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Biochimica et Biophysica Acta 1843 (2014) 1612–1619

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Review

Protein selection and export via outer membrane vesicles ☆

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ARTICLE INFO

Article history:
Received 1 October 2013
Received in revised form 13 December 2013
Accepted 17 December 2013
Available online 24 December 2013

Keywords:
Outer membrane vesicle
Secretion
Gram-negative bacteria
Bacterial envelope
Cargo selection

ABSTRACT

Outer membrane vesicles (OMVs) are constitutively produced by all Gram-negative bacteria. OMVs form when buds from the outer membrane (OM) of cells encapsulate periplasmic material and pinch off from the OM to form spheroid particles approximately 10 to 300 nm in diameter. OMVs accomplish a diversity of functional roles yet the OMV's utility is ultimately determined by its unique composition. Inclusion into OMVs may impart a variety of benefits to the protein cargo, including: protection from proteolytic degradation, enhancement of long-distance delivery, specificity in host-cell targeting, modulation of the immune response, coordinated secretion with other bacterial effectors, and/or exposure to a unique function-promoting environment. Many enriched OMV-associated components are virulence factors, aiding in host cell destruction, immune system evasion, host cell invasion, or antibiotic resistance. Although the mechanistic details of how proteins become enriched as OMV cargo remain elusive, recent data on OM biogenesis and relationships between LPS structure and OMV-cargo inclusion rates shed light on potential models for OM organization and consequent OMV budding. In this review, mechanisms based on pre-existing OM microdomains are proposed to explain how cargo may experience differing levels of enrichment in OMVs and degrees of association with OMVs during extracellular export. This article is part of a Special Issue entitled: Protein trafficking and secretion in bacteria. Guest Editors: Anastassios Economou and Ross Dalbey.
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1. Introduction

All Gram-negative bacteria studied to date release outer membrane vesicles (OMVs) in every stage of growth and in a range of different environmental conditions [1–4]. Originating from the cell envelope, OMVs are spheroid particles, approximately 10 to 300 nm in diameter, composed of a membrane bilayer enclosing a proteinaceous lumen [4]. Components of OMVs include lipopolysaccharide (LPS), phospholipids, DNA, RNA, as well as proteins localized to the cytoplasm, inner membrane (IM), periplasm, and outer membrane (OM).

Formation of vesicles is a ubiquitous process, occurring in liquid culture, solid culture, and in biofilms [5]. Notably, OMVs contain newly synthesized proteins and form in the absence of cell death or bacterial lysis [6–9]. OMVs are observed in the process of budding and pinching off from multiple sites on the bacterial OM by electron microscopy [1,3,7,10–12].

Much research has delved into the complex cellular regulation of OMV production. The regulation of OMV production seems to be dependent upon a number of factors including environmental conditions, pathogenicity, and the overall cellular metabolic state. Neither the rates of OMV production between various bacterial species nor the production rates for a single species between varying environmental conditions are uniform.

Quantitative analyses have demonstrated that *Escherichia coli* packages an estimated 0.2–0.5% of OM and periplasmic proteins into OMVs [6,13,14]. Approximately 1% of OM material is incorporated into vesicles for typical lab strains of *E. coli* and *Pseudomonas aeruginosa*, whereas log-phase cultures of *Neisseria meningitidis* incorporate 8–12% of total protein and endotoxin into vesicles [15–18]. The utilization of OMVs for protein export is suggested by experimental evidence of enrichment and exclusion of membranous and soluble cargo in vesicles as compared with their respective concentrations in whole bacteria, the periplasm, or the OM. As formation and release of OMVs results in the export of more cellular material than other methods of secretion, vesicle production is likely highly regulated and optimized for maximum functionality.

2. The OMV pathway in comparison with other methods of protein export

Protein export via OMVs offers several distinct advantages over other secretory mechanisms. Firstly, the protein cargo exits the cell in a medium inaccessible to extracellular degradative enzymes. Proteins located in the vesicle lumen are insensitive to protease treatment [19,20]. Accordingly, vesicles are thought to be capable of long-distance transport. OMV antigens disperse to sites far from initial colonization, such as the urine, blood, and a number of organs of *Borrelia burgdorferi*-infected mice, dogs, and humans [21].

Protein cargo bereft of a mechanism to self-direct to target cells may utilize adhesins or other surface-associated virulence factors present on the OMV surface for transport to an appropriate destination. For example, *Actinobacillus actinomycetemcomitans* leukotoxin or other unknown

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http://dx.doi.org/10.1016/j.bbamcr.2013.12.011

factors can target OMVs to host cells. Leukotoxin binds nucleic acid on bacterial membrane surfaces and interacts with the host cell surface receptor β-2 integrin [24,25,27,28]. However, the association of *A. actinomycetemcomitans* OMVs with the host cytoplasmic membrane may occur independently of leukotoxin [25]. *A. actinomycetemcomitans* vesicles are enriched not only in leukotoxin, but also in an OmpA homologue, an OM lipoprotein component of an ABC transporter, as well as a minor lipid species not detectable in the OM, and these factors may be critical in directing OMVs to designated host cells [24]. In *E. coli* K1, OmpA interacts with surface receptor Ecgp on brain microvascular endothelial cells to mediate invasion [29,30]. It is possible that OmpA in *A. actinomycetemcomitans* OMVs plays a similar role to mediate OMV invasion.

In regard to cargo, OMV-mediated secretion uniquely enables export of membrane-embedded proteins, membrane-associated proteins, and other proteins which lack canonical secretion signal sequences. *Acinetobacter baumannii* utilizes OMVs for secretion of AbOmpA, a porin which allows for the passage of small solutes across the OM [22]. This protein is abundant in culture supernatants and functions as a virulence factor by directly contributing to host cell death. Proteomic analysis of *A. baumannii* OMVs reveals the presence of several other virulence factors besides AbOmpA, including active AmpC β-lactamase, a putative hemolysin, and a Resistance-Nodulation-Cell Division (RND) superfamily transporter (which catalyzes substrate efflux via an H^+ antiport mechanism) [21]. In the absence of OMV formation, membrane proteins like OmpA must rely on bacterial-host cell contact for delivery. Vesicles, however, may adeptly accomplish interactions with target host cells, reaching even those cells residing in deep tissue often inaccessible to colonizing bacteria [5].

Additional advantages to packaging cargo into OMVs are that cargo molecules may reach their destination in a concentrated manner and/or simultaneously with other bacterial factors. *P. aeruginosa* vesicles concurrently deliver multiple virulence factors, including β-lactamase, Cif, hemolytic phospholipase, and alkaline phosphatase, to host cells in their fully-folded and enzymatically active forms [23]. The delivery of multiple virulence factors in a single concentrated bolus may allow pathogens to impact host cells in a more complex, nuanced manner.

Furthermore, the specific environment offered by a membranous compartment may allow for the establishment of a more potent or biologically active enzyme. *A. actinomycetemcomitans* utilizes OMVs for export of a Type-I secreted RTX family-member, leukotoxin [24,25]. Once inside human polymorphonuclear leukocytes and monocytes, toxin activity induces cell lysis. Leukotoxin is not only an enriched component of OMVs, it is also more potent when associated with vesicles than with the OM [24].

Similarly, OMVs could facilitate the formation of higher-order complexes from periplasmic or OM precursors. The pore-forming cytotoxin ClyA exemplifies this advantage as its oligomeric OMV-associated form exhibits higher cytotoxic activity than its monomeric periplasmic form [26].

3. Proteins which utilize the OMV pathway

Bacteria export an assortment of proteins via OMVs. Functionally, many vesicle-associated proteins are virulence factors, playing diverse roles in invasion, adherence, antibiotic resistance, damage to host cells, modulation of the host immune response, biofilm formation, and promotion of virulence. Other vesicle-associated proteins may perform roles in interspecies cooperation and intercellular communication. For concision, only bacterial products which have been functionally analyzed in association with OMVs will be discussed (see Table 1 for a comprehensive list).

3.1. Invasion

Proteins which enhance the invasive abilities of bacteria may associate with OMVs to increase the invasive abilities of cells or may simply promote more efficient OMV internalization. Vesicles containing Ail, a protein known to confer an invasive phenotype to laboratory *E. coli* strains, exhibit 10-fold higher association with host cells than vesicles without Ail [13,31]. In *Shigella flexneri*, host uptake of vesicles is predicted to be catalyzed by OM-localized invasins IpaB, C, and D [32].

3.2. Adherence

Components present in *Actinobacillus* vesicles enhance the ability of *Actinobacillus* cells to adhere to oral KB epithelial cells [33]. Exactly how OMV-localized material could influence the adherence of parent bacteria is unknown. Potentially, the OMVs could bind epithelial cells and bridge the interaction between bacterial and mammalian cell. Alternatively, internalized OMVs could initiate changes in epithelial cell surface receptor expression to enhance the ability of bacterial cell binding.

However, not all OMV-based adhesins positively impact bacterial adherence. For instance, the OM proteins OspA and OspB bind bacterial cell receptors on human umbilical vein epithelial, dendritic, and epithelial cells. As these proteins are present on the surface of both OM and vesicles, the vesicles of *B. burgforferi* compete for host cell binding with bacterial cells [34].

3.3. Antibiotic resistance

For most Gram-negative bacteria, β-lactamase activity plays a key role in raising the intrinsic level of resistance to penicillins and cephalosporins [35]. *P. aeruginosa* packages β-lactamase into the lumen of vesicles as a means of promoting resistance to β-lactam antibiotics [36]. Interestingly, the resistance gained from this type of OMV export could be considered a community good, as extracellular β-lactamase benefits the entire bacterial population and not just the cells which produce the resource. Additional components present in *P. aeruginosa* vesicles which are able to kill other species [37] may serve to reduce instances of social 'cheating,' or use of the public good without equal contribution.

3.4. Damage to host cells

Enterohemorrhagic *E. coli* O157:H7 and *Shigella dysenteriae* both package active Shiga toxin, Stx1 and Stx2, into OMVs [38,39]. Shiga toxin is an AB_5 family toxin with RNA-N-glycosidase activity which works to inhibit eukaryotic protein synthesis [40]. Shiga toxin appears to localize to both the OMV lumen and surface, as suggested by its partial protease sensitivity and partial protease protection [19,38,39].

The OMVs of enterotoxigenic *E. coli* (ETEC) contain a substantial portion of physiologically active heat-labile toxin (LT) [41]. A member of the AB_5 toxin family, LT uses the G_{M1} ganglioside binding capacity of its B subunit to enter intestinal epithelial cells before the enzymatic A subunit inactivates adenlyate cyclase, leading to increased cAMP levels and eventual chloride efflux. *Vibrio cholerae* has also been reported to secrete a portion of the highly related cholera toxin (CT) via OMVs [42]. Like LT, CT also seems to mediate host cell internalization of the pathogen-derived vesicles [13,42].

Campylobacter jejuni exports biologically active cytolethal distending toxin (CDT) in association with vesicles [43]. CDT is a genotoxin responsible for eukaryotic cell cycle arrest at the G2/M stage. Unlike most other Gram-negative pathogens, the *C. jejuni* genome gives no evidence of identifiable secretion systems for delivery of virulence factors into host cells [44]. Accordingly, export of CDT via OMVs may be the major mechanism for coordinated release of the tripartite toxin.

3.5. Modulation of host immune response

Vesicle-associated proteins possess the capability to enact either pro- or anti-inflammatory effects on host immune systems. *Porphyromonas gingivalis* preferentially packages its major virulence factors, a group of proteases named gingipains, into OMVs [45]. Gingipains degrade

Table 1
Comparison of OMV-associated proteins: Characteristics, alternative secretion mechanisms, and degree of OMV-association.

OMV-associated proteins		Complex	Protein type[1]/secretion pathway[2]	Enrichment/Surface exposure[3]	Number of exported forms[4] (% OMV-associated)	References
β-lactamase					2	[36]
Leukotoxin (Ltx)	*A. actinomycetemcomitans*		S/Type I	E/+		[22]
	C. jejuni		S/Type I	E/+	2	[73]
Shiga toxins (Stx, Stx1, Stx2)		AB_5	S/Type III		2	[37,38]
Heat-labile enterotoxin (LT)		AB_5	S/Type II	E/+	2 (95%)	[40]
Cholera toxin (CT)		AB_5	S/Type II		2	[41]
Cytolethal distending toxin (CDT)	*A. actinomycetemcomitans*	CdtABC	S + OMLP	E/+	2 (>95%)	[42,73]
	C. jejuni, *E. coli*	CdtABC	S + OMLP	E/+	2	[43,45,63,67]
Gingipains (RgpA, RgpB, Kgp)			S + OM	+	2	[44,80–82]
Vacuolating toxin (VacA)			S + OM/Type V	+	2 (25%)	[69]
α-Hemolysin (HlyC)			S/Type I	E	2 (2–66%)	[67]
Cytotoxin ClyA		multimer	S + OM	E		[30,53]
RTX (repeat-in-toxin) toxin			S + OM/Type I	E/+	2	[76]
Cytotoxicity-associated immunodominant antigen (CagA)			S/Type IV	N/+	2	[59]
Heme-utilization protein (HmuY)			OMLP			[48]
PhoP-activated gene C (PagC)			OMP	E	1	[54]
PhoP-activated gene J, K1, K2 (PagJ, PagK1, PagK2)			S		1	[52]
Surface protein A1,A2 (UspA1, UspA2)		oligomer	OMP		1	[55]
CFTR inhibitory factor (Cif)			S		2	[77,78]
Invasins (IpaB, IpaC, IpaD)			OMP		1	[32]
Outer surface protein A, B (OspA, OspB)			OMLP		1	[34]
Serralysin			S/Type I	E	2	[61]
High temperature requirement A protein (HtrA)		oligomer	S	E/+	2	[60]
Outer membrane porin A (OmpA)			OMP		1	[22]
Adhesins (BabA, SabA)			OMP	X/+	1	[60]
Aminopeptidase				E/+		[11,79]

[1] S: soluble protein; OMLP: outer membrane lipoprotein; OM: OM-association; and OMP: outer membrane protein.
[2] Secretion system (Types I–V) used by the protein to traverse the bacterial envelope is indicated.
[3] E: enriched as OMV cargo; N: neutral, neither enriched nor excluded as OMV cargo; X: excluded from OMV cargo; and +: Surface-exposed on OMVs.
[4] 1: OMV-associated form only; and 2: OMV-associated and OMV-independent forms.

cytokines in order to down-regulate host inflammation and immune response. In contrast, *C. jejuni* OMVs work to provoke immune response. OMV-mediated export of sixteen immunogenic N-linked glycoproteins delivers proteins normally localized to the *C. jejuni* periplasm to host cells [44].

Soluble ETEC LT elicits ultimately similar cytokine responses as OMV-associated LT but through different activation pathways [46]. However, comparison between the two export forms shows kinetic differences in CREB activation, possibly due to the manner in which the different presentations of the toxin are trafficked in the cell. In contrast, macrophage cells sense both lipid and protein components of *P. aeruginosa* OMVs to produce unique, strain-specific cytokine responses [47].

3.6. Biofilm formation

P. gingivalis OMVs contain the surface-exposed heme-utilization protein (HmuY), a putative heme-binding lipoprotein associated with the OM [48]. HmuY aids in biofilm formation and cell survival during periods of starvation characteristic of plaque conditions [48].

3.7. Promotion of virulence

OMV production levels increase during conditions that stress the bacterial envelope, such as host conditions during infection [49–51]. In *Salmonella typhimurium*, the two-component system PhoP/Q directly and indirectly regulates changes in LPS structure and expression of virulence-related proteins necessary for the bacteria to transition from an external environment to the host environment. A set of PhoP/Q-activated genes, including *pagC*, *pagK1*/*K2*, and *pagJ*, encode proteins which are associated with OMVs [52,53]. Although the function for PagC is currently unknown, a PagC-deficient strain displays decreased resistance to complement-mediated killing [54]. PagK and PagJ play unknown roles in virulence but as both proteins are only 39 amino acids long, it is unlikely that they possess enzymatic activity [52].

3.8. Interspecies cooperation

Moraxella catarrhalis OMVs export proteins that bind and inactivate the complement protein C3. This secretion, acting as a sort of 'community good', benefits *M. catarrhalis* and a co-inhabitant, *Haemophilus influenza*, by promoting survival [55].

4. Mechanistic models for protein export via OMVs

Electron microscopy data suggests a simplistic vesicle formation model: the OM blebs outwards and pinches off, encapsulating lumenal content derived from the periplasm. Yet the composition of the OMV membrane often differs from the cell OM; likewise, the OMV lumen differs from the cell periplasm, suggesting a specific and/or selective mechanism for OMV cargo inclusion.

Preferential packaging of specific OM lipid and protein components into vesicles may originate during the cell envelope biogenesis processes. Theoretically, compositionally distinct microdomains could result from the insertion of new membrane material by LPS and OM protein biogenesis mechanisms [56]. Addition of new membrane material is believed to occur in distinct patches which subsequently remain relatively stable due to the unique nature of the outer leaflet of the OM. Specifically, the outer bilayer LPS molecules and many OMPs are not observed to substantially diffuse (except by cell growth) [56]. Thus, OM microdomains formed during envelope biogenesis could possess compositional differences rendering certain areas more or less prone to form OMVs.

Sequestered into domains, particular LPS and OMP compositions (both dependent on environmental conditions) could function as 'signposts' for sites of vesicle initiation (green symbols, Fig. 1A). The 'signpost' properties may pertain to physical membrane attributes such as distinct curvature, fluidity, charge, and/or affinity. Appropriate inner leaflet composition could organize to complement these features, as phospholipids and lipoproteins within the inner leaflet likely experience greater freedom to diffuse. Potentially, interleaflet coupling with LPS acyl

chains and the locations of pre-existing covalent linkages to peptidoglycan could influence this process. Ultimately, the establishment of these OMV formation-competent domains may trigger lumenal cargo enrichment, detachment from the underlying peptidoglycan, and recruitment of any vesicle-formation machinery.

In the following section, multiple models are proposed which attempt to explain how the enrichment and exclusion of proteins and lipids occurs and ultimately results in OMV formation (Fig. 1). More mechanistic details of how envelope architecture impacts OMV formation have been comprehensively discussed in other reviews [57,58].

4.1. OMV cargo enrichment or exclusion

Notably, the mechanism by which certain protein cargo is enriched or excluded from vesicles remains elusive. Enrichment is achieved when the concentration of a certain protein cargo, normalized to major OMP concentration, lipid concentration, or total protein, is higher relative to that protein's concentration in the whole cell or a particular cellular compartment. Negative enrichment, where relative concentration of a protein is higher in the cell than in OMVs will be referred to as exclusion.

Evidence suggests that the cellular localization of a protein greatly affects its potential for inclusion into OMVs. For instance, in *Helicobacter pylori* the majority of OM proteins (77%) can be found in OMVs [59]. Even so, some highly abundant OM proteins are actively excluded from OMV export. For instance, the *Serratia marcescens* OMPs TolC, LptD, maltoporin, and YaeT are present in the OM yet are absent or undetectable in OMVs [60]. This type of exclusion observed by OMV proteomic studies is a theme throughout a wide range of Gram-negative species. Excluded OMPs, lacking the ability to diffuse, could act to

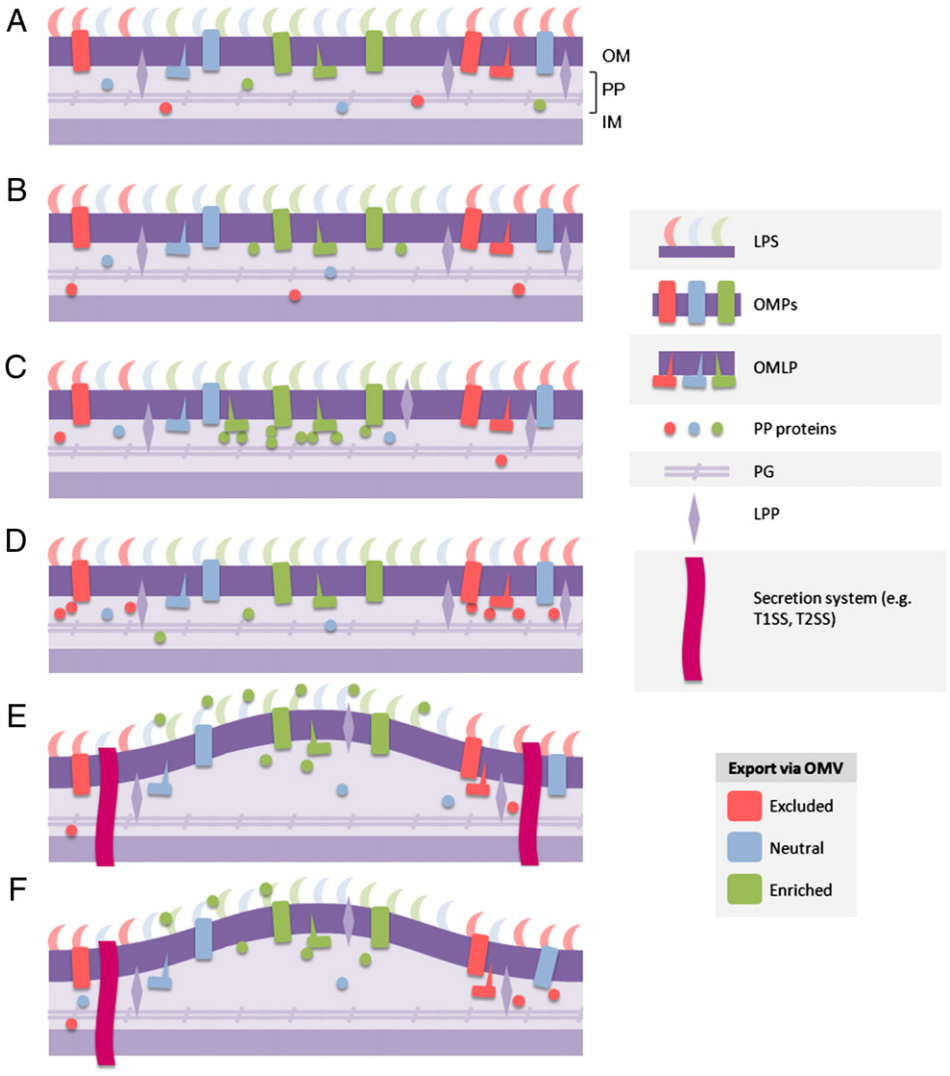

Fig. 1. Models of envelope organization prior to outer membrane vesicle (OMV) budding that could lead to cargo enrichment and exclusion. A. Outer membrane proteins (OMPs) and lipopolysaccharide (LPS) are likely found in pre-existing microdomains in the outer membrane, resulting directly from the membrane biogenesis processes. The content clustered in one area may be inherently more prone to budding. This organization can lead to the enrichment of particular OMPs into OMVs. Subsequent partitioning of envelope components can lead to enrichment of other envelope factors (B–F). Envelope features for parts B–F are as indicated in part A: outer membrane (OM); periplasm (PP); inner membrane (IM); and individual envelope components coded by shape (indicated in the side legends) and color (OMV excluded cargo: red symbols; enriched cargo: green symbols; neither enriched/excluded cargo: blue symbols). B. The localization of periplasmic proteins (PP proteins) to the external face of the inner membrane (IM) may lead to exclusion from OMVs while localization to the OM may enhance incorporation into OMVs. C. Recruitment factors, potentially OM lipoproteins (OMLPs) or OMPs (green symbols), may bind to or interact with OMV cargo to enhance its localization to future budding sites. D. Exclusion factors, such as OMPs or OMLPs excluded from OMVs (red symbols) may interact with or bind to PP proteins in order to hinder their incorporation into OMVs. E. Protein secretion apparatuses may exist near potential sites of budding. If their secretion substrate binds an OM component or OM-associated component, then these secreted proteins may cluster on the external surface. These areas of high local protein concentration could cause crowding and thereby provide the energy for establishment of positive curvature. F. A combination of all of the previously-described mechanisms most likely is at work to promote budding and OMV formation. In each of the models, lipoprotein (LPP)—peptidoglycan (PG) crosslinks are notably absent from potential OMV bud sites, either by an exclusion mechanism or by conformational changes to the LPP.

mark a region of OM for retention (red OMP symbols, Fig. 1A) and recruit other excluded membrane and periplasmic factors (red periplasmic proteins, OMLPs, and LPS, Fig. 1B–F).

In an intriguing juxtaposition, the *S. marcescens* OMP MipA is found exclusively in OMVs and is undetectable in OM [60]. In the case of proteins like MipA, absence from the OM suggests a mechanism whereby specific newly synthesized proteins are targeted for incorporation into the OM before immediate export via OMVs (green OMP symbols, Fig. 1A). In this scenario, the localization of active Bam complexes, protein complexes consisting of a single OMP and four OM lipoproteins (OMLPs) which catalyze the assembly of OMPs by an unknown mechanism, would predict locations of future budding OMVs [61]. Bulk flow of envelope content into these sites by random diffusion events would lead to neutral (neither enriched nor excluded) incorporation into OMVs.

A protein localized to the periplasmic space but tightly associated to the IM (red periplasmic proteins, Fig. 1B) will likely experience lesser incorporation into OMVs than an OM-associated protein (green periplasmic proteins, Fig. 1B). For instance, removal of 11C-terminal residues from ClyA abolishes its OMV-association as ClyA becomes associated with the IM [62]. In contrast, periplasmic proteins associated with the inner leaflet of the OM may display higher rates of incorporation into OMV. Localization of this sort plays a key role in the export of CDT. In *A. actinomycetemcomitans* and *C. jejuni*, the three subunits of CDT (CdtA, B, and C) are translocated to the periplasm. The "lipobox" contained within the N-terminal signal sequence of CdtA targets this subunit for lipid modification. This modification targets CdtA to the OM as a lipoprotein [63]. With CdtA acting as a spatial anchor, the full CDT complex forms in association with the OM prior to additional processing and eventual export via OMVs. It is possible that lipid-modifications and other interactions with OMLPs play a significant, but as of yet unidentified, role in the mechanism of cargo selectivity and vesicle formation. Study of OMLPs may reveal novel ties to OMV formation or production. Approximately 100 different OMLPs exist in the *E. coli* OM, yet very few have known functions [35].

OMV secretion or cellular retention decisions for envelope proteins may be governed by tags (Fig. 1C, D). In *S. typhimurium*, the N-terminal signal sequence from PagK is necessary for its periplasmic localization but is not sufficient for incorporation into OMVs [52]. However, the cytolysin ClyA of *E. coli* accumulates in the periplasmic space prior to surface exposure and OMV-export without the cleavage of any N-terminal signal sequence [62]. No common primary structure has been identified as a OMV sorting or exclusion signal, however it is possible that a more complex tag, utilizing properties from a protein's three-dimensional structure, could direct specific cargo into vesicles.

Cargo proteins could also directly promote their own export through localized, physical enhancement of OMV production (Fig. 1E). In *S. dysenteriae*, treatment with mitomycin C induces higher Shiga toxin expression. The increased production of Shiga toxin, a known OMV cargo protein, leads to increased OMV production, toxicity, and size [38]. Directed localization of secretion apparatuses and/or Bam complexes could promote high local concentrations of cargo protein within a region of periplasmic space, within the OM, or attached to the cell surface. A high local periplasmic or OM concentration of a cargo protein could accelerate OM budding. In the case of surface-associated proteins destined for OMV export, the high concentration of proteins bound to the membrane may impart a protein-protein crowding effect that accelerates formation of OM curvature appropriate for OMV formation (green external symbols, Fig. 1E).

Alternatively, sites of budding vesicles may contain protein and/or lipid recruitment factors which lead to the accumulation of appropriate OMV cargo. In *P. gingivalis*, mutations which alter LPS structure disrupt normal protein sorting into OMVs [44]. In *P. aeruginosa*, two classes of LPS, one negatively-charged and one neutral, exist in the OM, yet primarily negatively-charged LPS is found in vesicles [11]. This negatively-charged LPS is required for the formation of peroxide stress-induced OMVs [64].

In most cases, the mechanistic cause for differential degrees of enrichment or exclusion for various protein cargo in OMVs is unclear, and a combination of factors is likely important in promoting envelope distension and OMV formation (Fig. 1F).

4.2. OMV-associated versus OMV-independent export

For some OMV-associated proteins, extracellular release is only possible as OMV cargo. Most of these proteins either are not substrates for other secretion systems or are embedded in the OM. Alternatively, some proteins which utilize other secretion systems bind LPS with such a high affinity that the vast majority of extracellular protein observed is in an OMV-associated state. Such is the case for the heat labile enterotoxin (LT), with the vast majority (95%) of LT activity in ETEC culture supernatant detected in the OMV fraction [40]. The ability of LT to bind LPS allows it to remain associated with the bacterial OM. Accordingly, extracellular LT is found both on the surface and interior of OMVs. The toxin's ability to bind LPS as well as intestinal epithelial cell receptor G_{M1} ganglioside allows it to target LT-laden OMVs to host cells and initiate internalization [13].

The strength of a certain protein's association with OMVs may be related to the affinity of the protein for LPS. Many gingipains and RTX toxins (including leukotoxins and hemolysins) also bind or form high-molecular weight complexes with LPS [65,66]. However, not all of these proteins are found as highly-associated with OMVs as LT. Instead, both OMV-associated and OMV-independent forms coexist extracellularly. An in-depth study of the association of α-hemolysin with vesicles from different strains of *E. coli* reveals an important relationship between LPS structure and toxin enrichment. Extraintestinal *E. coli* exports α-hemolysin (HlyC), a fatty acid acyltransferase and RTX toxin family member, to the extracellular space through a contiguous protein transmembrane channel. Despite being a known Type I secretion substrate, a portion of extracellular HlyC is vesicle-associated. Four extraintestinal *E. coli* isolates (likely R1 core type) display from 1.7% to 31.4% OMV-associated HlyC in culture supernatants. Two *E. coli* K-12 derivatives (K-12 core type) expressing HlyC show 11% and 17% of toxin as OMV-associated, while in a previously-identified high OMV-producing mutant of MC1061 (galE15 galU galK16, conferring a truncated "rough" LPS core), 66% of the α-hemolysin is vesicle-associated. The differences in LPS structure between these strains may influence the association of α-hemolysin with OMVs and hint at a greater, but as of yet undiscovered, role for OM lipids and lipopolysaccharide in OMV formation [67].

However, both OMV-associated export and OMV-independent export also occur for many OMV-associated proteins which are not known to bind or associate with LPS. Functionally, multiple methods of release for a single protein into the external milieu could enhance different aspects of protein activity. In the case of two virulence factors of *H. pylori*, VacA and CagA, OMV-association affects function, targeting, and likelihood of delivery with other proteins. In highly virulent *H. pylori* strains, the oncoprotein CagA and vacuolating toxin VacA are involved in a functional relationship with one another thought to fine-tune the bacterial-host interaction. Both VacA and CagA are OMV-associated and found on the surface of vesicles [59,68–70]. The presence of VacA on *H. pylori* OMVs increases the rate of vesicle association with host cells, as VacA is known to bind sphingomyelin, glycosphingolipids, GPI-ancohored proteins, and receptor-like protein tyrosine phosphatase β [71]. However, VacA is not found exclusively associated with vesicles in culture supernatants. In two strains of *H. pylori*, only 25% of extracellular VacA is OMV-associated [69]. Likely the presence or absence of CagA in VacA-containing vesicles adds further complexity to the interaction of *H. pylori* with the host. Additionally, the OMV-associated toxin displays poor vacuolating activity, indicating that the two export methods of VacA, OMV-associated and OMV-independent, may play different pathobiological roles. This difference between OMV-associated and OMV-independent VacA may arise in part due to the necessity for urease, an enzyme secreted independently of OMVs by *H. pylori*, to

induce VacA toxicity [70]. Internalized VacA remains stable in eukaryotic cells and its toxicity may be induced days post-internalization [72].

4.3. OMV subpopulations

As vesicles serve many diverse functions for bacteria, a population of OMVs is likely varied in composition and size to more optimally accomplish a smaller number of these roles.

In *A. actinomycetemcomitans*, active CDT (as well as the previously-discussed leukotoxin) associates tightly with OMVs [73]. After density-gradient centrifugation, analysis of fractions for CdtB, LtxA, and OmpA reveals potential vesicle subpopulations. Higher density vesicles contain CDT while the vesicles from lower density fractions contain LtxA. Intermediate fractions display the presence of both CDT and LtxA, representing either a third population containing both toxins or an overlap between two content-disparate populations in size-profiles and lipid-to-protein ratios.

In *H. pylori* OMVs, analysis of density-gradient centrifugation fractions reveals an interesting relationship between OMV subpopulations and cargo enrichment. VacA, known to be enriched in vesicles, is present in all fractions in equivalent concentrations from fractions 5–11. CagA, which is neither enriched nor excluded from vesicles (by protein content) in comparison with its concentration in the OM, can only be observed in the higher density fractions (6–11) and its concentration appears to increase with increasing vesicle density. Two moderately-excluded adhesins, BabA and SabA, display similar profiles in the gradient, being present throughout fractions 3–11 with peaks in concentration around fraction 7 [59]. As both VacA and CagA also display adhesive properties, the lower concentration of BabA and SabA in the higher density vesicles which contain the most VacA and CagA may indicate that different populations of OMVs could be targeted to different host cells and contain finely-tuned ratios of virulence factors in order to play specialized roles in pathogenicity.

Further analysis of OMVs may be necessary in other Gram-negative species to ascertain the existence, relationship with protein enrichment, and function of multiple vesicle subpopulations.

4.4. Timing of OMV production

Proteomic analyses and many other studies have yet to fully account for the effect of the many cellular and environmental factors which feed into the complex process of OMV formation and regulation on sample preparation. Vesicles originate from a dynamic cell envelope with structure and composition constantly changing in response to cellular and environmental signals. As such, OMV samples collected from cultures grown overnight are beset with several problems which make it difficult to identify potential vesicle subpopulations and complicate the discovery of a single conserved "vesicle formation machinery."

Firstly, a culture grown overnight from initial lag phase to the eventual stasis in stationary phase will have undergone dramatic transitions in both peptidoglycan structure and OM composition. The peptidoglycan sacculus of exponentially growing and stationary-phase *E. coli* cells differs in glycan length, crosslinking, and covalent attachment of Lpp. Specifically, in stationary phase, glycan length decreases and both crosslinking and covalent attachment of Lpp increase [74].

Furthermore, OM protein composition may not remain constant during a cell's progression through growth phases. Fernando and Kumar demonstrated the correlation of *A. baumannii* bacterial growth phase with the varying expression levels of two OM porins [75]. Analysis of expression of *carO* and *oprD* genes via qPCR revealed a maximum for *carO* at higher optical densities whereas *oprD* expression peaked at early exponential phase [75]. For *V. cholerae*, the RTX toxin is found in both an OM-independent and OMV-associated form, the concentrations of which are dependent on complex regulatory mechanisms [76]. RTX toxin activity is influenced by the growth-phase-dependent regulation of the Type I secretion system which secretes the toxin, by decreased toxin expression in stationary phase, and by increased protease expression in stationary phase cells. Accordingly, while RTX toxin may be found abundantly in OMV-independent form during exponential growth, all remaining toxin is observed to be vesicle- or OM-associated in stationary phase [76].

5. Perspectives

The determination of whether a specific protein utilizes the OMV pathway for export in a substantial manner is a complex issue. Proteomic data generate insight on protein presence in a particular vesicle sample but can be misleading, as these do not reveal abundance (unless the analysis is painstakingly undertaken using a quantitative assay) and thus fail to expose whether a component is actively partitioned into vesicles.

However, determination of abundance and enrichment in comparison to other cellular compartments is only meaningful if the amount of an exported protein necessary to elicit a functional consequence is taken into consideration. Whether or not the OMV-associated protein is active, potent, in complex with other requisite factors, or found in OMV-independent forms should also be determined.

Additionally, if distinct subpopulations of vesicles are isolated and analyzed, greater insight into OMV formation mechanisms could be gained. Comparisons between functionally different vesicles subpopulations of a single species could reveal differences in lipid composition, as well as protein enrichment and exclusion. These features could then be related to the state of the envelope under different growth or inducing conditions to reveal how timing and environmental factors contribute to OMV production and function.

Accordingly, an exhaustive study on a specific protein exported by OMVs should address the following questions:

Number of export forms: How much protein is present in an OMV-independent form versus an OMV-associated state?

Enrichment/Exclusion: How much protein is present in OMVs in comparison to a relevant cellular compartment (e.g. periplasm, OM)?

Subpopulations: Is the exported protein only present in a subset of the total OMV population? What other proteins are co-exported within the overall population or subset?

Timing: Does protein expression correlate with export? Does OMV production and cargo incorporation change during different growth phases?

Activity: Is protein activity/potency variable in cell-associated, OMV-associated, to OMV-independent forms? Does OMV-association enahance protein complex formation?

Location: Is the protein present in the lumen and/or presented on the surface of OMVs?

Future studies of protein secretion via bacterial OMVs will likely benefit from the continually advancing technology in microscopy. As achievable optic resolution improves, along with the availability of new, more visible compartment markers and molecular tags, studies to determine protein location coincident with the dynamics of OM biogenesis and the cell envelope will soon be feasible.

Discovery of the mechanisms involved in the selectivity protein export via OMVs may be species-specific or rely on structural similarities unable to be identified by simple homology studies. Potential vesiculation machinery must be able to integrate and respond to multiple and diverse signals: OM homeostasis, growth stage, metabolic state, pathogenicity requirements, communication needs, and environmental conditions. Comparative studies of multiple species using multiple conditions will be important to elucidate the process.

Acknowledgements

This research was supported by NIH grant 5R01GM099471.

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