digested by trypsin and the subsequent peptides were analyzed using nanoLC-MS/MS with a high resolution LTQ-Orbitrap. Raw data were searched against a UNIPROT protein database of predicted *C. jejuni* proteins. The experiment was performed three times. A total of 283 vesicular proteins were observed in the purified OMV samples. 134 of these proteins were identified in two out three experiments with high confidence (Table S1) and these were functionally classified. The vesicular proteins were categorized by the predicted subcellular localizations and biological functions (Fig. 2A and B). We observed 48 membrane-associated proteins (36%), 15 periplasmic proteins (11%) and 43 cytosolic proteins (32%). Twenty eight (21%) of them remain unknown, but five of these were predicted to possess signal peptides, indicating that they are more likely periplasmic proteins. In addition, the functional groups ‘energy production and conversion’, ‘cell wall/membrane/envelope biogenesis’, and ‘cell motility’ were present. We also observed 64 enzymes including 20 oxidoreductases (15%), 18 transferases (13%), 5 hydrolases (4%), 7 lyases (5%), 6 isomerases (5%) and 8 ligases (6%), (Fig. 2C).

Functional annotation of the genes of the vesicular proteins

To interpret the functional relationship of the diverse set of genes identified from the OMVs, the vesicular proteins were functionally annotated by DAVID tools [33]. The 134 high-quality identifications from the OMV samples were compared to 774 genes that were identified from whole-cell lysate of *C. jejuni* in this study (data not shown). The lists of functional annotation terms of related genes and clusters of the annotated terms enriched in the OMV samples can be found in Table S2 and S3. Among the terms, OMV proteins were found to be enriched for terms related to ‘energy production’ and ‘respiratory metabolism’, indicating a potential role of OMVs in respiration. Flagellar assemble- and cell adhesion-related genes were also enriched in the OMV samples. Interestingly, some of the known N-linked glycoproteins, which are encoded by *cjaA*, *dsbI*, *kpsD*, *napA*, *Cj0089*, *Cj0114*, *Cj0152c*, *Cj0268c*, *Cj0404*, *Cj0511* and *Cj0694*, were found in the vesicles. The annotation terms, which were not clustered in this enrichment analysis, are listed in Table S4.

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Signal peptide and N-glycosylation motifs in vesicular proteins

Signal peptide predictors were used to further characterize the identified vesicular proteins of *C. jejuni* since most outer membrane and periplasmic proteins are known to have a signal peptide sequence in their N-termini that marks them for localization to the periplasm [34]. Prediction of signal peptides in the vesicular proteins was performed using diverse bioinformatics tools such as SignalP 4.1, PrediSi, PRED-TAT and TATFIND 1.4, then the signal motif for the twin-arginine translocation (Tat) pathway was discriminated from the signal peptide for the general secretory (Sec) pathway [35]. Finally, fifty three vesicular proteins (40% of the total) were predicted to carry a signal peptide or a TM domain. Among those, eight vesicular proteins (6% of the total) were predicted to use the Tat apparatus, while 35 proteins (26% of the total) were assumed to be localized *via* the Sec pathway (Fig. 3A). The substrates for the Tat pathway were classified in proteins that play a role in electron transports and/or anaerobic respiration. Predicted Tat substrates and their sequences are listed in Table 2.

In addition, the bacterial N-glycosylation motif (i.e. D/E-X_1-N-X_2-S/T) was searched from the list of vesicular proteins. Thirty five proteins had the motif in their sequences, but only 11 of them have been experimentally confirmed [36, 37]. Regardless of the presence or absence of signal peptides, the glycosylation motif was present in 35 proteins and certain proteins seem to be actually glycosylated (Fig. 3B and C).

Discussions

The analysis of the OMVs isolated from *C. jejuni* unveiled the vesicular protein pool providing insight toward the role of OMVs in bacterial physiology and pathology. We isolated a homogeneous population of spherical OMVs, identifying 134 vesicular proteins by mass spectrometry. These were categorized by their biological function and predicted cellular location. The most enriched terms were related to energy generations (i.e. aerobic respiration and TCA cycle) while the most abundant enzymes belonged to the oxidoreductase family. Flagellum assembly proteins were also highly represented.

Several of the observed enzymes, such as oxidoreductases, are known to be involved in the TCA cycle, generating energy by oxidation of certain substrates during respiration. Some respiratory enzymes such as nitrate reductase (NapA), nitrite reductase (NrfA) and *cbb3*-type oxidase (CcoN) in *C. jejuni* are known to play a significant role in host colonization [38]. The *napA* or *nrfA* deficient strain showed a dramatically reduced level of colonization, while the *ccoN* mutant completely lost the ability to colonize in chicks [38]. It is also known that, instead of oxygen, *C. jejuni* can use nitrate, formate, DMSO and/or trimethylamine N-oxide (TMAO) as a respiratory electron acceptor under anaerobic condition, which implies that the ability of the reductases to use a variety of alternative acceptors is crucial in the pathology and physiology [38, 39]. Recently, Liu and coworkers reported that the change of its respiration mode of *C. jejuni* by the expression levels of those respiratory chain-associated enzymes is closely associated with the bacterial survival in host environments [11].

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In the analysis of signal peptides, eight vesicular proteins were predicted to be localized *via* the twin-arginine translocation (Tat) pathway and were classified in cofactor-containing proteins involved in electron transport chains. This agreed well with the previous results that the Tat machinery in bacteria is mainly responsible for the transport of cofactor-binding folded proteins across the cytoplasmic membrane [40]. Although only eight vesicular proteins were predicted to possess the Tat signal peptide, the actual Tat substrates being found in OMVs might be more substantial as Tat substrates can be secreted as part of a complex [40]. We also observed that some proteins have Sec signal peptides that contribute to a diverse range of cellular processes such as flagellin assembly, signal transduction, amino acid metabolism and virulence. In contrast to the Sec-protein translocation, the Tat-derived protein transport seems to be more specifically used in adaptation and survival of *C. jejuni* in diverse environments. Thus, it is possible that OMVs can be used as an export route for these crucial enzymes into the environment, to assist in respiration; alternatively, it could be a route to secrete non-functioning enzymes.

This study also identified some cytoplasmic proteins in OMVs. While Horstman and Kuehn believe that cytoplasmic proteins should not be present in OMVs [41], many proteomics studies on OMVs confirmed their presence [18, 20, 42–45]. Further analyses are certainly necessary to confirm the presence or absence of cytoplasmic proteins in OMVs. It is nevertheless worth pointing out DNA and RNA is also present in OMV, indicating that some transcriptional and ribosomal proteins may be needed in OMVs [46–49].

Cell adherence is the first step in host-pathogen interactions and adhesion factors are important components of a bacterial infection [50]. In OMVs we find flagellar proteins, well-known adhesins [51, 52], such as flagellin A, B and flagellar hook proteins. Along with rhamnolipids, flagellar proteins of *Pseudomonas aeruginosa* are known to be a major inducing factor of psoriasis in human skin [53]. Therefore, the flagellin assembly proteins found in the OMVs of *C. jejuni* might have some role in bacterial host interaction.

Outer membrane proteins (OMPs), expected to be found in OMVs, were observed such as pore-forming proteins (PorA and Omp50), fibronectin-binding proteins (CadF and Cj1279c) and a surface antigen (CjaA), each known virulence factors [15, 54–60]. In addition, several putative lipoproteins (Cj0089, Cj0396c, Cj0406c and Cj0497) and putative membrane proteins (Cj0129c, Cj0152c, Cj0268c and Cj0404) were found that are additional virulence factor candidates.

C. jejuni protein N-glycosylation is associated with bacterial host interactions [61] and 11 N-glycoproteins are found in the OMVs suggesting a role in pathogenesis. Among those, the surface-exposed CjaA is a well-known antigenic molecule and its glycosylation is known to be related to virulence [54, 62, 63]. An additional set of periplasmic and transmembrane N-glycoproteins were also identified as OMV components.

Recently, Elmi and coworkers identified 151 vesicular proteins from a hypermotile strain *C. jejuni* 11168H and showed that the OMVs with functional virulence factors [i.e. cytolethal distending toxin (CDT)] can trigger host-immune responses [64]. We found 64 proteins in common from their protein list, but 70 proteins were unique (Fig. 4). This may represent the

dynamic nature of protein expression in *C. jejuni* regarding strain-dependency or growth conditions [57, 65]. OMVs prepared in the laboratory may not reflect the full proteome of OMVs from *C. jejuni* found in eukaryotic hosts due to the dynamic protein expression profile in response to environmental stimuli. The combined proteomic data provides valuable information toward understanding the pathogenesis and survival of *C. jejuni*.

We identified some lower confidence vesicular proteins of interest (Table S5), e.g. known toxins (CdtA and CdtB) and antigenic peptides (PEB1, PEB2 and PEB3) as well as multidrug efflux components (CmeA and CmeD). The virulence factor CDT complex consisting of CdtA, CdtB and CdtC is released via OMVs causing cell distending effects on host cells [24]. PEB1, a common antigen and cell-binding factor, can be used as a candidate vaccine [66]. CmeA and CmeD function as a multidrug efflux pump contributing to bacterial resistance to a broad range of antimicrobial agents [67, 68]. Some of these (i.e. CmeA and PEB3) are known to be N-glycosylated as well. As we observed in this study, the OMVs of *C. jejuni* possess a diverse set of proteins such as enzymes, adhesins, toxins etc., implying their latent functions in biological processes. Some of the vesicular proteins could be novel antibacterial targets, diagnostic markers of *C. jejuni*-mediated diseases and/or vaccine candidates. Therefore, further biological assays need to be performed to elucidate the biological roles of each protein in this context.

Recently, it was reported that the gram-positive pathogen *Stapylococcus aureus* produces membrane-derived vesicles (MVs) inducing apoptosis of host cells [69, 70] and a secreted vesicle was also observed from the solid spores of *Streptomyces coelicolor*, a kind of actinobacteria [71]. Taken together, most bacterial cells likely produce MVs; therefore, the secretion of bacterial MVs is likely a ubiquitously observed phenomenon. As the global vesicular proteome of human pathogens becomes clear we will better understand the various biological functions of OMVs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Lee EY, Choi DS, Kim KP, Gho YS. Proteomics in gram-negative bacterial outer membrane vesicles. Mass Spectrom Rev. 2008; 27:535–55. [PubMed: 18421767]
2. Mashburn-Warren L, McLean RJ, Whiteley M. Gram-negative outer membrane vesicles: beyond the cell surface. Geobiology. 2008; 6:214–9. [PubMed: 18459967]
3. Kuehn MJ, Kesty NC. Bacterial outer membrane vesicles and the host-pathogen interaction. Genes Dev. 2005; 19:2645–55. [PubMed: 16291643]

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4. Collins BS. Gram-negative outer membrane vesicles in vaccine development. Discov Med. 2011; 12:7–15. [PubMed: 21794204]
5. Kim JY, Doody AM, Chen DJ, Cremona GH, Shuler ML, Putnam D, et al. Engineered bacterial outer membrane vesicles with enhanced functionality. J Mol Biol. 2008; 380:51–66. [PubMed: 18511069]
6. Findlow J, Balmer P, Yero D, Niebla O, Pajon R, Borrow R. Neisseria vaccines 2007. Expert Rev Vaccines. 2007; 6:485–9. [PubMed: 17669003]
7. Findlow H, Southern J, Mabey L, Balmer P, Heyderman RS, Auckland C, et al. Immunoglobulin G subclass response to a meningococcal quadrivalent polysaccharide-diphtheria toxoid conjugate vaccine. Clin Vaccine Immunol. 2006; 13:507–10. [PubMed: 16603620]
8. Allos BM. Campylobacter jejuni Infections: update on emerging issues and trends. Clin Infect Dis. 2001; 32:1201–6. [PubMed: 11283810]
9. Fujimoto S, Amako K. Guillain-Barre syndrome and Campylobacter jejuni infection. Lancet. 1990; 335:1350. [PubMed: 1971411]
10. Pearson BM, Pin C, Wright J, I'Anson K, Humphrey T, Wells JM. Comparative genome analysis of Campylobacter jejuni using whole genome DNA microarrays. FEBS Lett. 2003; 554:224–30. [PubMed: 14596944]
11. Liu X, Gao B, Novik V, Galan JE. Quantitative Proteomics of Intracellular Campylobacter jejuni Reveals Metabolic Reprogramming. PLoS Pathog. 2012; 8:e1002562. [PubMed: 22412372]
12. Taboada EN, Acedillo RR, Carrillo CD, Findlay WA, Medeiros DT, Mykytczuk OL, et al. Large-scale comparative genomics meta-analysis of Campylobacter jejuni isolates reveals low level of genome plasticity. J Clin Microbiol. 2004; 42:4566–76. [PubMed: 15472310]
13. Cordwell SJ, Len AC, Touma RG, Scott NE, Falconer L, Jones D, et al. Identification of membrane-associated proteins from Campylobacter jejuni strains using complementary proteomics technologies. Proteomics. 2008; 8:122–39. [PubMed: 18095373]
14. Rathbun KM, Thompson SA. Mutation of PEB4 alters the outer membrane protein profile of Campylobacter jejuni. FEMS Microbiol Lett. 2009; 300:188–94. [PubMed: 19824902]
15. Moser I, Schroeder W, Salnikow J. Campylobacter jejuni major outer membrane protein and a 59-kDa protein are involved in binding to fibronectin and INT 407 cell membranes. FEMS Microbiol Lett. 1997; 157:233–8. [PubMed: 9435102]
16. Blaser MJ, Hopkins JA, Vasil ML. Campylobacter jejuni outer membrane proteins are antigenic for humans. Infect Immun. 1984; 43:986–93. [PubMed: 6365789]
17. Choi DS, Kim DK, Choi SJ, Lee J, Choi JP, Rho S, et al. Proteomic analysis of outer membrane vesicles derived from Pseudomonas aeruginosa. Proteomics. 2011; 11:3424–9. [PubMed: 21751344]
18. Ferrari G, Garaguso I, Adu-Bobie J, Doro F, Taddei AR, Biolchi A, et al. Outer membrane vesicles from group B Neisseria meningitidis delta gna33 mutant: proteomic and immunological comparison with detergent-derived outer membrane vesicles. Proteomics. 2006; 6:1856–66. [PubMed: 16456881]
19. Galka F, Wai SN, Kusch H, Engelmann S, Hecker M, Schmeck B, et al. Proteomic characterization of the whole secretome of Legionella pneumophila and functional analysis of outer membrane vesicles. Infect Immun. 2008; 76:1825–36. [PubMed: 18250176]
20. Lee EY, Bang JY, Park GW, Choi DS, Kang JS, Kim HJ, et al. Global proteomic profiling of native outer membrane vesicles derived from Escherichia coli. Proteomics. 2007; 7:3143–53. [PubMed: 17787032]
21. Mullaney E, Brown PA, Smith SM, Botting CH, Yamaoka YY, Terres AM, et al. Proteomic and functional characterization of the outer membrane vesicles from the gastric pathogen Helicobacter pylori. Proteomics Clin Appl. 2009; 3:785–96. [PubMed: 21136987]
22. Nally JE, Whitelegge JP, Aguilera R, Pereira MM, Blanco DR, Lovett MA. Purification and proteomic analysis of outer membrane vesicles from a clinical isolate of Leptospira interrogans serovar Copenhageni. Proteomics. 2005; 5:144–52. [PubMed: 15672460]
23. Pierson T, Matrakas D, Taylor YU, Manyam G, Morozov VN, Zhou W, et al. Proteomic characterization and functional analysis of outer membrane vesicles of Francisella novicida

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suggests possible role in virulence and use as a vaccine. J Proteome Res. 2011; 10:954–67. [PubMed: 21138299]

24. Lindmark B, Rompikuntal PK, Vaitkevicius K, Song T, Mizunoe Y, Uhlin BE, et al. Outer membrane vesicle-mediated release of cytolethal distending toxin (CDT) from Campylobacter jejuni. BMC Microbiol. 2009; 9:220. [PubMed: 19835618]
25. Manza LL, Stamer SL, Ham AJL, Codreanu SG, Liebler DC. Sample preparation and digestion for proteomic analyses using spin filters. Proteomics. 2005; 5:1742–5. [PubMed: 15761957]
26. Wisniewski JR, Zougman A, Nagaraj N, Mann M. Universal sample preparation method for proteome analysis. Nat Methods. 2009; 6:359–62. [PubMed: 19377485]
27. Kalli A, Hess S. Effect of mass spectrometric parameters on peptide and protein identification rates for shotgun proteomic experiments on an LTQ-orbitrap mass analyzer. Proteomics. 2012; 12:21–31. [PubMed: 22065615]
28. Sun N, Pan C, Nickell S, Mann M, Baumeister W, Nagy I. Quantitative proteome and transcriptome analysis of the archaeon Thermoplasma acidophilum cultured under aerobic and anaerobic conditions. J Proteome Res. 2010; 9:4839–50. [PubMed: 20669988]
29. Bagos PG, Nikolaou EP, Liakopoulos TD, Tsirigos KD. Combined prediction of Tat and Sec signal peptides with hidden Markov models. Bioinformatics. 2010; 26:2811–7. [PubMed: 20847219]
30. Rose RW, Bruser T, Kissinger JC, Pohlschroder M. Adaptation of protein secretion to extremely high-salt conditions by extensive use of the twin-arginine translocation pathway. Mol Microbiol. 2002; 45:943–50. [PubMed: 12180915]
31. Petersen TN, Brunak S, von Heijne G, Nielsen H. SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat Methods. 2011; 8:785–6. [PubMed: 21959131]
32. Hiller K, Grote A, Scheer M, Munch R, Jahn D. PrediSi: prediction of signal peptides and their cleavage positions. Nucleic Acids Res. 2004; 32:W375–9. [PubMed: 15215414]
33. Huang da W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc. 2009; 4:44–57. [PubMed: 19131956]
34. Wickner W, Schekman R. Protein translocation across biological membranes. Science. 2005; 310:1452–6. [PubMed: 16322447]
35. De Buck E, Lammertyn E, Anne J. The importance of the twin-arginine translocation pathway for bacterial virulence. Trends Microbiol. 2008; 16:442–53. [PubMed: 18715784]
36. Scott NE, Nothaft H, Edwards AV, Labbate M, Djordjevic SP, Larsen MR, et al. Modification of the Campylobacter jejuni N-linked glycan by EptC-mediated addition of phosphoethanolamine. J Biol Chem. 2012; 287:29384–96. [PubMed: 22761430]
37. Scott NE, Parker BL, Connolly AM, Paulech J, Edwards AV, Crossett B, et al. Simultaneous glycan-peptide characterization using hydrophilic interaction chromatography and parallel fragmentation by CID, higher energy collisional dissociation, and electron transfer dissociation MS applied to the N-linked glycoproteome of Campylobacter jejuni. Mol Cell Proteomics. 2011; 10:M000031-MCP201. [PubMed: 20360033]
38. Weingarten RA, Grimes JL, Olson JW. Role of Campylobacter jejuni respiratory oxidases and reductases in host colonization. Appl Environ Microbiol. 2008; 74:1367–75. [PubMed: 18192421]
39. Kurihara FN, Satoh T. A Single Enzyme Is Responsible for Both Dimethylsulfoxide and Trimethylamine-N-Oxide Respirations as the Terminal Reductase in a Photodenitrifier, Rhodobacter-Sphaeroides Fs Denitrificans. Plant and Cell Physiology. 1988; 29:377–9.
40. Lee PA, Tullman-Ercek D, Georgiou G. The bacterial twin-arginine translocation pathway. Annu Rev Microbiol. 2006; 60:373–95. [PubMed: 16756481]
41. Horstman AL, Kuehn MJ. Enterotoxigenic Escherichia coli secretes active heat-labile enterotoxin via outer membrane vesicles. J Biol Chem. 2000; 275:12489–96. [PubMed: 10777535]
42. Molloy MP, Herbert BR, Slade MB, Rabilloud T, Nouwens AS, Williams KL, et al. Proteomic analysis of the Escherichia coli outer membrane. Eur J Biochem. 2000; 267:2871–81. [PubMed: 10806384]
43. Henry T, Pommier S, Journet L, Bernadac A, Gorvel JP, Lloubes R. Improved methods for producing outer membrane vesicles in Gram-negative bacteria. Res Microbiol. 2004; 155:437–46. [PubMed: 15249060]

44. Wei C, Yang J, Zhu J, Zhang X, Leng W, Wang J, et al. Comprehensive proteomic analysis of Shigella flexneri 2a membrane proteins. J Proteome Res. 2006; 5:1860–5. [PubMed: 16889407]
45. Xu C, Lin X, Ren H, Zhang Y, Wang S, Peng X. Analysis of outer membrane proteome of Escherichia coli related to resistance to ampicillin and tetracycline. Proteomics. 2006; 6:462–73. [PubMed: 16372265]
46. Dorward DW, Garon CF, Judd RC. Export and intercellular transfer of DNA via membrane blebs of Neisseria gonorrhoeae. J Bacteriol. 1989; 171:2499–505. [PubMed: 2496108]
47. Kadurugamuwa JL, Beveridge TJ. Virulence factors are released from Pseudomonas aeruginosa in association with membrane vesicles during normal growth and exposure to gentamicin: a novel mechanism of enzyme secretion. J Bacteriol. 1995; 177:3998–4008. [PubMed: 7608073]
48. Kolling GL, Matthews KR. Export of virulence genes and Shiga toxin by membrane vesicles of Escherichia coli O157:H7. Appl Environ Microbiol. 1999; 65:1843–8. [PubMed: 10223967]
49. Yaron S, Kolling GL, Simon L, Matthews KR. Vesicle-mediated transfer of virulence genes from Escherichia coli O157:H7 to other enteric bacteria. Appl Environ Microbiol. 2000; 66:4414–20. [PubMed: 11010892]
50. Pizarro-Cerda J, Cossart P. Bacterial adhesion and entry into host cells. Cell. 2006; 124:715–27. [PubMed: 16497583]
51. McSweegan E, Walker RI. Identification and characterization of two Campylobacter jejuni adhesins for cellular and mucous substrates. Infect Immun. 1986; 53:141–8. [PubMed: 2873103]
52. Grant CC, Konkel ME, Cieplak W Jr, Tompkins LS. Role of flagella in adherence, internalization, and translocation of Campylobacter jejuni in nonpolarized and polarized epithelial cell cultures. Infect Immun. 1993; 61:1764–71. [PubMed: 8478066]
53. Meyer-Hoffert U, Zimmermann A, Czapp M, Bartels J, Koblyakova Y, Glaser R, et al. Flagellin delivery by Pseudomonas aeruginosa rhamnolipids induces the antimicrobial protein psoriasin in human skin. PLoS One. 2011; 6:e16433. [PubMed: 21283546]
54. Pawelec D, Rozynek E, Popowski J, Jagusztyn-Krynicka EK. Cloning and characterization of a Campylobacter jejuni 72Dz/92 gene encoding a 30 kDa immunopositive protein, component of the ABC transport system; expression of the gene in avirulent Salmonella typhimurium. FEMS Immunol Med Microbiol. 1997; 19:137–50. [PubMed: 9395059]
55. Pei Z, Blaser MJ. PEB1, the major cell-binding factor of Campylobacter jejuni, is a homolog of the binding component in gram-negative nutrient transport systems. J Biol Chem. 1993; 268:18717–25. [PubMed: 8360165]
56. Zhang Q, Meitzler JC, Huang S, Morishita T. Sequence polymorphism, predicted secondary structures, and surface-exposed conformational epitopes of Campylobacter major outer membrane protein. Infect Immun. 2000; 68:5679–89. [PubMed: 10992471]
57. Scott NE, Cordwell SJ. Campylobacter proteomics: guidelines, challenges and future perspectives. Expert Rev Proteomics. 2009; 6:61–74. [PubMed: 19210127]
58. Bolla JM, De E, Dorez A, Pages JM. Purification, characterization and sequence analysis of Omp50,a new porin isolated from Campylobacter jejuni. Biochem J. 2000; 352(Pt 3):637–43. [PubMed: 11104668]
59. Bacon DJ, Johnson WM, Rodgers FG. Identification and characterisation of a cytotoxic porin-lipopolysaccharide complex from Campylobacter jejuni. J Med Microbiol. 1999; 48:139–48. [PubMed: 9989641]
60. Konkel ME, Garvis SG, Tipton SL, Anderson DE Jr, Cieplak W Jr. Identification and molecular cloning of a gene encoding a fibronectin-binding protein (CadF) from Campylobacter jejuni. Mol Microbiol. 1997; 24:953–63. [PubMed: 9220003]
61. Szymanski CM, Burr DH, Guerry P. Campylobacter protein glycosylation affects host cell interactions. Infect Immun. 2002; 70:2242–4. [PubMed: 11895996]
62. Wyszynska A, Zycka J, Godlewska R, Jagusztyn-Krynicka EK. The Campylobacter jejuni/coli cjaA (cj0982c) gene encodes an N-glycosylated lipoprotein localized in the inner membrane. Curr Microbiol. 2008; 57:181–8. [PubMed: 18584243]
63. Muller A, Thomas GH, Horler R, Brannigan JA, Blagova E, Levdikov VM, et al. An ATP-binding cassette-type cysteine transporter in Campylobacter jejuni inferred from the structure of an extracytoplasmic solute receptor protein. Mol Microbiol. 2005; 57:143–55. [PubMed: 15948956]

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64. Elmi A, Watson E, Sandu P, Gundogdu O, Mills DC, Inglis NF, et al. Campylobacter jejuni outer membrane vesicles play an important role in bacterial interactions with human intestinal epithelial cells. Infect Immun. 2012; 80:4089–98. [PubMed: 22966047]

65. Zhang MJ, Xiao D, Zhao F, Gu YX, Meng FL, He LH, et al. Comparative proteomic analysis of Campylobacter jejuni cultured at 37C and 42C. Jpn J Infect Dis. 2009; 62:356–61. [PubMed: 19762984]

66. Du LF, Li ZJ, Tang XY, Huang JQ, Sun WB. Immunogenicity and immunoprotection of recombinant PEB1 in Campylobacter-jejuni-infected mice. World J Gastroenterol. 2008; 14:6244–8. [PubMed: 18985818]

67. Akiba M, Lin J, Barton YW, Zhang Q. Interaction of CmeABC and CmeDEF in conferring antimicrobial resistance and maintaining cell viability in Campylobacter jejuni. J Antimicrob Chemother. 2006; 57:52–60. [PubMed: 16303882]

68. Lin J, Michel LO, Zhang Q. CmeABC functions as a multidrug efflux system in Campylobacter jejuni. Antimicrob Agents Chemother. 2002; 46:2124–31. [PubMed: 12069964]

69. Gurung M, Moon DC, Choi CW, Lee JH, Bae YC, Kim J, et al. Staphylococcus aureus produces membrane-derived vesicles that induce host cell death. PLoS One. 2011; 6:e27958. [PubMed: 22114730]

70. Lee EY, Choi DY, Kim DK, Kim JW, Park JO, Kim S, et al. Gram-positive bacteria produce membrane vesicles: proteomics-based characterization of Staphylococcus aureus-derived membrane vesicles. Proteomics. 2009; 9:5425–36. [PubMed: 19834908]

71. Schrempf H, Koebsch I, Walter S, Engelhardt H, Meschke H. Extracellular Streptomyces vesicles: amphorae for survival and defence. Microb Biotechnol. 2011; 4:286–99. [PubMed: 21342473]

72. Ingmer H, Brondsted L. Proteases in bacterial pathogenesis. Res Microbiol. 2009; 160:704–10. [PubMed: 19778606]

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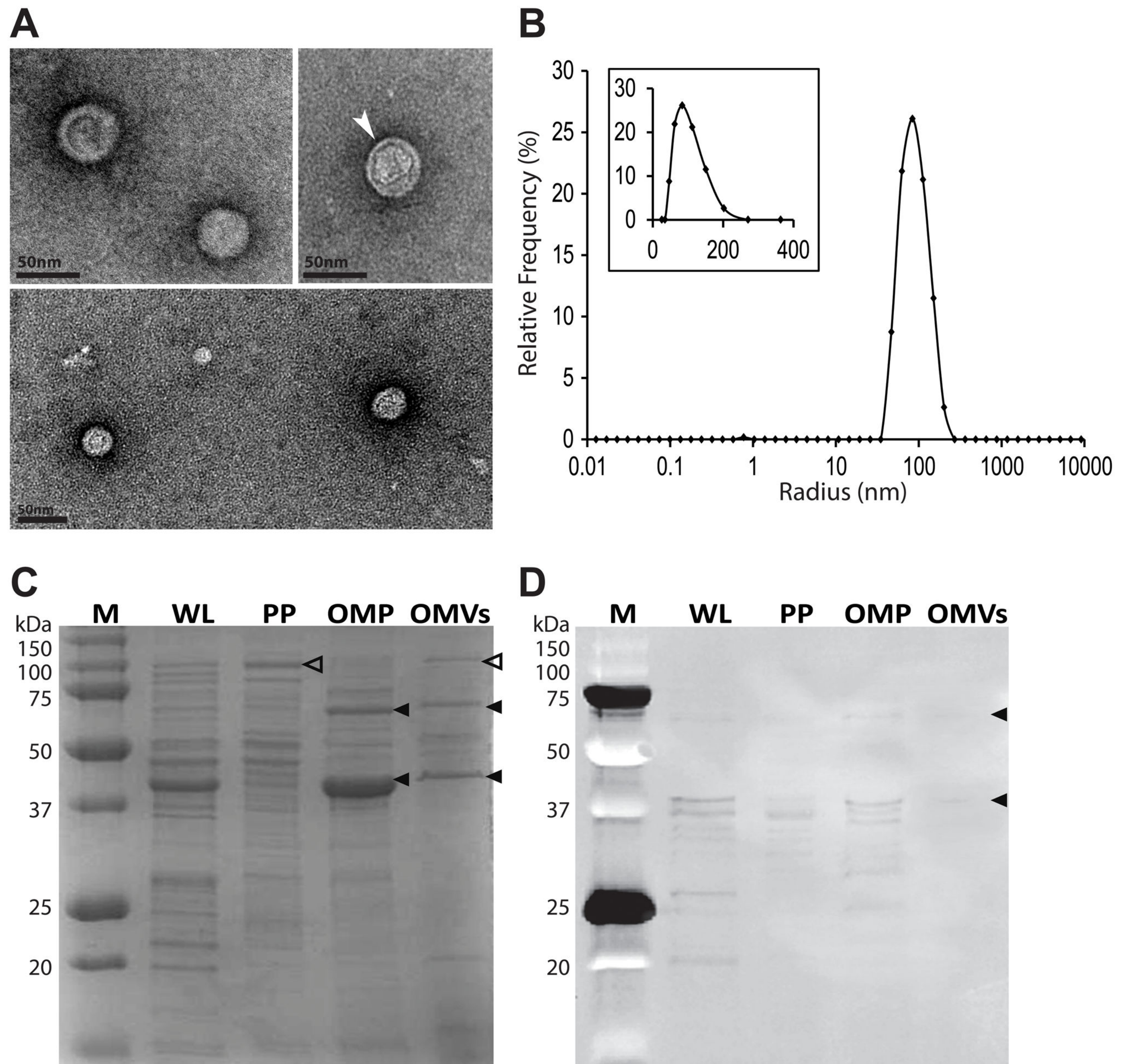

Figure 1.

Characterization of OMVs of *C. jejuni*. (A) Negative-staining TEM of OMVs. The mean diameter of those OMVs is ca. 50 nm. This TEM image shows the bilayered and spherical shaped vesicle (white arrow). (B) Size distributions of OMVs measured by DLS. The averaged hydrodynamic radius of OMVs is ca. 75 nm. (C) SDS-PAGE profiles of OMVs. M, protein marker; WL, whole-cell lysate; PP, periplasmic proteins; OMP, outer membrane proteins. Filled arrows indicate proteins observed in both OMP and OMVs, while empty arrows show proteins observed in both PP and OMVs. (D) Western Blot result probed with fluorescein-conjugated SBA lectin. Filled arrows indicate the SBA-reactive bands in the OMV sample.

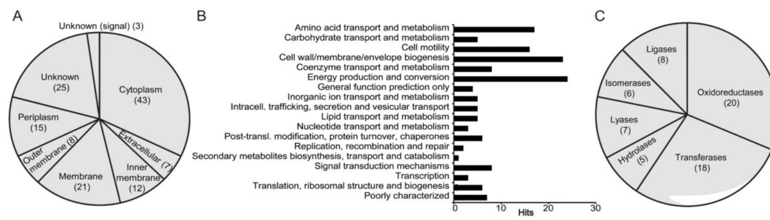

Figure 2.

Classification of cellular locations (A), functional annotation (B), and vesicular enzymes (C) of the identified proteins of OMVs. (B) The functional classification of the vesicular proteins was done based on the COG database (http://www.ncbi.nih.gov/COG).

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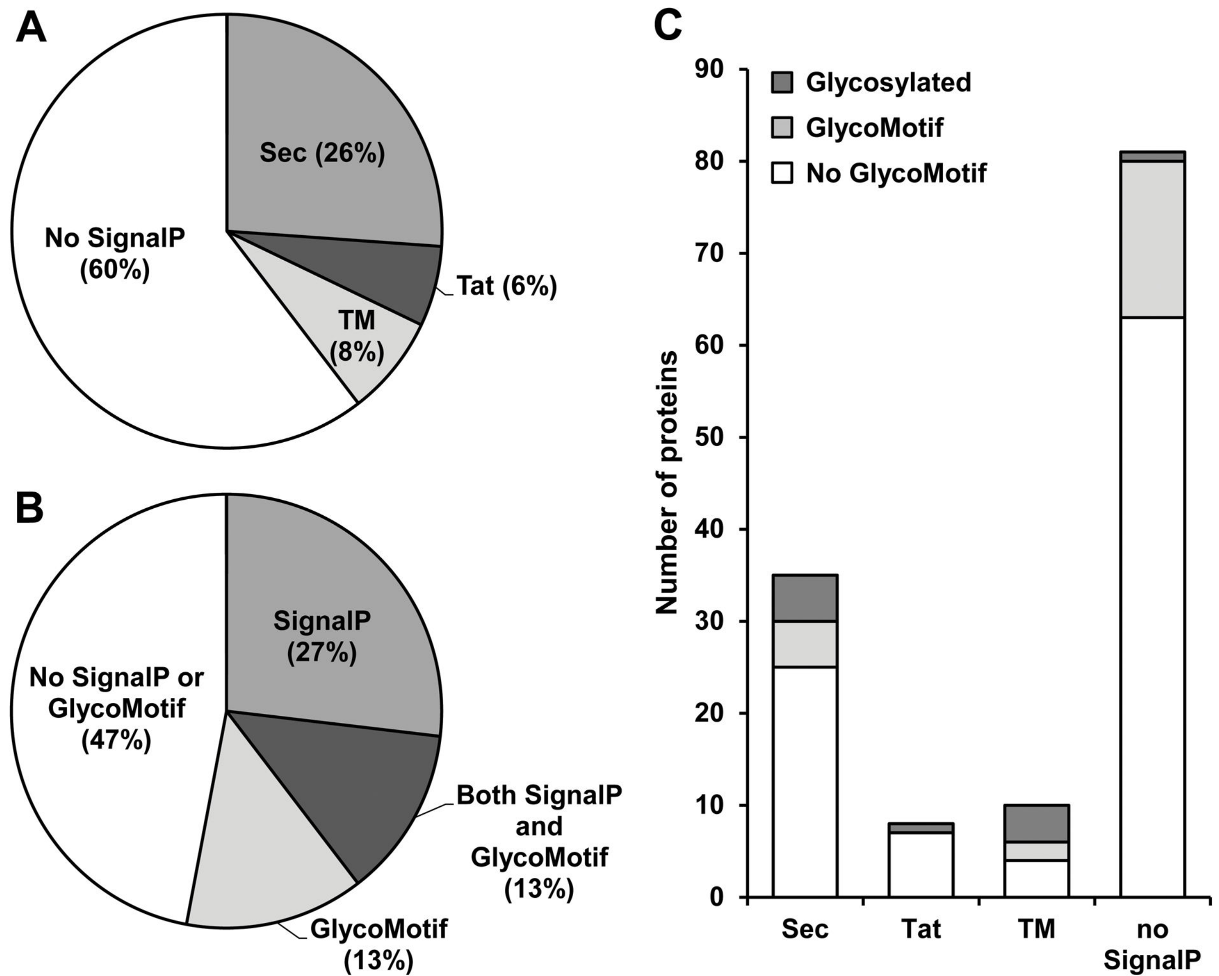

Figure 3.

Pie charts and a bar graph of the relative percentages of the vesicular proteins regarding a signal peptide (SignalP) and/or an N-glycosylation motif (GlycoMotif). (A) A SignalP for Sec or Tat pathways as well as a TM domain was separated from the proteins not carrying any SignalP. (B) SignalP and/or GlycoMotif-containing proteins were discriminated. (C) Actual glycoproteins were separated from the proteins-containing only GlycoMotif or not at all.

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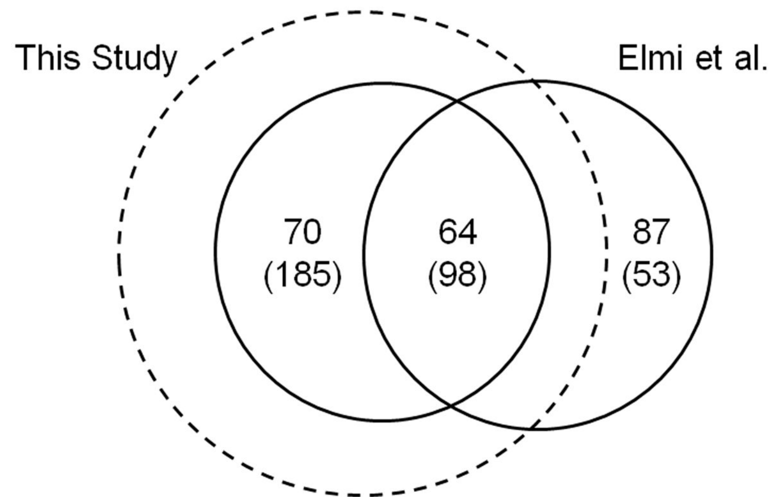

Figure 4.

Venn-diagram showing the number of vesicular proteins identified from this study and the previous study done by Elmi et al. [64]. Sixty four proteins were identified in both studies, while 70 and 87 proteins were observed in each study. Dotted circle presents the vesicular proteins even including low-confidence ones, then numbers in parentheses present proteins including the low-confidence proteins.

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Table 1

List of known N-glycoproteins, which were reported in literature [36, 37], identified from OMVs of *C. jejuni*.

Uniprot accession	Description	Gene	Subcellular localization	COG[a]	Signal peptide[b]	Related to[c]
Q0PB04	Putative secreted protease	Cj0511	Extracellular	O	Sec	Proteolysis[72]
Q0P8H0	Capsule polysaccharide export system periplasmic protein	kpsD	Membrane	M	Sec	
Q0PCA8	Disulphide bond formation protein	dsbI	Membrane	O	TM	
Q0PC48	Putative lipoprotein	Cj0089	Membrane	M	Sec	
Q0PBY5	Putative membrane protein	Cj0152c	Membrane	X		
Q0PBA6	Putative transmembrane protein	Cj0404	Membrane	M	TM	
Q0PBN7	Putative transmembrane protein	Cj0268c	Membrane	M	TM	
Q0P9S0	Putative amino-acid transporter periplasmic solute-binding protein	cjaA	Outer membrane	E	Sec	Highly immunogenic[54]
Q9PPD9	Periplasmic nitrate reductase	napA	Periplasm	P	Tat	Host colonization[38]
Q0PC23	Putative periplasmic protein	Cj0114	Periplasm	M	Sec	
Q0PAI5	Putative periplasmic protein	Cj0694	Periplasm	M	TM	

[a]COG, Clusters of orthologous groups of proteins. E, amino acid transport and metabolism; M, cell wall/membrane/envelope biogenesis; P, inorganic ion transport and metabolism; O, post-translational modification, protein turnover, chaperones; X, poorly characterized.

[b]Signal peptide motifs of the vesicular proteins were predicted using PRED-TAT. Sec, a signal peptide for the general secretory pathway; Tat, a signal peptide for the twin-arginine translocation pathway; TM, transmembrane domain.

[c]Known functions related to bacterial virulence or survival.

Table 2

List of predicted Tat signal substrates of the vesicular proteins by TATFIND 1.4.

Uniprot accession	Description	Gene	Predicted Tat motif[a]
Q9PPD9	Periplasmic nitrate reductase	napA	M**NRRDFIK***NTAIASAASVAGLSVPSS*
Q0PCC0	Molydopterin containing oxidoreductase	Cj0005c	MKQNDQKE**NRRDFLK***NIGLGLFGISVLSNFSF*
Q0PB73	Succinate dehydrogenase flavoprotein subunit	sdhA	MGEF**SRRDFIK***TACISVGALAASSSGVYA*
[*] Q0P8Y9	Ni/Fe-hydrogenase small chain	hydA	MIDYHQIESRLSALEKLPSLKDNDSITKALEKSGF **SRRDFMK***WAGAMTAFLALPASF*
Q0P8A3	Putative periplasmic oxidoreductase	Cj1516	M**NRRNFLK***FNALTLASMGVAYANPMH*
Q0P8A7	Putative formate dehydrogenase large subunit (Selenocysteine containing)	fdhA	MSSVGENIKL**TRRSFLK***MAALSSLATPLLA*
Q0P970	Ubiquinol-cytochrome c reductase iron-sulfur subunit	petA	MATSE**SRR_SFMG_**_FAFGSVAAVGGVFSLVAM_
Q0PBP1	Molybdopterin containing oxidoreductase	Cj0264c	ML**DRRKFLK***IGASLSALPLIPSLSAG*

[a]Sequence including predicted a Tat signal motif and a hydrophobic region. A Tat motif was bold and underlined. A hydrophobic region was italic and shaded.

[*]This Tat substrate was not predicted using TATFIND 1.4, but using PRED-TAT. Therefore, the Tat motif sequence was predicted manually.

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