Review

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Prokaryotic membrane vesicles: new insights on biogenesis and biological roles

Abstract: Biogenesis and trafficking of membrane vesicles are essential and well-studied processes in eukaryotes. In contrast, vesiculation in bacteria is not well understood. Outer membrane vesicles (OMVs) are produced in Gram-negative bacteria by blebbing of the outer membrane. In addition to the roles in pathogenesis, cell-to-cell communication and stress response, recent work has suggested that OMVs play important roles in immunomodulation and the establishment and balance of the gut microbiota. In this review we discuss the known and novel roles of OMVs and the different biogenesis models proposed, and address the evidence for cargo selection into OMVs. We also discuss the growing evidence for the existence of membrane vesicles in Gram-positive bacteria and Archaea. Due to their biological importance and promising applications in vaccinology, the biogenesis of OMVs is an important topic in microbiology.

Keywords: cargo selection; gut health; outer membrane vesicles; pathogenesis.

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Introduction

Secretion of membrane vesicles is an important biological process. The vesicles secreted by eukaryotic cells and most of their organelles have multiple well-known and -studied roles, such as the storage, trafficking (cell-cell and inter-organelar communications) and digestion of cellular components. The vesicles can be distinguished by their shape, size (between 30 nm and 1 μm) and cellular location based on the function that they perform (Nieuwland and Sturk, 2010). Similar to many other biological processes, vesicle secretion with physiological roles has long been considered an exclusive eukaryotic phenomenon. In prokaryotes, however, vesicle formation was reported several decades ago; first in Gram-negative and recently in Gram-positive bacteria and also in Archaea (Beveridge, 1999; Ellen et al., 2009; Rivera et al., 2010). The vesicles secreted by Gram-negative bacteria have regained interest due to the finding of multiple biological roles, especially focused on microbial pathogenesis (Mashburn-Warren and Whiteley, 2006). The proposed roles of prokaryotic vesicles resembled their eukaryotic counterparts. These vesicles can store and transport a broad repertoire of cargo such as virulence factors, protecting them from the environmental conditions (Kesty et al., 2004; Aldick et al., 2009). Furthermore, their role in cell-cell communication and modulation of the host’s immune system has been described (Mashburn and Whiteley, 2005; Mashburn-Warren and Whiteley, 2006; Ellis and Kuehn, 2010). In addition they could be considered as a defense mechanism by carrying away toxic compounds, phages or unfolded proteins after exposure to stressful conditions (McBroom and Kuehn, 2007; Ellis and Kuehn, 2010). Recently, an important role has been proposed for outer membrane vesicles (OMVs) in microbiota homeostasis (Elhenawy et al., 2014; Rakoff-Nahoum et al., 2014).

A vesicle is formed when a small portion of the membrane protrudes from the cell or organelle envelope and it is released. However, membrane bending is neither a spontaneous nor a random process. In eukaryotes, vesicle biogenesis is a selective process, where specific cell components are carefully chosen as cargo to perform a variety of cellular functions. This sorting process is the result of a highly tuned system that requires membrane reorganization or remodeling at the vesicle formation site, where...
membrane curvature has to be induced in order that a planar membrane becomes a spherical vesicle (Zimmerberg and Kozlov, 2006). Membrane curvature is an expensive process for both the eukaryotic or prokaryotic cell. The free energy (ΔG) required to form a spherical vesicle from a flat membrane is ΔG = 250–600 k_BT (where k_BT is the thermal energy), and is considered a non-spontaneous biological process (Bloom et al., 1991). Biological systems have developed different strategies to reduce the energy level needed for vesicle formation as well as how the cargo is sorted into the vesicles. The mechanisms better studied and explored are those found in eukaryotic biological systems. Unfortunately, the information available on prokaryotic vesicles is limited.

In this review, we will discuss the current knowledge about vesicle biogenesis and the protein cargo selection in Gram-negative bacteria. In addition, we will discuss the role of vesicles in bacterial-host interactions during pathogenesis and symbiosis. Unraveling OMV biogenesis would have an impact on the current view of bacterial pathogenesis and vaccine industry. Furthermore, we will briefly discuss OMV formation in Gram-positive bacteria and Archaea.

**Outer membrane vesicle secretion in Gram-negative bacteria**

The Gram-negative bacteria envelope is constituted of two bilayered membranes: the inner or cytoplasmic membrane and the outer membrane (OM) that encloses the periplasmic space and a thin peptidoglycan layer. The OM is an asymmetric lipid membrane. The lipopolysaccharide (LPS) is the most abundant lipid in the outer leaflet and the phospholipids in the inner leaflet. The LPS is constituted of the lipid A, a glycan core and a long polysaccharide side chain known as the O antigen (Raetz and Whitfield, 2002).

OMVs are 20–300 nm in diameter. These vesicles are secreted by pathogenic and non-pathogenic Gram-negative bacteria. OMV composition has been described and the identification of OM components such as LPS and OM proteins confirmed the OM origin (Grenier and Mayrand, 1987; Kadurugamuwa and Beveridge, 1995; Beveridge, 1999). OMVs are therefore formed when the OM bulges by a not fully characterized mechanism, encapsulating periplasmic components (Mayrand and Grenier, 1989; Kadurugamuwa and Beveridge, 1995).

In 1965, it was observed that *Escherichia coli* cells secreted cell-free LPS when they were grown under lysine-limiting conditions (Bishop and Work, 1965). Later, the presence of small spherical membrane structures in the secreted cell-free LPS samples was shown by electron microscopy and it was proposed that these structures were formed when the OM protruded (Knox et al., 1966). Even though OMV production was documented almost 50 years ago, the subject has been neglected and the OMVs were often regarded as broken cells or artifacts. When the protein composition of the purified OM and OMVs of several Gram-negative bacteria was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by protein staining, a different protein pattern was observed in both fractions (Grenier and Mayrand, 1987; Kadurugamuwa and Beveridge, 1995; Horstman and Kuehn, 2002; Kato et al., 2002; Wai et al., 2003; Sidhu et al., 2008; Frias et al., 2010; Kahnt et al., 2010; Haurat et al., 2011; Lappann et al., 2013; McNaught et al., 2013; Aguilera et al., 2014; Elhenawy et al., 2014; Jang et al., 2014). Certain proteins were more abundant in the OMVs than in the OM fraction and others were missing in one of the fractions. If OMV production was a random process or the result of cell lysis, their protein composition should have been identical to the OM of intact cells. However, it is important to mention that the detection of some proteins in OMVs, for example flagellin or some secreted proteins, may be due to their association with vesicles rather than selective sorting. In these conditions, density gradient may be due to their association with vesicles rather than selective sorting. In these conditions, density gradient should be employed to obtain cleaner OMV preparations. Nevertheless, there is increasing evidence that, at least in some bacterial species, OMV formation is the result of a selective process.

**OMV roles**

**Roles in pathogenesis**

OMVs contribute in bacterial pathogenesis because adhesins, toxins and immunomodulatory compounds have been identified as OMV cargo (Figure 1) (Grenier and Mayrand, 1987; Kadurugamuwa and Beveridge, 1995; Horstman and Kuehn, 2002; Wai et al., 2003; Kesty et al., 2006; Chitcholtan et al., 2008). Heat labile enterotoxin (enterotoxigenic *E. coli*), VacA (*Helicobacter pylori*), Shiga toxin (*Shigella dysenteriae* and enterohemorrhagic *E. coli*) and ClyA (enterohemorrhagic *E. coli*) are some examples of the several toxins that have been reported to be associated with OMVs (Horstman and Kuehn, 2000; Horstman and Kuehn, 2002; Wai et al., 2003; Dutta et al., 2004; Kesty et al., 2004; Chitcholtan et al., 2008). Also, the enteric...
pathogen, Salmonella typhi was shown to induce hemolysin packing into OMVs in response to norepinephrine (Karavolos et al., 2011). These toxins packed in OMVs have several advantages over their soluble or cell-associated counterparts. Some OMVs are packed with adhesins that help them interact with the host plasma membrane, and either fuse with the membrane delivering their cargo into the eukaryotic cells or promote OMV uptake by the host cells (Kesty et al., 2004; Bomberger et al., 2009; Furuta et al., 2009). OMVs may also provide optimal conditions for folding of some toxins. For example, inactive ClyA monomers are found in the periplasm while the active ClyA oligomers are in the OMVs, which provide the right redox environment for the monomer’s polymerization (Wai et al., 2003). OMVs constitute a protective mechanism against the host proteases and antibodies, increasing the half-life of those toxins packed in them (Aldick et al., 2009).

Interestingly, some bacteria can use OMVs to interfere with host trafficking pathways. For example, Pseudomonas aeruginosa promotes cystic fibrosis transmembrane conductance regulator (CFTR) degradation through the OMV-packed toxin Cif. CFTR is required for mucociliary clearance. Cif is delivered into host cells after OMV fusion with lipid rafts where it inhibits the deubiquitination of CFTR leading to its lysosomal degradation (Bomberger et al., 2009, 2011). Different groups demonstrated the ability of OMVs from different bacteria to activate the immune system in various ways (Schild et al., 2008; Ellis et al., 2010; Vidakovic et al., 2010; Nakao et al., 2011; Schaar et al., 2011; Elmi et al., 2012; Lee et al., 2012; Pollak et al., 2012; Roier et al., 2012, 2013; Jun et al., 2013; Kim et al., 2013; Deknuydt et al., 2014). Bordetella pertussis causes an increase in intracellular cAMP levels of the host cells through the delivery of its adenylate cyclase toxin as OMV cargo (Donato et al., 2012). OMVs not only deliver proteins into the host cells; some species such as Borrelia burgdorferi employ OMVs to deliver lipids that aid in modulating the immune response (Crowley et al., 2013).

Recent findings indicate that bacteria can modify their OMV cargo in response to the surrounding environment. Cytotethal distending toxin from S. typhi was detected in OMVs when bacteria were grown in conditions mimicking those of Salmonella-containing vacuole (Guidi et al., 2013). In this work, the cytotoxic distending toxin was detected in vivo co-localizing with bacterial LPS, which was proposed to be OMV.
Roles of OMVs in microbiota homeostasis

The role of OMVs produced by members of the human microbiota has received much attention in recent years. Members of genus *Bacteroides* are well known for their active contribution to the gut health. This role is not only restricted to their ability to digest a wide variety of polysaccharides but extends to immunomodulation. Polysaccharide A (PSA) synthesized by *Bacteroides fragilis* was found to activate interleukin-10 secretion via regulatory T-cells, which was found to be important for the immunotolerance of the host towards the symbiont (Mazmanian et al., 2008). Recently, *B. fragilis* OMVs were found to be the vehicle for PSA delivery to dendritic cells (Shen et al., 2012). PSA carried by OMVs resulted in a different cascade of immune response compared to pure PSA. This shed light on the importance of OMVs as the delivery tools of microbiota modulators in the gut. Another interesting example of the OMV role in microbiota-host communication is *Bacteroides thetaiotaomicron* OMVs, where an active homologue of the eukaryotic inositol phosphate phosphatase (MINPP), an enzyme involved in intracellular Ca²⁺ signaling and with implications in cancer, was detected (Stentz et al., 2014). It was suggested that the enzyme is packed into OMVs in order to be protected from proteases in the medium. Recently, two groups employed totally different approaches, arriving to the same conclusion that *Bacteroides* OMVs are involved in a complex network dedicated to processing of nutrients in the human gut. Elhenawy et al. employed a proteomic approach to demonstrate the selective packing of a large number of carbohydrate and protein hydrolases exclusively in the OMVs of both *B. fragilis* and *B. thetaiotaomicron* (Elhenawy et al., 2014). Some of these hydrolases were shown to be active in *vitro* and were enriched in OMVs in response to external stimuli. It was proposed that the OMVs carry a ‘social’ function, as the oligo-, monosaccharides and amino acids resulting from the activity of the hydrolytic enzymes would be available for other bacteria to utilize (Figure 1) (Elhenawy et al., 2014). The elegant work by Rakoff-Nahoum et al. showed that these OMV-packed hydrolases play an important ecological role in the gut. Members of the *Bacteroides* genus are capable of digesting different polysaccharides via the hydrolases contained in OMV. The product of the digestion of a polysaccharide by an OMV secreted by one species can support the growth of other species that is unable to degrade the polysaccharide, contributing to the homeostasis of the gut. Therefore, the OMV-packed hydrolases are ‘public goods’ for other gut bacterial species. It was concluded that this OMV-based network likely represents foundational relationships creating organized ecological units within the intestinal microbiota (Rakoff-Nahoum et al., 2014). These two complementary articles strongly support a key role of OMVs in the establishment and balance of the gut microbiota.

Other roles of OMVs

An increase in OMV production after exposing the bacterial cells to toxic compounds such as antibiotics, stressor substances, phages or the host environment at the infection site have been observed, probably constituting a new defense mechanism (Loeb, 1974; Grenier and Belanger, 1991; Allan and Beveridge, 2003; Neves et al., 2006; Irazoqui et al., 2010). Under these harsh conditions, bacterial cells were able to secrete unnecessary or unwanted material, such as unfolded or over-expressed proteins, to reduce the envelope stress (Figure 1). However, it is unknown how this process is controlled by any of the stress response pathways described in *E. coli* (McBroom and Kuehn, 2007). In *Serratia marcescens*, however, the production of OMVs appears to be a thermoregulated process. Under laboratory conditions, *Serratia* produces a significant number of OMVs at 22°C or 30°C and negligible quantities formed at 37°C. Inactivation of the synthesis of the enterobacterial common antigen (ECA) resulted in hypervesiculation, supporting the notion that OMVs are produced in response to stress. The hypervesiculating phenotype caused by the mutation in the ECA was reversed upon inactivation of the Rcs phosphorelay response regulator RcsB, suggesting a role for the Rcs phosphorelay in the production of OMVs in this organism (McMahon et al., 2012). Interestingly, OMVs were found to be involved in interbacterial killing. For example, OMVs are utilized by *Myxococcus* during its predation on other bacteria (Whitworth, 2011). Moreover, competitive microorganisms found in the same ecological niche secrete antimicrobials inside the OMVs, selectively killing cells from other species (Kadurugamuwa et al., 1998; Li et al., 1998). Additional functions of OMVs include the stimulation of biofilm formation as OMVs from a biofilm-forming *H. pylori* strain induced biofilm formation in another strain (Yonezawa et al., 2009).

The OMVs provide a protective environment for the cargo, like hydrophobic quorum-sensing molecules involved in cell-cell communication, such as 2-heptyl-3-hydroxy-4-quinolone (*Pseudomonas* quinolone signal or PQS) (Mashburn and Whiteley, 2005; Aldick et al., 2009). OMVs carrying PQS were able to complement a *P. aeruginosa* PQS-deficient strain, indicating that OMVs have carried and secreted a bioactive compound (Mashburn and Whiteley, 2005). Furthermore, the DNA found...
in the OMV has been successfully transferred into other bacterial cells, which may constitute a new DNA delivery system (Figure 1) (Deich and Hoyer, 1982; Dorward et al., 1989; Renelli et al., 2004; Yang et al., 2008).

An important biotechnological application of the OMVs is their use as vaccines (OMV-based vaccines) (Bjune et al., 1991; Sierra et al., 1991; de Moraes et al., 1992). An example is the commercially-available vaccine against Neisseria meningitidis serogroup B (MeNZB) developed by Novartis Vaccine (Davenport et al., 2008). Recently, another OMV-based vaccine, Besxero has been approved in Europe and other countries (Tani et al., 2014).

Several benefits make OMVs an attractive alternative to conventional vaccines. They have been successfully used during outbreaks because they can be easily purified (Oster et al., 2005; Holst et al., 2009). Furthermore, they are natural liposomes due to the lipid composition and size, having adjuvant properties that increase the immune response while reducing the amount of antigen needed (Muralinath et al., 2011). Another intriguing feature of OMV-based vaccines is that they can be engineered to allow the presentation of foreign antigens (Chen et al., 2010; Baker et al., 2014).

**Biogenesis of OMV**

Bacteria cells have to be growing in order to secrete OMVs, therefore OMV formation is a process that may require energy (Mug-Opstelten and Witholt, 1978). Still is not known how OMVs are generated and how the cargo is selected. However several models have been proposed based on the components identified as OMV cargo.

**Peptidoglycan fragments accumulation**

Generally, low molecular weight peptidoglycan fragments accumulate in the periplasm before being translocated into the cytoplasm by an oligopeptide permease during the peptidoglycan turnover. Low molecular weight muramyl peptides have been identified inside OMVs (Zhou et al., 1998; Kaparakis et al., 2010; Bielig et al., 2011). The accumulation of the peptidoglycan fragments in the periplasmic space could therefore exert a turgor pressure strong enough to bend the OM and produce OMVs (Figure 2A) (Zhou et al., 1998). An increase in OMV production was observed when peptidoglycan fragments accumulated because of the incomplete degradation of the peptidoglycan in a Porphyromonas gingivalis autolysin mutant strain (Hayashi et al., 2002). Autolysins are murein hydrolases that cleave covalent bonds in the peptidoglycan and they are responsible for the cell wall remodeling, peptidoglycan turnover, cell division and peptidoglycan cell wall reparation (Shockman, 1994). In agreement with their role in cell wall remodeling, this autolysin mutant strain exhibits impaired cell division (Hayashi et al., 2002). The imbalance between cell wall turnover and OM biosynthesis could be responsible for the increased OMV secretion (Hayashi et al., 2002). The peptidoglycan fragment accumulation should be localized in order to generate OMVs with enriched proteins; otherwise composition of the OMVs would resemble the one of the OM. Unfortunately, the OMV protein composition has not been analyzed in the P. gingivalis autolysin mutant strain.

An increase in peptidoglycan fragments in the periplasm does not indicate that they are the driving force for OMV biogenesis. The OMV production could again be the result of a defense mechanism to release the stress generated in the periplasmic space, as previously reported for the accumulation of unfolded proteins in that compartment (McBroom and Kuehn, 2007). The bacterial OM can tolerate up to 3 atm of turgor pressure and the concentration of these fragments in the periplasm should be considerably higher in order to exceed this limit and force vesicles to be released (Koch, 1998). Unfortunately the concentration of peptidoglycan fragments was not determined in the autolysin mutant strain or in the wild-type strain and therefore it is not possible to estimate whether the emerging turgor pressure could be enough to produce hypervesiculation.

Alternatively, the peptidoglycan fragments could be encapsulated inside of the OMV to be secreted as virulence factors and to induce a NOD1- and NOD2-dependent pro-inflammatory response (Kaparakis et al., 2010; Bielig et al., 2011). H. pylori secretes peptidoglycan fragments into the host cell via the type IV secretion system to modulate the host immune responses (Viala et al., 2004). OMVs could therefore constitute an alternative vehicle for bacterial strains to modulate host responses in the absence of a type IV secretion system.

**OM peptidoglycan interaction**

The cross-link between murein and lipoproteins is important to maintain OM integrity. E. coli OMV contains fewer lipoproteins than its OM (Hoeckstra et al., 1976). Therefore, if the OM grows faster than the underlying cell wall, the cross-linking can be missing, allowing the OM to protrude (Figure 2B) (Wensink and Witholt, 1981). The observation...
that *P. aeruginosa* OM possesses a lower lipoprotein content than *E. coli* OM could explain why *P. aeruginosa* cells produce more OMV than *E. coli* (Martin et al., 1972; Mashburn-Warren and Whiteley, 2006). Mutant strains in genes encoding OM lipoproteins and proteins that are associated with the peptidoglycan layer, such as OmpA, Lpp, ToIB and Pal, secrete several-fold more vesicles than their respective wild-type strains (Bernadac et al., 1998; Cascales et al., 2002; McBroom et al., 2006; Iwami et al., 2007; Song et al., 2008; Deatherage et al., 2009). It is important to highlight that the OM integrity of all those mutant strains was compromised and a non-continuous wavy OM was observed by electron microscopy (Rolhion et al., 2005; Iwami et al., 2007). As in the case of the peptidoglycan accumulation, it is expected that mutations affecting the OM integrity produce more vesicles simply as a mechanism to release surface stress. In *Vibrio cholera* OMV production is increased by down-regulation of OmpA levels by the expression of the small RNA VrrA. Environmental conditions such as stress induce the expression of Vrra, which represses OmpA synthesis and therefore stimulates OMV production (Song et al., 2008). These results suggested that OMVs might be generated in OM regions with relaxed OM-peptidoglycan interaction and that the proteins that favor that interaction would be excluded from the OMV (Figure 2B).

### O antigen charge repulsion

A third mechanism has been proposed for *P. aeruginosa* vesicle formation. Only the B-band LPS, which is
negatively charged, has been detected in the \textit{P. aeruginosa} vesicles. This model is based on the selective packing of LPS moiety in the vesicles (Kadurugamuwa and Beveridge, 1995). Thus, OMVs could be generated in regions where the B-band moiety is more abundant, bending the OM to release the charge repulsion generated by the negatively charged O antigen (Figure 2C) (Kadurugamuwa and Beveridge, 1995). The role of the O antigen in OMV biogenesis was analyzed in several \textit{P. aeruginosa} strains displaying different LPS phenotypes on their surfaces: A'B', AB', A'B' and AB (Nguyen et al., 2003). The strain that secreted the fewest OMVs was the one expressing only A-band and, interestingly, the double mutant strain hypervesiculated (Nguyen et al., 2003). Although, similar results were observed for other bacterial strains lacking O antigen or the core, this effect was not observed in \textit{P. gingivalis} O antigen mutant strains (Smit et al., 1975; McBroom et al., 2006; Haurat et al., 2011). This bacterium also synthesizes two different O-antigen moieties, a negatively-charged LPS (known as A-LPS) and a neutral LPS (O-LPS) and mutations affecting the A-LPS synthesis or the attachment of both O-antigens onto the lipid A-core did not affect the OMV biogenesis (Paramonov et al., 2005, 2009; Rangarajan et al., 2008; Haurat et al., 2011). Contrary to \textit{P. aeruginosa}, it appears that both O-antigen moieties are packed in \textit{P. gingivalis} OMV (Haurat et al., 2011). It seems that the charge repulsion model would only apply in certain bacteria producing neutral and negatively-charged O antigens. For this reason other mechanisms should take place in bacteria displaying only neutral surface glycans.

LPS contains negatively-charged phosphate groups in the lipid A and it is known that divalent cations such as Ca$^{2+}$ and Mg$^{2+}$ are important in forming salt bridges with the LPS' negative charges. The generation of these salt bridges helps to stabilize the OM. Several compounds can sequester these divalent cations and destabilize the OM, contributing to the generation of OMVs to release the charge repulsion (Mashburn and Whiteley, 2005). Furthermore, molecules that are able to change OM fluidity were found to stimulate vesiculation. For example, PQS, a quorum-sensing molecule found in the OMVs, interacts with lipid A and induces the OMV production (Mashburn-Warren et al., 2008; Mashburn-Warren et al., 2009).

\section*{Cargo recruitment in OMV}

OM and OMV protein compositions have been shown to differ in Gram-negative bacteria (Grenier and Mayrand, 1987; Kadurugamuwa and Beveridge, 1995; Horstman and Kuehn, 2002; Kato et al., 2002; Wai et al., 2003; Sidhu et al., 2008; Frias et al., 2010; Kahnt et al., 2010; Haurat et al., 2011). Only a selected set of proteins was packed in the OMVs (Haurat et al., 2011). In \textit{P. gingivalis} gingipains, important virulence factors, were selected as OMV cargo, while other more abundant OM proteins involved in nutrient uptake were excluded (Haurat et al., 2011). These results indicate that OMV formation is the result of a directed process, where specific exclusion and/or inclusion cues may dictate the proper sorting of the proteins into the OM and OMVs. Further results have revealed that the cargo selection of OMVs depends on proper O antigen assembly (Haurat et al., 2011). \textit{P. gingivalis} O antigen mutant strains presented an aberrant cargo selection; proteins excluded from the OMV in the wild-type strain were instead packed into OMVs in LPS mutants (Haurat et al., 2011). These results suggested that the O antigen has a role in the selection process of the protein cargo. Two models could explain these findings (Figure 3). In the first model the cargo proteins may interact directly with the O antigen (Figure 3A). The cargo proteins may have a domain to recognize and interact with the O antigen, promoting the compartmentalization of the OM. In \textit{P. gingivalis} such a domain may recognize the long A-LPS molecules enriched in the OMVs (Haurat et al., 2011). Proteins not having this domain could be excluded from the microdomains where the OMVs are formed. In the second model a sorting factor may recognize the cargo proteins and the O antigen (Figure 3B). This sorting factor would associate the protein to the O antigen moiety enriched in the OMV. The latter model reminds us of the role of galectins in sorting proteins in the exosomes (Delacour et al., 2007). Some proteins were excluded from the OMV, however, even in the O antigen mutant strains, indicating that certain OM retention signals might be present on these proteins (Haurat et al., 2011). It is possible that the two mechanisms are not mutually exclusive. In this scenario, some proteins might be sorted into the OMVs by one of the proposed models (OMV positive selection) and others might be sorted out of the OMVs with the help of retention signals (OMV negative selection). In agreement with the sorting model, during the proteomic analysis of \textit{B. fragilis} and \textit{B. thetaiotaomicron} OMV a large subset of OMV-unique proteins were found to be acidic (negatively charged) while the majority of the OM proteins had high pIs (they were positively charged) (Elhenawy et al., 2014). This suggests the presence of a yet uncharacterized factor responsible for recruiting proteins into OMVs based on their charge and/or structure. Although there are many studies that have analyzed the OMV proteomes from different bacteria, this is the first time OMV proteins have displayed a common
feature. This suggests that bacteria have evolved different ways of recruiting cargo into OMVs.

The OM and OMVs were found to differ in protein and lipid composition. LPS is one of the lipids that were differentially distributed between the OM and OMVs. *P. gingivalis* OMVs were enriched in high molecular weight O antigen-containing LPS molecules and both O antigen structures are probably packed in the OMVs. Interestingly, lipid A of *P. gingivalis* OMV was found to be deacylated compared to OM (Haurat et al., 2011). As mentioned before, preferential packing of a particular LPS molecule has previously been reported in *P. aeruginosa* (Kadurugamuwa and Beveridge, 1995). In addition, the phospholipid composition has been characterized in *P. aeruginosa* OM and OMVs and although phosphatidylethanolamine (PE), peptidoglycan (PG) and phosphocholine (PC) were detected in the OM and OMVs, they are differently distributed in both cellular fractions (Tashiro et al., 2011). PE, PG and PC are, in decreasing order, the most abundant OM phospholipids, whereas in the OMV the PG was the most abundant phospholipid followed by PE and PC (Tashiro et al., 2011). Moreover, whereas the unsaturated and saturated fatty acids were equally represented in *P. aeruginosa* OM, the saturated fatty acids were over-represented in the OMVs (Tashiro et al., 2011). Similarly, cardiolipin was enriched in *Actinobacillus actinomycetemcomitans* OMVs (Kato et al., 2002). These differential lipid compositions identified in different bacterial species might support the hypothesis that the OMV are formed at specific OM regions as a result of a compartmentalization or remodeling of the OM. In further agreement, the formation of lipid domains in bacterial cells has been reported (Matsumoto et al., 2006; Lopez and Kolter, 2010). However, membrane vesicles from *E. coli* showed similar lipid composition to the OM, which indicates that lipid compartmentalization may be involved in OMV formation in some, but not all, Gram-negative bacteria (Hoekstra et al., 1976).
Vesicle formation in Gram-positive bacteria

The Gram-positive bacteria envelope consists of plasma membrane and a thick peptidoglycan layer. The plasma membrane is an asymmetric bilayer, containing teichoic acids, lipoteichoic acids and phospholipids in the outer leaflet and phospholipids and cardiolipin in the inner leaflet. Vesicle production is not a common phenomenon in Gram-positive bacteria, and for this reason it was thought for a long time that they were not able to produce and secrete vesicles. To date, Gram-positive vesicle production has been reported in only a few cases, *Staphylococcus aureus*, *Bacillus* spp., *Streptomyces coelicolor*, *Clostridium perfringens*, *Listeria monocytogenes*, *Thermoanaerobacterium thermosulfurogenes* and *Mycochromatium* spp. (Dorward and Garon, 1990; Mayer and Gottschalk, 2003; Lee et al., 2009, 2013; Rivera et al., 2010; Gurung et al., 2011; Prados-Rosales et al., 2011; Schrempf et al., 2011; Thay et al., 2013; Jiang et al., 2014). One reason for this very limited number of reports could be the low vesicle yield, only 200 μg (wet weight) of vesicles were obtained from 1 l of *S. aureus* culture, compared to the 1 mg (dry wet) of OMVs obtained from 10 ml *P. gingivalis* culture (Lee et al., 2009; Haurat and Feldman unpublished results).

These Gram-positive vesicles are 20–250 nm in diameter (Lee et al., 2009; Schrempf et al., 2011). Cytoplasmic membrane and extracellular proteins have been identified in them (Lee et al., 2009; Rivera et al., 2010; Schrempf et al., 2011). The nature of the proteins identified suggests that Gram-positive bacteria secrete vesicles with similar roles to their Gram-negative counterparts. Enzymes involved in peptidoglycan degradation, antibiotic degradation and virulence (anhydrolysin, antraxxin components, coagulases, hemolysins and lipases) and immunologically-active compounds have been identified (Lee et al., 2009; Rivera et al., 2010; Schrempf et al., 2011). The finding of penicillin-binding proteins, β-lactamases and the global regulator MsrR (which confer resistance to meticillin) could explain the increment of multidrug-resistant *S. aureus* infections (Lee et al., 2009). Furthermore, *M. tuberculosis* releases vesicles inside macrophages and the amount is increased under iron limitation (conditions found in the granuloma), indicating a role in virulence (Prados-Rosales et al., 2011, 2014). Moreover, it has been suggested that vesiculation in *M. tuberculosis* is a regulated process (Rath et al., 2013). As OMVs, the Gram-positive vesicles could be used as vaccine in the future. Immunization with *Bacillus anthracis* vesicles has prolonged the survival of *B. anthracis*-challenged mice (Rivera et al., 2010). It is premature to suggest that vesicles could be formed as the result of membrane remodeling processes, although some evidence supports this hypothesis. Proteins detected in *S. coelicolor* vesicles had an N-terminal signal peptide with the twin-arginine motif and another undetermined signal peptide, which suggests an enrichment of proteins in vesicles based on a common signal recognized by the vesiculation machinery (Schrempf et al., 2011). Furthermore, in *B. anthracis* palmitic and stearic acids were the most abundant fatty acids detected in both fractions. However, the composition of the less abundant lipids in the vesicle was enriched in unsaturated fatty acids (Lee et al., 2009; Rivera et al., 2010). Interestingly, *M. tuberculosis* vesicles are enriched in diacyl and triacylglycerides and PE, but none of the characteristic lipids found in the mycomembrane were detected in them (Prados-Rosales et al., 2014). A more comprehensive characterization of lipid and protein composition of vesicles is needed, but it could be hypothesized that vesicles are formed when plasma membrane microdomains protrude. The thick peptidoglycan would have to be degraded before the vesicles are released into the extracellular medium, either prior to or during the vesicle formation, by the vesicle peptidoglycan degrading enzymes. Supporting the former hypothesis, when the *T. thermosulfurogenes* is grown under starch limitation, the peptidoglycan layer is degraded and vesicles are formed from the plasma membrane (Mayer and Gottschalk, 2003).

Vesicle formation in archaea

The archaeal cell envelope is remarkably different from bacterial envelopes. With the exception of *Ignicoccus* spp. (which has an OM), archaeal species have only a single cytoplasmic membrane, which is usually enclosed by a protein crystal structure known as S-layer (Ellen et al., 2010; Albers and Meyer, 2011). Archaeal lipids are isoprenyl repeating units ether-linked to L-glycerol-3-phosphate backbones; whereas in eukaryotic and bacterial membranes the lipid units are ester-linked to a D-glycerol-1-phosphate moiety (Albers and Meyer, 2011). The archaeal lipids can form diether or tetraether lipid structures (Ellen et al., 2010).

Vesicles secreted by the archaean *Sulfolobus* spp. Are 90–230 nm in diameter and coated with S-layer. Archaeal vesicles can be differentiated from the parental cells in their lipid and protein compositions (Ellen et al., 2009). The archaeal tetraether lipids (glycerol dialkyl glycerol tetraethers (GDGTs) and a glycerol trialkylglycerol tetraether) are more abundant in the vesicles than in the cells. B. anthracis-challenged mice (Rivera et al., 2010). It is premature to suggest that vesicles could be formed as the result of membrane remodeling processes, although some evidence supports this hypothesis. Proteins detected in *S. coelicolor* vesicles had an N-terminal signal peptide with the twin-arginine motif and another undetermined signal peptide, which suggests an enrichment of proteins in vesicles based on a common signal recognized by the vesiculation machinery (Schrempf et al., 2011). Furthermore, in *B. anthracis* palmitic and stearic acids were the most abundant fatty acids detected in both fractions. However, the composition of the less abundant lipids in the vesicle was enriched in unsaturated fatty acids (Lee et al., 2009; Rivera et al., 2010). Interestingly, *M. tuberculosis* vesicles are enriched in diacyl and triacylglycerides and PE, but none of the characteristic lipids found in the mycomembrane were detected in them (Prados-Rosales et al., 2014). A more comprehensive characterization of lipid and protein composition of vesicles is needed, but it could be hypothesized that vesicles are formed when plasma membrane microdomains protrude. The thick peptidoglycan would have to be degraded before the vesicles are released into the extracellular medium, either prior to or during the vesicle formation, by the vesicle peptidoglycan degrading enzymes. Supporting the former hypothesis, when the *T. thermosulfurogenes* is grown under starch limitation, the peptidoglycan layer is degraded and vesicles are formed from the plasma membrane (Mayer and Gottschalk, 2003).
Detailed analysis of the GDGT composition has shown that the amount of GDGTs with five and six cyclopentanes in their structure represented 13% of the total amount of GDGTs and the one containing two to four cyclopentanes was 84% in the vesicles, whereas in the cells their values were 27% and 69% respectively (Ellen et al., 2009).

The proteins identified as vesicle cargo can be classified as transport, energy and metabolism, cell surface, stress and structural. Interestingly, three archaeal ESCRT-III like proteins (scaffold proteins of the endosomal sorting complex for transport) and a homolog to the ATPase Vps4 were identified in the vesicles (Ellen et al., 2009). The role of the ESCRT-III-like proteins in archaeal vesicle formation and how the proteins are sorted into these vesicles are still unknown. The cytoplasmic membrane could protrude with the help of the ESCRT-III-like proteins and that the Vps4 homolog would provide the energy required for this process (Ellen et al., 2010). In this case the vesicles would be the result of an outward process and the ESCRT-III-like proteins would be inside the vesicle, whereas in eukaryotic vesicles the ESCRT-III components are on the outside. Furthermore, the ESCRT-III complex is also involved in eukaryotic cytokinesis and archaeal cell division. For this reason vesicles could be released during cell division (Makarova et al., 2010; Caballe and Martin-Serrano, 2011). However, the physiological role of these vesicles is still unknown.

**Conclusion**

The study of OMVs was halted for several decades but the pioneering work of the Beveridge and the Kuehn labs has opened new avenues for the study of these vesicles. Over the years, many researchers remained skeptical about the existence of true vesiculation process in bacteria. There is now an extensive body of work that indicates, at least in some Gram-negative species, OMVs are the result of a directed and selective cellular process. Nevertheless, many aspects about the biogenesis of OMVs remain unanswered. Is there a universal mechanism for OMV formation? What is the energy source for the vesiculation process in bacteria? How is protein cargo selected? Is OMV formation an essential process in Gram-negative bacteria? How do OMVs from bacterial pathogens deliver their toxic cargo into the host cells?

All the mechanisms for vesicle formation summarized here could be applied to all bacterial species. However all of them require, to some extent, membrane compartmentalization. Regardless of whether they involve the recruitment of a scaffold machinery or membrane remodeling, certain proteins and lipids are sorted in and out of the vesicle formation site. To the best of our knowledge, membrane-coated vesicles have only been reported in eukaryotes. This could suggest that they constitute a new event in the evolution of vesicle production. Interestingly, there are some common features in vesiculation among bacteria and archaea (Table 1). Glycans displayed on the bacterial surface could have a role in the membrane compartmentalization. It is unknown, however, how glycans perform this membrane reorganization in bacterial cells. This may be done by direct interaction between the proteins and the glycans or through an unidentified sorting factor recognizing both the glycans and the proteins to be sorted. In addition, the proteins might have a sorting signal in their sequences, e.g., their pl, indicating whether they are to be excluded or not from the OMV formation site.

Customized OMVs carrying specific cargo and detoxified LPS could be used to improve the current vesicle-based vaccines. Thus, the understanding of how proteins are sorted into vesicles and the role that lipids play in OMV biogenesis will have a great impact in the field of bacterial pathogenesis as well as in the development of biotechnological applications.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Comparison of the vesicles produced by membrane remodeling in different biological systems.</th>
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<tbody>
<tr>
<td><strong>Gram negative bacteria</strong></td>
<td><strong>Gram positive bacteria</strong></td>
</tr>
<tr>
<td>Vesicle membrane composition</td>
<td>Different to OM</td>
</tr>
<tr>
<td>Protein sorting</td>
<td>Yes</td>
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<tr>
<td>Glycans involved in protein sorting</td>
<td>Yes</td>
</tr>
<tr>
<td>Vesicle lipid composition*</td>
<td>Decylated lipid A, higher phosphatidylglycerol, cardiolipin and saturated FA content than the OM</td>
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*FA, fatty acid; OM, outer membrane.
References


M. Florencia Haurat studied biotechnology at the National University of Rosario, Argentina. Later, she joined as PhD student to the laboratory of Dr. Feldman at University of Alberta, Canada. The main focus of her PhD thesis was to investigate the secretion of outer membrane vesicles in Gram-negative Bacteria. Actually she is working as a Postdoctoral fellow in the laboratory of Dr. Sonja V. Albers at the Max Planck Institute for Terrestrial Microbiology, Germany. There she is interested in elucidate the mechanisms involved the regulation of motility in Archaea.

Wael Elhenawy earned his BSc in pharmaceutical sciences from Cairo University in 2006. Upon joining the Feldman laboratory in 2010 as a PhD student, Wael started studying the outer membrane vesicles formation in Gram-negative bacteria, particularly *Bacteroides fragilis*. Membrane vesicles of *B. fragilis* were shown to play an important role in gut homeostasis. Using biochemical and genetics-based approaches, Wael is trying to understand how *Bacteroides* selectively packs proteins into membrane vesicles. These studies will bring us closer to unravel the mechanisms by which members of genus *Bacteroides* interact with the human host to establish lifetime symbiosis.

Mario F. Feldman obtained his PhD in Argentina and carried out post-doctoral work in Belgium and Switzerland. In 2006, he moved to Edmonton, Alberta, and established himself as independent scientist. Mario is now Associate Professor at the Department of Biological Sciences at the University of Alberta. He has pioneered the field of “bacterial glycoengineering”. In recent years, he has investigated the composition of OMV in different organisms, establishing that in some species, OMV biogenesis is an active process, resulting in OMBV with specific protein cargo. He has published articles in journals like Science, PNAS, PLoS Pathogens, Molecular Microbiology, and The Journal of Biological Chemistry, and authored several patents.