

# Chapter 2

## Biogenesis of Gram-Negative OMVs



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**Abstract** Initially dismissed as artifacts during sample preparation or as cell debris in bacterial cultures, outer membrane vesicles (OMVs) are now widely accepted as facsimiles of the outer membrane naturally secreted by Gram-negative bacteria. Within the last decades, several studies focused on OMV biogenesis resulting in different, in part complementary models to explain OMV release. Notably, vesicle formation seems to be an essential process as neither a bacterial species nor a mutant lacking vesicle release has been reported so far. Based on the complex physiological roles discussed for OMVs, it is likely that parallel strategies for their production have evolved to ensure their release in diverse conditions. Although, we are still far from a comprehensive mechanistic understanding of OMV release, several studies shed some insights in the processes driving the liberation of OMVs from the bacterial surface. Within this chapter, we will discuss the observations resulting in the current models for OMV formation including their regulation and relevance for bacterial physiology.

### 2.1 Introduction

Over the last years, the biogenesis of Gram-negative outer membrane vesicles (OMVs) has been investigated by several groups. This has resulted in a range of models for OMV release from bacteria (Fig. 2.1). Indeed, it is likely that bacteria encompass several complementary mechanisms for vesicle biogenesis.

First reports indicated that mutants harboring a loss or reduction of linkages between the outer membrane and the periplasmic peptidoglycan layer exhibit increased vesiculation (Deatherage et al. 2009; Suzuki et al. 1978; Schwechheimer

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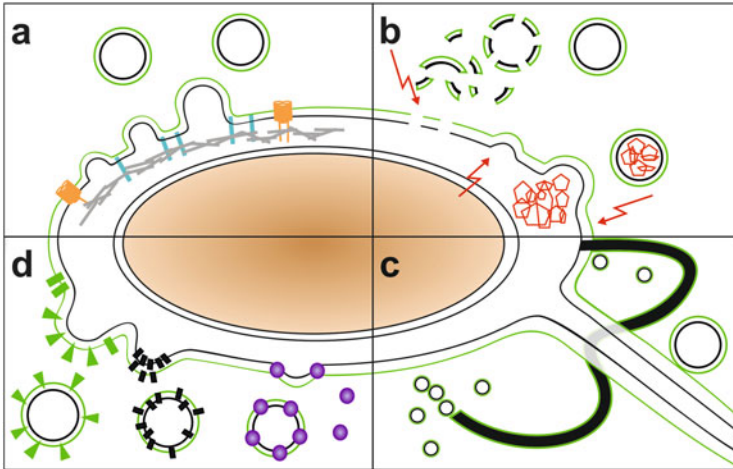
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**Fig. 2.1** Schematic overview of OMV biogenesis routes discussed in this chapter. Shown are models of OMV formation based on: modulation of outer membrane–peptidoglycan linkages (**a**), bacterial stress responses (**b**), filamentous and tubular surface structures (**c**), and modulation of outer membrane components or outer membrane composition (**d**). In detail, (**a**) shows the mechanisms of OMV biogenesis due to loss of linkages between the outer membrane [outer leaflet composed of LPS (green) and inner leaflet composed of phospholipids (black)] and the peptidoglycan layer (gray) either by porins (orange) or proteins (light blue). (**b**) shows OMV formation due to stressors (red arrows) ranging from membrane attacking substances, high temperature, DNA damage or bacteriophages and accumulation of osmolytes (red). (**c**) shows OMV formation associated to surface structures like the sheathed flagellum (black) as well as tube-like outer membrane extensions such as nanopods, nanotubes, or nanowires. (**d**) indicates the mechanism of OMV formation via outer membrane modulation ranging from LPS modulation (green rectangles to triangles), intercalation of curvature-inducing molecules (purple circles), and modulation of phospholipid composition (black rectangles)

et al. 2013; Iwami et al. 2007; Moon et al. 2012; Turner et al. 2015; Llamas et al. 2000; Yeh et al. 2010; Mitra et al. 2016; Bernadac et al. 1998). Although these observations were predominantly achieved by loss-of-function mutants, current data suggest that bacteria can rearrange outer membrane–peptidoglycan linkages or regulate their abundance thereby affecting OMV production (Song et al. 2008; Choi et al. 2017; Schwechheimer et al. 2013).

Envelope stress is a general trigger for OMV release, highlighted by induction of vesiculation upon the presence of physical, chemical, and biological membrane stressors (Kadurugamuwa and Beveridge 1997). Moreover, accumulation of misfolded proteins and metabolites (e.g., peptidoglycan breakdown products) in the periplasm can result in increased OMV secretion, most likely via increased turgor pressure and bulging of the outer membrane (Schwechheimer et al. 2014; Hayashi et al. 2002). This could represent a response to harmful environments to increase the bacterial fitness and allow effective removal of undesired factors.

More recent studies revealed OMV secretion processes independent of mutations and presence of stressors, but allow refined regulation by the bacteria (Mashburn-

Warren et al. 2008; Tashiro et al. 2011; Roier et al. 2016; Bonnington and Kuehn 2016; Elhenawy et al. 2016). A first report in this direction investigated small bacterial molecules produced during defined stages of the bacterial life cycle, which induce surface curvature via intercalation into the outer membrane and consequently promote vesiculation (Mashburn-Warren et al. 2008). So far, this strategy seems restricted to certain species as curvature-inducing molecules have only been identified in *Pseudomonas aeruginosa* (Mashburn and Whiteley 2005). However, alternative OMV biogenesis models relying on modulation of the outer membrane composition that are applicable to a wide range of Gram-negative bacteria have been described. This includes several studies demonstrating that OMV release is affected by alterations in the lipopolysaccharide (LPS) composition, LPS modifications or phospholipid accumulation in the outer membrane via silencing of a retrograde lipid trafficking system (Tashiro et al. 2011; Roier et al. 2016; Bonnington and Kuehn 2016; Elhenawy et al. 2016; Sabra et al. 2003; Haurat et al. 2011). As LPS as well as the retrograde lipid trafficking system are quite conserved among Gram-negative species, these models probably reflect general mechanisms broadly applicable to a diverse set of bacteria.

OMV isolation and quantification protocols have improved within the last years. As OMV research spans over decades, different approaches were used to identify and characterize mechanisms of OMV biogenesis such as phenotypical analyses of OMV formation under differential cultivation conditions or random and site-directed mutants. Furthermore, standard operating procedures for isolation and quantification of bacterial membrane vesicles are currently lacking. Methods for OMV analyses range from protein measurement (e.g., Bradford assay, SDS, BCA assay), dry weight measurement, immune-based assays (e.g., ELISA, immunoblot, dotblot), lipid measurements (e.g., FM4-46, phospholipid measurement with ammonium ferrothiocyanate), LPS quantification (e.g., purpald assay, mass spectrometry analyses), or microscopical analyses (e.g., fluorescence microscopy, electron microscopy, nanosight). Thus, comparison of the results from different OMV biogenesis studies may require careful evaluation of the diverse methodologies used to isolate and quantify OMVs. We kindly refer the interested readers to the original articles cited throughout the chapter.

## 2.2 Modulation of Outer Membrane–Peptidoglycan Linkages

The cell envelope of Gram-negative bacteria is composed of two bilayered membranes. The so-called inner and the outer membranes are separated by the periplasm, which contains a thin layer of peptidoglycan. While the inner membrane has phospholipids in both leaflets, the outer membrane is composed of phospholipids in the inner leaflet and predominantly LPS in the outer leaflet. This asymmetric structure functions as a selective barrier against the extracellular environment (Ruiz

et al. 2006). The peptidoglycan is covalently linked to the outer membrane and determines the shape of the cell's envelope. Moreover, it prevents Gram-negative cells from lysis due to osmotic changes or mechanical stress (Vollmer and Bertsche 2008). The peptidoglycan is a highly dynamic polymer, which consists of glycan chains that are crosslinked by short peptides (Vollmer and Bertsche 2008). Outer membrane proteins can interact with the peptidoglycan layer, thereby stabilizing the cell envelope by crosslinking the inner and outer membranes with the peptidoglycan. Among these, the main players regarding envelope cross linkage are the Braun's lipoprotein (Lpp), outer membrane porin A (OmpA) and the Tol-Pal complex. Several reports revealed that modulation of these covalent cross-linkages reduces outer membrane integrity and induces vesiculation, which will be discussed within this chapter.

### 2.2.1 Braun's Lipoprotein (Lpp)

Lpp represents the Braun's lipoprotein in the outer membrane of *Escherichia coli*, with homologs found in a variety of Gram-negative species including all *Enterobacteriaceae* (Chang et al. 2012; Deatherage et al. 2009). It is the most abundant lipoprotein in *E. coli* and was shown to exist in a free or bound form, whereby the latter is covalently linked to the peptidoglycan (Braun 1975). About one third of bacterial Lpp is connected to peptidoglycan via the C-terminal lysine residue, thereby the outer membrane is anchored to the peptidoglycan via Lpp (Braun and Rehn 1969; Braun 1975). While Lpp is quite abundant in the outer membrane of *E. coli*, its OMVs contain relatively low levels of Lpp (Wensink and Witholt 1981; Hoekstra et al. 1976). In fact, OMVs contained almost none of the peptidoglycan-bound Lpp and only 35% of the free form compared to the corresponding bacterial outer membrane (Wensink and Witholt 1981). These results led to early models of OMV formation, wherein membrane blebbing occurs when the outer membrane expands faster than the underlying peptidoglycan or in surface areas with reduced prevalence of covalent linkages between Lpp in the outer membrane and peptidoglycan (Wensink and Witholt 1981; Hoekstra et al. 1976). Consequently, deletion of *lpp* leads to increased OMV formation in *E. coli* and in *Salmonella typhimurium* (Deatherage et al. 2009; Suzuki et al. 1978). Notably, loss of Lpp is associated with an impairment of the structural integrity of the outer membrane and cellular leakage (Suzuki et al. 1978; Deatherage et al. 2009). Thus, elevated OMV production might simply result from elevated cellular disintegration of *lpp* mutants. However, Lpp-dependent OMV formation might be more precisely regulated in wild-type cells. This regulation could be based on the reduction of Lpp crosslinks via the modulation of peptidoglycan or a change in the overall amount of Lpp via the small RNA Reg26 (Schwechheimer et al. 2013). Noteworthy, an uneven distribution of (bound) Lpp resulting in outer membrane areas with reduced Lpp amounts, as suggested for cell division sites (Hoekstra et al. 1976), might be more relevant for OMV formation than the overall Lpp cross-linkages as almost no Lpp is found in *E. coli* OMVs (Schwechheimer et al. 2014; Wensink and Witholt 1981).

### 2.2.2 Outer Membrane Protein A (OmpA)

OmpA is a major porin in the outer membrane of many Gram-negative species (Palva 1983; Wang 2002). Through its ability to non-covalently bind peptidoglycan via the conserved residues D271 and R286 of the C-terminus, it contributes to the structural integrity and stability of the cell envelope as it connects the peptidoglycan with the outer membrane (Samsudin et al. 2016; Iwami et al. 2007). Indeed, loss of OmpA results in an increase of OMV formation in *S. typhimurium*, *Porphyromonas gingivalis*, and *Acinetobacter baumannii* (Iwami et al. 2007; Moon et al. 2012; Deatherage et al. 2009). Both, Lpp and OmpA interactions, support membrane integrity. Consequently, OMV release by the *lppompA* double mutant is significantly increased compared to each single mutant in *S. typhimurium* (Deatherage et al. 2009). In a similar manner, Sonntag and coworkers could demonstrate that an *lppompA* double mutant in *E. coli* produced more OMVs than a *lpp* single mutant (Sonntag et al. 1978). Notably, Lpp-peptidoglycan linkages seem to be a dominant factor for increased OMV production in most bacteria probably due to the higher abundance of Lpp in the outer membrane (Deatherage et al. 2009). Concordantly, the impact of an *ompA* deletion in *S. typhimurium* is less pronounced as for *lpp* mutants and the loss of OmpA-peptidoglycan linkages was not necessarily associated with reduced membrane integrity (Deatherage et al. 2009). Noteworthy, bacteria can regulate *ompA* expression. For example, the small RNA VrrA of *Vibrio cholerae* interferes with *ompA* translation and its overexpression leads to increased vesiculation (Song et al. 2008). Similar results were obtained in *E. coli* and *S. typhimurium* by MicA, a homologue of VrrA, which downregulates *ompA* expression, thereby inducing OMV production in both organisms (Choi et al. 2017). As highlighted above, a similar regulation has also been observed for Lpp via the small RNA Reg26 (Schwechheimer et al. 2013). Interestingly, these small RNAs (VrrA, MicA, and Reg26) are under positive control of  $\sigma^E$ , an alternative sigma factor induced upon membrane stress, linking OMV formation to the envelope stress response (See Sect. 2.3.1) (Schwechheimer et al. 2013; Udekwi and Wagner 2007; Song et al. 2008).

### 2.2.3 Tol-Pal Complex

The Tol-Pal complex is widely conserved among Gram-negative bacteria and is crucial for multiple physiological roles including the maintenance of membrane integrity. In *E. coli* the apparatus consists of five proteins (TolQ-R-A-B and Pal) (Lazzaroni et al. 1999). While TolQ, TolR, and TolA form a complex in the cytoplasm, the periplasmic protein TolB interacts with Pal, which is non-covalently linked to the peptidoglycan (Lazzaroni et al. 1999; Parsons et al. 2006). Moreover, TolB and Pal interact with Lpp and OmpA at a protein level (Clavel et al. 1998; Cascales and Lloubes 2004), indicating that the Tol-Pal complex belongs to a protein network, which connects the periplasmic peptidoglycan layer with the outer membrane.

Accordingly, *tol-pal* mutants exhibit elevated OMV formation associated with loss of membrane integrity and leakage of periplasmic proteins into the extracellular milieu in *E. coli* (Bernadac et al. 1998). Concordantly, *tolQ*-, *tolR*-, and *tolA*-mutants in *Pseudomonas putida* produce more OMVs, loss of *tolA* resulted in more vesicle protein amount in *Shigella boydii*, deletion of *tolB* in *Helicobacter pylori* resulted in increased vesiculation and in *Caulobacter crescentus* extensive blebbing of OMVs were observed upon mutation of *pal*, *tolA*, or *tolB* (Turner et al. 2015; Llamas et al. 2000; Yeh et al. 2010; Mitra et al. 2016). In *tol-pal* mutants of *E. coli* and *C. crescentus*, a substantial fraction of OMVs originate from the cell poles and division sites where Tol-Pal usually accumulates in wild-type cells. Notably, *tol-pal* mutants fail to connect the outer membrane and peptidoglycan during cell division, which could explain the increased OMV release (Gerding et al. 2007; Yeh et al. 2010).

## 2.3 Bacterial Stress Responses Affecting Vesiculation

Facing the outside world, the cell envelope of bacterial cells is exposed to varying environmental conditions, which can be detrimental for microbial viability. Variations in temperature, pH, or the exposure to antimicrobial or toxic compounds can cause impaired protein folding and may result in cell death. Thus, the ability to rapidly sense and respond to these adverse conditions is essential for the survival of bacterial cells. Therefore, bacteria have developed regulatory networks to control gene expression according to their specific requirements. Some of them have been linked to increased vesiculation, which can enhance fitness under stressful conditions.

### 2.3.1 Envelope Stress Response

In *E. coli*, adaptation to harsh environments is mediated by the activation of the signal transduction pathways of the bacterial envelope stress responses (Raivio 2005). One of them is the  $\sigma^E$  stress response activated via DegS, a periplasmic sensor protease, which recognizes misfolded outer membrane proteins. Upon its activation, DegS cuts the anti-sigma factor RseA, which is again cleaved by RseP and ClpXP and finally releases the alternative sigma factor  $\sigma^E$ . Liberated  $\sigma^E$  subsequently induces the transcription of genes involved in outer membrane protein and cell wall biogenesis, including DegP, a temperature-dependent periplasmic chaperone and protease (Hayden and Ades 2008). Recently, studies in *E. coli* and *S. typhimurium* linked the  $\sigma^E$ -mediated stress response to OMV biogenesis demonstrating increased vesiculation for mutants of the  $\sigma^E$  pathway such as *degP*-, *degS*-, and *rseA*-mutants for *E. coli* and the *degP* mutant in case of *S. typhimurium* (McBroom and Kuehn 2007; McBroom et al. 2006). The authors concluded that the impairment of the functionality of the  $\sigma^E$  pathway leads to an accumulation of

misfolded products in the periplasm causing increased turgor pressure, which can be resolved by vesiculation.

The importance of OMV formation as a stress relief mechanism in *E. coli* was confirmed by mutation of *nlpA* (resulting in reduced vesiculation) in combination with *degP* (resulting in accumulation of periplasmic misfolded proteins) (Schwechheimer and Kuehn 2013). The reduced vesiculation of the *nlpA/degP*-mutant compared to a *degP* mutant caused a severe growth defect upon higher temperatures as misfolded proteins accumulated in the periplasm and could not be removed via vesiculation (Schwechheimer and Kuehn 2013). By the construction of a fusion protein, which mimicked an unfolded outer membrane protein, McBroom and coworkers could reinforce the model that vesiculation might be an alternative stress relief mechanism as this protein was significantly enriched in OMVs compared to other periplasmic proteins (McBroom and Kuehn 2007). Thus, they introduced OMV formation as a stress response of Gram-negative bacteria, which supports the cell's efforts to reduce the consequences of unfolded proteins.

Furthermore, the model of OMV formation as a stress relief mechanism could be extended from misfolded proteins to other compounds accumulating in the periplasm. For example, vesiculation was also increased in *E. coli* upon deletion of *ampG*, which is an inner membrane permease and *amiD*, an amidase breaking down large peptidoglycan fragments as well as upon deletion of the autolysin homologue *ami* in *P. gingivalis*. These mutations resulted in the accumulation of peptidoglycan fragments in the periplasm and a subsequent increase of turgor pressure (Schwechheimer et al. 2014; Hayashi et al. 2002). In a similar manner, OMV formation was increased in *rfaC* or *rfaG* deletion strains in *E. coli*. Both mutations led to an accumulation of LPS in the periplasm, due to an impaired LPS maturation in the outer membrane (Schwechheimer et al. 2014). Consequently, OMVs of *rfaC* or *rfaG* deletion mutants in *E. coli* exhibited an increase of lipid-to-outer membrane protein ratio (Schwechheimer et al. 2014). The detrimental effect of excessive membrane products was demonstrated by deletion of *yciM* in *E. coli*, which increases LPS production and its periplasmic accumulation. Interestingly, *E. coli* strains lacking *yciM* required specific suppressor mutations for survival, either involved in LPS synthesis (*lpxA*, *lpxC*, or *lpxD*) or enhance vesiculation (*lpp*, *tolA*, *pal*, *galU*) (Mahalakshmi et al. 2014; Kulp et al. 2015).

The Kuehn lab extended their OMV biogenesis model by showing that the  $\sigma^E$ -homologue AlgU of *P. aeruginosa* also plays a major role in OMV formation (Macdonald and Kuehn 2013). In concordance with earlier observations in *E. coli*, impairment of the envelope stress response in *P. aeruginosa* also resulted in enhanced OMV formation (Tashiro et al. 2009; Macdonald and Kuehn 2013). Interestingly, hypervesiculating mutants often showed no altered  $\sigma^E$  activity in *E. coli* and deletion of *algU* in *P. aeruginosa* did not alter high vesiculation phenotypes of stressed cells, indicating that various mechanisms exist for OMV formation (Macdonald and Kuehn 2013; McBroom and Kuehn 2007). In the case of *P. aeruginosa*, these additional mechanisms might be modulations of surface structures or composition (see Sects. 2.5.1 and 2.5.2.3).



### 2.3.2 Cell Wall-Directed Agents

Various chemical and biological substances targeting the outer membrane have been shown to stimulate OMV formation. Increased OMV formation upon presence of sublethal concentrations of membrane-attacking substances (e.g., polymyxin B, LL-37 and colistin) was shown to be beneficial for bacterial survival as OMVs can act as sink for these antimicrobial compounds (Manning and Kuehn 2011; Duperthuy et al. 2013). The increased vesiculation induced by polymyxin B and colistin is based on the displacement of  $Mg^{2+}$  and  $Ca^{2+}$  cations in the outer membrane, which destabilize the electrostatic interactions between negatively charged LPS molecules (Moore and Hancock 1986; Storm et al. 1977). Upon treatment with polymyxin B, increased OMV levels were observed for *P. aeruginosa* (Macdonald and Kuehn 2013), *E. coli* and enterotoxigenic *Escherichia coli* (ETEC) (Manning and Kuehn 2011) as well as enterohemorrhagic *Escherichia coli* (EHEC) (Bauwens et al. 2017a; Bauwens et al. 2017b). Exposure to colistin resulted in elevated OMV formation in *E. coli* (Manning and Kuehn 2011). Similarly, the human cathelicidin LL-37, a cationic antimicrobial peptide was shown to induce pore formation in the outer membrane (Brogden 2005) and increases OMV formation in EHEC (Urashima et al. 2017). Presence of bile salts, acting as an emulsifier of biological membranes, also promotes vesicle formation in *Campylobacter jejuni* (Taheri et al. 2018). Moreover, gentamicin was shown to increase OMV formation in *P. aeruginosa* via destabilization of the outer membrane by binding to the LPS (Kadurugamuwa and Beveridge 1997).

Furthermore, highly hydrophobic carbon sources such as hexadecane or phenanthrene were reported to interact with the outer membrane of Gram-negative bacteria and are thought to induce vesiculation. Hexadecane stimulates OMV release in *Acinetobacter calcoaceticus* and phenanthrene induces vesiculation in *Delftia acidovorans* (Shetty and Hickey 2014; Borneleit et al. 1988). Although this is not a comprehensive list of cell wall-directed agents, it still indicates that substances that disturb or disrupt the outer membrane of Gram-negative bacteria are connected to OMV formation.

It is likely that several other cell wall-directed agents destabilize the outer membrane by similar modes of action as described above and thereby increase bacterial vesiculation. Elevated OMV levels caused by cell wall-directed agents could rather be a consequence of physical or chemical membrane damage, than a controlled OMV formation process actively regulated by the bacterium. Indeed, vesicles can reassemble from membrane material originating from lysed cells (Sych et al. 2018; Turnbull et al. 2016). However, cell wall-directed agents might activate the  $\sigma^E$  pathway. As a matter of fact, polymyxin B was shown to induce *micA* in a  $\sigma^E$ -dependent manner in *S. typhimurium* (Papenfort et al. 2006) and  $\sigma^E$  in *V. cholerae* (Mathur et al. 2007). Moreover, bile induces the SOS response in *E. coli* and *S. typhimurium* (Prieto et al. 2006). Thus, OMV formation by sublethal concentrations of cell wall-directed agents could be linked to regulatory pathways implicated in OMV biogenesis (see Sects. 2.3.1 and 2.3.3).



### 2.3.3 SOS Response and Bacteriophages

In *E. coli*, one regulatory network facilitating bacterial survival upon DNA damage is the so-called SOS response, representing an inducible DNA repair system (Simmons et al. 2008). Along the SOS response two regulatory proteins, the LexA repressor and the RecA protein, modulate the expression of more than 50 genes comprising, for example, *sulA*, an inhibitor of cell division; *uvr* genes, which are involved in DNA repair or *sbmC*, a DNA gyrase inhibitor (Simmons et al. 2008). While LexA inhibits the expression of the SOS genes during normal cell growth, RecA binds to single-stranded DNA caused by DNA damaging agents (e.g., antibiotics or UV radiation). Binding of RecA to single-stranded DNA activates RecA and stimulates the autocatalytic cleavage of LexA, thereby relieving the repressor from the SOS genes and leading to the expression of LexA repressed genes (Simmons et al. 2008). Recently, some studies have shown that the SOS response is closely linked to OMV biogenesis inducing OMV formation. Such an upregulation of OMV formation has been observed for EHEC and *Shigella dysenteriae* upon treatment with SOS response stimulating substances like ciprofloxacin or mitomycin C (Bauwens et al. 2017a; Dutta et al. 2004). In *P. aeruginosa*, Maredia and coworkers demonstrated that the OMV amount was higher under ciprofloxacin treatment compared to non-treated wild-type cells (Maredia et al. 2012). Since ciprofloxacin is an efficient SOS response stimulator, a noncleavable LexA strain (no induction of the SOS response) was used to test if the enhanced vesiculation can be solely attributed to the SOS response. Under ciprofloxacin treatment, OMV levels were higher in wild-type cells than in the noncleavable LexA strain. However, an induction of vesiculation upon ciprofloxacin treatment was also detected in the noncleavable LexA strain, indicating that vesiculation is linked to the SOS response, but also to alternative pathways (Maredia et al. 2012). Moreover, the study of Toyofuku and coworkers confirmed the association of the SOS response with enhanced OMV formation in *P. aeruginosa* as an increase of the OMV amount was also observed under denitrifying conditions, which induces the SOS response (Toyofuku et al. 2014). Although a concise mechanism is lacking, Maredia and coworkers speculated that the enhanced OMV formation is a consequence of the delay in cell division and cell surface alterations during the SOS response (Maredia et al. 2012).

DNA damage also activates lysogenic bacteriophages, which infect, parasitize, and lyse their host cell (Weinbauer 2004). These phages integrate their genome into the bacterial chromosome and replicate through the cell cycle of their host. Upon stressors such as UV radiation or antibiotics, resulting in bacterial DNA damage and induction of the SOS response, lysogenic phages become activated causing excision from the chromosome, proliferation of new phages, and lysis of the bacterial cell (Weinbauer 2004; Feiner et al. 2015). Recently, a novel OMV formation mechanism based on a phage effector was proposed for *P. aeruginosa* in biofilms (Turnbull et al. 2016). Turnbull and coworkers demonstrated that the SOS-dependent activation of the cryptic phages endolysin, which degrades the bacterial peptidoglycan, results in

explosive cell lysis and high amounts of liberated OMVs. These OMVs are formed by shattered membrane fragments of the exploding bacteria, which self-assemble to vesicle structures (Turnbull et al. 2016). Notably, activation of the SOS response in *Stenotrophomonas maltophilia* triggers formation of vesicles containing outer and inner membranes (Devos et al. 2017). Such cell lysis-induced outer–inner membrane vesicles were also reported for *P. aeruginosa*, *Pseudoalteromonas marina*, and *Shewanella vesiculosa* (Perez-Cruz et al. 2013; Hagemann et al. 2014; Kadurugamuwa and Beveridge 1995). A similar mechanism based on cell lysis was shown to induce vesicle formation in the Gram-positive bacterium *Bacillus subtilis* mediated by the SOS-dependent activation of an endolysin encoded by a defective prophage (Toyofuku et al. 2017).

In contrast, lytic phages immediately replicate their genome upon infection until the bacterial cell bursts, thereby transferring the phages to new cells. During this process, increased vesicle formation was observed for *Yersinia pseudotuberculosis* upon infection with a pseudotuberculous diagnostic bacteriophage and for *E. coli* upon infection with the T4 phage, which is thought to occur due to increased osmotic stress and subsequent self-assembly of outer membrane fragments (Byvalov et al. 2018; Tarahovsky et al. 1994). Prophage(-like) elements are frequently found in bacterial genomes and lytic phages are constantly attacking bacterial cells (Casjens 2003; Weinbauer 2004). Thus, the induction of vesicle formation by phages might be a ubiquitous mechanism for the production of membrane vesicles ranging from Gram-negative to Gram-positive bacteria. Similar to cell wall-directed agents (see Sect. 2.3.2), phage-induced vesicle formation likely represents less of an active mechanism than a physical or chemical consequence of the self-assembly properties of biological membranes, which re-organize themselves to vesicles and vesicle-like structures (Sych et al. 2018).

## 2.4 Filamentous or Tubular Surface Structures

Recently, new mechanisms of OMV biogenesis have been addressed by several studies dealing with the outer membrane sheathed flagellum or nanotube-like structures of Gram-negative bacteria. Several bacterial species like *Brucella melitensis*, *Vibrio* spp., or *H. pylori* assemble flagella covered with an outer membrane-derived sheath (Fuerst and Perry 1988; Geis et al. 1993; Ferooz and Letesson 2010). Interestingly, for *Vibrio fischeri*, *B. melitensis*, and *H. pylori* vesicle-like structures were found along the sheath, frequently localized at the distal tip of the flagella (Ferooz and Letesson 2010; Millikan and Ruby 2004; Qin et al. 2016). In the case of *V. fischeri*, rotation of the flagellum results in release of more LPS molecules, which were later shown to be shed via OMVs (Brennan et al. 2014; Aschtgen et al. 2016). Interestingly, Aschtgen and coworkers also observed different OMV sizes depending on the presence and functionality of the bacterium's sheathed flagellum. A hyperflagellated mutant produced a higher proportion of smaller OMVs whereas a nonmotile mutant produced fewer OMVs that tend to be slightly larger in electron

microscopy than those generated by wild-type cells (Aschtgen et al. 2016). Hampton and coworkers reported similar observations for *Vibrio vulnificus* comparing the amount and size of OMVs via electron microscopy derived from motile and nonmotile strains (Hampton et al. 2017). Notably, recent studies show that the bacterial growth phase determines OMV size and protein composition in *H. pylori*, a pathogen with growth phase-dependent expression of flagellar genes (Niehus et al. 2002; Zavan et al. 2019). Taken together, these results not only demonstrate a novel mechanism of OMV formation by bacteria with sheathed flagella, but also raise the question if at least two OMV populations exists, being either released from the cellular body or from the flagellar sheath. Due to their different site of origin, these vesicles may differ in their size and composition (Aschtgen et al. 2016).

Recently, studies showed that bacteria are also able to produce other outer membrane structures such as nanotubes, nanopods, or nanowires, which stay connected to their originating cell and enable them to interact directly with distant surfaces. Among them, the presence of nanopods and nanowires has just recently been reported (Shetty et al. 2011). Nanopods are described as outer membrane tubes filled with OMVs and may represent a new bacterial organelle, still their biogenesis and regulation are currently not well defined (Shetty et al. 2011). In *D. acidovorans* nanopods were shown to play an important role in the biodegradation of the highly hydrophobic phenanthrene as cells producing almost no nanopods were massively attenuated upon growth on this carbon source (Shetty and Hickey 2014).

*Shewanella oneidensis*, a metal-reducing bacterium was found to produce extensions of the outer membrane and periplasm called nanowires, which were shown to enable the cells to link the respiratory chain to external (metal) electron acceptors (Pirbadian et al. 2014).

Nanotubes have been described as tube-shaped membranous structures and are considered to be a specialized form of OMV production (McCaig et al. 2013). It has been suggested that these structures connect neighboring cells thereby playing important roles in cell–cell communication or the exchange of molecules in *Myxococcus xanthus*, enteropathogenic *Escherichia coli* (EPEC), *V. vulnificus*, and *Francisella novicida* (Remis et al. 2014; Hampton et al. 2017; McCaig et al. 2013; Pal et al. 2019). Though their nature is not completely understood, in EPEC nanotubes are thought to be evolutionary related to the injectosome and the flagellum (Pal et al. 2019). A first step toward a regulatory mechanism is a study showing that nanotube production is induced upon amino acid starvation in *F. novicida* indicating their importance for nutrient acquisition (Sampath et al. 2018).

Taken together, these findings suggest that nanotubes, nanopods and nanowires represent a specialized type of OMVs that stay connected to the cell, thereby facilitating nutrient degradation and acquisition of highly specialized bacteria, aid in energy production cycles or enable cell to cell communication.

## 2.5 Modulation of Outer Membrane Components or Composition

All domains of life share a bilayered membrane mainly composed of phospholipids in both leaflets or at least in the inner leaflet (i.e., outer membrane of Gram-negative bacteria). For eukaryotic cells, the curvature alteration of membranes plays an important role in defining the cell morphology, organelles, and local subdomains and is therefore crucial for compartmentalizing proteins and enzymatic reactions (McMahon and Boucrot 2015). Besides changes in the phospholipid composition, curvature induction in eukaryotes is predominately mediated via proteins, which may be integral components of the membrane or are able to induce curvature via binding from the inside or outside of the membrane (Zimmerberg and Kozlov 2006; McMahon and Boucrot 2015). Notably, mechanisms for the alteration of membrane curvatures have also been identified in bacteria, which range from the insertion of curvature-inducing small molecules (e.g., *Pseudomonas* quinolone signal PQS), modification of membrane components (e.g., LPS), or alteration of the membrane composition (e.g., phospholipid accumulation) (Mashburn-Warren et al. 2008; Tashiro et al. 2011; Roier et al. 2016; Bonnington and Kuehn 2016; Elhenawy et al. 2016). All of these processes have also been demonstrated to affect OMV formation, which will be discussed in this chapter.

### 2.5.1 *Pseudomonas* Quinolone Signal (PQS)

The quorum sensing signal molecule 2-heptyl-3-hydroxy-4-quinolone (*Pseudomonas* quinolone signal; PQS) is involved in communication and growth phase coordination of *P. aeruginosa*. The inter-bacterial delivery of this hydrophobic molecule in aqueous solution was not understood until Mashburn and coworkers demonstrated that PQS is transferred via OMVs (Mashburn and Whiteley 2005). Aside from its role in quorum sensing signaling, PQS also induces OMV formation. In *P. aeruginosa* it seems to be a dominant biogenesis mechanism as a *pqsA* mutant, unable to produce PQS, exhibits massively reduced OMV levels (Mashburn and Whiteley 2005). Due to its chemical properties, it is able to interact with the acyl chains of lipid A, a component of LPS, bringing them into a more ordered gel-like state. Thereby PQS reduces membrane fluidity, which facilitates bulging of the membrane and OMV release. Importantly, the high fluidity of the *P. aeruginosa* outer membrane likely prevents OMV blebbing without PQS (Schertzer and Whiteley 2012; Mashburn and Whiteley 2005; Mashburn-Warren et al. 2008). Consistent with the observation that PQS is strictly produced under aerobic growth, OMV formation under anaerobic conditions is massively reduced (Schertzer and Whiteley 2013). Interestingly, vesicle formation can also be induced upon the addition of PQS to cultures of other Gram-negative or Gram-positive bacteria including *E. coli*, *Burkholderia cepacia*, and *B. subtilis* and is thought to interact with their membranes (Mashburn-Warren et al. 2008; Tashiro et al. 2010).

This fascinating observation implicates a unique strategy allowing interspecies controlled induction of vesicle formation in bacteria.

## 2.5.2 LPS Modifications

LPS is a major component of the outer membrane of Gram-negative bacteria and constitutes its outer leaflet. This molecule consists of three components and is highly variable between species. The O-antigen is the outermost part of the LPS with the highest diversity and is mainly recognized by the host immune system. The core region is in parts highly conserved and plays an important role in outer membrane stability. The lipid A, or so-called endotoxin, represents the membrane anchor of the LPS. In many species, it is a target molecule for defined, inducible modifications to increase resistance against antimicrobial factors, which need to penetrate through the outer membrane or directly attack the outer membrane integrity (Hankins et al. 2012; Gunn 2001). Due to its central role in the bacterial surface it is not surprising that modifications in all three parts of the LPS molecule have been reported to affect OMV formation in various species, which will be discussed below.

### 2.5.2.1 Lipid A Modifications

The biosynthesis of lipid A is a multistep process wherein many modifications can be introduced such as glycinations, changes in acylation patterns or the attachment of phosphoethanolamine or aminoarabinose (Hankins et al. 2012; Raetz et al. 2007; Brozek and Raetz 1990; Gunn et al. 1998). These lipid A modifications have been shown to promote resistance to antimicrobial peptides, reduce host immune system activation, change in membrane stability, or change in vesicle formation (Raetz et al. 2007; Kawasaki et al. 2004b; Gwozdziński 2018).

A recent study showed that the addition of a phosphoethanolamine group onto the N-acetylglucosamine disaccharide of lipid A via *mcr-1* in *E. coli* increases OMV formation (Gwozdziński 2018). The current model suggests this modification causes a charge repulse inducing vesiculation (Gwozdziński 2018). In *S. typhimurium* PagL can deacetylate lipid A to downregulate the host immune response. PagL is activated via the two-component system PhoPQ upon entering the macrophages (Kawasaki et al. 2004a; Trent et al. 2001). The deacylation in the 3-position of the lipid A also increases OMV formation inside of macrophages, where OMVs are thought to interfere with host cellular pathways. The loss of the acyl chain is thought to generate a positive curvature of the outer membrane by changing the shape of the lipid A and thereby induce vesiculation (Kawasaki et al. 2004a; Elhenawy et al. 2016).

### 2.5.2.2 LPS Core Modifications

Mutations in the core region of the LPS can have a massive impact on the bacterial surface structure, colony morphology, and outer membrane integrity. Several LPS core mutants showed elevated OMV levels in *P. aeruginosa* (Ruhel et al. 2015; Salkinoja-Salonen and Nurmiaho 1978). Similar results were observed in *E. coli* (Nakao et al. 2012; Schwechheimer et al. 2014). These studies often relied on mutants lacking the majority of the LPS core and, as a consequence, the entire O-antigen. As full length LPS has a pivotal impact on outer membrane stability, such core mutants have an impaired outer membrane integrity, which could explain the increased liberation of outer membrane material. Yet, a study by Schwechheimer and coworkers described a mechanism for the increased vesiculation observed in LPS core mutants (Schwechheimer et al. 2014). According to their model, enhanced vesiculation in *E. coli* *rfaC* or *rfaB* mutants lacking full-length LPS results from accumulation of periplasmic LPS and subsequent increase in turgor pressure (Schwechheimer et al. 2014).

### 2.5.2.3 O-Antigen Modifications

*P. aeruginosa* was shown to express two distinct types of LPS varying in the O-antigen composition. One is a homopolymer of D-rhamnose and is named common polysaccharide antigen (CPA, formerly termed A-band), the other is a heteropolymer of three to five distinct sugars in its repeat units known as O-specific antigen (OSA, formerly termed B-band). *P. aeruginosa* mutants lacking OSA or CPA were shown to produce OMVs with altered size and protein composition (Murphy et al. 2014). While OSA is more immunogenic, CPA plays an important role in binding to human cells (Lam et al. 2011). Interestingly, *P. aeruginosa* OMVs were shown to be enriched with the negatively charged OSA (Kadurugamuwa and Beveridge 1995). Enrichment of OMVs with OSA is believed to be the consequence of the charge repulsive forces between adjacent OSA and other LPS molecules in the outer membrane. Interestingly, exposure to oxidative stress increased the abundance of negatively charged OSA and OMV formation (Sabra et al. 2003). Thus, increased vesiculation during stress conditions might be induced by the AlgU pathway (see Sect. 2.3.1) and alterations in O-antigen composition to facilitate survival of *P. aeruginosa*.

*P. gingivalis* also produces two classes of LPS, carrying either neutral (O-LPS) or negatively charged (A-LPS) O-antigen chains (Paramonov et al. 2001; Rangarajan et al. 2008; Paramonov et al. 2005). OMVs of this human oral pathogen are enriched in A-LPS, which may implicate a role for this O-antigen species in OMV release (Haurat et al. 2011). Indeed, for species with neutral and negatively charged O-antigens, the latter has been found to be enriched in OMVs. Thus, it could be suggested that an interaction between negatively charged O-antigen species contributes to OMV formation comparable to the destabilization of the OM of *P. aeruginosa* by negatively charged gentamicin (see Sect. 2.3.2; Kadurugamuwa and Beveridge 1997).

### 2.5.3 Modulation of Phospholipid Composition

The outer membrane of Gram-negative bacteria is composed of an asymmetric bilayer with phospholipids in the inner leaflet and LPS on the outside. This asymmetry is maintained by a putative retrograde phospholipid trafficking system transporting phospholipids from the outer to the inner membrane (Malinverni and Silhavy 2009). This system was first described in *E. coli*, but is highly conserved among Gram-negative bacteria (Roier et al. 2016; Malinverni and Silhavy 2009). In *E. coli*, the system is composed of the outer membrane lipoprotein VacJ (MlaA), the periplasmic binding/transport protein YrbC (MlaC), and a permease complex consisting of YrbB (MlaB), YrbD (MlaD), YrbE (MlaE), and YrbF (MlaF) (Malinverni and Silhavy 2009).

Deletion or downregulation of this retrograde phospholipid transport system has been shown to increase OMV formation in multiple species including *E. coli*, *Haemophilus influenzae*, *V. cholerae*, and *Neisseria gonorrhoeae* (Roier et al. 2016; Baarda et al. 2019). In *H. influenzae*, comprehensive lipid analyses revealed a higher phospholipid abundance in the OMVs derived from *yrbE* and *vacJ* deletion mutants compared to wild type (Roier et al. 2016). This is accompanied by distinct changes of the lipid species composition, such as the enrichment of short-chain fatty acids, in the outer membrane and OMVs of *yrbE* and *vacJ* mutants compared to wild type (Roier et al. 2016). Together with the observation that the fatty acid composition of *P. aeruginosa* OMVs differs from the outer membrane, it can be hypothesized that arrangement of certain phospholipid might promote OMV biogenesis (Tashiro et al. 2011). Generalized models based on accumulation of defined lipid species are hampered by the heterogeneous phospholipid compositions found in the outer membrane of Gram-negative bacteria.

Interestingly, the phospholipid transport system was shown to be transcriptionally silenced upon iron limitation in a Fur (ferric uptake regulator)-dependent manner. As iron limitation is a signal for host environment for many bacteria, it is very likely that vesiculation plays a major role in colonization strategies of pathogenic bacteria (Roier et al. 2016). Along this line, an increased *in vivo* fitness upon deletion of this system has been reported for *P. aeruginosa* and *N. gonorrhoeae* (Shen et al. 2012; Baarda et al. 2019). In contrast, other studies report that mutants in the retrograde lipid transporter of *Shigella flexneri* and *Haemophilus parasuis* are attenuated *in vivo* (Carpenter et al. 2014; Zhao et al. 2017; Suzuki et al. 1994), indicating a current lack of understanding of the complex physiological processes involved. Another example of increased vesiculation due to phospholipid accumulation was observed in *Neisseria meningitidis*, where higher production of phospholipids triggered by sulfate depletion led to increased OMV production. Moreover, sulfate depletion is associated with oxidative stress, which has already been shown to be a signal for increased vesiculation in *P. aeruginosa* (see Sect. 2.5.1; Gerritzen et al. 2019; Macdonald and Kuehn 2013).



## 2.6 Conclusion

Long neglected within the scientific community, the vital role of membrane vesicles is nowadays increasingly studied and has been proven for all branches of life ranging from Gram-negative and Gram-positive bacteria to archaea and even eukaryotic cells (Deatherage and Cookson 2012). Among them, OMVs of Gram-negative bacteria have been most extensively studied. Pioneer work of the Beveridge and Kuehn labs on OMV physiology and biogenesis provided basic knowledge on the existence and multiple physiological roles of these spherical surface derivatives, which ultimately opened this field to become a striving research topic (Horstman and Kuehn 2000; Kadurugamuwa et al. 1993). Noteworthy, a lack of vesiculation has neither been observed for the investigated bacterial species nor any mutants, implicating that outer membrane vesiculation might be an essential process. This chapter provided a concise overview of the most prominent models of OMV formation of Gram-negative bacteria, which have been reported so far (Fig. 2.1). Importantly, all of these mechanisms could act in concert without excluding each other.

While vesiculation due to the loss of outer membrane and peptidoglycan interaction has mostly been observed in mutant strains lacking important structural proteins, recent studies showed that bacteria may actively regulate the abundance of such integral molecules via small RNAs (Song et al. 2008; Choi et al. 2017; Schwachheimer et al. 2013). In a similar manner, bacteria can modulate their surface composition to alter vesiculation. For example, vesiculation models based on modulation of phospholipid abundance or LPS act on structures ubiquitously present in Gram-negative bacteria (Gerritzen et al. 2019; Roier et al. 2016; Kawasaki et al. 2004a; Elhenawy et al. 2016). In contrast, the only curvature-inducing molecule known to date (PQS) is exclusively produced by *P. aeruginosa* although it induces vesicle formation in other strains including Gram-positive bacteria (Mashburn-Warren et al. 2008; Tashiro et al. 2010). Vesiculation due to external stressors like cell wall-directed agents, UV radiation or high temperature is not only a consequence of bursting cells or increased turgor pressure, but can also be induced by modulation of the  $\sigma^E$  pathway or the SOS response (McBroom and Kuehn 2007; McBroom et al. 2006; Maredia et al. 2012). Indeed, the increased release of vesicles upon exposure to hostile conditions can be beneficial for bacterial cells due to their abilities to act as decoys or relief of turgor pressure.

The multitude of OMV biogenesis mechanisms as well as the lack of strains showing no vesiculation emphasizes an important role of these surface facsimiles for bacterial fitness. Interestingly, OMVs play an important role in host–pathogen interactions including toxin delivery, immunomodulation, binding of antimicrobial factors as well as the acquisition of nutrients. Thus, it could be speculated that pathogenic or human-associated bacteria are generally prone to high vesiculation in order to aid in pathogenesis.

Recent work has already revealed quite comprehensive and complementary OMV biogenesis models. It is likely that in the same species, several OMV biogenesis mechanisms are simultaneously active or conditionally induced. Thus, one

species might produce a variety of OMV types of different sizes or composition, reflecting diverse OMV biogenesis routes. The fast progression of techniques to study OMVs ranging from their isolation, composition analyses, and microscopic visualization will drive future investigation of OMV biogenesis, morphology, and content.

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