Outer Membrane Vesicles of Bacteria: Structure, Biogenesis, and Function

Armaity Nasarabadi, James E. Berleman, and Manfred Auer

Abstract

Extracellular membrane vesicles (*EMVs*), a characteristic present across each domain of life, are subcellular shuttles of biologically active cargo that have a variety of functions ranging from cell-to-cell communication to predatory behavior. Mechanism(s) governing EMV biogenesis remain elusive; however, several initiators have been determined such as stress stimuli, sensing a potential prey or intruder, and signaling molecules. Regardless of function, increased membrane curvature and bulging is a key characteristic that leads to budding and release. This chapter highlights the differences between biogenesis processes of the bacteria, archaea and eukarya. We then focus on the outer membrane vesicles (*OMVs*) specific to Gram-negative bacteria, including several mechanism(s) that potentially explain how the loss of crucial OM-peptidoglycan (*PGN*) and OM-PGN-inner membrane (*IM*) interactions can destabilize the OM to result in OMV biogenesis. Despite gaps present in the current understanding of these novel organelles, OMVs are one mechanism that allow microbial cells to function as multicellular organisms, as pathogens, and act as key predators in their environment. We discuss the importance in better understanding OMV biogenesis for greater insight into how this form of membrane architecture can be utilized for vaccines and targeted/specific treatments for infections.

A. Nasarabadi (✉) • M. Auer
Lawrence Berkeley National Laboratory, Berkeley, CA, USA
e-mail: anasarabadi@lbl.gov; mauer@lbl.gov

J.E. Berleman
Lawrence Berkeley National Laboratory, Berkeley, CA, USA

Saint Mary's College of California, Moraga, CA, USA
e-mail: jeb8@stmarys-ca.edu

© Springer International Publishing AG (outside the USA) 2017

O. Geiger (ed.), *Biogenesis of Fatty Acids, Lipids and Membranes*, Handbook of Hydrocarbon and Lipid Microbiology, DOI 10.1007/978-3-319-43676-0_44-1

Contents

1 Introduction 2
2 EMVs Across the Tree of Life 3
3 OMV Structures, Composition, and Function 7
4 Mechanism(s) of OMV Biogenesis 8
5 Regulation of OMV Biogenesis 10
6 OMV Functions 10
6.1 Biofilm Formation 11
6.2 Pathogenesis 11
6.3 OMV Associated with Predatory Behavior 12
7 Future Research Need 13
References 13

1 Introduction

After initial controversy about their in vivo existence and functional relevance for microbial physiology, outer membrane vesicles (*OMVs*) in recent years have risen to high prominence as they mediate remote cell-to-cell interactions in biofilms, allowing for the exchange of macromolecules (lipids and proteins) and small molecules including quorum signals (*QS*) and toxins (Mashburn-Warren et al. 2008b; reviewed in Kulp and Kuehn 2010; Berleman et al. 2014; Rivera et al. 2010; Remis et al. 2014). The triggers for OMV formation and fusion are still being elucidated, as are the (macro-)molecular mechanisms underlying these mechanisms. Once formed, OMVs reside in the extracellular space, where they may immediately promote extracellular activities or later fuse to another cell (Kulp and Kuehn 2010; Mashburn and Whiteley 2005; Kadurugamuwa and Beveridge 1996; Li et al. 1996, 1998). Because of their hydrophobic nature, the molecules packed within OMVs are not diffused throughout an aqueous environment, but instead serve as a discrete package of goods that another cell, perhaps much later, may receive. It is not clear if different messages packaged in OMVs are targeted for intra- or interspecies delivery, but the complex array of cell surface proteins and lipopolysaccharide modifications may constitute a specific “bar code” or constrain the distribution of uptake. There are intriguing examples of OMV potential, from the motility proteins shared as a community resource through OMV transfer between cells of *Myxococcus xanthus* to the QS-induced OMVs in *Pseudomonas aeruginosa* biofilms (reviewed in Berleman and Auer 2013; Remis et al. 2014; Schaber et al. 2007; Mashburn and Whiteley 2005; Palsdottir et al. 2009). OMVs may also contain a lethal cargo, killing the receiving cell upon vesicle fusion and corresponding uptake of an undiluted chemical dose (Mashburn-Warren et al. 2008a). This mode of a contact-only delivery system clearly has stood the test of time, and we review here the current state of the field in understanding the biogenesis of these structures, their roles in nature and the applications that OMVs may have.

Gram-negative bacteria have a dual membrane envelope, with distinctive inner membrane (*IM*) and outer membrane (*OM*) structures (Fig. 1a). But Gram-negative bacteria are not monophyletic, and it is not clear whether there exists one OMV

biogenesis mechanism for all Gram-negative bacteria or a variety of mechanisms that vary significantly from clade to clade. The OM is a unique envelope structure, with an inner leaflet of phospholipid and an outer leaflet composed of lipopolysaccharides (*LPS*), and is far more complex in its composition (Fig. 1a). While membrane vesicle formation is best documented and understood in Gram-negative bacteria, species with other cell architectures also release extracellular membrane vesicles (*MVs*) (Fig. 1b). Gram-positive bacteria and archaea have also been shown to produce extracellular MVs that serve similar functions as OMVs in Gram-negative bacteria (reviewed in Deatherage and Cooksona 2012; Rivera et al. 2010). Despite having only a single membrane and their extensive envelope with a thick cell wall (peptidoglycan or S-layer protein coat/pseudopeptidoglycan, respectively), MVs are still observed. For Gram-negative bacteria, with their clearly defined inner membrane (*IM*) and outer membrane (*OM*) systems, OMVs bud off the OM with the IM typically held intact. But in the case of Gram-positive bacteria, MVs must originate from the cytoplasmic membrane. These MVs lack LPS and consist predominantly of a phospholipid bilayer. Similarly, archaeal MVs reflect the unique biochemistry of archaeal cytoplasmic membranes, containing ether linkages, isoprenoid chains, and transmembrane phospholipids. Extracellular secretion of vesicles also exists in eukaryotes, where they are known as exosome vesicles (*EVs*). Such EVs have been shown to play a vital role in red blood cell maturation, including the sequestration of certain membrane proteins and nucleic acids breakdown fragments of the cell nuclear chromatin. Exosomes also have been implemented in cancer development, as EVs are capable to remove unwanted chemicals from the cancer cell.

We will focus here mostly on OMVs, where a host of functions have evolved for optimal survival, ranging from indirect cell–cell communication, acquisition of nutrients, evasion of the host's immune system and functioning as pathogens. For examples, soil bacteria live in a dynamic and complex environment that constantly changes with respect to nutrient availability and competition from other species. Communication is key in such environments, and in order to maintain their territory and avoid annihilation, a concerted response mediated by OMVs is needed, for example, coordinating a targeted and lethal attack and alerting other biofilm members to the threat.

2 EMVs Across the Tree of Life

Secreted vesicles are found in Gram-negative and Gram-positive bacteria, archaea, as well as eukaryotic cell systems. The lumen or membrane envelope of OMV often contains toxins, small signaling molecules and biologically active proteins, including virulence factors (Fig. 1c). Gram-negative bacterial OMVs have been extensively studied, whereas the ability of Gram-positive bacteria to produce MVs, that are similar in size to OMVs (20–100 nm), has been more recently reported (reviewed in Deatherage and Cooksona 2012; Lee et al. 2008, 2009; Rivera et al. 2010). *Bacillus* MVs can be double-layered (Lee et al. 2008, 2009; Rivera et al. 2010)

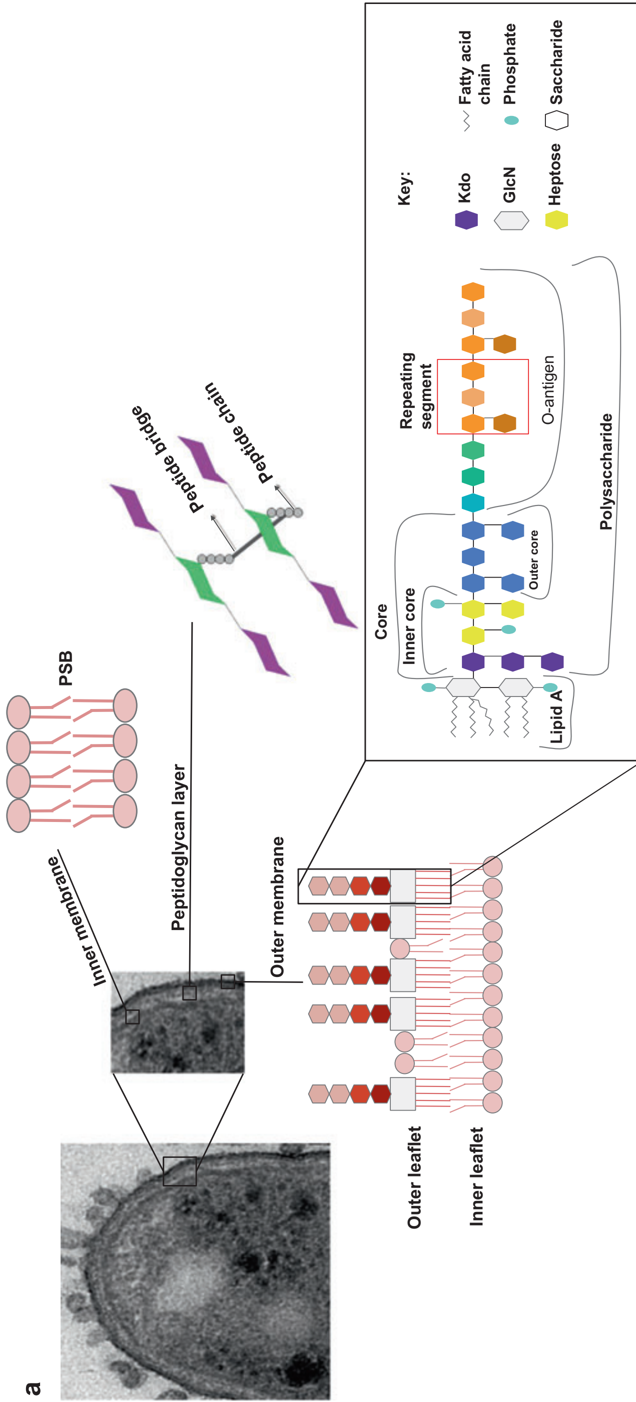

Fig. 1 (continued)

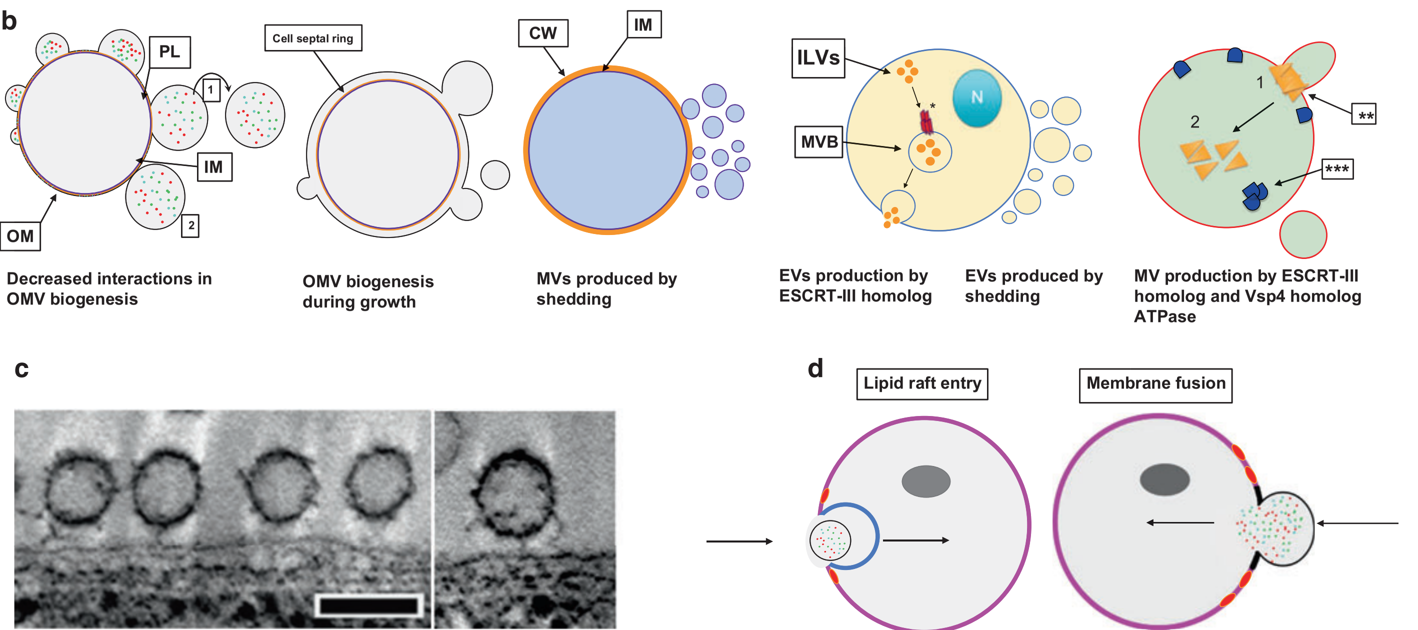

Fig. 1 Gram-negative bacteria membrane structure, MV biogenesis and OMV acceptance in target cell: Gram-negative bacteria make OMVs from the OM, which either stay connected or bud off (**a**, *far left panel*) (Images adopted from Berleman and Auer 2013). The inner membrane is composed of phospholipids, but the OM inner leaflet is composed of phospholipids and the outer leaflet is mainly LPS. LPS is composed of a polysacchride chain and lipid body, with the *O*-antigen being presented to the extracellular matrix (Key: Kdo, 3-deoxy-D-*manno*-oct-2-ulopyranosonic acid). The *O*-antigen is responsible for inducing the endotoxic response in the host's immune system. The peptidoglycan layer makes up the bacterial cell wall and is composed of strands of alternating *N*-acetylmuramic acid (*green*) and *N*-acetylglucosamine (*purple*) which are connected by peptide bridges at the peptide chains that extends from *N*-acetylmuramic acid.

and contain deadly cargo, such as the anthrax toxin (Rivera et al. 2010). Plausible mechanisms that govern Gram-positive bacteria membrane vesicle formation are largely unknown. However, there is experimental evidence that suggest vesicle shedding as a mechanism (Lee et al. 2009).

Archaeal and eukaryotic microbes produce vesicles that are of somewhat similar size to bacteria, 90–230 nm and 40–100 nm, respectively (Prangishvili et al. 2000; Ellen et al. 2008; reviewed in Deatherage and Cooksona 2012). Archaea related MV production appears to be created by pinching off the cell membrane and outer S-layer of the cell wall, whereas eukaryotic microbes utilize exocytosis of intraluminal vesicles (*ILVs*) for EV formation, a process more similar to the production of OMVs by bacteria and the shedding microvesicles (*SMVs*) of eukaryotes (Deatherage and Cooksona 2012) (Fig. 1b). Both archaeal and eukaryotic microbes are suspected to utilize *e*ndosomal *s*orting *c*omplex *r*equired for *t*ransport (*ESCRT-III*) and *v*acuolar *s*orting *p*rotein (*Vps4*) homologues in the biogenesis of their membrane vesicles (Deatherage and Cooksona 2012; Ellen et al. 2008), since this class of machinery is conserved between both domains (reviewed in Deatherage and Cooksona 2012). In eukaryotic cells, ILVs are packaged in larger membrane vesicle congregates called *m*ulti *v*esicular *b*odies (*MVBs*) by ESCRT-III homologues, which can be thought of as a larger vesicle holding smaller vesicles (Keller et al. 2006; Deatherage and Cooksona 2012) (Fig. 1b). MVBs fuse to the plasma membrane and release of ILVs as exosomes

Fig. 1 (continued) EMV formation occurs in all three domains of life (**b**). Gram-negative (*two light grey cells to the left*) bacteria are observed making OMVs by two mechanisms: reducing the number of OM-PL (*light blue lines*) and OM-PL-IM (*purple lines*) interactions in areas around the cell in response to stimuli and at points called nanoterritories or reducing the interactions previously mentioned through cell growth since the cell wall grows at a slower rate than the OM at the septum of the dividing cell. OMVs can either be released from the cell (*1*) or remain attached (*2*). Once removed, OMVs are found to have a variety of contents, including proteins, secondary metabolites and nucleic acids. Gram-positive bacteria (*center left blue cell*) have been observed producing MVs, which is suspected to be done by shedding, similar to eukaryotic microbes, at areas of weakened or absent cell wall. Eukaryotic microbe cells (*two center right beige cells*) are also observed producing EVs by the gathering of ILVs in MVBs by ESCRT-III homolog mediation which then fuse to the cell membrane and release its contents into the extracellular matrix. Archaea (*light green cell far right*) utilize a ESCRT-III homolog to make the membrane bulge to produce the MV, which is released through ESCRT-III membrane complex disassembly by Vsp4 homolog ATPase. Note that the archaeal cell membrane is shown above, but the S-layer and cell wall would be present during MV genesis, as well. Electron density increases from the center of the vesicles to its edge and are observed aligned on the surface of a *Myxococcus xanthus* cell (imaged by cryo-tomography) with cargo or an underlying protein organization (**c**). Scale bar, 100 nm (Palsdottir et al. 2009) (Images adopted from Berleman and Auer 2013; Palsdottir et al. 2009). OMVs enter host cells by two methods: lipid rafts or membrane fusion (**d**). Lipid rafts are areas that are rich in sphingolipids and cholesterol, causing the membrane to invaginate (*blue*) at areas of dense lipid packing and allow OMVs to enter the cell whole. OMV fusion to the target cell's membrane also leads to OMV contents entering the cell, despite variations in membrane structure, at colocalized lipid raft rich areas. Note abbreviations and symbols: *PL* peptidoglycan layer, *IM* inner membrane, *OM* outer membrane, *PSB* phospholipid bilayer, *CW* cell wall, * (ESCRT-III homolog), ** (Vsp4 homolog ATPase assembled)

(Deatherage and Cooksona 2012). Eukaryotic microbes utilize similar machinery which allows the release of ILVs in the form of exosomes. Production of exosomes for eukaryotic microbes is the result of shedding, similar to bacteria, although the exact mechanisms are still unknown.

Archaea utilize ESCRT-III homologue proteins to form membrane vesicles and appears to require Vsp4 homologs for vesicle release (Ellen et al. 2008; Deatherage and Cooksona 2012). ESCRT-III homolog proteins are thought to congregate at the plasma membrane surface to create a bulge, with pinching off of the vesicle occurring when Vps4 homolog ATPases leads to archaeal ESCRT-III homolog disassembly (Ellen et al. 2008; Deatherage and Cooksona 2012). ESCRT-III homolog and Vps4 homolog ATPase proteins are membrane associated, which upon vesicle release mostly stay with the cell (Deatherage and Cooksona 2012; Ellen et al. 2008). While the detailed mechanisms between bacterial and archaea/eukaryotes appear to be different, it would seem that neither process is random or passive but highly orchestrated and controlled.

3 OMV Structures, Composition, and Function

OMVs were first observed with *Escherichia coli* when it was placed in growth limiting nutritional conditions resulting in OMVs that appeared as protrusions on the outer surface of cells, still attached in many cases (Bishop and Work 1965; Knox et al. 1966). As the nutrient concentration decreased, vesicle growth seemed to increase (Knox et al. 1966; Work et al. 1966). Analysis initially done by Bishop and Work (1965) showed that LPS was incorporated into the vesicles. The origin of the vesicles was identified using time lapse imaging and initially it was hypothesized that an excess of membranes due to stalled growth of *E. coli* was the reason for vesicle formation (Work et al. 1966). Biochemical analysis of vesicles and outer membrane revealed an identical LPS signature, which supported an early hypothesis, that the vesicles solely originated from the outer membrane (Katsui et al. 1982; Hoekstra et al. 1976).

The cytotoxic protein ClyA in an *E. coli* K-12 derivative strain accumulates initially as periplasm-folded ClyA monomers underneath OM bulges (Wai et al. 2003; reviewed in De Geyter et al. 2016) in regions of reduced peptidoglycan linkages. Oligomerization of ClyA occurs in OMVs forming pores which contribute to the infection of erythrocytes and other mammalian cells (Hunt et al. 2010; Wai et al. 2003). When observed by TEM imaging, vesicles appear hollow, and devoid of major macromolecular proteins and complexes, in accordance with the hypothesis of their origins (Katsui et al. 1982). These structures have been observed being relatively spherical in shape, and the size reported was typically between 30 and 80 nm or larger in diameter (Lee et al. 2008, 2009; Rivera et al. 2010; Kadurugamuwa and Beveridge 1996; Palsdottir et al. 2009).

Recent proteomics studies support the idea that OMVs share compositional characteristics with the outer membrane, but are not an exact replica (Kulp and Kuehn 2010; Berleman et al. 2014; Loeb and Kilner 1979). Certain proteins are only found in

OMVs but not in the OM, whereas others are shared between OMVs and the OM; supporting the notion that there are possible organization or selection mechanisms that determine outer membrane vesicle composition (reviewed in Kulp and Kuehn 2010; Berleman et al. 2014). In addition to OMV-OM-shared and OMV-unique proteins, periplasmic proteins, inner membrane proteins and cytoplasmic components have also been found in OMVs as well (Berleman et al. 2014; Roier et al. 2016; Deatherage and Cooksona 2012; Kulkarni and Jagannadham 2014; Deatherage et al. 2009; Schwechheimer and Kuehn 2015), and they do not appear to represent mere contamination issues as certain other high abundance proteins were not found.

OMVs have been found to enter mammalian cells by cholesterol and sphingolipids-rich areas that form lipid rafts and fusion to a target membrane at colocalized areas of lipid rafts (O'Donoghue and Krachler 2016) which result in the content to be released into its cytoplasms (Fig. 1d). However as a word of caution, LPS serves as an endotoxin that elicits an immune response in humans. Though, upon entry into eukaryotic cells, the content of OMVs is eventually released and may contain secondary metabolites and proteins that maybe have a broad cytotoxic effect and can induce cell apoptosis.

4 Mechanism(s) of OMV Biogenesis

While the biological processes governing the formation of OMVs have yet to be fully understood, differences in OMV and OM composition suggest that OMV formation is not a passive process, but is somehow regulated (Berleman et al. 2014; Wensink and Witholt 1981; Kato et al. 2001; Hoekstra et al. 1976; Deatherage and Cooksona 2012). Given the different roles OMV play in bacterial physiology, ranging from mediating benign and supportive interactions such as the sharing and exchange of OM proteins, to hostile and detrimental interactions, such as the delivery of toxins, one would expect that both the cargo-load as well as the targeting mechanisms are well controlled and adjusted to the respective situation. However, none of the proteins in this sorting machinery have been identified.

In order for the vesicles to exhibit any kind of function they first have to be secreted. Three mechanisms for OMVs formation has been proposed (reviewed in Berleman and Auer 2013): (I) in the first mechanism small molecules or membrane proteins associate with the outer membrane to induce curvature that then subsequently leads to budding of the vesicle; (II) in the second mechanism, the accumulation of periplasmic proteins pushes the outer membrane out to form a vesicle; and (III) in the third mechanism the contact between the OM and peptidoglycan layer is lost, leading to the buildup of lipids, and subsequent vesicle formation. In this scenario links between the OM and the cell wall are severed (Schwechheimer and Kuehn 2015). Either such links are actively broken, or there may exist as nanoterritories, or areas in the membrane, with reduced interactions (e.g., near the poles) (Schwechheimer and Kuehn 2015). Recent studies of OMV formation in *Haemophilus influenza*, suggests the involvement of an ABC transporter protein for the accumulation of lipids in the outer leaflet of the outer membrane (Roier et al. 2016). These proposed mechanisms

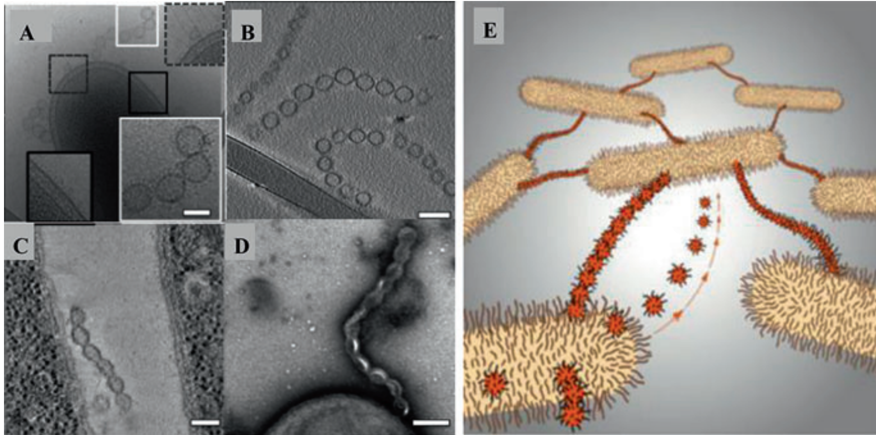

Fig. 2 Structural analysis of *M. xanthus* vesicle chains and model of vesicle facilitated interactions in biofilm formation. *M. xanthus* forms both single vesicles and vesicle chains. Cryo-fixed *M. xanthus* cells were imaged by TEM to view OMVs that have a single membrane (**a**, *white bordered square*) when compared to the parent cell double-membrane structure (**a**, *black line border square*). Vesicles were discernible from pili (**a**, *black dotted square*). Vesicle chains prepared by fast-frozen methods (**b**) were observed being of similar size in a single chain, with size variation observed between chains. Continuous lumen was observed in constricted vesicle chains of a slice through a 3D tomogram of a HPF/FS, resin-embedded sample and negatively stained whole mount sample (**c**, **d**, respectively). (**e**) Remis et al. propose that connections between individual cells within a biofilm are maintained by extracellular appendages including free diffusing vesicles and vesicle chains. These appendages are coated in carbohydrates (i.e., LPS and OM lipids) utilized for cell-cell recognition and communication through the transfer of intercellular matter. This model shows individual cells being organized into a highly integrated multicellular community through free vesicles diffusion and direct intercellular connections by vesicle chains. Scale bars represent: 50 nm (**a**) and 100 nm (**b**, **c**, **d**) (Images adopted from Remis et al. 2014)

are not mutually exclusive and may all in some part contribute to OMV biogenesis. Proteomics analysis has revealed that more than half of the proteins found in *Myxococcus* OMVs have no known function (Berleman et al. 2014). Comparison to other Gram-negative OMV, proteomics studies (Kulp and Kuehn 2010; Deatherage and Cooksona 2012) have not yielded a clearly defined set of proteins that is always present and thus evolutionary conserved.

From an ultrastructural point of view, the center of the vesicles is found to be relatively hollow with a single membrane bilayer surrounding the luminal content (Knox et al. 1966; Work et al. 1966; Kadurugamuwa and Beveridge 1996). Electron tomographic analysis (Palsdottir et al. 2009) of resin-embedded samples confirmed a relatively sparse occupancy of the lumen (Fig. 1c). OMVs were often found to be tethered to the membrane, and even tethered to one another, with OMVs often forming a tight linear like pearl array on a string (Fig. 2). At this point it remains uncertain, whether there exists a cage-like structure just underneath the membrane, not unlike clathrin in eukaryotes, but experimental validation, for example, by electron tomographic imaging, remains to be carried out.

5 Regulation of OMV Biogenesis

Outer membrane vesicle biogenesis occurs under normal growth conditions but is upregulated upon encountering environmental stress (Kulp and Kuehn 2010; Berleman and Auer 2013; Baumgarten et al. 2012; Deatherage and Cooksona 2012; Roier et al. 2016). These stressors include salinity, acidity, temperature, and nonrelated microbial communities occupying the same space (Kulp and Kuehn 2010; Baumgarten et al. 2012; Katsui et al. 1982; Deatherage and Cooksona 2012; Roier et al. 2016). Such external stressors can even alter the bacterial envelope physiology, for example, *Pseudomonas putida* DOT-T1E increases OMV formation and renders the hydrophobicity of the cell surface to facilitate biofilm formation (Baumgarten et al. 2012). *P. aeruginosa* increases production of membrane vesicles after exposure to the antibiotic gentamicin with an altered contents and integrated membrane composition when compared to normal OMV biogenesis (Mashburn-Warren et al. 2008a; Kadurugamuwa and Beveridge 1995). OMV production is also altered by the exogenous introduction of a quorum signaling molecule (such as PQS), which induces curvature and perturbation of the OM (Mashburn and Whiteley 2005; Mashburn-Warren et al. 2008b; Schertzer and Whiteley 2012). It is thought that the interaction of PQS with the outer LPS leaflet of the OM drives the biogenesis of vesicles (Mashburn and Whiteley 2005; Mashburn-Warren et al. 2008b; Schertzer and Whiteley 2012). When confronted with another species, *M. xanthus* will form OMVs that are filled with a lethal load resulting in the lysis of the opposing colony and the uptake of nutrients (Berleman et al. 2014; Evans et al. 2012; reviewed in Keane and Berleman 2016).

6 OMV Functions

Outer membranes vesicles are present during basal activity and often upregulated in times of stress, suggesting that they carry a variety of functions. The formation of OMVs was observed by Knox et al. (1966) when imaging *E. coli* under stress due to the lack of lysine in the growth medium. Since then, OMVs have been observed in pathogenic bacteria, as a hunting and defense mechanism and appear to play a role in biofilm formation. The exact mechanism of delivery remains to be determined; one can envision autolysis in the vicinity of the target cell (Kulp and Kuehn 2010; Mashburn-Warren et al. 2008a; Kadurugamuwa and Beveridge 1996; Li et al. 1998) or the fusion of the vesicle with its target cell, and thus release of the content into its periplasm (Kulp and Kuehn 2010; Mashburn and Whiteley 2005; Kadurugamuwa and Beveridge 1995; Li et al. 1996, 1998), possibly disrupting the peptidoglycan structure and leaving the bacterium vulnerable. Results from our and other labs support the fusion model.

6.1 Biofilm Formation

Biofilms are constructs of large microbial colonies that emerge in order for cells to communicate and serve as protection against harsh environments. *P. aeruginosa* biofilms have also been observed in chronic infections and in burn wounds, with vesicles found in the extracellular matrix surrounding cells (reviewed in Berleman and Auer 2013; Schaber et al. 2007). PQS facilitates the formation of OMVs, and while in itself it is not sufficient for biofilm formation, it affects group dynamics and triggers PQS-related group behavior (Mashburn and Whiteley 2005). Presence of PQS leads to the formation of vesicles that contain quinolone/quinolines that have active antimicrobial properties (Mashburn and Whiteley 2005; Mashburn-Warren et al. 2008b). Within the biofilm borders of *M. xanthus*, it was found that OMVs fill available space that cell bodies do not occupy (Palsdottir et al. 2009), providing ultrastructural evidence that OMVs are closely interacting with the OM of cells via proteinaceous tethers. Thus, OMVs are not only utilized for the initial formation of biofilms but are prominently found in mature biofilms. The upregulation of OMV secretion in *M. xanthus* biofilms compared to its planktonic state further emphasizes the key role they play in intraspecies, intercellular communication.

Among the more surprising findings of the last few years is that OMVs are not only secreted as individual vesicles, but they often form more complex structures, such a membrane vesicles chains, regular-interval constricted membranes tubes, as well as membrane tubes with a uniform circular or oval-shaped diameter (Remis et al. 2014). Dubbed as "bacterial social networks," they can be viewed as a stealth intercellular communication and molecule-sharing system, where large numbers of cells are physically connected at the level of their respective periplasmic space (Fig. 2). Such interconnection of cells in a biofilm would allow *Myxococcus* biofilms to behave like a complex multicellular organism with a spatially organized division of labor, not unlike one that is found in plants or animals. This type of network would also allow regions of the biofilms that are far away from a competing species to be alerted in time and thus prepare for such threats or opportunities.

6.2 Pathogenesis

Biofilms have been found in infected wounds or vulnerable tissue (lungs of individuals with cystic fibrosis), with pathogenesis apparently correlating with OMV presence. In order for the pathogenicity to occur, a microbial community/biofilm has to be established, that conducts an attack while shielding itself. OMVs may have two functions, communication between colonies of microbes and attacking cells. The packaging of virulence and pathogenic factors into OMVs presents different advantages: first, concentrating virulence factors to the target cell for more efficient delivery, and second, virulence factors would be protected against degradation and recognition by the host and other microorganisms (Mashburn-Warren et al. 2008a). When harmful antimicrobial agents (including gentamicin) are presented to *P. aeruginosa* OMV formation is induced, leading to the packaging of

P. aeruginosa antimicrobial agents (Kadurugamuwa and Beveridge 1996; Mashburn-Warren et al. 2008a), including autolysins, hydrolytic enzymes, phospholipase C, proteases, alkaline phosphatases and hemolysins (Mashburn-Warren et al. 2008a; Kadurugamuwa and Beveridge 1995).

OMV mediated toxin attack is facilitated by endocytosis into eukaryotic cells or fusion with the plasma membrane (Kulp and Kuehn 2010; Furuta et al. 2009). OMVs by *Acinetobacter baumannii* cause host cells to undergo apoptosis through an OmpA channel protein-mediated process that disrupts the host membrane (Jin et al. 2011). *Aggregatibacter actinomycetemcomitans* uses OMVs to deliver the cytolethal toxins to both HeLa cells and human gingival fibroblasts (Rompikuntal et al. 2012). While the mode of producing a cytotoxic effect on these two eukaryotic target cells differs, in both cases OMVs utilized to evade the host's immune response and deliver the toxic agent.

6.3 OMV Associated with Predatory Behavior

In addition to biofilm formation and pathogenesis, OMV also play a role in predation, as "predatory [O]MVs" (Mashburn-Warren et al. 2008a; Mashburn and Whiteley 2005; Kadurugamuwa and Beveridge 1996), which may fuse with both Gram-negative bacteria and eukaryotic cells (Kadurugamuwa and Beveridge 1996; Evans et al. 2012). Predatory [O]MVs contain enzymes that disrupt the OM and peptidoglycan walls, thus killing a wide spectrum of bacteria (Li et al. 1998). Salt bridges forming stable interactions between the OMV and the Gram-positive cell wall have been proposed, which may deform OMVs resulting in OMV bursting in close proximity to the target cell (Kadurugamuwa and Beveridge 1996). Likewise scavenging and predatory soil bacteria take advantage of this long-range, but targeted mode of attack and/or defense.

M. xanthus, a Gram-negative bacterium, is a key predator species in the soil that utilizes OM vesicles not only to maintain its territories, but also to acquire essential nutrients. *M. xanthus* requires close contact with prey cell to induce prey death, without invasion or engulfment, and OMVs have been found to facilitate predation (Keane and Berleman 2016; Berleman et al. 2014; Evans et al. 2012). OMV content analysis revealed the presence of 16 molecules with antibiotic activities and several homologs of hydrolytic enzymes. Proteomics analysis revealed 234 OMV-enriched proteins, with 46 specific to OMVs and 188 shared with the OM (Berleman et al. 2014). Enrichment suggests a selection mechanism for the packing of the OMV, which may suggest that OMV formation may not be a rather passive process, but entails a high level of control. Furthermore, it is possible that OMVs contain a type of "zip-code" (suggested by Evans et al. 2012), that ensures proper targeting of the OMV content, which could be protein- or more interestingly LPS-based.

Whatever the mode of targeting, delivering an undiluted lethal cargo to a targeted cell is an effective way to avoid resistance that may otherwise occur since dilution of the antimicrobial molecules would reduce their efficacy, which in by itself may justify the energy cost of OMV biogenesis.

7 Future Research Need

Many advances have been made in understanding the biological processes governing the formation of vesicles in the extracellular environment. We still lack an understanding of the exact mechanisms of OMV formation and their molecular triggers. We also have little insight about the control of vesicle content, but discrepancies in OM and OMV macromolecular composition suggest that OMV formation is not a passive but instead a highly controlled active process. The question of a possible ZIP code that may be protein and/or polysaccharide based is a particularly intriguing one, as it would allow control over specificity of messaging. Given the success of OMVs in communication and predation, and the fact that targeted species have not developed resistance despite millions to billions of years of coevolution, OMVs may be an intriguing novel approach for antibiotics development. Packaging of one or several antibiotic/antimicrobial (macro-)molecules into vesicles offers protection and stability.

Given their ubiquitous presence in extracellular fluids, their apparent biostability, and their small size, OMVs may serve as noninfectious vehicles to deliver small molecules or macromolecules to different human cells and tissues and to initiate or suppress a host immune response (Ellis and Kuehn 2010). Such "designer OMVs" are likely to be taken up by microbes and human cells in ways similar to pathogens (Kulp and Kuehn 2010; Furuta et al. 2009; Ellis and Kuehn 2010). An OMV-based vaccine for *Neisseria meningitidis* is the most successful case of OMVs initiating an immune response with moderately high efficacy (Acevedo et al. 2014). OMV vaccines for *N. meningitidis* were proposed as an alternate source due to a lower risk of potentially developing autoimmunity (reviewed in Acevedo et al. 2014).

OMV formation could also be an important drug target, as interference in this process may stall biofilm formation and pathogenesis altogether. Clearly, it is early in the days of OMV research, as further research is needed to better understand the various biological processes involved in OMV formation and molecular packing, so that ultimately we may control this process and design OMVs with specific characteristics, content and ZIP-code for the control and treatment of infectious diseases or for other cell-specific cargo-delivery (e.g., lung diseases, cancer, or immune response abnormalities).

References

Acevedo R, Fernández S, Zayas C et al (2014) Bacterial outer membrane vesicles and vaccine applications. Front Immunol 5:121. doi:10.3389/fimmu.2014.00121

Baumgarten T, Sperling S, Seifert J et al (2012) Membrane vesicle formation as a multiple-stress response mechanism enhances *Pseudomonas putida* DOT-T1E cell surface hydrophobicity and biofilm formation. Appl Environ Microbiol 78(17):6217–6224. doi:10.1128/AEM.01525-12

Berleman J, Auer M (2013) The role of bacterial outer membrane vesicles for intra- and interspecies delivery. Environ Microbiol 15(2):347–354. doi:10.1111/1462-2920.12048

Berleman JE, Allen S, Danielewicz MA et al (2014) The lethal cargo of *Myxococcus xanthus* outer membrane vesicles. Front Microbiol 5:1–11. doi:10.3389/fmicb.2014.00474

Bishop DG, Work E (1965) An extracellular glycolipid produced by *Escherichia coli* grown under lysine-limiting conditions. Biochem J 96(2):567–576. doi:10.1042/bj0960567

Deatherage BL, Cooksona BT (2012) Membrane vesicle release in bacteria, eukaryotes, and archaea: a conserved yet underappreciated aspect of microbial life. Infect Immun 80 (6):1948–1957. doi:10.1128/IAI.06014-11

Deatherage BL, Lara JC, Bergsbaken T, Barrett SLR, Lara S, Cookson BT (2009) Biogenesis of bacterial membrane vesicles. Mol Microbiol 72(6):1395–1407. doi:10.1111/j.1365-2958.2009.06731.x.Biogenesis

De Geyter J, Tsirigotaki A, Orfanoudaki G, Zorzini V, Economou A, Karamanou S (2016) Protein folding in the cell envelope of *Escherichia coli*. Nat Microbiol 1:16107. doi:10.1038/nmicrobiol.2016.107

Ellen AF, Albers SV, Huibers W et al (2008) Proteomic analysis of secreted membrane vesicles of archaeal *Sulfolobus* species reveals the presence of endosome sorting complex components. Extremophiles 13(1):67–79. doi:10.1007/s00792-008-0199-x

Ellis TN, Kuehn MJ (2010) Virulence and immunomodulatory roles of bacterial outer membrane vesicles. Microbiol Mol Biol Rev 74(1):81–94. doi:10.1128/mmbr.00031-09

Evans A, Davey H, Cookson A, Currinn H, Cooke-Fox G, Stanczyk P, Whitworth D (2012) Predatory activity of *Myxococcus xanthus* outer-membrane vesicles and properties of their hydrolase cargo. Microbiology 158(11):2742–2752. doi:10.1099/mic.0.060343-0

Furuta N, Takeuchi H, Amano A (2009) Entry of *Porphyromonas gingivalis* outer membrane vesicles into epithelial cells causes cellular functional impairment. Infect Immun 77 (11):4761–4770. doi:10.1128/IAI.00841-09

Hoekstra D, van der Laan JW, de Leij L, Witholt B (1976) Release of outer membrane fragments from normally growing *Escherichia coli*. Biochim Biophys Acta 455:889–899

Hunt S, Green J, Artymiuk P (2010) Hemolysin E (HlyE, ClyA, SheA) and related toxins. Adv Exp Med Biol 677:116–126

Jin JS, Kwon S-O, Moon DC et al (2011) *Acinetobacter baumannii* secretes cytotoxic outer membrane protein A via outer membrane vesicles. PLoS One 6(2):e17027. doi:10.1371/journal.pone.0017027

Kadurugamuwa JL, Beveridge TJ (1995) Virulence factors are released from *Pseudomonas aeruginosa* in association with membrane vesicles during normal growth and exposure to gentamicin: a novel mechanism of enzyme secretion. J Bacteriol 177(14):3998–4008

Kadurugamuwa JL, Beveridge TJ (1996) Bacteriolytic effect of membrane vesicles from *Pseudomonas aeruginosa* on other bacteria including pathogens: conceptually new antibiotics. J Bacteriol 178(10):2767–2774

Kato S, Kowashi Y, Demuth DR (2001) Outer membrane-like vesicles secreted by *Actinobacillus actinomycetemcomitans* are enriched in leukotoxin. Microb Pathog 32(1):1–13. doi:10.1006/mpat.2001.0474

Katsui N, Tsuchido T, Hiramatsu R, Fujikawa S, Takano M, Shibasaki I (1982) Heat-induced blebbing and vesiculation of the outer membrane of *Escherichia coli*. J Bacteriol 151 (3):1523–1531

Keane R, Berleman J (2016) The predatory life cycle of *Myxococcus xanthus*. Microbiology 162 (1):1–11. doi:10.1099/mic.0.000208

Keller S, Sanderson MP, Stoeck A, Altevogt P (2006) Exosomes: from biogenesis and secretion to biological function. Immunol Lett 107(2):102–108. doi:10.1016/j.imlet.2006.09.005

Knox KW, Vesk M, Work E (1966) Relation between excreted lipopolysaccharide complexes and surface structures of a lysine-limited culture of *Escherichia coli*. J Bacteriol 92(4):1206–1217

Kulkarni HM, Jagannadham MV (2014) Biogenesis and multifaceted roles of outer membrane vesicles from Gram-negative bacteria. Microbiology 160(2014):2109–2121. doi:10.1099/mic.0.079400-0

Kulp A, Kuehn MJ (2010) Biological functions and biogenesis of secreted bacterial outer membrane vesicles. Annu Rev Microbiol:163–184

Lee E, Choi D, Kim K, Cho Y (2008) Proteomics in Gram-negative bacterial outer membrane vesicles. Mass Spectrom Rev 27:535–555

Lee E-Y, Choi D-Y, Kim D-K et al (2009) Gram-positive bacteria produce membrane vesicles: proteomics-based characterization of *Staphylococcus aureus*-derived membrane vesicles. Proteomics 9(24):5425–5436. doi:10.1002/pmic.200900338

Li Z, Clarke AJ, Beveridge TJ (1996) A major autolysin of *Pseudomonas aeruginosa*: subcellular distribution, potential role in cell growth and division and secretion in surface membrane vesicles. J Bacteriol 178(9):2479–2488. http://www.ncbi.nlm.nih.gov/pubmed/8626312

Li Z, Clarke AJ, Beveridge TJ (1998) Gram-negative bacteria produce membrane vesicles which are capable of killing other bacteria. J Bacteriol 180(20):5478–5483

Loeb MR, Kilner J (1979) Effect of growth medium on the relative polypeptide composition of cellular outer membrane and released outer membrane material in *Escherichia coli*. J Bacteriol 137(2):1031–1034

Mashburn LM, Whiteley M (2005) Membrane vesicles traffic signals and facilitate group activities in a prokaryote. Nature 437(7057):422–425. doi:10.1038/nature03925

Mashburn-Warren L, McLean RJC, Whiteley M (2008a) Gram-negative outer membrane vesicles: beyond the cell surface. Geobiology 6(3):214–219. doi:10.1111/j.1472-4669.2008.00157.x

Mashburn-Warren L, Howe J, Garidel P et al (2008b) Interaction of quorum signals with outer membrane lipids: insights into prokaryotic membrane vesicle formation. Mol Microbiol 69 (2):491–502

O'Donoghue EJ, Krachler AM (2016) Mechanisms of outer membrane vesicle entry into host cells. Cell Microbiol:1508–1517. doi:10.1111/cmi.12655

Palsdottir H, Remis JP, Schaudinn C et al (2009) Three-dimensional macromolecular organization of cryofixed *Myxococcus xanthus* biofilms as revealed by electron microscopic tomography. J Bacteriol 191(7):2077–2082. doi:10.1128/JB.01333-08

Prangishvili D, Holz I, Stieger E, Nickell S, Kristjansson JK, Zillig W (2000) Sulfolobicins, specific proteinaceous toxins produced by strains of the extremely thermophilic archaeal genus *Sulfolobus*. J Bacteriol 182(10):2985–2988. doi:10.1128/JB.182.10.2985-2988.2000

Remis JP, Wei D, Gorur A et al (2014) Bacterial social networks: structure and composition of *Myxococcus xanthus* outer membrane vesicle chains. Environ Microbiol 16(2):598–610. doi:10.1111/1462-2920.12187

Rivera J, Cordero RJB, Nakouzi AS, Frases S, Nicola A, Casadevall A (2010) *Bacillus anthracis* produces membrane-derived vesicles containing biologically active toxins. Proc Natl Acad Sci U S A 107(44):19002–19007. doi:10.1073/pnas.1008843107

Roier S, Zingl FG, Cakar F et al (2016) A novel mechanism for the biogenesis of outer membrane vesicles in Gram-negative bacteria. Nat Commun 7:10515. doi:10.1038/ncomms10515

Rompikuntal PK, Thay B, Khan M et al (2012) Perinuclear localization of internalized outer membrane vesicles carrying active cytolethal distending toxin from *Aggregatibacter actinomycetemcomitans*. Infect Immun 80(1):31–42. doi:10.1128/IAI.06069-11

Schaber JA, Triffo WJ, Sang JS et al (2007) *Pseudomonas aeruginosa* forms biofilms in acute infection independent of cell-to-cell signaling. Infect Immun 75(8):3715–3721. doi:10.1128/IAI.00586-07.1

Schertzer J, Whiteley M (2012) A bilayer-couple model of bacterial outer membrane vesicle. MBio 3(2):e00297–e00211. doi:10.1128/mBio.00297-11.Editor

Schwechheimer C, Kuehn MJ (2015) Outer-membrane vesicles from Gram-negative bacteria: biogenesis and functions. Nat Rev Microbiol 13(10):605–619. doi:10.1038/nrmicro3525

Wai SN, Lindmark B, Söderblom T et al (2003) Vesicle-mediated export and assembly of pore-forming oligomers of the enterobacterial ClyA cytotoxin. Cell 115(1):25–35. doi:10.1016/S0092-8674(03)00754-2

Wensink J, Witholt B (1981) Outer-membrane vesicles released by normally growing *Escherichia coli* contain very little lipoprotein. Eur J Biochem 116:331–335

Work E, Knox KW, Vesk M (1966) The chemistry and electron microscopy of an extracellular lipopolysaccharide from *Escherichia coli*. Ann N Y Acad Sci 133:438–449. doi:10.1111/j.1749-6632.1966.tb52382.x