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# Bacterial Outer Membrane Vesicles: From Discovery to Applications

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## Keywords

OMV, outer membrane vesicle, secretion system, pathogenesis, vaccines, commensalism

## Abstract

Secretion of cellular components across the plasma membrane is an essential process that enables organisms to interact with their environments. Production of extracellular vesicles in bacteria is a well-documented but poorly understood process. Outer membrane vesicles (OMVs) are produced in gram-negative bacteria by blebbing of the outer membrane. In addition to their roles in pathogenesis, cell-to-cell communication, and stress responses, OMVs play important roles in immunomodulation and the establishment and balance of the gut microbiota. In this review, we discuss the multiple roles of OMVs and the current knowledge of OMV biogenesis. We also discuss the growing and promising biotechnological applications of OMV.

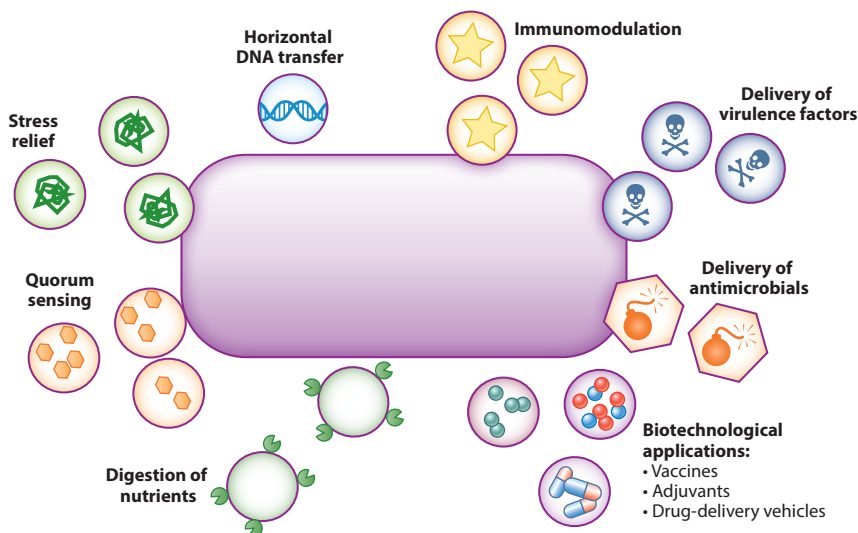
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## 1. INTRODUCTION

Secretion of cellular components across the plasma membrane is an essential process that occurs in all life-forms, enabling organisms to interact with their environments. One way cells accomplish this is by secreting vesicles, which are spherical, nanosized structures derived from lipid membranes of the cell surface (21, 38). Production of extracellular vesicles is a well-documented process that takes place in gram-positive and gram-negative bacteria. Outer membrane vesicles (OMVs), which are released from the cell envelope of gram-negative bacteria, have been studied for more than 50 years. However, little is known about OMV biogenesis. In this review, we discuss the current knowledge of the biogenesis of OMVs in gram-negative bacteria, consider their roles in bacterium-host interactions, and discuss their biotechnological applications. Finally, we briefly address some of the reasons why some researchers remain skeptical about the physiological roles of OMVs.

OMV production was first observed in 1965 in an auxotrophic *Escherichia coli* strain that released significant amounts of cell-free lipopolysaccharides (LPS) under lysine-limiting growth conditions (17). Later, Knox and collaborators showed by electron microscopy that these secreted cell-free LPS elements were part of membrane structures and proposed that these vesicles were derived from the outer membrane (OM) (86). Rothfield & Pearlman-Kothencz (125) followed up these observations and showed that chloramphenicol exposure and amino acid starvation promote the secretion of these OM blebs in *E. coli*. Subsequent studies reported the observation and isolation of OMVs from different gram-negative bacteria, like *Veillonella parvula* (106), *Vibrio cholerae* (28), and *Salmonella enterica* ser. Typhimurium (125). Despite the increasing evidence of OMV production by bacteria, OMVs were considered mere growth artifacts or cell lysis by-products for several years. Later, OMVs were observed in cerebrospinal fluid samples from patients with acute meningitis, suggesting that OMVs were not generated only in lab conditions (41). Since then, the biogenesis of OMVs and their roles have gained interest.



**Figure 1**

Roles of outer membrane vesicles (OMVs) in gram-negative bacteria. OMVs, which are released from the cell envelope, range in size from 20 to 300 nm and function as a versatile secretion and transport mechanism. OMV roles include intracellular and extracellular communication, quorum sensing, horizontal gene transfer, interbacterial killing, toxin delivery, nutrient hydrolysis, and stress responses.

OMVs range between 20 and 300 nm and function as a versatile secretion and transport mechanism for bacterial cells (63). OMV composition in several species has been described and includes lipids, LPS and OM proteins as well as encapsulated periplasmic content. The presence of cytoplasmic elements, like DNA and RNA, has also been reported, but it is unclear how these elements are transported into the periplasm to be packed into OMVs (18, 36). Analysis of OM and OMV fractions from different microorganisms revealed a distinct enrichment of proteins and lipids in each fraction (1, 44, 62, 65, 71, 77, 92, 105, 122, 132). These findings favor the hypotheses that bacteria possess specific sorting mechanisms and that OMV formation is a directed process and not the result of cell lysis.

## 2. OMV ROLES

OMVs have been implicated in an array of physiological processes, including intracellular and extracellular communication, quorum sensing, horizontal gene transfer, interbacterial killing, toxin delivery, polysaccharide hydrolysis, and stress responses (63, 66, 102, 130) (**Figure 1**).

### 2.1. OMVs in Pathogenesis

OMVs have been linked to pathogenesis. They can serve as long-distance delivery vehicles that can promote host colonization and immune evasion. They carry an array of immunogenic molecules, such as LPS, flagellin, and peptidoglycan, which stimulate the host immune system through Toll-like receptors (TLRs) (13, 23). OMV-associated molecules include virulence factors related to adherence, invasion, antimicrobial resistance, and modulation of host immunity. To highlight the role of OMV in pathogenesis, we discuss examples in further detail below.

Enterotoxigenic *E. coli* (ETEC) is an important diarrheal pathogen that secretes several toxins, including heat-labile enterotoxin (LT) (71, 145). This toxin disrupts the electrolyte balance in

the gut endothelium and has been detected in OMVs (71, 85). ETEC OMVs can also deliver the pore-forming cytotoxic protein cytolysin A (ClyA) (144). ClyA incorporated into OMVs has higher cytotoxicity compared to the purified toxin in a mammalian cell model (144). This finding has been attributed to an effect of the redox environment within the vesicles that allows ClyA oligomerization (144). Cytotoxic necrotizing factor type 1 (CNF1) is a virulence factor produced by uropathogenic *E. coli* contained in OMVs (87). Once this toxin reaches the host cells, it alters actin cytoskeleton and promotes bacterial invasion of endothelial cells of the blood–brain barrier.

Early studies in the human dental pathogen *Porphyromonas gingivalis* also linked OMV secretion to pathogenesis. The main virulence factors of *P. gingivalis*, gingipains, are enriched in OMVs, which contributes to impairment of host cell function (65, 143). Moreover, hemagglutinins and heat shock proteins, which are mainly involved in host cell attachment and invasion, are also secreted as OMV cargo (10). Consequently, OMV production increases bacterial adherence to host cells, stimulating bacterial aggregation and leading to the formation of dental plaque (45, 62, 79).

*Pseudomonas aeruginosa* is an opportunistic pathogen that causes nosocomial infections mainly in immunocompromised patients (97). Its OMVs carry multiple virulence factors, including degradative and pore-forming molecules such as peptidoglycan hydrolase, phospholipase C, alkaline phosphatase, protease, elastase, and hemolysin (33, 77). Additionally, *P. aeruginosa* OMVs facilitate bacterial competition during infection, being able to kill gram-negative and gram-positive competitors in cocultures (78). Furthermore, *P. aeruginosa* elicits a potent destructive inflammatory response via combined sensing of both LPS and protein components (47). In vitro assays carried out in macrophages showed that OMVs led to a significant increase of MIP-2, TNF- $\alpha$ , IL-1, and IL-6 transcriptional levels compared to exposure to LPS alone.

*S. Typhimurium*, the leading cause of gastroenteritis, also exploits OMVs to transport virulence factors. This bacterium is able to produce OMVs even during its intracellular life, secreting OMVs packed with cytolethal distending toxin inside infected epithelial cells (64). Interestingly, OMV biogenesis in *S. Typhimurium* inside infected macrophages is triggered by the two-component system PhoPQ (43).

*V. cholerae*, the causative agent of cholera, produces cholera toxin (CT) as its main virulence factor (27). Although CT is primarily secreted by the type II secretion system (T2SS), OMVs serve as a secondary mechanism by which CT is secreted (27). In addition to CT, several other virulence factors have been linked to OMVs, such as the pore-forming toxin *Vibrio* cytolysin (118) as well as various serine proteases and metalloproteases (57, 124). These compounds were found to cause cytotoxicity and induce an inflammatory response in host cells (109). Moreover, this bacterium can alter the immunogenicity of OMV cargo by decreasing the expression of virulence factors through quorum sensing (16).

## 2.2. OMVs in Commensalism

Gut microbiota-derived vesicles are an emerging topic of study and were first reported less than ten years ago (80, 131). The functionality of microbiota-derived OMVs varies greatly depending on the species releasing them. Proteomic studies on OMVs from gut commensal strains suggest that OMV-associated proteins contribute to modulating host immunity, promote host colonization, and act as public goods by degrading various carbon sources in the gut (1, 44, 120, 148).

*Bacteroides* is well known for secreting large amounts of OMVs and actively contributing to gut symbiosis (68, 131). These bacteria secrete glycosylases and proteases, which degrade complex polysaccharides and mucins (44, 68, 120). The generated degradation products can then be utilized as nutrient sources by any other members of the gut microbiota (44, 68, 120). Thus, OMVs

are considered public goods: Once OMVs are released, any member of the gut microbiota can benefit from their degradative activity. However, not all the OMV cargo fulfills this altruistic role. Recently, a family of peptide toxins that has broad-spectrum activity against *Bacteroides* has been found as OMV cargo. The genes encoding these toxins are widely distributed throughout the human gut microbiota (29, 35).

OMVs from commensal bacteria can also modulate the host innate immune system. For example, *Bacteroides fragilis* polysaccharide A triggers TLR-mediated signaling that attenuates host immune responses and promotes commensal gut colonization (103, 126). The constant and controlled immune stimulation mediated by OMVs from commensals in the gut is linked to intestinal health (23, 108). This cross talk between microbiota and host cells constitutes a key process in maintaining gut homeostasis.

### 2.3. Production of OMVs by Plant Pathogens

OMVs have been investigated primarily in human pathogens and commensals. However, recent studies show that OMVs produced by plant pathogens perform similar functions (72, 83, 135). Plant pathogens deliver virulence factors, such as T2SS effectors and xylanase, as OMV cargo (72, 112, 135). The innate immune system of plants recognizes and responds to purified OMVs from plant pathogens (7). Thus, when *Arabidopsis thaliana* seedlings were incubated with OMVs purified from several *Xanthomonas* species, three defense responses (defense gene activation, reactive oxygen species burst, and medium alkalization) were modulated (7). Still, it remains unexplored whether OMVs play a role in plant symbiosis or in interkingdom cell-to-cell cross talk.

### 2.4. Other OMV Roles

OMV production is increased when cells are subjected to physical or chemical stress (3, 61, 95, 104). *E. coli* mutants lacking the proteases DegS and DegP exhibited a hypervesiculating phenotype, and the amount of vesicles released correlated with the level of protein accumulated in the cell envelope (104). Furthermore, the increased vesiculation enhanced bacterial survival upon challenge with stressing agents. These observations led to the hypothesis that vesicle overproduction is linked to the maintenance of the cell envelope (104, 137). Temperature is another factor that can modulate the amount of OMVs generated. In vitro, *Serratia marcescens* produced significant amounts of vesicles at 22°C or 30°C, whereas it produced negligible quantities at 37°C (131). Moreover, inactivation of the synthesis of the enterobacterial common antigen resulted in hypervesiculation in this strain, and this hypervesiculating phenotype was reverted upon inactivation of the response regulator RcsB (131). Nutrient limitations have been also associated as a trigger for OMV production. For example, under sulphate-depleted conditions, *Neisseria meningitidis* increased its OMV biogenesis, while *Haemophilus influenzae*, *V. cholerae*, and *E. coli* increased OMV production under iron-limited conditions (56, 122).

OMVs can also act as decoys to confront and attenuate antibiotic activity. *E. coli* OMVs contributed to protection against the membrane-targeting antibiotics colistin and melittin (90). The protective effect was not limited to *E. coli*, as these purified *E. coli* OMVs provided protection against those antibiotics to *P. aeruginosa* and *Acinetobacter radioresistens* strains (90). However, the protective effect of the OMVs could not be extended to other antibiotics, like ciprofloxacin, streptomycin, and trimethoprim, indicating that OMVs appear to protect the bacterial community mainly against antibiotics targeting the membranes (90), possibly by sequestering and reducing the availability of the antibiotic. *P. aeruginosa* and other bacteria also secrete antibiotic-degrading enzymes like  $\beta$ -lactamases in OMVs (34, 58). Similarly, OMVs secreted by *V. cholerae* acted as decoys to increase resistance to antimicrobial peptides, such as polymyxin B and LL-37 (42).

Furthermore, OMVs act as decoys during phage infections. OMVs irreversibly bind the phage, highly reducing their ability to infect the bacterial cell (95, 101).

Recent studies have suggested that OMVs can harbor DNA and different types of RNAs, mostly noncoding RNAs (18, 36, 59, 134). Intriguingly, many of these sequences align to intergenic noncoding regions of human DNA (26). The delivery of bacterial RNA through OMVs could exert epigenetic changes in host transcription (26). However, it remains unclear how these RNA molecules reach the periplasm prior to being packed inside OMVs.

OMVs are also pivotal in bacterial communication. Certain bacteria can regulate gene expression in response to quorum sensing. *P. aeruginosa* is able to secrete quinolone signal compounds [*Pseudomonas* quinolone signal (PQS)] through OMVs, which provide a protective environment to these highly hydrophobic molecules (102). PQS simultaneously stimulates OMV production by intercalating into the OM, generating a positive-feedback loop (51). As PQS is found at high concentrations in OMVs, the fusion of a single vesicle with a bacterial cell is enough to trigger the quorum sensing response. Hydrophobic signal molecules related to quorum sensing were also detected as OMV cargo in other microorganisms (20), thus suggesting a novel OMV-based mechanism for hydrophobic signal molecule trafficking.

### 3. REGULATION AND BIOGENESIS OF BACTERIAL OMVs

Bacterial OMVs have been studied extensively for decades. However, researchers have not yet elucidated a definitive or universal mechanism to explain OMV production. Here, we address several biogenesis mechanisms proposed to explain how OMVs are formed (**Figure 2**).

#### 3.1. Membrane Cross-Links and OMV Production

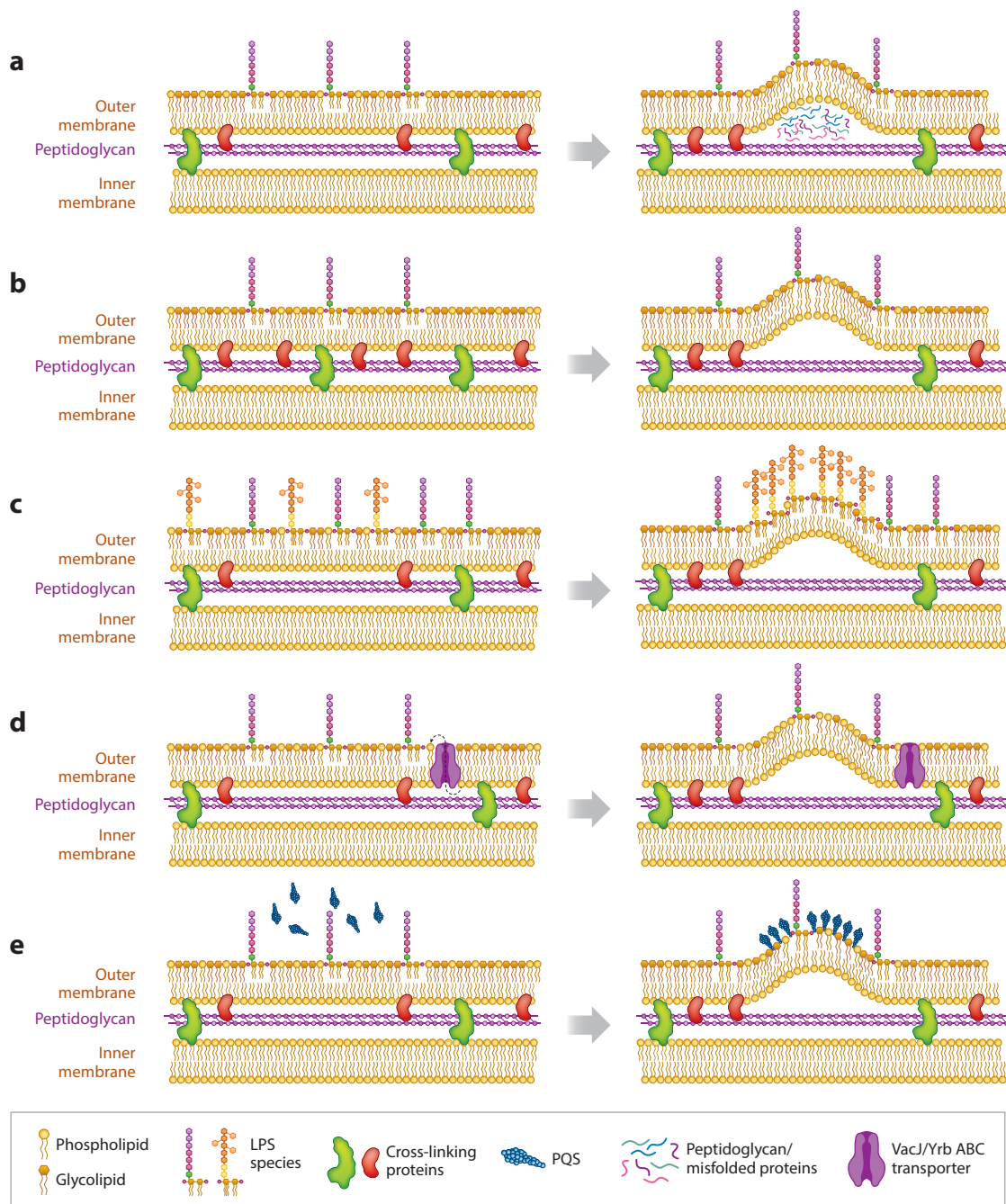
One of the earliest models for OMV formation linked OMV biogenesis to a decrease in OM-peptidoglycan cross-links at the site of vesicle formation (**Figure 2b**). Braun's lipoprotein (Lpp), outer membrane protein A (OmpA), and components of the Tol-Pal complex are OM proteins found to be involved in this process. Lpp is an abundant OM protein in some bacteria that acts as a molecular staple, linking the OM to the peptidoglycan layer. Inactivation of *lpp* results in increased OMV production in *P. aeruginosa*, *E. coli*, and *S. Typhimurium* (12, 146). However, *lpp* mutants have defects in the integrity of their OMs, so it is difficult to distinguish OMV production from cell damage.

OmpA is another OM protein that associates noncovalently with the underlying peptidoglycan layer. OmpA is a common component of OMVs (114, 140, 146). Regulation of OmpA impacts OMV production, and its deletion induces hypervesiculation in many bacterial species (114, 140, 146). In *V. cholerae*, the small noncoding RNA VrrA is suggested to modulate OMV production by acting as a negative regulator of OmpA. *vrpA* mutants were found to display a hypervesiculation phenotype likely due to decreased OM-peptidoglycan interactions (136).

The Tol-Pal complex spans the inner membrane and OM of gram-negative bacteria and consists of five proteins, TolA, TolB, TolQ, TolR, and Pal (55). TolA, TolQ, and TolR form a complex in the inner membrane, whereas TolB is a periplasmic protein that interacts with Lpp, OmpA, and Pal (147). Pal is localized to the inner leaflet of the OM, where it interacts directly with the peptidoglycan layer and promotes membrane stability (55, 147). The Tol-Pal complex is proposed to have many functions; however, its best-characterized role is in cell division (55, 147). Tol-Pal proteins localize at invagination sites during cell division, and inactivation of the genes encoding these proteins leaves the cell unable to divide (55, 147). However, Tol-Pal mutants displayed increased bleb formation rates around invagination sites. It has been reported that the Tol-Pal complex is involved in producing OMVs at sites of cell division and that these OMVs are distinct from those generated at other sites on the cell (39).

### 3.2. Periplasmic Accumulation and OMV Production

It has been proposed that accumulation of periplasmic contents can induce vesicle formation (Figure 2a). For example, peptidoglycan fragments liberated in the periplasm during growth could exert turgor pressure on the OM, leading to vesicle production (150). Studies in



(Caption appears on following page)

**Figure 2** (Figure appears on preceding page)

Mechanisms of OMV production in gram-negative bacteria. (a) Curvature of the OM is induced by the local accumulation of peptidoglycan fragments or misfolded proteins in the periplasm. (b) Removal of proteins anchoring the OM to the underlying peptidoglycan increases OM fluidity, enabling the membrane to bend and form vesicles. (c) Owing to charge repulsion, local enrichment of LPS species with anionic charges induces curvature of the OM and subsequent vesicle formation. Enrichment in deacylated LPS species also promotes membrane curvature because lipid A deacylation changes the shape of LPS from cylinder-like to an inverted cone. (d) The VacJ/Yrb ABC transporter is involved in retrograde transport of phospholipids from the OM. Downregulation of this transporter leads to the accumulation of phospholipids in the outer leaflet of the OM. This causes the outer leaflet to expand rapidly compared to the inner leaflet, which leads to membrane curvature and vesicle formation. (e) Once PQS is produced, it is secreted from the cell and subsequently intercalated into the outer leaflet of the OM owing to its interaction with lipid A and phospholipids. Insertion of PQS into the OM causes outer leaflet expansion that increases OMV production. Abbreviations: ABC, ATP-binding cassette; LPS, lipopolysaccharide; OM, outer membrane; OMV, OM vesicle; PQS, *Pseudomonas* quinolone signal.

*P. gingivalis* demonstrated that mutants lacking an autolysin displayed increased OMV production. Researchers explained this phenotype by claiming that the absence of the autolysin activity prevented *P. gingivalis* from degrading periplasmic peptidoglycan fragments, which accumulated in the periplasms and were therefore expelled via OMVs (67). Misfolded proteins might also induce OMV production by exerting force on the membrane of bacteria with mutations in envelope stress pathways, since these cells were unable to degrade the proteins (129). This claim was supported by the fact that mutants grown at lower temperatures (30°C or 34°C), which cause less protein misfolding, produced OMVs at comparable rates to wild-type cells (104).

### 3.3. Lipopolysaccharide Remodeling and OMV Production

Several reports have shown that altering LPS content impacts OMV production (Figure 2c). *P. aeruginosa* produces two LPS types containing different O-polysaccharides, the A-band LPS (neutral charge) and the B-band LPS (anionic charge). Remarkably, only B-band LPS has been detected in the vesicles (77). This finding has led to the hypothesis that OMVs are generated in regions where B-band LPS is more abundant and that the OM bends to alleviate the charge repulsion between them (77). Mutants that only produce B-band LPS were found to produce more OMVs than wild-type organisms and mutants only able to produce A-band LPS (115). However, the OMVs secreted by the wild-type and these mutant strains are different sizes, which indicates that the overproduction of OMVs by strains producing only the B-band LPS could be the result of an envelope stress-coping mechanism (111).

*P. gingivalis* also synthesizes two different O-antigen chains, the A-LPS (anionic charge) and the O-LPS (neutral charge). Contrary to LPS in *P. aeruginosa*, both LPS types are packed into *P. gingivalis* OMVs (65). Mutations affecting A-LPS synthesis or attachment of both O-antigens onto the lipid A core did not affect OMV biogenesis, and all bacteria with LPS mutations produced OMVs comparable in size to wild-type OMVs (65). Even though *P. gingivalis* packs both types of LPS into OMVs, the lipid A compositions of the OM and OMVs are different (65). The lipid A sorted into OMVs is deacylated compared to lipid A in the OM, suggesting that OMVs are generated in specific OM regions because of compartmentalization or remodeling of the OM (66). In this model, lipid A deacylation alters the configuration of LPS, increasing the membrane curvature and consequently inducing OMV production. When the lipid A deacylase PagL was overexpressed in *S. Typhimurium*, deacylated lipid A was preferentially packed into OMVs, which subsequently increased OMV production (43). PagL is tightly regulated and is expressed inside macrophages (113). Upon macrophage infection, PagL is required for OMV formation by intracellular *S. Typhimurium* (43). Given that most of the lipid A-modification enzymes, such as PagL, are regulated and not expressed in normal lab conditions, which is the setting for extracting samples for

lipid A analysis, it is possible that other bacteria produce OMVs with deacylated lipid A during infection.

### 3.4. OMV Production and the Bilayer-Couple Model

The bilayer-couple model describes a mechanism where membrane curvature is initiated by the insertion of biomolecules into the outer leaflet of the OM (**Figure 2d,e**). This is proposed to cause the outer leaflet to expand faster than the inner leaflet, resulting in blebbing of the OM and formation of OMVs (127). This model has been extensively studied in *P. aeruginosa* (**Figure 2e**); however, a similar mechanism involving VacJ/Yrb ATP-binding cassette (ABC) transport has been investigated in other species (**Figure 2d**).

As previously mentioned, the molecule PQS is enriched in OMVs produced by *P. aeruginosa*, and reduced OMV production was found in PQS-deficient mutants (102). Subsequent studies found that PQS stimulates OMV production when it is secreted from the cell and intercalated into the OM (51). PQS insertion causes the OM to expand rapidly, resulting in vesicle formation (127) (**Figure 2e**). This mechanism appears to be limited to *P. aeruginosa* and related species that produce PQS (70).

*V. cholerae* OMVs are enriched in phospholipids and carry large amount of enzymes associated with phospholipid biosynthesis (122). It has been proposed that OMVs are formed as a result of accumulation of phospholipids in the OM, forcing blebbing of the membrane (122). The VacJ/Yrb ABC transport system is involved in the carriage of phospholipids from the OM (99). Disruption of this system promotes OMV overproduction (99, 122). Similar results have been reported for *Chromobacterium violaceum*, where VacJ/Yrb mutants hypervesiculated (9). Loss of VacJ/Yrb causes an accumulation of phospholipids in the outer leaflet of the OM, which leads to OM expansion and, subsequently, vesicle formation (122) (**Figure 2d**). Accordingly, OMVs from VacJ/Yrb mutants contain twice as many phospholipids as wild-type OMVs (122). In addition, during iron-limited conditions, the VacJ/Yrb ABC transporter is downregulated by the ferric uptake regulator, causing a hypervesiculation phenotype in *H. influenzae*, *V. cholerae*, and *E. coli* (122). Moreover, regulation of VacJ/Yrb modulated OMV production in the presence of the bile salt sodium taurocholate (37, 48). Since the VacJ/Yrb pathway has been shown to impact OMV production under various conditions and in various organisms, this mechanism of OMV biogenesis could serve as a general way to stimulate OMV production.

### 3.5. Proposed OMV Cargo Selection Mechanisms

Enrichment of specific protein and lipid cargo is an essential characteristic of OMVs. Vesicles from pathogenic bacteria like *P. aeruginosa*, *V. cholerae*, *P. gingivalis*, and ETEC have been shown to selectively pack virulence factors (19, 71, 77). OMVs from commensal *Bacteroides* are enriched with glycoside hydrolases that enable the degradation of environmental polysaccharides (44). Similarly, those from the predatory bacterium *Myxococcus xanthus* are enriched in acidic hydrolases and alkaline phosphatases, which aid the microbe in killing other environmental bacteria (11). The observed OMV cargo selection requires extensive OM compartmentalization to generate patches in the OM regions from which OMVs are secreted. In *P. gingivalis*, intact LPS is required to achieve proper cargo selection (65). Mutant strains deficient in the synthesis of A-LPS produced OMVs with aberrant cargo, packing a few additional proteins. Interactions of proteins with A-LPS could shape the OM by partitioning proteins into OMVs or excluding them from OMVs (65). This interaction may be direct, in which case the OMV-specific proteins could have a domain to recognize and interact with the O-antigen, promoting compartmentalization of the OM. Alternatively, the interaction could be mediated by a yet unknown sorting factor, which would recruit the protein

to the O-antigen-enriched region in the OMV. This proposed mechanism has an uncanny resemblance to the role of galectin in protein sorting in exosomes (40). In agreement with the sorting model, *B. fragilis* and *Bacteroides thetaiotaomicron* utilize conserved protein sequences to specifically direct proteins to OMVs (44). The OMVs of these organisms are enriched in a large subset of OMV-unique proteins, which were found to be acidic lipoproteins (negatively charged), while the majority of the OM proteins,  $\beta$ -barrel proteins, are positively charged (44). In *Bacteroidetes*, lipoproteins that are abundant in OMVs were found to contain a conserved, negatively charged amino acid motif known as the LES (lipoprotein export signal) domain (141). This domain is required for surface exposure of lipoproteins in *Bacteroidetes* (93). When it was mutated, lipoproteins were less efficiently packaged into OMVs and were retained in the OM fraction (141). Although many studies have analyzed the OMV proteomes from different bacteria, this is the first time that a specific signal was shown to be linked to an OMV protein-sorting mechanism (141).

#### 4. MECHANISMS OF OMV ENTRY INTO HOST CELLS

OMVs are long-distance delivery systems; however, the molecular mechanisms underlying OMV internalization and cargo delivery into host cells are not fully understood. Two pathways have been proposed: (a) direct cargo delivery via fusion to host cell membranes and (b) OMV internalization via the endocytic pathway (19, 53, 85, 116). During direct cargo delivery, OMV fusion to host cell lipid rafts induces actin remodeling to allow OMV soluble cargo to diffuse directly into the host cytoplasm. This is the mechanism proposed for *P. aeruginosa*, *Legionella pneumophila*, and *Aggregatibacter actinomycetemcomitans* OMVs (19, 75, 123). Alternatively, delivery of OMVs is facilitated by diverse mechanisms of endocytosis, including clathrin-mediated endocytosis, caveola-mediated endocytosis, and clathrin- and caveola-independent endocytosis. After OMVs have bound to lipid rafts, they are internalized through one of many endocytic pathways (19, 53, 85, 117). The pathway employed is organism-, or even strain-dependent (19, 53, 85, 117). For example, OMVs derived from *Helicobacter pylori*, *E. coli* O157, non-O157 enterohemorrhagic *E. coli* (EHEC), and nonpathogenic *E. coli* strains are internalized by clathrin-mediated endocytosis (15, 49, 91), whereas the internalization of OMVs from ETEC, *V. cholerae*, *P. aeruginosa*, and *A. actinomycetemcomitans* depends on caveola-mediated endocytosis (19, 27, 81, 85, 123). Finally, *P. gingivalis* OMVs are internalized via clathrin- and caveola-independent endocytosis (53). Upon entry, the OMV can follow multiple fates inside the host trafficking network. ETEC OMVs containing LT accumulate in nonacidified compartments (85), whereas *P. gingivalis* and *E. coli* O157 OMVs and nonpathogenic OMVs are routed to the early endosome, followed by sorting to lysosomal compartments (14, 53, 74).

OMV size and cargo could determine which mechanism(s) of entry into the host cells is employed (76, 82, 104, 138). OMVs from pathogenic *E. coli* strains are internalized approximately 30% more rapidly and efficiently than those of nonpathogenic *E. coli* strains (117). Similarly, OMVs produced and secreted by mutant strains lacking one virulence factor, such as LT, Cif, or gingipains, cannot fuse with or enter host cells (19, 53, 85). Furthermore, a reduced OMV entry ratio was observed in strains lacking O-antigen (117). Smaller OMVs preferentially enter epithelial cells via caveola-mediated endocytosis (138). These findings suggest that the kinetics of entry are cargo dependent; proteins and/or LPS carried by OMVs probably interacts with or bind to receptors present on the lipid rafts that promote OMV attachment and entry (19, 53, 85, 117).

#### 5. OMVs AS A PLATFORM FOR BIOTECHNOLOGICAL APPLICATIONS

In recent years, OMVs have gained attention as a platform for biotechnological applications. Such applications take advantage of the fact that OMVs contain bacterium-derived antigens

## OVERVIEW OF OMV-BASED MenB VACCINES

For most *N. meningitidis* serogroups, the available vaccines consist of capsule polysaccharides coupled to a carrier protein. However, the serogroup B capsule is homologous to molecular structures present in the human brain, making it impossible to produce a glycoconjugate vaccine for this serogroup (69). Thus, OM proteins such as PorA were considered as vaccine candidates. PorA, the main immunogenic protein in *N. meningitidis* and also present in OMVs, is highly variable between strains (69). To overcome the problem of strain specificity, a novel MenB vaccine using OMVs derived from bioengineered strains expressing multiple PorA variants has been developed in the Netherlands (69, 142). Most available OMV-based MenB vaccines (VA-MENGOC-BC, MenBvac, and MenZB) have succeeded in combating specific outbreaks of MenB-caused meningitis (in Cuba, Norway, and New Zealand), with an efficacy of at least 70% (69). In 2016, the BEXSERO vaccine was approved for human use by the European Medicines Agency and the FDA, as it provides protection against endemic disease (69).

and multiple pathogen-associated molecular patterns (PAMPs) and can modulate the immune system. The best-known biotechnological application is the use of OMVs as vaccines (142, 107). However, other applications have been proposed, such as drug-delivery systems (94).

### 5.1. OMVs as Vaccines

By mimicking a pathogen but not causing the related diseases, vaccines induce humoral (antibody-mediated) and/or cellular (immune cell- and cytokine-mediated) immunity and dramatically decrease infection and illness rates. As OMVs are nonreplicative entities that mimic immunogenic properties of the producing bacteria, they are an attractive vaccine platform. Further advantages of OMVs are their size, which enables their entry into lymph vessels and uptake by antigen-presenting cells (APCs) (47). In addition, OMVs have natural adjuvant properties that strongly stimulate the innate and, more importantly, the adaptive immune responses (46, 142). The high stability of OMVs upon exposure to high temperatures and several chemical treatments further points to them as attractive vaccine candidates (4). Two types of OMV-based vaccines have been described: natural OMV and bioengineered OMVs.

**5.1.1. Naturally secreted OMV-based vaccines.** The vaccines against *N. meningitidis* group B (MenB) are the most representative and successful OMV-based vaccines developed to date. In the last 25 years, these vaccines have been developed and employed to combat outbreaks in various countries (69) (see the sidebar titled Overview of OMV-Based MenB Vaccines). OMV-based vaccines against *V. cholerae*, *S. Typhimurium*, and *Shigella flexneri* have been investigated in animal models, but none has progressed to clinical trials (2, 22, 128).

OMVs have the intrinsic capacity to act as adjuvants. The immunogenic properties of OMV cargo lead to protective mucosal and systemic bactericidal antibody responses. OMVs can easily be phagocytized and processed by APCs, including dendritic cells (47). Then, the OMV-delivered antigens are presented by APCs to CD4<sup>+</sup> T cells, leading to the generation of antigen-specific B cell responses (46, 100, 107, 142). LPS, a major component of OMVs, is a potent activator of immune cells, such as monocytes/macrophages. Specific recognition by the TLR4/MD2 receptor on these cells triggers NF- $\kappa$ B- and IRF3-dependent gene expression (98, 100). However, this inflammatory activation can also result in high vaccine reactogenicity. In addition to LPS, OMVs carry lipoproteins that are recognized by TLR2 and also modulate and activate innate immunity (88, 100).

Despite a few successful examples, some disadvantages of vaccines based on naturally released OMVs need to be overcome before they are widely employed. The main concern is safety. OMVs contain lipid A, an endotoxic component of LPS that can provoke a severe, even lethal, inflammatory response in the host (113). Furthermore, OMV cargo varies between strains, which may in some cases limit their applicability to a specific subset of strains. Finally, many strains secrete low amounts of OMVs, which means that large volumes of pathogenic bacteria are needed. In the last decade, bioengineering of OMV-producing bacteria has substantially progressed to address these concerns.

**5.1.2. Bioengineered OMV-based vaccines.** Bioengineered OMV-carrying, heterologous recombinant proteins expressed in lab strains have shown potential. One strategy to effectively direct heterologous antigens to the surface of the OMV is to fuse them with native OMV proteins. Many groups have selected ClyA from *E. coli* as the fusion partner to deliver exogenous proteins to OMVs (30, 54, 144). For example, ClyA has been fused to green fluorescent protein (GFP), to the ectodomain of the influenza A matrix protein 2 (M2), and to the domain 4 moiety of *Bacillus anthracis* protective antigen (30, 54, 121). As an alternative, antigens can be overexpressed and directed to the periplasmic space, where they will be entrapped and packed into the OMV lumen (84, 110). Several proteins from *Streptococcus* have been fused to the signal peptide of *E. coli* OmpA, and once in the periplasm, they were successfully packed into OMVs (50). A similar approach was employed to generate *E. coli* OMVs carrying the *Chlamydia muridarum* protein HtrA (8). Even though these proteins were not surface exposed, they were able to elicit antigen-specific antibody responses (8, 50, 110). The IgG antibodies generated had excellent functional activity in terms of bacterial opsonophagocytic killing and protection in murine lethal infectious challenge assays (50, 110).

Capsular polysaccharides (CPS) and LPS, which decorate the cell surfaces of pathogenic bacteria, are also good vaccine antigens. Unfortunately, vaccines consisting solely of polysaccharides typically promote T cell-independent immune responses, which do not include IgM-to-IgG class switch and fail to generate immunological memory (6). A common strategy to trigger immunological memory is to covalently couple the polysaccharide to a carrier. Capsule and O-antigen biosynthesis gene clusters from pathogenic bacteria can be transferred into *E. coli* lab strains. These glycoengineering techniques can be employed to display the glycans of choice as recombinant LPS in nonpathogenic bacteria. The glycoengineered OMVs can be purified and employed directly as conjugate vaccines. *Streptococcus pneumoniae* CPS (Sp-CPS) gene cluster was expressed in *E. coli*. There, Sp-CPS was attached to the lipid A core and displayed on the bacterial surface and glycoengineered OMVs (119). These glycoengineered OMVs raised specific IgG antibodies against Sp-CPS in immunized mice and were shown effective in opsonophagocytosis assays (119). In another example, the *Francisella tularensis* O-antigen polysaccharide gene cluster was also successfully expressed in *E. coli* to produce glycoengineered OMVs decorated with *F. tularensis* O-antigen (31). Mice immunized with these *F. tularensis* glycoengineered OMVs were protected against lethal challenge with several strains of *F. tularensis* (31). Furthermore, to highlight the versatility of this vaccine technology, glycosyltransferases and the enzymes from various organisms required for the synthesis of nucleotide-activated sugars can be expressed in *E. coli* cells to produce customized glycoengineered OMVs (139).

**5.1.3. OMV-based vaccine detoxification alternatives.** Several strategies have been developed to obtain OMVs with a low level of LPS toxicity. The first method is to reduce LPS content by treating purified OMVs with detergents, such as deoxycholate and polyoxyethylene 10 oleyl ether (Brij-96) (69, 142). However, this approach has the disadvantage of a loss of

lipoproteins, which are TLR agonists, thus reducing the adjuvant properties of OMVs (100). A second detoxification method is based on the fact that less-acylated lipid A has reduced toxicity (133). Monophosphoryl lipid A is a safe and effective vaccine adjuvant that is approved by the US Food and Drug Administration for human use (25). Some naturally occurring *Neisseria* strains produce penta-acylated LPS due to a mutation in the lipid A biosynthesis lauroyl acyltransferase, encoded by *lpxL1*. Their secreted OMVs do not need the detergent extraction step because of low endotoxic activity of the penta-acylated lipid A (52). Therefore, by modifying genes responsible for lipid A synthesis, it is possible to obtain genetically detoxified strains and, consequently, detoxified OMVs (100, 113). Inactivation of the genes encoding lipid A acyltransferases, such as *msbA*, *msbB*, *lpxL1*, and *lpxM*, and/or overexpression of genes encoding lipid A deacyltransferases, such as *pagP*, results in lipid A lacking an acyl chain and the concomitant reduced activation of TLR4/MD2 (5, 43, 73, 113). An additional fine-tuning step can be accomplished by introducing lipid A phosphatases, such as LpxE, to generate monophosphorylated penta-acylated lipid A (31, 113). Another alternative is to engineer nonpathogenic bacteria that naturally produce OMVs that only pack monophosphorylated penta-acylated lipid A. *Bacteroides* OMVs contain only monophosphorylated penta-acylated lipid A moieties, and these organisms can be engineered to deliver antigens and drugs (24, 44). A balanced response that generates sufficient adjuvant activity but prevents harsh side effects can be achieved by adopting any of the methods described above.

## 5.2. OMVs as Cancer Immunotherapy Agents

In the last decades, multiple preclinical and clinical studies involving cancer vaccines have been performed. Unfortunately, these vaccines have had an overwhelmingly low rate of efficacy (less than 5%) (149). However, promising results for the use of liposomes have been reported lately (89). Considering the immunomodulatory role of OMVs, they can be engineered to express cancer-specific epitopes or to carry small noncoding RNAs (60, 149). Furthermore, OMVs induced a durable antitumor immune response that inhibited tumor growth in multiple tumor models (32, 149). The caveat, again, is the toxic effect of LPS. However, some of the detoxification methods mentioned above are promising and worthy of exploration to improve the cancer therapies available.

## 6. SKEPTICISM ABOUT OMVs AND THEIR PHYSIOLOGICAL RELEVANCE

OMVs were described for the first time in the 1960s (17, 86). However, for several reasons, a number of scientists are still skeptical about their biological relevance. The story of OMVs resembles that of eukaryotic extracellular vesicles, or exosomes, which have also been a matter of controversy. Many concerns stem from the fact that despite more than 50 years of research, we have yet to elucidate the mechanism(s) for their formation. Pieces of the puzzle regarding OMV biogenesis have been uncovered in different species, however, and none of the working models has been confirmed to be universal.

It has been proposed that DNA and RNA are packed inside OMVs (18, 36, 134). None of the current models for OMV biogenesis specifically accounts for the packing of nucleic acids, as these molecules would need to be translocated into the periplasm and across the peptidoglycan layer to be included in an OMV. On the contrary, RNA and DNA are released upon cell lysis and could easily contaminate OMV preparations. A feasible explanation for how DNA or RNA is transported into the bacterial periplasm would support the biological function of these molecules in OMVs.

Multiple mutant strains presenting hypervesiculating phenotypes have been described (104, 117, 140, 146). Although some of these mutations appear to regulate OMV formation, many mutations also affect membrane stability. Consequently, the material produced by these strains that is considered OMVs may result from lysis or membrane destabilization. No mutants unable to produce OMVs have been identified. Those who believe in the relevance of OMVs explain this by postulating the essentiality of OMV biogenesis. Because vesicles formed as by-products of lysis have the same composition as the membrane from which they are derived, the fact that protein and lipid cargo selection has been shown argues against the notion that OMVs are simply bacterial debris. Many secreted proteins, such as flagellins, pilins, and T3SS effectors, may aggregate and copurify with OMVs. Therefore, claims of cargo selection have to be considered with caution when these are the proteins that are presumably enriched in OMV preparations. In any case, cargo selection has been conclusively demonstrated in a few bacterial species, with proteomics indicating that, at least in these species, OMVs do not result from lysis (19, 44, 65, 71, 77, 141, 143).

Numerous functions have been proposed for OMVs. In most reports appearing in the literature, OMVs are purified from bacteria cultured in large volumes under standard lab conditions with regard to media, temperature, and aeration (27, 47, 91, 96, 109, 134). In some studies, OMVs were obtained from up to 1.5 L of culture and concentrated by a factor in the hundreds before their biological properties were tested. Although this might suggest that OMVs are produced in negligible amounts, OMV biogenesis is likely stimulated *in vivo*. However, the results of these experiments have to be carefully interpreted, as the composition of OMVs produced during infection can be very different from that of OMVs produced in culturing media.

Part of the problem is that the vesicle biogenesis process is very difficult to investigate. *Bona fide* OMVs and lysis-derived vesicle-like structures are extremely difficult to differentiate by biophysical and biochemical methods. We recognize the limitations of current studies involving the biogenesis and functions of OMVs. It is possible that some of the published works contain artifacts. However, there is substantial evidence to assert that at least some bacteria under particular conditions produce *bona fide* OMVs as a result of an orchestrated process. Given recent technical advances in mass spectrometry and microscopy, it will be possible to investigate the vesiculation process in relevant clinical strains and in infection models to decipher OMV biogenesis mechanisms and establish their true biological significance.

### SUMMARY POINTS

1. Outer membrane vesicles (OMVs) constitute a universal secretion system that is found in all gram-negative bacteria and that results from a directed and selective cellular process.
2. OMV composition can be differentiated from that of the outer membrane, as OMVs are enriched with a specific subset of proteins and lipids.
3. At least five mechanisms have been proposed to explain OMV formation.
4. OMVs can have offensive and defensive roles in bacterium–bacterium and bacterium–host interactions.
5. Engineered OMVs carrying customized cargo and detoxified lipopolysaccharide could be used to improve current vesicle-based vaccines and drug-delivery platforms.

## FUTURE ISSUES

1. Is there a universal mechanism for OMV biogenesis?
2. How is protein cargo selected?
3. What is the composition of the cargo of OMVs secreted in vivo?

## DISCLOSURE STATEMENT

M.F.F. has a patent granted for the use of glycoengineered OMVs as vaccines (US9526775B2, “Glycoengineered outer membrane vesicles and use thereof as vaccines”). All other authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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