Outer membrane vesicles (OMVs) from Gram-negative bacteria were first considered as artifacts and were followed with disbelief and bad reputation. Later, their existence was accepted and they became characterized as bacterial bombs, virulence bullets, and even decoys. Today, we know that OMVs also can be involved in cell–cell signaling/communication and be mediators of immune regulation and cause disease protection. Furthermore, OMVs represent a distinct bacterial secretion pathway selecting and protecting their cargo, and they can even be good Samaritans providing nutrients to the gut microbiota maintaining commensal homeostasis beneficial to the host. The versatility in functions of these nanostructures is remarkable and includes both defense and offense. The broad spectrum of usability does not stop with that, as it now seems that OMVs can be used as vaccines and adjuvants or vehicles engineered for drug treatment of emerging and new diseases not only caused by bacteria but also by virus. They may even represent new ways of selective drug treatment.

Keywords: outer membrane vesicles; offense; defense; versatility in functions

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Outer membrane vesicles (OMVs) are released from Gram-negative bacterial cells, as well as from Archaea, fungi, and parasites. The production of OMVs was first reported more than 40 years ago (1) but their full biological significance was first recognized recently, particularly in Gram-negative bacteria. Membrane vesicle (MV) production in Gram-positives was long overlooked (2) but has now been demonstrated in *Streptococcus pneumoniae*, *Streptococcus mutans*, *Staphylococcus aureus*, and *Bacillus subtilis* (2–5). OMVs range in size from 20 to 300 nm in Gram-negative bacteria. MVs in Gram-positive bacteria are somewhat smaller. Due to their small size they have been characterized as nanovesicles (6, 7). The vesicle membrane in Gram-negative bacteria is somewhat different from that of the outer membrane (OM) although there are great similarities (8). It is still unclear how OMVs are generated in detail. They are formed when the OM bulges and encapsulates periplasmic components (9–11) (Fig. 1) which involve membrane remodeling (8).

The present review will deal mainly with OMVs from Gram-negative bacteria where they can be derived from both pathogenic and non-pathogenic species. OMVs used to have a disreputable past, first being considered as debris or artifacts (12). Today, we know that OMVs have very diverse functions. They are involved in both defense and offense. The field is quite extensive and only major functions will be dealt with. Functions mentioned for OMVs of the periodontopathogenic *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* are listed in Table 1.

**OMVs as communication tools**

**OMVs as secretion system**

OMVs enable secretion of insoluble or hydrophobic material such as lipids, membrane proteins, and signaling molecules (13). They have a number of advantages over simple secretion systems in bacteria because the cargo is protected inside the lumen of the vesicle which can be targeted to specific destinations through receptors (14). In the case of gentamicin-containing OMVs, these OMVs may help eradicate even intracellular pathogens not reached by gentamicin in external body fluids (15). Different toxins can be transported by OMVs in different
bacteria. In *A. actinomycetemcomitans*, biologically active cytolethal distending toxin (CDT) depended on vesicle transport into HeLa cells and human gingival fibroblasts (16). OMVs were internalized in these cells by fusion with lipid rafts in the plasma membrane and the active toxin unit, CdtB, was localized inside the nucleus of the intoxicated cells. It has been suggested that OMVs, due to their preponderance in biofilms, could be used to deliver cell toxins which would affect only the intended target (7). OMVs can also be internalized in host cells with the result that intracellular constituents can become degraded leading to cellular malfunction (17).

Many organisms use OMVs to secrete virulence factors. Examples are cytotoxin Cly from *Escherichia coli* and *Salmonella enterica* serovar Typhi, the heat-labile enterotoxin of enterotoxigenic *E. coli* and the vacuolating cytotoxin VacA of *Helicobacter pylori* (reviewed by Ref. 18). Notably, bacteria actively regulate their OMV cargo to manipulate the host-pathogen interplay.

Gentamicin has been shown to induce *Pseudomonas aeruginosa* to generate OMVs containing this aminoglycoside (19). The OMVs were similar to natural ones except that they contained small amounts of gentamicin. The synergistic effect of antibiotic plus autolysin in gentamicin-containing OMVs can suggest a novel strategy as how to deal with hard-to-kill pathogens (19). OMVs can also deliver enzymes and lipopolysaccharide (LPS) in high concentration to the target as a package (11) excreting membrane-perturbing substances from the cell. Dorvard et al. (20) proposed that OMVs can function as a mechanism of genetic exchange between cells because they are efficient mediators of genetic transformation. Indeed DNA in OMVs has been successfully transferred into other bacterial cells, even between cells of different species (20–22). This may represent a so far little recognized DNA delivery system for bacteria (20, 23, 24).

OMVs can contain β-lactamases which may protect bacterial species against the stress of antibiotics (25–28). This could represent a new form of drug delivery (29–31). When bacterial cells are among non-competitors their OMVs probably deliver ‘benign’ messages, whereas the cargo may change when the cells are faced with stress, competition, or prey (7). To be able to differentiate between messages from a friend or foe OMVs would probably need a barcode-like recognition system (7).

**Effect on innate and adaptive immune system**

Through delivery of enzymes, toxins, communication signals, and antigens, OMVs can influence the innate and adaptive immune systems (18). In bacterial OMVs, Toll-like receptor ligands such as LPS and lipoproteins stimulate maturation of and cytokine release by macrophages and dendritic cells (DCs). This may promote the pathogenesis of infections. Furthermore, peptidoglycan-containing OMVs upregulated nuclear factor-kappaB (NF-kB) and nucleotide-binding oligomerization domain (NOD)1-dependent responses *in vitro* (32, 33). This was suggested as a new mechanism whereby Gram-negative bacteria can deliver peptidoglycan to cytosolic NOD1 in host cells and thereby promote inflammation and pathology.

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**Table 1. Functions of OMVs from *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* mentioned in this review**

| Function/Effect                                      | *P. gingivalis* | *A. actinomycetemcomitans* |
|-----------------------------------------------------|-----------------|-----------------------------|
| OMVs as communication tools                          | Ref             | Ref                         |
| OMVs as a secretion system                           | 17              | 16                          |
| Effect on innate and adaptive immunity               |                 | 34                          |
| Ecological determinants                               | 42, 43, 45,     | 48, 51, 57                  |
|                                                     | 46, 47, 52,     |                             |
|                                                     | 53, 54          |                             |
| OMVs as offensive weapons                             |                 |                             |
| Adhesion and invasion                                 | 61, 62, 63,     | 64                          |
| Virulence bullets/bacterial bombs                     | 76, 77, 78,     | 81, 82, 83                  |
| Sepsis                                               | 93, 94          |                             |
| OMVs as possible good Samaritans                     |                 | 97                          |
| Vaccines/adjuvants                                   |                 |                             |
| Classification                                       |                 | 99                          |

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Fig. 1. OMVs observed at the outer cell membrane in *P. gingivalis* with transmission electron microscopy. A: Membrane-blebbing OMVs in strain ATCC 33277 T (type I fimA strain). B: OMVs through the blebbing and pinching-off of the outer membrane in strain TDC 60 (type II fimA strain). Bars = 200 nm.
OMVs-containing antigens such as surface proteins and LPS are probably potent stimulators of the adaptive immune response and both B and T cell antigens have been identified in OMVs (18). In *A. actinomycetemcomitans*, OMVs after internalization into human cells, acted as a trigger of innate immunity by carrying NOD1 and NOD2-active pathogen-associated molecular patterns into host cells (34). It was suggested that OMV internalization can represent an important mechanism for intracellular exposure of antigens for *A. actinomycetemcomitans*. Interestingly, because *A. actinomycetemcomitans* triggers bone resorption mainly via NOD1, intracellular delivery of NOD1 via OMVs may induce periodontal bone loss (34).

Antigen decoys
OMVs may act as decoys *in vivo* meaning that they redirect the antibody response making antibodies ineffective for clearance of intact organisms (18). Thus, proteins and carbohydrates in OMVs may act as additional and significant antigen sources beyond those provided by the organism. This was seen in *Moraxella* that avoided direct interaction with host B cells by redirecting the adaptive humoral immune response by using superantigen-bearing OMVs as decoys resulting in the production of antibodies ineffective for elimination of intact organisms (35). *Moraxella catarrhalis* can actually direct the humoral immune response away from itself. In this bacterium, OMV secretion probably represents a sophisticated mechanism to modify host immune response avoiding direct contact between bacterium and host (36). Microbes can also modulate the host response to suit their lifestyle while staying inside the host, and they can modify the microbial surface to avoid immune detection. Also, OMVs can act as a decoy target for phages (37, 38). In biofilms OMVs have been suggested to serve as decoys for bacteria growing there (39). OMVs can also alleviate stress caused by peptide antibiotics by acting as decoys and transporting these molecules away from the parent cells (38).

Cross-kingdom dialogs
OMVs can mediate intercellular exchange such as cell-cell signaling. In the gut, a bacterial homolog of a eukaryotic inositol phosphate signaling enzyme (InsP6 phosphatase or MINPP) was found to mediate cross-kingdom dialog (40). It was demonstrated that MINPP from *Bacteroides thetaiotaomicron* (BtMinpp) was packaged inside OMVs protecting the enzyme from degradation by external bacterial proteases. Furthermore, BtMinpp-OMVs interacted with intestinal epithelial cells to promote intracellular Ca2+ signaling. In other words, a bacterial enzyme was used to mediate dialog between gut bacteria and the human host. This is a good example of how the microbiota can serve human gastrointestinal physiology and how commensal gut bacteria can use OMVs in a manner that is beneficial to the host.

Intermicrobial communication
OMVs are known to exert important functions not only in inter-kingdom communication but also in intercellular and inter-species communication (18). Intermicrobe cross talk is promoted by OMV release. Examples are *P. aeruginosa* that releases quorum-sensing signaling molecules pqs (*Pseudomonas* quinolone signal) and *Haemophilus influenzae* that releases DNA in OMVs (transformasomes) (18). They are effective mediators of communication even at long distances (41).

Ecological determinants
OMVs, being able to specifically concentrate the release of a large number of its virulence factors into the environment (42) could probably regulate the ecology at the site they are acting against promoting disease, for example, early onset periodontitis.

OMVs can be involved in coaggregation of bacterial cells (43–47) and in adhesion of bacteria, for example, *A. actinomycetemcomitans* to KB epithelial cells (subline of the ubiquitous keratin-forming tumor cell line HeLa) (48). OMVs from a biofilm-forming *H. pylori* strain stimulated biofilm formation in another strain (49). OMVs of *P. gingivalis* contained multiple complexes of adhesins which caused cellular aggregation, autoaggregation, and coaggregation with a number of oral bacteria thereby enabling formation of dental plaque and influencing its ecology (43, 45). In *P. gingivalis*, OMVs promoted adherence between homologous cells and also mediated attachment between non-aggregating bacterial species (43). Kamaguchi et al. (45) found that OMVs from *P. gingivalis* have the ability to aggregate with a wide range of *Streptococcus* species, *Fusobacterium nucleatum*, *Actinomyces naeslundii*, and *Actinomyces viscosus*. When *P. gingivalis* OMVs were present, *S. aureus* also coaggregated with *Streptococcus* species and the mycelial form of *Candida albicans*. It was suggested that strains of *S. aureus*, even the methicillin resistant *S. aureus* (MRSA) type, could adhere to subgingival plaque with *P. gingivalis* present. It has also been shown that *P. gingivalis* or its OMVs can increase attachment and invasion of *Tannerella forsythia* to epithelial cells (46). The mixed interaction of the two red complex bacteria *P. gingivalis* and *T. forsythia* may increase periodontitis pathogenesis through OMV action. The HGP 17 domain was found to be responsible for *P. gingivalis* OMV-mediated aggregation with *Prevotella intermedia* (47).

OMVs from *A. actinomycetemcomitans* promote damage to the sulcular/periodontal epithelium by transporting CDT and LPS to the subgingival area (50, 51). OMVs can therefore act as a transport system to bring virulence factors into host cells affecting the microbial ecology of these cells. OMVs from *P. gingivalis* mediated coaggregation and piggybacking of *Treponema denticola* and...
Lachnoanaerobaculum saburreum (52). This may be a mechanism that provides access of non-motile bacteria to new niches where they might not otherwise be able to penetrate. P. gingivalis OMVs also mediated coaggregation between Capnocytophaga ochracea and L. saburreum (43). Besides, OMVs from P. gingivalis bound to and aggregated A. viscosus and A. naeslundii cells (53). They also attached to serum-coated hydroxyapatite (54). In the OMV-cell recognition, LPS has been suggested to play a role (7).

OMVs can also facilitate transport of material between bacteria to maintain the microbiota (36). They can transfer antibiotic-resistance plasmids among Gram-negative bacteria (20) and P. aeruginosa may deliver antibiotic-resistance enzymes to other bacteria (55). Delivery of murein hydrolase was demonstrated when P. aeruginosa OMVs fused with E. coli and associated with S. aureus (19). It has been suggested that murein hydrolases in OMVs can be an effective way of bringing enzymes to the surfaces of other cells. This could imply fusion of OMVs with foreign membranes (56, 57). Vesicles from Shigella flexneri and P. aeruginosa rapidly fused with the OM of other Gram-negative bacteria (58). Fusion may cause incorporation of vesicle components directly into the cytoplasmic membrane or the cytoplasmic lumen of host cells.

Bacteroides OMVs may have a 'social' function since oligosaccharides, monosaccharides, and amino acids resulting from the activity of their containing hydrolytic enzymes could be made available for other bacteria (59). OMV-packed hydrolases from this bacterium could play an important role in the microbial ecology of the gut (60). Also, digestion of polysaccharides by hydrolases present in OMVs could support the growth of bacteria that are unable to degrade polysaccharides, thereby contributing to the gut homeostasis. This could create balanced ecological units within the gut microbiota (60).

\[ \beta \text{-lactamases in } M. \text{ catarrhalis OMVs were found to enhance survival of its own species and also promote infection with co-inhabiting pathogens such as } H. \text{ influenzae and } S. \text{ pneumoniae (25). This clearly demonstrated} \]

the role of OMVs as an ecological determinant and that can be used by bacteria to establish a colonization niche (36).

OMVs as offensive weapons

Adhesion and invasion

P. gingivalis OMVs swiftly adhered to human gingival epithelial cells in a fimbrriae-dependent manner, and then entered via a lipid rafts-dependent endocytic pathway through the assembly of actin filaments (Fig. 2). The OMVs were routed to early endosomes and thereafter sorted to proteolytic lysosomes (17). Following cell entry, P. gingivalis OMV-associated gingipains degraded cellular functional molecules causing cellular impairment, which included the cellular transferrin receptor and paxillin (integrin-related signaling molecule)/focal adhesion kinase. This caused depletion of intracellular transferrin and inhibition of cellular migration (17).

It has been shown that microspheres coated with P. gingivalis vesicles are adhesive and interact with both bacteria and host cells (61–63). They could even invade host cells and cause cell death (62, 63). Recently, it was demonstrated that minor components of long fimbriae (FimC, D and E) but not FimA were involved in the invasive activities of OMVs from P. gingivalis (64). Notably, P. gingivalis strains that lacked or had a reduced FimA expression exhibited a significant reduction in vesiculation suggesting that production and pathogenicity of P. gingivalis vesicles may depend to a large extent upon expression of the fim locus. Invasion by OMVs could be a new mechanism for P. gingivalis in periodontitis enabling their gingipain content to degrade a range of intracellular functional molecules, resulting in cellular impairment (65).

Bacterial defense

A significant task of OMVs is to remove agents that can harm the cell-surface of the parent bacterium, for example, antimicrobial peptides and T4 bacteriophages (38). When bacterial cells were treated with lytic phage

Fig. 2. Entry of OMVs isolated from P. gingivalis ATCC 33277 into immortalized gingival epithelial cells. The cells were incubated with OMVs (30 μg/ml) for 15 min, then further incubated for the indicated times. For fluorescence microscopy, the cells were processed for staining for OMVs (green) and actin (Alexa Fluor 568-conjugated phalloidin red).
OMV production increased the survival of the cells (38). The number of OMVs released from bacteria seems to be related to stress which may promote biofilm formation and biofilm-specific antibiotic tolerance and resistance (66–68). As mentioned OMVs can probably deliver antibiotics in high concentrations to the target as a package (11) and the biofilm mode of growth can protect them against antimicrobial substances (68). OMVs also have a role in antimicrobial peptide resistance (69). They protect against host antibodies and proteases thereby increasing the half-life of toxins packed inside (70, 71). Besides, they can adsorb antibiotics and complement (72).

**Virulence bullets/bacterial bombs**

OMVs have been designated both as virulence bullets and bacterial bombs. Because a distinction is not easy to make these terms will be used together.

OMVs can be involved in cell–cell inhibition and killing among competing bacterial cells. Thus, they can carry antimicrobials that selectively kill cells from other species (15, 73). In *A. actinomyctemcomitans* OMVs, a leukotoxin kills lymphocytic and monomyelocytic cell lineages which should defend the periodontal pocket against infection. OMVs from the highly leukotoxic strain JP2 were 5- to 10-fold more toxic than vesicles from the minimally toxic strain 652 (74). The vesicles of JP2 were also 4- to 5-fold more toxic than their OM preparations. Therefore, formation of OMVs in *A. actinomyctemcomitans* involved enrichment of leukotoxin. OMVs of *P. gingivalis* contain gingipains which remodel the normally symbiotic microorganism into a pathogenic one by C5 convertase activation (75). Grenier and Bélanger (76) suggested that OMVs and LPSs contain gingipains which can selectively kill cells from other species. In *P. gingivalis* OMVs, which are enriched in serotype specific antigen (B-band) in the O-antigen portion of LPS. This was in contrast to the lipid composition of the OM. Bacteria can even modify their OM cargo according to the environment implying that the OMV components and cargo might be actively sorted by the producing cell (14). In *P. gingivalis*, gingipains which are major virulence factors were selectively sorted out as OMV cargo whereas other abundant OM proteins, for example, those involved in the nutrient uptake, were excluded but remained in the OM (80). Accordingly, OMV production was a result of a directed process where specific events were involved in specific exclusion and/or inclusion of protein sorting into the OM and OMVs (8).

Bacteria have different methods for recruiting cargo into their OMVs (8). The O-antigen of LPS can have a role in the selection of the protein cargo. In *P. gingivalis*, which produces two classes of LPS with either neutral (O-LPS) (81) or negatively charged (A-LPS) O-antigen chains (82, 83), the cargo proteins may have a domain that recognizes the long A-LPS molecules enriched in the OMVs (80). Virulence factors of ecologic importance in *P. gingivalis* are gingipains (RgpA/B and Kgp) which are among the favored OMV cargo (42, 80). This implies that *P. gingivalis* has the ability to selectively sort its C-terminal domains proteins into OMVs.

In Gram-negative bacteria OMVs can be enriched in toxins, quorum-sensing molecules, misfolded proteins, and DNA (8). Proteins of the OMVs are sorted and also glycolipids can be involved in the sorting. In *A. actinomyctemcomitans* a subpopulation of OMVs was found with slight variation in the protein composition (34). Actually, OMVs from *A. actinomyctemcomitans* could deliver multiple proteins simultaneously including OmpA and biologically active cytolethal toxin into HeLa cells and gingival fibroblasts (16). Also in *S. pneumoniae* OMVs, many reported immunogenic protein antigens were found (2), including toxin Ply which is its most widely studied virulence factor. In cystic fibrosis, cystic fibrosis transmembrane conductance regulator (CFTR) is required for mucociliary clearance. *P. aeruginosa* promotes degradation of CFTR through the OMV-packed toxin Cif (CFTR inhibitory factor) (8). Cif is brought to host cells after OMV fusion with lipid rafts causing lysosomal degradation of CFTR (56).

OMVs from one bacterium can kill competing microbes in its vicinity (73). Killing was most efficient if the target bacteria possessed peptidoglycan similar to the OMV donor. If the peptidoglycan hydrolases were similar to those of the target strain they were unable to cleave the peptidoglycan layer (13). This may change if they fuse with cells of a different strain. In that case they can degrade the cell wall and kill the target cell (84). OMVs from bacteria can also fuse with OM of other bacteria. This may release vesicle-encapsulated autolysin to the periplasm thereby causing lysis of the target organisms. This predatory response may allow microcolonies to live in a biofilm at the expense of neighboring cells (85, 86).
Sepsis
Oral bacteria can be associated with systemic diseases (87). Bacteria frequently disseminate through the blood, particularly in periodontitis. OMVs are important sources of inflammatory stimulants both locally and systemically when entering the circulation (88). They induce a robust systemic inflammatory response causing organ damage and death in animals (89, 90). By initiating an inflammatory response, they can induce sepsis in rats even when the cells from which they were derived are absent (89). OMVs also influence the inflammatory and coagulation cascade and may thus contribute to the hypercoagulable response in sepsis (91). Important in this context is their high content of LPS which is a potent proinflammatory trigger. OMVs from different bacteria can activate the immune system in different ways (reviewed by 8) and stimulate the production of proinflammatory mediators such as IL-8, IL-6, IL-12, and TNF-α (92). P. gingivalis OMVs regulate cells that participate in immune responses (93) and even have a crucial role in mucosal immunogenicity (94).

OMVs as possible good Samaritans

Good Samaritans
OMVs can contribute to gut health via immunomodulation of host responses or by providing nutrients to members of the gut microbiota (8). Polysaccharide capsular antigen (PSA) from Bacteroides fragilis reduced inflammation in animals by inducing immune tolerance (95). It seems that OMVs delivered this PSA directly to its host through DCs (96). OMVs that are internalized by DCs induce tolerogenic DCs that generate interleukin 10 (IL-10) which in turn drives the development of IL-10 producing regulatory T-cells (TREGS). Therefore, PSA programs DCs to change into an anti-inflammatory profile that can lead to T-cell-mediated tolerance and protection from experimental disease such as colitis, inflammatory bowel disease, and Crohn’s disease (96). It is likely that delivery of PSA by OMVs contributes to the probiotic properties of B. fragilis in the gut enabling communication between the microbiota and the immune system during host-bacterial mutualism (96). In this interplay PSA of B. fragilis is an archetypical symbiosis factor. Accordingly, OMVs can be important tools for modulators of the microbiota in the gut and in this sense they act as good Samaritans (12). Whether similar modification of the oral microbiota occurs is not known. However, it is likely (by extrapolation) that ‘good Samaritan’ activities might be delivered by vesicles in the mouth that are similar to those reported for the gut ecosystem. It is not clear what maintains the balance between OMVs causing destructive events intracellularly and those that induce a benefit. In the case of B. fragilis, it should be kept in mind that this bacterium belongs to the commensal microbiota of the gut and therefore probably represents no threat to Gram-negatives trying to induce a dysbiotic gut microbiota.

Vaccines/adjuvants
Application of OMVs as vaccine antigens after intranasal immunization of BALB/c mice gave high levels of P. gingivalis-specific IgA in nasal washes and saliva and in serum IgG and IgA. This suggested a potential role of P. gingivalis OMVs as non-replicating mucosal immunogens for vaccines against periodontal disease. The range of virulence factors enriched in P. gingivalis OMVs may make them particularly suited for a periodontal disease vaccine (97). Neisserial OMVs are considered a successful vaccine immunogen against bacterial meningitis (98) such as the commercially available vaccine against Neisseria meningitidis serogroup B. It is thought that OMVs are safer as antigens than whole bacterial cells and can harbor a number of cell-surface markers such as LPS and proteins to stimulate an immune response (31). OMVs have also been manipulated to act as adjuvants while displaying foreign epitopes of interest resulting in some success for producing a single vaccine against both (14).

Classification
OMVs have proved useful for classification of members of the Actinobacillus-Haemophilus-Pasteurella group (99) where several belong to the oral ecosystem.

Concluding remarks
OMVs have a number of functions as described in the current review: virulence bullets, bacterial bombs, decoys, vehicles for cell–cell signaling, mediators of immune regulation and disease protection, unique secretion systems, good Samaritans, and so on. It is amazing how these small vesicles can serve functions that are good to themselves and their parent bacteria and even to the host. However, they have also clear detrimental effects towards other bacteria and the host. There is no question; these structures are both offensive weapons and good Samaritans. Their diversity in function is remarkable. We should probably try to modify them for the benefit of the host, for example, as therapeutics against disease. A specific field of interest is new and emerging bacterial and virus infections where engineered OMVs containing proteins might be used as decoys and vaccines.

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