

Review

Bacterial Outer Membrane Vesicles as a Delivery System for Virulence Regulation

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Outer membrane vesicles (OMVs) are spherical nanostructures that are ubiquitously shed from gram-negative bacteria both in vitro and in vivo. Recent findings revealed that OMVs, which contain diverse components derived from the parent bacterium, play an important role in communication with neighboring bacteria and the environment. Furthermore, nanoscale proteoliposomes decorated with pathogen-associated molecules attract considerable attention as a non-replicative carrier for vaccines and drug materials. This review introduces recent advances in OMV biogenesis and discusses the roles of OMVs in the context of bacterial communication and virulence regulation. It also describes the remarkable accomplishments in OMV engineering for diverse therapeutic applications.

Keywords: Outer membrane vesicle, virulence, pathogen, biogenesis

Introduction

Outer membrane vesicles (OMVs) have been observed in diverse bacterial taxa, but their importance has only recently received attention. Gram-negative bacteria secrete nanoscale vesicles of 20 to 250 nm in diameter under a variety of conditions from planktonic to sessile biofilm states, and in environments from marine ecosystems to mammalian cells. Bilayered spherical vesicles containing proteins, DNA, and RNA of the parent bacterium deliver their cargo at a distance without the need for contact. OMV-associated proteins with enzymatic activities provide commensal bacteria with additional nutrients by degrading uncommon substrates such as polysaccharides [15], and also protect co-existing bacteria by detoxifying antimicrobial molecules [4, 16]. The incorporation of cytoplasmic DNA into vesicles enables intraspecies DNA exchange [29]. In the case of pathogenic bacteria, their OMVs enriched with lipopolysaccharide (LPS) and membrane-bound proteins exhibit immune-stimulatory capabilities and facilitate establishment of infection and inflammation. Despite the multitude of functions of OMVs, the molecular mechanisms underlying their formation and secretion, and the basis of cargo selectivity are not fully understood. Further advances

in OMV biogenesis will facilitate exploitation of nano-sized OMVs as a biomedical delivery system.

OMV Biogenesis

OMVs are spherical structures that pinch off from the cell surface and enclose a broad range of molecules such as lipoproteins, phospholipids, and LPS, which are distributed in the outer membrane. Therefore, in principle, the outer membrane becomes dissociated from the underlying peptidoglycans and protrudes outward, forming a sphere-like compartment [13]. Mass spectrometry-based OMV profiling in diverse gram-negative bacterial taxa has provided evidence regarding the formation and release mechanism, since OMVs appear to be enriched with proteins at the sites of envelope bulges. However, no specific component was conserved among OMVs of several bacterial taxa according to proteomic analyses. This is likely due to the compositional diversity of bacterial species in various environments [22]. Despite using identical bacteria, the protein compositions of OMVs vary according to the isolation conditions used (Fig. 1). *Salmonella* Typhimurium OMVs were abundant in membrane proteins associated with nutrient transport when harvested under limiting nutritional conditions mimicking

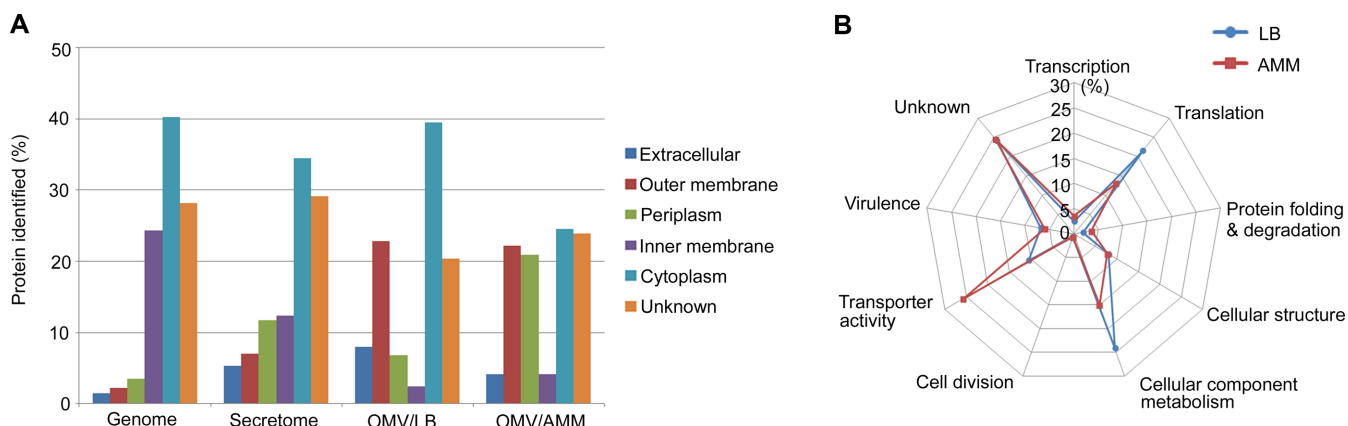


Fig. 1. *Salmonella* OMV contents according to growth conditions (Images adapted from Bai *et al.* [2]).

(A) The subcellular location of OMV-associated proteins was predicted using PSORTb (<http://www.psort.org/psortb/>). OMVs were isolated under nutrient-abundant (LB) or nutrient-limiting (acidic MgM; AMM) conditions. Proteins identified through *S. Typhimurium* genome annotation and secretome analysis were subjected to subcellular location prediction and compared in parallel. (B) OMV-associated proteins were classified using Gene Ontology-based functions. The OMV-associated proteins unique to the LB or acidic MgM (AMM) conditions were grouped based on their biological processes and molecular functions, and their proportions under each condition are plotted.

intracellular environments, but were enriched with cytosolic proteins involved in translation and cellular metabolism under nutrient-abundant conditions [2]. Although no proteins have been determined to be closely associated with OMV formation, it is generally believed that bacteria shed vesicles in response to membrane stresses [3]. The envelope of gram-negative bacteria is composed of an outer membrane and an inner membrane, and a thin layer of peptidoglycan in the periplasmic space links the two membranes. Lpp is an abundant outer membrane lipoprotein distributed evenly throughout the cell surface and covalently cross-links the outer membrane and the peptidoglycan layer, providing structural integrity to the envelope. When the cross-links bridging the outer membrane to peptidoglycans are locally broken by an altered balance between peptidoglycan breakdown and synthesis, small portions of the outer membrane bulge away from the cell, leading to vesiculation [21]. Another model of OMV formation involves nanoterritories, where misfolded proteins or aberrant envelope components accumulate. Abnormal build-up of cellular components in the periplasm in response to environmental stresses decreases envelope integrity and extrudes a portion of the outer membrane outward, being detached from the peptidoglycan layer and the inner membrane [20]. Additionally, it is hypothesized that altered biophysical characteristics of the outer membrane may induce vesiculation [18]. Incorporation of specifically modified LPS or phospholipids into the outer membrane leads to changes in membrane flexibility and fluidity, resulting in

curvature of the membrane and hence budding off of OMVs. Besides these three mechanisms, many other factors are speculated to influence the size, production rate, and constituents of OMVs. Further comparative analysis of vesicle proteomic data would improve our understanding of the universal biogenesis of OMVs.

OMVs as a Vehicle for Intra- and Inter-Species Communication

OMVs are exploited as a multifaceted delivery system for intraspecies and interspecies interactions. As common resources, OMVs benefit commensal bacteria as well as the producing bacteria. Vesicles harboring scavenging enzymes such as proteases and cellulases are disseminated into barren environments and aid in nutrient acquisition, thereby providing a survival advantage for both the producing bacteria and bystander, non-OMV-producing bacteria. *Bacteroides ovatus* OMVs carry inulin-degrading enzymes and support the growth of non-utilizer *B. vulgatus* in defined inulin medium, demonstrating a role for OMVs in ecological interactions among *Bacteroides*, the major genus of the human gut microbiota [15]. OMVs produced from *Mycobacterium tuberculosis* carry the siderophore mycobactin, an iron acquisition system, which facilitates capture of this essential nutrient at a distance in the hostile host environment [14]. In the context of interspecies interaction via OMVs, it should be noted that OMVs mediate both beneficial and harmful effects. Some bacteria deliver

hydrolytic and proteolytic enzymes among competing bacterial species through vesicles and take advantage of nutrients liberated from the lysed prey bacteria [5]. Recent findings revealed the importance of OMVs in antimicrobial resistance. Vesicles containing β -lactamase confer resistance not only to the producer but also to other bacteria, including commensal and pathogenic bacteria co-occurring with the producer at the site of infection (Table 1) [17, 26]. Vesicle-associated antibiotic resistance is also accomplished through DNA exchange. *Acinetobacter* spp. OMVs can transfer double-stranded DNA to the same species or other genera, such as *Escherichia coli* [6]. OMVs isolated from the foodborne pathogen *E. coli* O157:H7 transfer genetic materials such as virulence genes to recipient bacteria, *Salmonella enteric* serovar Enteritidis or *E. coli* JM109, and render the recipient bacteria more cytotoxic to host cells [32]. OMV-mediated DNA transfer suggests an important role for OMVs in bacterial evolution in mixed microbial communities from host animals to diverse ecosystems.

OMVs as a Delivery Vehicle for Virulence Factors

Accumulating data demonstrate that many bacterial pathogens utilize vesicles to deliver virulence determinants to host cells at local and distal sites, which in turn compromise the host defense system and manipulate the immune responses to facilitate infection of the host. In parallel, OMVs produced by commensal bacteria play a role in facilitating maturation of the immune system [24]. OMVs from various mucosal pathogens display numerous

pathogen-associated molecular patterns, including LPS, lipoprotein, DNA, and RNA. Their interaction with pattern-recognition receptors (PRRs) of host epithelial cells stimulates the production of cytokines and chemokines in the mucosal epithelial surface, the first line of host defense. OMVs released from *E. coli* and *Pseudomonas aeruginosa* engage Toll-like receptor 4 on epithelial cells as a PRR and provoke the production of cytokines, chemokines, myeloid differentiation primary response protein 88, and nuclear factor- κ B, leading to inflammation [25, 34]. Involvement of OMVs in the interaction with immune cells—including macrophages, neutrophils, and dendritic cells—results in modulation of pathology in various ways, depending on their bacterial origin and content, by either stimulating or limiting the activation of these myeloid cell subsets. OMVs from *Salmonella* spp. and *Porphyromonas gingivalis* activated murine macrophages, inducing the production of the proinflammatory mediators of tumor necrosis factor, nitric oxide (NO), and inducible NO synthase [1, 8]. Meanwhile, *H. pylori* OMVs increased the production of immunosuppressive cytokines in peripheral blood mononuclear cells, which limits inflammation and aids immune evasion by the pathogen [31]. In addition to the ubiquitous OMV constituents, such as LPS, bacterial pathogens deliver specific virulence factors to host cells via OMVs without cell-cell interactions. Virulence factors encompassed within OMVs can be delivered at a distance, and so are not compromised by dynamic physical and biochemical stresses, and they can translocate across the plasma membrane of host cells [11, 23]. *S. Typhimurium* utilizes OMVs to translocate a set of virulence factors of the PhoP/PhoQ regulon—including PagC, PagK1/K2, and PagJ—into the cytoplasm of host cells; a mutant lacking the cognate virulence determinants exhibit attenuated virulence in mice [33]. *Legionella pneumophila* secretes OMVs containing a variety of virulence factors within *Legionella*-specific phagosomes. The cargo proteins—including Mip, IcmK/IcmX, flagellin, and destructive enzymes—facilitate bacterial proliferation by degrading host local matrices and promoting bacterial migration [7]. In addition, *Moraxella catarrhalis* delivers a superantigen *Moraxella* immunoglobulin D-binding protein to B cells via OMVs and activates the production of polyclonal immunoglobulin M in B cells. This delays the production of specific antibodies against *M. catarrhalis* and so benefits its survival in the host [30].

Table 1. Cross-protection between intra- and inter-species by OMVs against cefotaxime (data adapted from Stentz *et al.* [26]).

Recipient ^a	OMV donor ^b	Resistance or susceptibility ^b
<i>Salmonella</i>	<i>B. thetaiotaomicron</i>	R
Typhimurium	<i>B. thetaiotaomicron</i> Δ cepA	S
	<i>Bacteroides dorei</i>	S
	<i>B. fragilis</i>	R
	<i>Bacteroides ovatus</i>	S
	<i>B. stercoris</i>	R
	<i>B. xylanisolvens</i>	S
<i>Bacteroides breve</i>	<i>B. thetaiotaomicron</i>	R
	<i>B. thetaiotaomicron</i> Δ cepA	S

^aInter (*Salmonella* Typhimurium) and intra (*Bacteroides breve*) species treated with *Bacteroides* spp. OMVs.

^b*Bacteroides* species used in OMV isolation; *B. thetaiotaomicron* Δ cepA, a mutant unable to degrade cefotaxime.

^cResistance of recipient species against cefotaxime at 10 mg/l.

Biomedical Applications of OMVs

OMVs, which retain the physicochemical characteristics of

the parent bacteria but are not capable of replication, have been a fascinating subject in vaccine development studies. For instance, *Neisseria meningitidis* OMVs stimulate a strong immune response and have been developed into a vaccine formula, Bexsero (Novartis), which was approved in Europe. However, the lack of information on OMV biogenesis hinders modification for large-scale OMV production, and the excessive toxicity of pathogen-derived OMVs prevents their clinical application. This review introduces recent achievements in OMV bioengineering. Targeting heterologous proteins of interest to different compartments of OMVs (*i.e.*, the outer leaflet surface, the membranous space and the lumen) is based in principle on general genetic and biomolecular techniques. Proteins tagged with signal peptides of the general secretory or twin-arginine translation system at their N-termini are thought to be translocated across the inner membrane and eventually are targeted into the vesicle lumen [10]. When a protein is required to be exposed on the exterior of OMVs to enable its interaction with a receptor on the plasma membrane of host cells, for instance, it is genetically fused with a membrane protein anchor that directs the fusion partner to the outer membrane of the parent bacterium and thereby to the membranous structure of OMVs [12]. Autotransporter systems can be exploited to promote “flip-flop” of a target protein to the surface of the outer leaflet of OMVs. Their N-terminal passenger domains linked to a protein of interest would automatically cross the membrane through pores formed by their C-terminal β -barrel domains [9, 19]. ClyA, a pore-forming cytotoxin located on the surface of *E. coli* OMVs, has been utilized to target heterologous proteins directly to bacterial OMVs. Diverse proteins—including β -lactamase, organophosphorus hydrolase, a single-chain Fv antibody, and GFP—were fused with the N- or C-terminus of ClyA and displayed on the exterior of OMVs with their biological activities retained [12]. For the biomedical application of OMVs (*e.g.*, as drug delivery vehicles), detoxification of these nanoparticles is important, since the outer membrane compartment is enriched with LPS, which stimulates inflammation and modulates immune responses. Attenuation of LPS toxicity can be accomplished in part by genetic modification. LPS consists of membrane-embedded endotoxin lipid A, a core oligosaccharide, and the O-antigen, a polysaccharide exposed on the outer membrane surface. A typical lipid A moiety of pathogens such as *E. coli* and *Salmonella* spp. possesses six fatty-acyl chains. *N. meningitidis* mutants lacking LpxL produce penta-acylated lipid A instead of hexa-acylated lipid A and exhibit less toxicity than wild-type strains while retaining

the desirable adjuvant activity [28]. The LPS structure can also be altered by introducing heterologous LPS-modifying enzymes. Expression of *H. pylori* Hp0021, a lipid A 1-phosphatase, in *E. coli* resulted in biosynthesis of monophosphorylated lipid A instead of the natural diphosphorylated form in *E. coli*. This would likely lead to less stimulation of the host innate immune response [27].

Concluding Remarks and Outlook

OMVs merit further exploration for biomedical applications owing to their nanosize and robust bilayered structures. However, despite the multiple benefits ascribed to OMVs, many questions regarding OMV biogenesis and cargo selectivity remain to be answered. Accumulation of OMV proteome information and genome engineering will reveal the mechanism(s) of OMV biogenesis and facilitate OMV bioengineering for their application in biotechnology and biomedicine.

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