Roles of Outer Membrane Vesicles (OMVs) in Bacterial Virulence

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Outer membrane vesicles (OMVs) are ubiquitous membranous structures in all Gram-negative bacteria, including pathogens and non-pathogens. Gram-positive bacteria also release membrane-derived vesicles (MV). Originating from the cell envelope, OMVs are enriched with bacterial antigen molecules that conduct multiple functions as decoys to manipulate the host immune system. Besides, OMVs and their components play diverse roles in nutrient acquisition, biofilm formation, and resistance to antibiotics. Despite the diverse benefits ascribed to OMVs, many questions remain unanswered with regard to OMV biogenesis and cargo selectivity. In this report, we review the advantages of vesiculation in the context of all bacteria and then focus on additional benefits acquired by OMVs in pathogenic bacteria.

Key Words: Outer membrane vesicle (OMV), Pathogen, Virulence

I. INTRODUCTION

In response to diverse stressful conditions, bacteria have evolved elaborate mechanisms to evade hostile stresses and thrive in their preferred niches. Outer membrane vesicles (OMVs) are an alternative vehicle for bacterial communication with the surrounding environment. Bacterial vesicles were identified several decades ago (1), but their importance has recently gained recognition. OMVs are ubiquitous structures released from all Gram-negative bacteria studied to date and deliver a variety of molecules that benefit the bacteria. These extracellular organelles can perform a variety of functions, including protection of the cell from antimicrobials, delivery of toxins, transfer of proteins and nucleic acids between bacterial cells, and secretion of virulence factors. In view of the diverse roles and constitutive production of OMVs, modulation of their formation and functions may provide an alternative solution for controlling bacterial prevalence (2). In this review, we focus on the roles of OMVs and their constituents in the regulation of virulence in pathogenic bacteria. Understanding the diverse roles of OMVs may allow us to disarm pathogens and exploit these multifunctional extracellular vesicle organelles as delivery tools for useful materials, such as vaccines.

II. What are Outer Membrane Vesicles (OMVs)?

As electron micrography of Gram-negative bacteria revealed the frequent presence of spherical blebs (3), outer
membrane vesicle (OMV) production has been considered an evolutionarily conserved process among Gram-negative bacteria (4, 5). OMVs have been observed in all stages of bacterial growth and under a variety of growth conditions, including planktonic growth in fluid environments and static growth as biofilms on solid surfaces (6, 7). OMVs are spherical structures 10–300 nm in diameter that pinch off from the cell surface and encompass a broad range of molecules derived from the outer membrane, including OM-anchored lipoprotein, OM phospholipids, and lipopolysaccharide (LPS). However, the protein and lipid contents of OMVs are not identical to those in the outer membrane. Some proteins are selectively trapped in OMVs while others are excluded from OMVs in comparison to their abundance in the whole cell. Furthermore, OMVs are generally devoid of inner membrane and cytosolic constituents, although they are enriched with proteins present in the outer membrane and the periplasm. No signal sequence is required for the localization of proteins into vesicles since heterologous proteins can be entrapped in the vehicles (8–10). Interestingly, some bacteria secrete DNA and RNA fragments derived from the chromosome and plasmids into OMVs (11, 12).

Why do bacteria transport entire portions of their envelope to vesicles? Diverse functions have been ascribed to OMVs, and the underlying common attribute is related to the bacterial dynamic interaction with the environment. Vesiculating bacteria may acquire a growth advantage by OMV secretion in a mixed bacterial population where nutrients are limiting. Pseudomonas aeruginosa secretes periplasmic peptidoglycan hydrolases through vesicles and eliminates neighboring Gram-negative and -positive bacteria for its proliferation (13, 14). Furthermore, Bacteroides (Fibrobacter) succinogenes, an anaerobic bacterium from the gastrointestinal tract, releases cellulase and xylanase into vesicles, which can aid in nutrient acquisition and thereby provide a growth advantage (15).

OMVs may also protect bacteria from bactericidal antibiotics. P. aeruginosa packages β-lactamase into the lumen of vesicles and promotes resistance to penicillin and cephalosporin treatment (16). In terms of OMV-mediated interspecies communication in bacteria, extracellular β-lactamase secreted by vesicles benefits not only the OMV-producing strain but also co-colonizing species in a mixed population.

Vesicles facilitate the formation of biofilms that provide bacteria with a resistant shelter against environmental stimuli such as antibacterials. LPS-abundant in the outer membrane and OMVs play an important role in cellular survival, adaptation, and virulence, and its location in the outer membrane compartment allows LPS to directly influence surface characteristics and the ability of cells to bind to various substrates (17). P. aeruginosa modulates the LPS composition to determine lifestyles between planktonic and static states (18). Hence, OMVs enriched with LPS have been identified as an important component of the extracellular matrix of bacterial biofilms and contribute a large portion of the protein content of this extracellular niche. Porphyromonas gingivalis, a major etiological agent of chronic periodontitis, produces OMVs harboring a putative heme-binding lipoprotein (HmuY) associated with the outer membrane, which aids in biofilm accumulation on abiotic surfaces and cell survival under starved plaque conditions (19).

Considering cell-to-cell signaling via vesicles, OMVs traffic quorum-sensing molecules. Bacteria utilize quorum-sensing signals to communicate and coordinate social activities. P. aeruginosa secretes the signaling molecule 2-heptyl-3-hydroxy-4-quinolone (PQS) into membrane vesicles and delivers the signal within mixed populations of bacteria (20). The addition of PQS to growth medium also stimulates OMV production, indicative of an intimate correlation between vesicle formation and quorum sensing. Removal of these vesicles from the bacterial population abolishes PQS-controlled group behavior.

**III. Questions Regarding OMV Biogenesis**

Numerous studies based on mass spectrometry have determined the OMV composition in various species and conditions and revealed diverse roles of vesicles in bacteria. However, the mechanism and regulation of OMV biogenesis...
remains unclear. OMVs appear to trap the cargo proteins and other molecules that are abundant at the site of budding. Therefore, their constituents may provide information regarding the formation and release mechanisms. Proteins identified repeatedly in proteomic analysis of OMVs from diverse species are thought to be involved in OMV biogenesis. To date, no conserved components have been identified by numerous proteomics studies (21–23).

The envelope of Gram-negative bacteria consists of an outer membrane, an inner membrane, and a periplasm with a thin layer of peptidoglycan between the two membranes. Lpp, an abundant lipoprotein of *Escherichia coli* and many other Gram-negative bacteria, covalently cross-links the outer membrane and the peptidoglycan layer, providing structural integrity in the envelope. Lpp exists in two forms: free and bound. The free form of Lpp is mainly anchored at the outer membrane moiety, whereas the bound form cross-links between the outer membrane and the peptidoglycan. Extensive vesicle formation has been observed in bacteria with mutations and deletions in Lpp (24, 25). Moreover, the released vesicles contain only 35% free Lpp and almost none of the bound form when compared with cellular outer membranes (24). These observations suggest that OMVs may easily pinch off from the outer membrane areas lacking lipoproteins (24, 26).

OMV biogenesis is also attributable to LPS. LPS consists of three parts including lipid A, core oligosaccharide, and O-antigen. The O-antigen moiety comprises the outermost domain of the LPS molecule and is exposed on the outer surface of the bacterial envelope. *P. aeruginosa* concomitantly produces two types of LPS, referred to as the A band and B band, depending on the constituents of O-antigen. The A-band LPS contains a conserved O-antigen region composed of D-rhamnose, while the B-band O-antigen is a heteropolymer composed of di- to pentasaccharide repeats, showing the serotype-specific structure among the 20 O serotypes of *P. aeruginosa*. *P. aeruginosa* strain PAO1, which expresses two O-antigen side chains, releases vesicles enriched with the highly charged B-band LPS (6, 27, 28). A region of the outer membrane enriched with the highly charged B-band form may be deformed and extrude outward due to the repulsing B-band LPS molecules (13, 29).

Hypervesculation phenotypes may be achieved through a defective Tol-Pal system (25). The Tol-Pal system is organized into two protein complexes: an inner membrane complex composed of the TolA, TolQ, and TolR proteins, and another complex associated with the outer membrane. The three proteins TolA, TolQ, and TolR interact with each other via their transmembrane domains, and Pal protein anchored to the outer membrane interacts with the peptidoglycan layer through TolB and TolA, forming the Tol-Pal system across the envelope. Pal is also known to independently interact with the outer membrane proteins of Lpp and OmpA (30). A general prerequisite for vesicle blebs appears to be deformation of the envelope structure to extrude a portion of the outer membrane outward, being detached from the peptidoglycan layer and the inner membrane. Dissociation between the outer membrane and the peptidoglycan layer may be accomplished through biased localization of Pal (31) as well as downregulation of Lpp and LPS (32).

**IV. Roles of OMV in Pathogenic Bacteria**

One important function attributed to OMVs in pathogens is that they function as a delivery system for virulence determinants. As a tool to facilitate their interaction with host cells, a number of pathogenic bacteria release vesicles enclosing mixtures of toxins, degradative enzymes, virulence factors, and proinflammatory molecules, and disseminate the contents into the residing environment (Table 1). The released OMVs benefit pathogens in offensive and defensive ways. Virulence factors enclosed in OMVs can be delivered at a distance, protecting themselves against diverse environmental stresses, and the vesicles containing virulence determinants integrate into the host plasma membrane, trafficking the contents directly into host cells (33, 34). The delivered virulence factors mediate host cell apoptosis and manipulate host immune responses (4, 35). As a defensive strategy, OMVs serve as bacterium-like decoys that protect the cells by absorbing antibacterial agents and neutralizing host immune responses (36, 37).
1. OMV-associated virulence factors

Pathogenic bacteria have been shown to increase vesicle production during infection (38, 40). *Salmonella* Typhimurium possesses a specialized apparatus called the type III secretion system (T3SS) and secretes more than 40 virulence factors. Table 1 lists a selection of OMV-associated virulence factors.

| OMV-associated proteins | Species | Activity | Reference |
|-------------------------|---------|----------|-----------|
| Apx toxin | *Actinobacillus pleuropneumoniae* | Hemolysis, Cytolysis | (59) |
| BabA, BabA | *Helicobacter pylori* | Adhesin | (60) |
| CagA | *Helicobacter pylori* | Cytotoxicity-associated immunodominant antigen | (61) |
| Cholera toxin (CTX) | *Vibrio cholera* | Adenylate cyclase activation | (62) |
| Cif | *Pseudomonas aeruginosa* | Cystic fibrosis transmembrane conductance regulator (CFTR) inhibition | (63) |
| Cytotoxic distending toxin (CDT) | *Aggregatibacter actinomycetemcomitans, Campylobacter jejuni, Escherichia coli* | DNA damage, Cell death | (64, 65) |
| Cytotoxic necrotizing factor type 1 (CNF1) | *Uropathogenetic Escherichia coli* | Cytotoxic | (66) |
| Cytotoxin ClyA | *Escherichia coli O111:H*- | Pore-forming | (48) |
| Gingipains (RgpA, RgpB, Kgp) | *Campylobacter jejuni, Porphyromonas gingivalis* | Trypsin-like cysteine proteinases | (22, 67) |
| Heat-labile enterotoxin (LT) | *Enterotoxigenic Escherichia coli* | Enterotoxic and vacuolating activities | (68) |
| HmuY | *Porphyromonas gingivalis* | Sequestering heme from host carriers | (19) |
| HtrAb | *Borrelia burgdorferi* | Proteolytic activity | (69) |
| IpAβ, IpAC, IpAD | *Shigella flexneri* | Invasins | (70) |
| Leukotoxin (Ltx) | *Aggregatibacter actinomycetemcomitans, Campylobacter jejuni* | Pore-forming | (71, 72) |
| NarE | *Neisseria meningitidis* | Iron-containing ADP-ribosyltransferase | (73) |
| OmpA | *Aggregatibacter actinomycetemcomitans* | Adhesin/invasion, immune evasion, biofilm formation | (71) |
| OspA, OspB | *Borrelia burgdorferi* | Outer membrane surface antigens | (74) |
| PaAP | *Pseudomonas aeruginosa* | Aminopeptidase | (36) |
| PagC | *Salmonella enterica serovar Choleraesuis* | Required for survival | (75) |
| PagJ, PagK1, PagK2 | *Salmonella enterica serovar Typhimurium* | Required for survival | (45) |
| PorA | *Neisseria meningitidis* | Outer membrane protein (OMP) antigens | (73) |
| RTX (repeat-in-toxin) toxin | *Vibrio cholera* | Cross-linking of actin cytoskeleton | (76) |
| Serralysin | *Pseudomonas aeruginosa* | Extracellular protease | (60) |
| Shiga toxins (Stx, Stx1, Stx2) | *Pseudomonas aeruginosa, E. coli* | Protein synthesis inhibition | (13, 77) |
| UspA1, UspA2 | *Moraxella catarrhalis* | Surface adhesion protein | (39) |
| VacA | *Helicobacter pylori* | Vacuolating cytotoxin | (78) |
| α-Hemolysin (HlyC) | *Enterohemorrhagic E. coli* | Acryltransferase inducing hemolysin | (68) |
| β-Lactamase | *Pseudomonas aeruginosa* | Antibiotics resistance | (79) |
virulence factors to interact with host cells. Secreted virulence factors play diverse roles in pathogenicity including actin rearrangement, induction of the inflammatory response, and modulation of vesicular trafficking (41–43). Recently, it was found that a set of virulence factors of the PhoP/PhoQ regulon, including PagC, PagK1/K2, and PagJ, were associated with OMVs in *S. Typhimurium* and translocated into the cytoplasm (Fig. 1) (44, 45). Although the functions of these OMV-associated virulence factors remain unclear, their absence attenuated *Salmonella* survival inside the host. The PhoP/PhoQ two-component regulatory system likely controls the secretion of virulence factors via OMVs as well as the modulation of LPS structure in response to the extracellular environments encountered during infection.

*Legionella pneumophila* also utilizes OMVs to disseminate virulence effectors into phagosomes to inhibit phagolysosome fusion (46). *Legionella* uses the Dot/Icm type IV secretion system to translocate virulence effectors. However, these two systems are independent of each other with respect to the contents delivered, but probably contribute synergistically to bacterial infection. OMVs are produced intracellularly within *Legionella*-specific phagosomes and deliver a variety of virulence factors including Mip, IcmK/IcmX, flagellin, and destructive enzymes to promote bacterial proliferation by degrading host local matrices and facilitating bacterial migration (47).

**Figure 1.** Translocation of PagK1/PagK2/PagJ into the host cytoplasm. Translocation of PagK1, PagK2, and PagJ into the cytoplasm was examined using CCF4-AM cleavage (45). PagK homologue proteins were fused with β-lactamase (Bla), which can cleave CCF4-AM to change its emission spectrum from green to blue when it is translocated into the cytoplasm. RAW264.7 cells were infected with wild-type *Salmonella* and Bla fusion strains for 18 hours. As a positive control, SseJ (a well-studied virulence effector) was tagged with Bla and examined together. Cells were loaded with CCF4-AM for 2 h and the cleavage of fluorescent substrates was investigated at emission wavelengths of 528 nm (green) and 457 nm (blue). *Salmonella* expressing Tomato fluorescence (pWKS30-Tomato) are shown in red.
2. OMVs as toxin transporters

Some pathogenic bacteria release toxins using vesicles. In many cases, toxins delivered by vesicles are more active than the toxins alone when applied to host cells because vesicles produced from pathogenic bacteria contain not only toxins but also other bacterial components such as LPS. Besides, vesicles provide favorable conditions to stabilize the activity of toxins. ClyA, a pore-forming cytotoxin, is active when delivered via OMVs in E. coli (48). ClyA present in the periplasm is oxidized at the cysteine residues and becomes inactive. Reduction of the cysteines is critical for the conversion of ClyA into active oligomers. The lumen of OMVs has a redox state different from that of the periplasm, and ClyA proteins enclosed in vesicles return to active oligomeric structures.

Heat-labile enterotoxin (LT) is secreted first through the outer membrane by the type II secretory pathway in E. coli, and then becomes associated with LPS on the extracellular surface of the outer membrane and is re-located in the lumen and external surface of vesicles when OMVs pinch off from the outer membrane (49, 50). More than 95% of secreted LT activity is accomplished by vesicles harboring LT (50). Purified LT-containing vesicles specifically bind, enter, and deliver the toxin into epithelial cells (33, 51).

3. OMV-mediated adherence and invasion into host cells

Since Gram-negative pathogens often localize adhesins on the surface, vesicles composed of a subset of outer membrane proteins are also adhesive. Accordingly, vesicles with surface adhesins can mediate bacterial adhesion to host cells as well as subsequent internalization of vesicle material (52). It remains unclear how OMV-localized material influences the adherence of parent bacteria because fimbriae, the dominant bacterial adhesins, have not been detected in purified vesicles. OMVs may bind epithelial cells and bridge the interaction between bacterial and host cells. Alternatively, OMV materials internalized into host cells may promote morphological changes of the host cells to enhance bacterial binding. An adherent-invasive strain of E. coli became noninvasive with a defect in vesicle formation (53), and it is believed that OMVs translocate effectors into the host cell to facilitate bacterial uptake. The vesicles isolated from the wild-type strain enabled the noninvasive mutant strain to enter host cells in trans.

4. Manipulation of the host immune response by OMV

Vesicles may mimic bacteria and enable pathogens to evade immune detection during colonization. Vesicles pinching off from the outer membrane contain numerous natural adjuvants, such as LPS and surface-localized bacterial antigens, which are recognized by host cells in the innate and adaptive immune response pathways. OMVs containing LPS, lipoproteins, and other antigens stably in the lumen or on the surface interact with Toll-like receptors (TLRs) and trigger the migration of white blood cells, mimicking pathogenic bacteria. Salmonella OMVs activate macrophages and dendritic cells to increase the expression of major histocompatibility complex (MHC) class II, and also stimulate the secretion of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-12 (IL-12) (54). Accordingly, OMVs induce CD4+ T-cell activation, indicating that bacterial antigen molecules delivered by vesicles are successfully processed and presented by the antigen-presenting cells. Mice infected with S. Typhimurium activate CD4+ T cells, which recognize antigens in Salmonella vesicles (55).

A proinflammatory response to bacterial OMVs has been reported frequently. Neisseria meningitides treated with detergent produce vesicles, which provoke neutrophils to produce proinflammatory cytokines including TNF-α, IL-8, IL-1β, and macrophage inflammatory proteins 1β (MIP-1β) (56). Campylobacter jejuni also packages diverse immunogenic N-linked glycoproteins in vesicles and delivers the contents to stimulate immune responses (22).

P. gingivalis OMVs appear to downregulate and stimulate host inflammatory responses. A group of gingipain proteases delivered via vesicles degrade CD14 protein in human macrophage cells, which may compromise the immune system in periodontal disease (57, 58).
V. CONCLUSION

OMVs, a constitutive secretion system ubiquitous in all Gram-negative bacteria, accomplish diverse roles in pathogenic and non-pathogenic bacteria. By virtue of their small sizes and solid bilayered structures, OMVs can be applied to develop a delivery vehicle. OMV-based carrier systems may provide many benefits, including protection of cargo from proteolytic degradation, application as complexes with other cooperating materials, bacteria-free long-distance delivery, and modulation of the immune response. For the versatile utilization of OMV structures, it is important to define the mechanism of vesicle formation and release and the basis of cargo selectivity. Comparative multi-omics approaches for OMVs of multiple species under diverse environmental conditions may be used to explore the universal biogenesis of OMV.

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