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Outer-membrane vesicles from Gram-negative bacteria: biogenesis and functions

Carmen Schwechheimer and **Meta J. Kuehn**

Department of Biochemistry, Box 3711, Duke University Medical Center, Durham, North Carolina 27710, USA

Abstract

Outer-membrane vesicles (OMVs) are spherical buds of the outer membrane filled with periplasmic content and are commonly produced by Gram-negative bacteria. The production of OMVs allows bacteria to interact with their environment, and OMVs have been found to mediate diverse functions, including promoting pathogenesis, enabling bacterial survival during stress conditions and regulating microbial interactions within bacterial communities. Additionally, because of this functional versatility, researchers have begun to explore OMVs as a platform for bioengineering applications. In this Review, we discuss recent advances in the study of OMVs, focusing on new insights into the mechanisms of biogenesis and the functions of these vesicles.

In all domains of life — Eukarya, Archaea and Bacteria — cells produce and release membrane-bound material, often termed membrane vesicles, microvesicles, exosomes, tolerasomes, agrosomes and virus-like particles. Outer-membrane vesicles (OMVs), which are derived from the cell envelope of Gram-negative bacteria, have been observed and studied for decades. All types of Gram-negative bacteria have been seen to produce OMVs[1–3] in a variety of environments, including planktonic cultures, fresh and salt water, biofilms, inside eukaryotic cells and within mammalian hosts[4–9]. Over the years, the study of OMVs has generally focused on the function of these vesicles, particularly as it relates to bacterial pathogenesis. Only recently have genetic and biochemical analyses led researchers to begin to elucidate mechanistic aspects of OMV production, as well as to appreciate aspects of OMV production by non-pathogenic bacteria. Notably, OMVs from non-pathogenic bacteria mediate functions similar to those mediated by other extracellular vesicles, such as cellular communication, surface modifications and the elimination of undesired components[10]. Furthermore, it is becoming clear that multiple mechanisms can lead to the production of OMVs in bacteria.

As OMVs are derived from the cell envelope of Gram-negative bacteria, it is important to consider the unique architecture of this bacterial component in order to understand the mechanisms that are involved in OMV budding and detachment (FIG. 1). The Gramnegative envelope consists of two membranes, the outer membrane and the cytoplasmic membrane,

Correspondence to M.J.K. meta.kuehn@duke.edu.

Competing interests statement

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and the periplasmic space in between, which contains a layer of peptidoglycan (PG)[11]. The outer membrane is a fairly unusual outermost cell barrier, being composed of an interior leaflet of phospholipids and an exterior leaflet of lipopolysaccharide (LPS; also known as endotoxin). The cytoplasmic membrane consists of a typical phospholipid bilayer that serves as an electrochemical barrier[11]. The periplasm is an oxidative environment that promotes protein folding but does not contain nucleotide sources of energy, such as ATP or GTP[12]. The net-like PG layer within the periplasm gives bacteria their shape and imparts protection from osmotic changes and sheer stress. Envelope proteins are either soluble (periplasmic proteins), membrane-associated, integral or anchored into the leaflet of either membrane via covalently attached lipid appendages (lipoproteins) (FIG. 1).

For most Gram-negative bacteria, envelope stability comes from different envelope crosslinks: the covalent crosslinking of Braun's lipoprotein (Lpp) in the outer membrane with the PG sacculus[13–15]; the non-covalent interactions between the PG and outer-membrane protein A (OmpA), which is an outer-membrane porin; and the non-covalent interactions between the PG and the Tol–Pal (peptidoglycan-associated lipoprotein) complex, which spans the envelope from the outer membrane across the periplasm to the cytoplasmic membrane[16,17]. As OMVs are spherical portions of the outer membrane, ~20–250 nm in diameter, that contain periplasmic luminal components and that bud and detach from the cell during active growth, and not as a by-product of cell lysis[18], OMV biogenesis presumably must rely on the dissociation of the outer membrane from the underlying PG in areas devoid or depleted of attachments, followed by fission without compromising envelope integrity (see below).

Although initial publications that demonstrated the presence of vesicular material or 'blebs' were actually reporting on lysed bacterial debris, several subsequent analyses revealed that particular OMV contents (such as lipids or proteins) can be enriched or depleted as compared with their prevalence in the bacterial envelope, and that OMV production occurs in the absence of lysis or cell leakiness. The selectivity of OMV cargo revealed that OMV biogenesis is a deliberate process, rather than a stochastic event. Furthermore, vesiculation levels can be altered by factors such as temperature, nutrient availability, oxidation, quorum sensing and envelopetargeting antibiotics[19–24]. In addition, subpopulations of OMVs with distinct compositions may exist within a bacterial culture, although OMV population heterogeneity is a facet of the field that has yet to be explored in much depth. Importantly, although the field has suffered from scepticism, often as a result of unreliable terminology — initially, vesicles that were the result of bacterial lysis were not differentiated from intact OMVs — careful experimentation on OMV production and cargo, and on the mechanisms regulating these processes, now supports the concept that OMV production is a bona fide bacterial secretion process.

Functionally, OMVs have been determined to have diverse roles, depending on the OMV-producing species and the culture conditions. This is unsurprising, as bacterial gene expression, and consequently envelope composition, is highly variable between species and is influenced by the bacterial environment. Owing to the diversity of functions mediated by OMVs, it has become important to focus on the question of why bacteria produce OMVs. In general, it appears that bacteria can utilize OMVs to improve their chances for survival and

to induce changes within their environment[2,19,25–27]. For example, OMVs can deliver virulence factors and modulate the host immune system during pathogenesis; they can aid in nutrient acquisition and ecological niche protection; and they can help provide structural support in multispecies environments such as biofilms[1,28,29].

In this Review, we discuss recent advances in the field regarding OMV biogenesis and cargo selection, as well as the functions of OMVs during nutrient and iron acquisition, interbacterial communication, stress relief and pathogenesis. It has become apparent that these multifaceted particles are challenging to analyse but that the outcome is worthwhile, contributing to our understanding of basic bacterial physiology as well as our ability to engineer vesicles to carry out desirable functions.

OMV biogenesis

In principle, for an OMV to form, the outer membrane must be liberated from the underlying PG and bulge outwards until the budding vesicle membrane undergoes fission and detaches. Therefore, understanding how, when, where and why covalent crosslinks in the bacterial envelope change without causing membrane instability (thus preserving bacterial viability) is essential if we are to understand OMV biogenesis. In addition, biophysical properties of the outer-membrane lipids and their interaction with proteins or other molecules that influence membrane bending are likely to have fundamental roles in OMV biogenesis. Here, we discuss novel contributions to the current models for the mechanism of OMV biogenesis (reviewed in more detail in REF 2). Progress in this area has resulted from the careful evaluation of bacterial mutants that carry modifications or deletions in genes encoding envelope components. Strains with mutations in envelope components often have lytic phenotypes, which make comparisons with wildtype bacteria challenging; additionally, genetic complementation often alters the expression levels of these genes, which can affect the envelope by activating stress pathways that alter OMV levels (see below).

Modulation of envelope crosslinks

The crosslinks bridging the outer membrane to PG have been extensively studied in *Escherichia coli*, although little is known about the dynamics of their formation and destruction or the homogeneity and dynamics of their distribution. OmpA is an outer-membrane porin that contains a periplasmic binding site for diaminopimelic acid (DAP), a component of PG[30]. The Tol–Pal complex is a cell-division component that aids in invagination of the outer membrane and in membrane stability, and also interacts with PG[16,31,32]. Lpp is an extremely abundant outer-membrane lipoprotein, one-third of which is covalently crosslinked to PG[33]. Lpp is evenly distributed throughout the entire cell wall, whereas Pal is preferentially located at the cell poles[31,32].

E. coli, Salmonella spp. and *Acinetobacter baumannii* mutants lacking OmpA display increased OMV production[34–37], most probably as a consequence of decreased crosslinking between PG and the outer membrane[30]. Interestingly, although modulation of this type of envelope crosslink also seemed to lead to hypervesiculation in *Pseudomonas aeruginosa* mutants lacking OprF (an OmpA homologue[38]), in this case hypervesiculation is actually a

consequence of an increase in the levels of *Pseudomonas* quinolone signal (PQS), which stimulates OMV production in *P. aeruginosa*[39] (see below).

To examine the role of the covalent and highly abundant Lpp–PG crosslinks in vesiculation, past studies have characterized the effects of null mutations that cause a complete lack of these crosslinks, but such null mutations can also cause problems with membrane integrity. Indeed, deletions and mutations eliminating either the Lpp membrane anchor or the covalent crosslink between PG and Lpp lead to membrane instability associated with cellular leakage[16,35,37,40,41]. During OMV formation, either a temporary decrease in overall crosslink abundance or a localized displacement of crosslinks is thought to occur[1,18,38,42]. It is worth noting that periplasmic enzymes that liberate Lpp from its covalent bond with PG have not yet been found.

New studies correlating vesiculation levels with more subtle changes in covalent envelope crosslinking have revealed distinct, and hopefully more physiological, envelope conditions that control OMV production. In some cases, the overall number of Lpp–PG crosslinks inversely correlates with OMV production. For instance, the amount of Lpp crosslinked to PG in the hypervesiculating *nlpI* mutant was approximately 40% lower than that in wild-type *E. coli*[43]. NlpI is an outermembrane lipoprotein that participates in cell division[44] and was recently discovered to control the activity of Spr (also known as MepS), a PG endopeptidase that cleaves peptide crosslinks in PG[43–45]. Therefore, it is postulated that an altered balance of PG breakdown and synthesis in the *nlpI* mutant prevents the formation of proper crosslinks between PG and Lpp and indirectly leads to increased OMV production. By contrast, the loss of the minor DAP–DAP peptide crosslinks increased the levels of PG–Lpp crosslinking and resulted in hypovesiculation[46]. Production of OMVs in *Neisseria meningitidis* also seems to be affected by PG architecture, as OMVs from this bacterium were found to contain lower levels of the three lytic transglycosylases MltA, MltB and Slt[47]. Together, these data support a model in which OMVs bud off at sites with locally decreased levels of crosslinks between the outer membrane and PG, and with locally reduced PG hydrolase activity (FIG. 2a,b). Therefore, wild-type cells may modulate OMV production by controlling the number of Lpp–PG crosslinks through the regulation of PG remodelling.

Although Lpp seems to play a crucial part in regulating OMV biogenesis, it was revealed that the overall amount of Lpp–PG crosslinks did not change in all cases of increased OMV production in *E. coli.* The data suggested the existence of a second route of OMV biogenesis, in which vesicle production is independent of the total level of PG-bound Lpp. This mechanism was discovered from investigating mutants that hypervesiculate as a consequence of a general stress response to misfolded proteins or owing to high concentrations of envelope proteins, PG fragments and/or aberrant LPS[46]. These findings led to a model in which these envelope components accumulate in nanoterritories that are relatively free of bound Lpp, although the overall level of Lpp–PG crosslinks throughout the envelope as a whole remains constant. Following accumulation of this cargo in these regions, the outer membrane could bulge outwards and bud off, effectively removing the undesirable envelope components from the cell (FIG. 2c).

Lipids and lipid-binding molecules

The biophysical characteristics of membrane lipids dictate membrane curvature and fluidity and thus probably have a key role in OMV biogenesis. However, defining the role of lipids in OMV production is complicated by the technical challenges involved in generating liposomes that simulate the asymmetrical leaflet composition of the outer membrane for *in vitro* studies. Furthermore, when the lipid content of the outer membrane is altered via genetic modifications, there is an indirect but considerable and often overlooked effect on the composition, organization and biophysical properties of the membrane and membrane-associated proteins.

Temperature-dependent differences in OMV production are likely to reflect the modulation of membrane lipid dynamics, but these effects are species specific. For example, in *E. coli*, as temperature increases, so does the amount of vesiculation[19], presumably owing to increasing membrane fluidity. However, in *P. aeruginosa*, neither a 12°C nor a 14°C increase in temperature affected vesiculation levels[20], whereas in the cold-adapted bacterium *Shewanella livingstonensis*, the soil bacterium *Serratia marcescens* and the pathogen *Bartonella henselae*, lower temperatures resulted in increased OMV production[21–23]. Biophysical analyses and assessments of protein composition will be necessary in order to conclude whether the observed temperature-dependent effects are protein mediated or lipid mediated.

The involvement of particular lipid species in OMV biogenesis has been proposed on the basis of enriched and excluded OMV components. For example, in the Antarctic bacterium *Pseudomonas syringae*, even-numbered carbon chain fatty acids were highly enriched in OMVs (making up more than 80% of the fatty acids present in the OMVs)[48]. Accordingly, it was hypothesized that increased membrane flexibility from areas of enrichment of these lipids may have a role in promoting OMV biogenesis. Furthermore, protein components of the LPS machinery were found very rarely in OMVs, suggesting that mature LPS, rather than nascent LPS, is preferentially shed in OMVs[48] (FIG. 2d). Additionally, the presence of unsaturated and branched-chain fatty acids in the OMVs from *P. syringae* suggests that lipids which increase membrane fluidity are shed via OMV production[49]. By contrast, analyses of the fatty acid composition of OMVs from *P. aeruginosa* revealed that these OMVs were enriched in longer and more saturated fatty acids compared with the outer membrane, suggesting that the more rigid regions of the outer membrane are prone to forming OMVs[50]. These relationships between fatty acid composition, membrane fluidity and propensity to form OMVs seem contradictory; however, it should be noted that the differences in lipid enrichment in OMVs from these bacteria may reflect differences in the habitats of these species, and thus these hypotheses require further testing.

LPS subtypes can be enriched in OMVs and can directly or indirectly influence OMV composition and outer-membrane curvature. For instance, *P. aeruginosa* expresses two different forms of LPS: a neutral form and a charged polysaccharide form that is enriched in OMVs[51]. Alterations in LPS affect both the size and protein profile of *P. aeruginosa* OMVs; cells that express only neutral LPS produce smaller OMVs with a protein composition that is more distinct from wild type than the protein composition of OMVs from cells expressing only charged LPS[52]. *P. aeruginosa* lacking the ability to express either polysaccharide

produces larger OMVs but, unexpectedly, these have a composition more similar to the wild type. *Porphyromonas gingivalis* also contains two forms of LPS, neutral and negatively charged anionic LPS (A-LPS). Gingipains, which are extracellular proteases and virulence factors of *P. gingivalis*, are processed and anchored by anionic polysaccharide[53]; thus, it was not surprising that a mutation that disrupts the synthesis of anionic polysaccharide and therefore eliminates A-LPS from *P. gingivalis* was reported to cause decreased levels of OMV-associated gingipains[54]. However, the lack of A-LPS also caused an increase in OMV incorporation of envelope proteins such as RagA and RagB, which are normally excluded from the OMVs of wild-type *P. gingivalis* by an as-yet-unidentified mechanism[54,55]. These findings suggest that microdomains of charged LPS can influence OMV size, and that certain proteins predominantly localize to these regions and thereby become enriched in OMVs, whereas proteins that associate with uncharged LPS are retained in the outer membrane (FIG. 2d.f).

The impact of changes in the curvature of the outer membrane in OMV biogenesis has been explored in detail with the OMV-promoting and LPS-binding molecule PQS, which is produced by *P. aeruginosa.* Exogenously added PQS promotes the generation of OMVs by *P. aeruginosa* as well as other Gram-negative organisms, and also perturbs the ultrastructure of pure preparations of LPS[56–58]; PQS incorporation into and/or fusion with LPS aggregates was shown to depend on the alkyl side chain and third-position hydroxyl of PQS[58]. $MgCl_2$ represses *E. coli* OMV production in either the absence or the presence of PQS, suggesting that PQS enhances anionic repulsion on the surface of *E. coli* and that this leads to OMV production[57]. These data support a model of bilayer budding by outer-leaflet expansion, in which the concentration of an amphiphilic molecule in one membrane leaflet causes that leaflet to expand relative to the other, resulting in curvature of the whole membrane[39] (FIG. 2e). Notably, however, PQS can act on membranes independently of its ability to bind LPS, as PQS is also incorporated into phospholipid liposomes made from zwitterionic phosphatidylethanolamine and negatively charged phosphatidylglycerol[58], and it induces both membrane vesicle formation in Gram-positive organisms[57] and membrane curvature of erythrocyte membranes[39] (neither of which contain LPS). Indeed, magnesium chloride did not repress vesicle production from *Bacillus subtilis*, suggesting that PQS has a different mode of action on Gram-positive membranes[57]. Furthermore, as OMVs are still produced by *P. aeruginosa* grown in conditions that inhibit PQS synthesis and by *P. aeruginosa* mutants that cannot produce PQS[20,24,27], an alternative pathway (or pathways) must allow OMV production not only by non-PQS-producing Gram-negative organisms, but also by wild-type *P. aeruginosa.* OMV populations from the same strain that are generated by different pathways would be expected to have distinct compositions, and future work to identify these differences could provide substantial insight into their distinct biogenesis mechanisms.

OMV protein cargo

Some of the most important, but reasonably rare, investigations in the field focus on how cargo is selected for enrichment in OMVs. These studies can provide critical mechanistic and functional insight into OMV biogenesis. However, our ability to draw meaningful conclusions from these data is complicated by the fact that OMV proteome reports are often inaccurate, typically owing to inexact purification methods (see REF. 1 for a comprehensive

review covering aspects of OMV purification). In most cases, in addition to outer-membrane components, OMV proteomes include inner-membrane and cytoplasmic proteins[52,59–63]; in fact, it is exceptional if no inner-membrane and cytoplasmic components are found[64]. Importantly, even in those cases in which the samples were rigorously prepared, purified and analysed, some inner-membrane and cytoplasmic components were still detected in OMVs[65,66]. It is still unclear how and why these components enter and/or associate with OMVs, although this is of particular interest, both functionally and mechanistically.

Virulence factors

The selective export of virulence factors into OMVs is thought to have evolved as a benefit for pathogenic bacterial species. Intriguingly, both positive and negative selection of virulence factors into OMVs has been reported (FIG. 2f). For example, in *Helicobacter pylori*, the type IV secretion system component VirD4 was completely excluded from OMVs[67], suggesting that this selectivity benefits the parent cell, in which an intact secretion system is needed for virulence. Notably, these OMVs were enriched in the protease HtrA, which is critical for bacterial survival under conditions of misfolded-protein accumulation[42,67]; moreover, in its secreted form, HtrA plays a part in pathogenesis in enteropathogenic *E. coli, Shigella flexneri* and *Campylobacter jejuni* by mediating E-cadherin cleavage and thereby disrupting the epithelial barrier[68]. The selectivity of envelope protein cargo was also investigated in the human pathogen *N. meningitidis*[47], and outer-membrane proteins that were enriched in OMVs included almost all the *N. meningitidis* autotransporters, regulatory proteins involved in iron and zinc acquisition, and two-partner secretion systems. By contrast, OMVs showed reduced levels of the outer-membrane porins PorA and PorB, the PG-binding protein RmpM, the efflux pump channel MtrE and the pilus pore protein PilQ. The functional consequences of this selectivity are still unknown.

It has been postulated that virulence factors involved in adhesion would be excluded from OMVs, as this could be important for pathogens that specifically benefit from direct contact with the host cells: if OMVs from these bacteria also carry the same adhesins, they would compete with the bacteria themselves for direct bacterium-host interactions. This notion is supported by the observation that in *H. pylori*, blood group antigenbinding adhesin (BabA) and sialic acid-binding adhesin (SabA), two adhesins that contribute to bacterial colonization, were found to be less abundant in OMVs than in the outer membrane[67].

In a few cases, specific bacterial factors have been identified which contribute to the incorporation of virulence factors into OMVs or to the association of soluble virulence determinants with the external leaflet of OMVs. For example, in meningitis-causing *E. coli*, the targeting of cytotoxic necrotizing factor 1 (CNF1) beyond the periplasm and into OMVs somehow depends on YgfZ[69–72]. YgfZ is a folate-binding protein that is predominantly located in the periplasmic space and may associate with the inner-membrane iron sulphur protein ferredoxin. The ability of YgfZ to target CNF1 to OMVs may be indirect, as the two proteins could not be co-precipitated. As discussed above, LPS can also contribute to the selective recruitment of virulence factors to OMVs. For example, numerous *P. gingivalis* virulence factors that are enriched in OMVs, including gingipains, are anchored extracellularly to the outer leaflet of the outer membrane via interactions with A-LPS[54,55,73].

Sorting of soluble proteins into OMVs

OMV cargo selectivity is also observed in non-pathogenic species, and identifying and understanding how these cargoes are enriched or excluded will shed light on the basic physiological mechanics and functions of OMVs. The relative abundance of macromolecules in OMVs compared with the bacterial envelope reflects their abundance at sites of OMV budding, the ability of bacteria to selectively export damaged or toxic molecules and/or the ability of bacteria to conserve resources by excluding from OMVs those macromolecules that are energetically costly or scarce and are needed in the OMV-producing cell.

As is the case for virulence factors, the sorting of soluble bacterial proteins into OMVs may result from interactions with the periplasmic face of particular outer-membrane proteins, outer-membrane-associated proteins or lipids (FIG. 2f). For example, a selective packaging signal for soluble cargo was identified in *E. coli*: when the carboxy-terminal sequence that triggers the envelope σ^E heat shock response was appended onto a soluble protein, this not only stimulated OMV production, but also led to an approximately tenfold enrichment of the chimeric soluble cargo in OMVs[19]. As discussed in more detail below, misfolded outer-membrane proteins and mislocalized LPS in the periplasm activate the σ^E heat shock response to manage the potential toxic biological consequences of these damaged envelope components[74]. An enrichment of misfolded native proteins in OMVs has also been observed; misfolded luminal outer-membrane proteins were enriched in OMVs produced by a strain lacking the envelope stress-inducible periplasmic chaperone-protease DegP[42], although the molecular interactions promoting this enrichment remain unknown.

OMV functions in bacterial physiology

The contribution of OMVs to bacterial pathogenesis obviously remains a topic of great interest (see below); however, the benefit of OMV production for non-pathogenic bacteria has also recently begun to be appreciated. Despite the energetic cost that is required for the secretion of these large macromolecular complexes, under many circumstances OMV production seems to offer advantages for the producing bacteria. Here, we summarize studies of OMVs in bacterial responses to stress conditions, in regulating complex bacterial communities, and in lipid acquisition and exchange (FIG. 3).

OMVs in stress responses

As mentioned above, vesiculation functions as an envelope stress response, and increasing OMV production aids in bacterial survival under stress conditions[19,20,22,24–27,42,75,76] (FIG. 3a, b). For example, a study showing that increased OMV production correlated with mutations in the σ^E heat shock response in *E. coli*[19] resulted in a hypothesis that OMV production increases when misfolded toxic products or highly overexpressed proteins accumulate in the periplasm (FIG. 3 a). The utility of OMVs in promoting envelope homeostasis and preventing toxicity appears to be supplemental to the other transcriptionally controlled stress response pathways. More recently, it was discovered that increased levels of AlgU (also known as σ^H), which is the σ^E (also known as RpoE) homologue in *P. aeruginosa*, correlated with increased OMV production, and that the loss of AlgU resulted in

a hypervesiculation phenotype, which is consistent with a role for OMVs in providing envelope stress relief[20].

The hypothesis that vesiculation serves as an important means to dispose of envelope 'garbage' was first evaluated with regards to proteinaceous waste accumulation using the *E. coli* Δ*degP* strain. Misfolded proteins are not degraded in this strain because it lacks the chaperone–protease DegP, and such substrates can cause lethality at high temperatures, when protein misfolding is more likely to occur[77]. Notably, reduced vesiculation in the context of high levels of periplasmic protein waste impaired bacterial growth, and the lumen of OMVs produced by the Δ*degP* strain contained misfolded outer-membrane proteins, which are DegP substrates[42]. Subsequently, a similar response was observed for other types of envelope garbage: the accumulation of both PG fragments (anhydrous tri- and tetrapeptides)[78] and LPS resulted in hypervesiculation, and in the case of LPS, accumulation was lethal in a hypovesiculating mutant[46,79,80]. Interestingly, unassembled protein and LPS components of the electron-dense surface layer (EDSL) accumulate in the periplasm in EDSL maturation mutants of *P. gingivalis*[54,73]. The EDSL is a 20 nm wide extracellular layer composed of 33 proteins that contain a C-terminal signal and are covalently anchored to the cell surface by anionic LPS. Based on the effects discussed above for the accumulation of other types of envelope components, it is predicted that the observed accumulation of unassembled EDSL components could drive hypervesiculation in these mutants.

Finally, vesiculation has been shown to play a part in the response to oxidative stress. For example, OMV production in *P. aeruginosa* increases on treatment with ciprofloxacin, an antibiotic that leads to DNA damage and results in the activation of the SOS response[26]. Notably, vesicle production was impaired in an antibiotic-treated SOS response mutant, suggesting a link between the SOS response genes and the vesiculation machinery (FIG. 3b). In another study, OMV production was found to significantly increase after hydrogen peroxide treatment[20]. This oxidationinduced increase in vesiculation by *P. aeruginosa* was found to be dependent on the ability of the bacterium to synthesize B-band LPS; this type of LPS carries the longer and highly charged form of O antigen and had previously been shown to be enriched in constitutively produced *P. aeruginosa* OMVs[51]. Furthermore, these data provided some insight into the reason for the previously observed hypersensitivity of O antigen mutants to oxidative stress: as these mutants are unable to synthesize B-band LPS, their ability to produce OMVs is impaired-resulting in increased sensitivity to oxidative stress[81].

OMVs and bacterial communities

Evidence supporting versatile roles for OMVs in promoting nutrient acquisition in bacterial communities include the preferential packing of glycosidases and proteases into OMVs (see above)[64], the use of OMVs to prey on competitors within mixed bacterial communities[82] and the ability of OMVs to serve as nutrient sources[6] (FIG. 3c). For example, the ability of *Myxococcus xanthus* OMVs to lyse *E. coli* and the almost exclusive packaging of alkaline phosphatase into these OMVs suggest that *M. xanthus* OMVs are important to liberate phosphate — a vital nutrient during the development of a multicellular community —

following *E. coli* lysis[82]. It is anticipated that the hydrolytic and proteolytic enzymes that were detected within *M. xanthus* OMVs, as well as the secondary metabolites with antibiotic activities (cittilin A, myxovirescin A, myxochelins and myxalamids), contribute to the killing of *M. xanthus* microbial prey[66]. Similarly, OMVs from the marine cyanobacterium *Prochlorococcus* were shown to support growth of the heterotrophs *Alteromonas* and *Halomonas* as the sole carbon source[6]. These data suggest that OMV-associated DNA and proteins function as a source of nitrogen and phosphorous for bacterial growth, but further work is needed in order to establish a general role for OMVs as a nutrient source in the biosphere. In addition, the secretion of OMVs carrying enolase by *Borrelia burgdorferi* suggests that OMVs can provide bacterial nutrients for pathogens during colonization of a host[83]. The catalytic product of the enolase is phosphoenolpyruvate, a receptor for the host glycoprotein plasminogen, which is proteolytically activated into the protease plasmin; this protease can aid the pathogen by degrading matrix proteins, enabling the spreading of the pathogen and possibly also generating nutrients that can be used for growth by the bacterium.

OMVs also seem to be involved in bacterial iron acquisition (FIG. 3d). Iron is an essential metal for nearly all organisms, and bacteria have evolved three mechanisms to obtain iron from sequestered iron stores within the host environment: siderophores, haem-scavenging proteins and haem acquisition systems. OMVs from a variety of species contain iron acquisition proteins and bacterial cell surface receptors that recognize haem groups. For example, OMVs from *N. meningitidis* are enriched in iron acquisition proteins, such as the iron-transporter components FetA and FetB[47]. Similarly, OMVs from *P. gingivalis* are enriched in HmuY, iron haem transport B (IhtB; also known as FetB) and gingipains[55], which have all been shown to play a part in haem or iron acquisition from haemoglobin[84–85]. Similarly, the surface receptors transferrin-binding protein B (TbpB) and CopB, along with the haem chaperone CcmE, are involved in iron acquisition by OMVs from *Moraxella catarrhalis*[86–88]. These data, along with studies of vesicles from Gram-positive bacteria (BOX 1), suggest a crucial role for vesicles in iron acquisition in a wide variety of bacterial species. This is not necessarily surprising, as vesicles are smaller and more readily diffusible than cells and are thereby able to cover larger areas to bind the vital metal for the bacteria. Although these findings imply that OMVs are able to capture iron and deliver it back to the bacteria that need it, thus far, this has only been shown to occur for *Mycobacterium tuberculosis* vesicles (BOX 1). Notably, the OMVs from *N. meningitidis* are also enriched in the zinc acquisition proteins ZnuA and ZnuD[47], suggesting that the role of OMVs in metal acquisition is not restricted to iron (FIG. 3d).

Considering the dispersive and functional aspects of OMVs, it can be postulated that OMVs act as ‘public goods’ that benefit both the producing bacteria and bystander, non-OMV-producing bacteria. Indeed, the addition of *E. coli* OMVs increased the survival of an *E. coli* population that was challenged with antimicrobial peptides and bacteriophages, as the OMVs acted as ‘decoy’ targets (see below; FIG. 4a)[25]. Similarly, OMVs produced by species of the *Bacteroides* genus (members of which are some of the main components of the human gut microbiota) were identified as vehicles that can distribute, at a cost to the producer, hydrolases and polysaccharide lyases, which serve as public goods that allow

bacteria that do not produce these enzymes (termed cheaters) to metabolize polysaccharides for nutrient acquisition[89] (FIG. 3c).

The potential for OMVs to mediate bacterial transformation has been studied for at least two decades, and the data implicate OMVs in the spread of antibiotic resistance within heterogeneous bacterial communities[90] (FIG. 4b). Recent studies have shown that *A. baumannii* OMVs can mediate the transfer of carbapenem resistance through both vesicle-associated antibiotic resistanceencoding DNA and enzymatic antibiotic resistance activity[91,92]. Typically, OMV-associated DNA is thought to be indiscriminately and externally bound to the vesicle. However, in a study investigating the capacity of *Acinetobacter baylyi* OMVs to enable horizontal gene transfer to *A. baylyi* and *E. coli* cells[93], the location of DNA was carefully tracked using an antibody against double-stranded DNA. This analysis revealed that the DNA translocated from the cytoplasm into the periplasm and subsequently into OMVs, and that successful gene transfer required competence proteins in the recipient cell, suggesting that the OMVs lysed before DNA uptake by the recipient. Further work is required to generate a more molecular, mechanistic basis to support these observations, but these data suggest that *A. baylyi* is able to regulate the DNA content of OMVs via an unusual translocation process.

Bacterium-associated OMVs

In some cases, OMVs remain associated with the producer cell, such as in the case of nanopods and nanowires. For example, *Delftia acidovorans* Cs1–4 benefits from the production of nanopods, which are proteinaceous surface-layer (S-layer) extensions filled with OMVs[94]. An increase in the number of nanopods and, consequently, in OMV production was observed when the bacteria were cultured in the presence of phenanthrene, a polycyclic aromatic hydrocarbon composed of three fused benzene rings. Bacteria with a substantial reduction in their production of nanopods and OMVs were unable to grow effectively in the presence of phenanthrene. The nanopods contain proteins that are encoded by genes close to the phenanthrene biodegradation locus, although the activity of these proteins in phenanthrene biodegradation has not yet been directly assessed. These data suggest that the OMVs enable the biodegradation of phenanthrene and that the OMV-containing nanopods are a mechanism to counteract the problems of limited diffusion in solid (that is, non-fluid) environments. Additionally, nanowires have been implicated in the ability of bacteria to scavenge and transport electrons — which serve as an energy source — from the environment over long distances, as demonstrated for the metal-reducing marine bacterium *Shewanella oneidensis*[95]. Bacterial nanowires, which are composed of outer-membrane lipids, multihaem cytochrome envelope protein complexes and periplasm, are thought to be an extracellular electron transport (EET) pathway linking bacteria to the external solid-phase iron and manganese minerals that can serve as terminal electron acceptors for respiration. The production of nanowires coincided with the formation of OMVs and may be the result of OMV extension and/or fusion. Indeed, microscopy studies suggest that chains of OMVs from *S. oneidensis* can smooth into tubes and form intercellular connections, indicating that these filaments could facilitate cell–cell electron signalling, similar to the connectivity observed for *M. xanthus*[96] and *Chlorochromatium aggregatum*[97].

OMVs in bacteria-host lipid exchange

Some bacteria require cholesterol in the outer membrane for optimal fitness, but lack the biosynthetic pathway for cholesterol synthesis and instead acquire cholesterol from the culture medium or their animal host. Interestingly, cholesterol exchange seems to be bidirectional, as labelled cholesterol and cholesterol glycolipids that had been incorporated into *B. burgdorferi* membranes were transferred to host cells, both via direct contact and via OMVs[98]. Furthermore, clustering of cholesterol into lipid rafts was observed in the outer membrane of *B. burgdorferi.* As cholesterol promotes curvature in asymmetrical bilayers[99], these raft regions could be favoured for OMV biogenesis. Similar observations have been made for *H. pylori*[100], but it remains to be determined whether the involvement of OMVs in this two-way lipid exchange is frequently used by other bacteria.

OMVs in pathogenesis

Compared with soluble secretion methods, OMVs provide a uniquely beneficial secretion option for pathogens, as OMVs can protect virulence determinants from host proteases and concentrate them for host cell delivery (FIG. 4c,d). In addition, OMVs can simultaneously deliver multiple virulence factors, OMV-associated adhesins can provide tissue-tropic delivery of OMV content, and OMVs can confer antibiotic resistance (FIG. 4). It should be appreciated that other basic physiological functions of OMVs (see above) and their association with biofilms (BOX 2) also contribute to bacterial pathogenesis by increasing bacterial adaptation and survival in the hostile host environment. Intriguingly, both harmful and beneficial effects have been linked to OMV production by bacteria present in the host gut microbiota.

Resistance to antimicrobials

OMVs can help bacteria to battle antibiotics in ways beyond the spread of antibiotic resistance genes (see above; FIG. 4b). For example, OMVs can provide immediate protection for the bacteria well before the bacteria can adapt by modifying or mutating antibiotic targets, because the OMVs act as decoys that bind or absorb antibiotics and toxins (FIG. 4a). In *E. coli*, the addition of OMVs or the use of a hypervesiculating mutant increased immediate resistance to the antimicrobial peptides polymixin B and colistin[25]. Similarly, OMVs from *P. syringae* reduced the levels of colistin and melittin (another antimicrobial peptide) in solution by sequestering these compounds[49]. In a mechanism that promotes cross-resistance, pre-incubation of *Vibrio cholerae* with sublethal amounts of polymixin B was found to result in the generation of OMVs with a modified protein composition, and these modified OMVs had an increased capacity to bind another antimicrobial peptide, defensin LL-37 (REF. 101).

Phages are also a type of antimicrobial, and both the addition of OMVs and a hypervesiculation mutation increased the viability of *E. coli* cultured with the lytic T4 phage, as OMVs irreversibly bound and inactivated the phage[25] (FIG. 4a). This *in vitro* observation is likely to reflect interactions that occur in the biosphere, as phage-OMV complexes have been observed in marine samples[6].

Finally, OMV-mediated absorption was also shown to mediate the resistance of *B. henselae* against haem toxicity. *B. henselae* resides in its haematophagous arthropod vector, the cat flea, and *B. henselae* OMVs containing hemin-binding protein C (HbpC) can sequester haem, which increases bacterial resistance to haem toxicity. This physiological role for *B. henselae* OMVs is further supported by data showing that OMV production by the bacterium was higher at 28°C, the temperature in arthropods, than at 37°C, the temperature in mammalian hosts[21].

Beyond reducing the effective antibiotic concentration in culture by adsorption, OMVs can also carry enzymes that mediate antibiotic protection (FIG. 4b). It was demonstrated about a decade ago and recently confirmed for Gram-positive bacteria (BOX 1) that β-lactamase can be packaged into OMVs and that co-incubation of these vesicles with β-lactam-sensitive species improves resistance to these antibiotics[102,103]. Recently, OMVs from amoxicillin-resistant *M. catarrhalis* were found to carry active β-lactamase and to protect amoxicillinsensitive *M. catarrhalis* from antibiotic-induced killing. Furthermore, OMVs from amoxicillin-resistant *M. catarrhalis* also improved the amoxicillin resistance of non-typeable *Haemophilus influenzae* and *Streptococcus pneumoniae*, two bacteria that typically co-infect the respiratory tract[104].

Delivery of virulence factors

It has long been appreciated that OMVs can also work as a toxin shuttle for pathogens (reviewed in REF. 105) (FIG. 4c). Furthermore, recent reports have started to elucidate how OMV-transported factors manipulate the host cell trafficking machinery. For example, *M. catarrhalis* OMVs act as immunomodulatory molecules, delivering the outer-membrane-bound superantigen *Moraxella* immunoglobulin D-binding protein (MID) into B cells. *M. catarrhalis* OMVs are internalized via receptor clustering in lipid rafts, and they activate the B cells via OMV-associated DNA, which induces Toll-like receptor 9 (TLR9) signalling[106]. OMV-mediated delivery of MID increases the survival of *M. catarrhalis*, as B cell activation leads to polyclonal immunoglobulin M (IgM) production, potentially delaying the production of specific antibodies. In *P. aeruginosa*, the OMV-associated toxin CFTR-inhibitory factor (Cif) controls the host deubiquitylating enzyme USP10 to cause increased ubiquitylation of cystic fibrosis transmembrane conductance regulator (CFTR), which is involved in mucus production. Ubiquitylation of CFTR leads to its degradation in lysosomes and results in increased bacterial survival owing to decreased chloride secretion by the host cells and decreased mucociliary clearance of *P. aeruginosa*[107].

In addition, some OMVs have now been identified as genotoxins. For example, *H. pylori* OMVs cause the formation of micronuclei (in which chromosomes are not correctly distributed), as well as alterations in iron metabolism and oxidative stress associated with genomic damage in gastric epithelial cells. These effects are dependent on the OMV-associated cytotoxin VacA[108]. VacA was also found to increase OMV association with host cells, most probably allowing OMVs to gain access to multiple internalization pathways[109]. VacA somehow increases the rate of cholesterol-independent OMV uptake, possibly by interacting with cell surface receptors and increasing the likelihood for secondary adhesin–receptor interactions to occur. Purified VacA cytotoxin reduces glutathione, and as

glutathione peroxidases utilize glutathione in the breakdown of hydrogen peroxide, the VacA associated with OMVs may increase the opportunity for hydrogen peroxidepromoted DNA damage, either alone or in combination with redox-active iron. Additionally, OMVs isolated from three different *E. coli* strains (avirulent DH5α, pathogenic adherent-invasive *E. coli* (AIEC), and enterohaemorrhagic *E. coli* (EHEC)) were discovered to be genotoxic to human enterocyte-like cells, causing double-stranded DNA breaks, increased proliferation and multinucleation of the enterocyte-like cells[110]. However, the particular component (or components) of *E. coli* OMVs that are responsible for this genotoxicity remain unidentified.

Microbiota-produced OMVs

New health-related properties of OMVs are also being uncovered in the context of the human microbiome. Delivery of an OMV-associated antigen in a sulfatase-dependent manner was recently reported to occur in the intestinal tract of a mouse model that is genetically prone to colitis[4]. Notably, orally gavaged *Bacteroides thetaiotaomicron* produced OMVs that were found to traverse the gut mucosal barrier and access the gut epithelial cells and the underlying intestinal macrophages in a sulfatase-dependent manner, initiating intestinal inflammation (FIG. 4d). By contrast, OMV-associated capsular polysaccharide from *Bacteroides fragilis* was reported to modulate the immune system to prevent colitis[111]. Therefore, microbiota-derived OMVs seem to both promote and prevent intestinal inflammation, and these effects appear to depend on host susceptibility factors as well as specific OMV components.

Outlook

The field of bacterial vesicle research has long experienced a wealth of information on the individual aspects of OMVs generated by a large variety of bacteria, but in-depth studies focused on identifying molecules that are crucial for the biogenesis or function of OMVs have been lacking. This is both exciting and frustrating. Excitement is generated by the ubiquitous nature of OMV production and the unique capabilities of each type of OMV, suggesting that there are even more widely diverse functions of these particles than is currently appreciated. However, frustration has mounted because there is not a single complete picture that explains either functional or mechanistic aspects of OMV production. Nevertheless, it is important to step back and consider whether such expectations for this field are just. As neither bacterial composition nor even the composition of the bacterial envelope is conserved among Gram-negative species, and these factors further depend on growth and environmental conditions, it is naive to consider that a single mechanism exists or should exist that can generate OMVs. In a close parallel to the study of OMVs, the field of exosomes and microvesicles is challenged with defining both the origin and composition of these ubiquitous and multifaceted extracellular particles, as these factors depend on the cell type, the status of the cell (for example, whether it is stressed, infected, cancerous, and so on) and the cell environment[112,113]. Nevertheless, understanding how diverse bacteria achieve a common mechanistic outcome involving vesicle budding, cargo selection, vesicle detachment and vesicle stability will benefit both the OMV field and biology in general. Likewise, common as well as distinguishing functional features of OMVs will enlighten our understanding of how bacterial products influence their environment.

Vesicle biogenesis, its mechanisms of regulation and the factors influencing cargo selection used to be a ‘black box’. However, common mechanisms are starting to emerge, and these insights will need to be extended over the coming years. Numerous basic unanswered questions are still left in the field. Which envelope factors lead to outer-membrane fission and OMV release? What signals and pathways regulate OMV biogenesis? How is LPS-independent enrichment and exclusion of soluble cargo achieved? Which processes are conserved across different Gramnegative species? An understanding of these and other concepts will be critical for the future development of OMVs as therapeutically potent delivery tools (BOX 3). Future studies will hopefully begin to address these fundamental questions as well as increase our appreciation of the unique capabilities of these complex and multifaceted entities.

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Glossary

Outer-membrane vesicle (OMVs)
Spherical portions (approximately 20–250 nm in diameter) of the outer membrane of Gram-negative bacteria, containing outer-membrane lipids and proteins, and soluble periplasmic content. OMVs are not the products of cell lysis

Biofilms
Heterogeneous bacterial communities that are adherent to a surface and often resistant to antibiotics and other chemical disruptants. The attachments between biofilm bacteria and their substrates are typically mediated by extracellular proteins, DNA, polymeric fibres and carbohydrates

Lipopolysaccharide (LPS)
A glycolipid found exclusively in the outer leaflet of the outer membrane of Gram-negative bacteria. LPS has a phosphorylated diglucosamine backbone that is typically hexa-acylated and modified with a variable core oligosaccharide and a highly variable O antigen oligosaccharide or polysaccharide chain

Envelope crosslinks
Covalent and non-covalent links between the peptidoglycan layer and the outer membrane of Gram-negative bacteria

OMV cargo, (Outer-membrane vesicle cargo)
Molecules that are within or associated with outer-membrane vesicles

Vesiculation
The process of producing vesicles. This process may be upregulated (hypervesiculation) or downregulated (hypovesiculation)

***Pseudomonas* quinolone signal (PQS)**

2-heptyl-3-hydroxy-4-quinolone, an extracellular, hydrophobic quorum sensing signalling molecule that is produced by aerobically grown *Pseudomonas aeruginosa.* PQS production starts during early stationary phase and is maximal during late stationary phase

Peptide crosslinks, In the context of this Review
common peptide bonds between the third and fourth residues of two peptide tails in peptidoglycan, catalysed by D,D-transpeptidases and generating D-Ala–*meso*-diaminopimelic acid crosslinks

DAP–DAP peptide crosslinks (Diaminopimelic acid–diaminopimelic acid peptide crosslinks)
Fairly uncommon peptide bonds between the third residues of two peptide tails in peptidoglycan, catalysed by L,D-transpeptidases and generating *meso*-DAP–*meso*-DAP linkages

Lytic transglycosylases
Muramidases which cleave the β-(1,4) glycosidic bond between *N*-acetylmuramic acid and *N*-acetylglucosamine and participate in cell wall turnover

Nanoterritories
Portions of the cell envelope with distinct compositions or biophysical characteristics owing to their specific protein and/or lipid compositions

Microdomains
Portions of the cell membrane with distinct compositions or biophysical characteristics owing to their specific protein and/or lipid compositions

Type IV secretion system
A multicomponent protein and DNA translocation complex that traverses the cell envelope and is evolutionarily related to the conjugation system

Autotransporters
Outer-membrane proteins composed of a carboxy-terminal β-barrel translocator domain and an amino-terminal passenger domain that passes through the interior of the barrel to face the external environment

Adhesins
Surface-associated bacterial molecules that act as ligands or receptors for receptors or ligands on the mammalian host cell, respectively. Typically, adhesins are lectins that bind specific carbohydrate moieties of mammalian glycoproteins and glycolipids

σ^E heat shock response
A transcriptional cascade involved in the maintenance, adaptation and protection of the bacterial envelope. The pathway is induced by envelope stress and is mediated by the activation of the σ-factor σ^E on degradation of the anti-σ-factor in the cytoplasmic membrane, RseA

SOS response

The coordinated DNA repair pathways that are induced by bacteria in response to DNA damage

Microbiota
A community of microorganisms that inhabit a particular site

Bacterial transformation
The stable genetic modification of bacteria with foreign DNA

Carbapenem resistance
The efflux or enzymatic destruction of carbapenems, a class of broad-spectrum β-lactam antibiotics that inhibit cell wall synthesis

Surface-layer (S-layer)
An outermost envelope layer that commonly occurs in archaea and is also found in bacteria. This layer is composed of a single type of protein or glycoprotein that self-assembles into a crystalline or lattice monomolecular structure

Haem toxicity
The generation of highly reactive hydroxyl radicals owing to the production of ferrous iron from haem within the reducing environment of cells

Genotoxins
Substances that induce cellular damage through interactions with DNA

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Box 1

Other bacterial membrane vesicles

The production of membrane vesicles is not exclusive to Gram-negative bacteria, as other bacteria are able to produce similar structures. Notably, despite the different membrane compositions of these other bacteria, several studies have highlighted the functional commonality of bacterium-derived vesicles in mediating toxicity, antibiotic resistance, immunomodulation and nutrient acquisition (reviewed in REF. 114). The distinct membrane composition of these other microorganisms implies that the vesicle biogenesis mechanisms are necessarily distinct from those of Gram-negative bacteria, but these other mechanisms have remained relatively unstudied.

Gram-positive bacteria

The production of membrane vesicles in Gram-positive bacteria is a fairly recent discovery, but the number of studies on this subject is increasing[115–117]. Membrane vesicles derived from *Staphylococcus aureus* (which are 20–130 nm in diameter) are produced *in vivo* and induce host cell death, and entry of these membrane vesicles into the host cytosol depends on cholesterol-rich membrane microdomains[118], as had previously also been shown for some Gram-negative outer-membrane vesicles (OMVs)[28,91]. Cytosolic entry of *S. aureus* membrane vesicles is necessary for their cytotoxicity[118].

The cargo of membrane vesicles from Gram-positive bacteria includes virulence factors and other components that, when released, enhance bacterial survival. For example, multiple components of the anthrax toxin of *Bacillus anthracis* are associated with membrane vesicles and are delivered to host cells via these structures, leading to a robust immune response[116]. *S. aureus* membrane vesicles contain active β-lactamase, which confers resistance to certain antibiotics. These vesicles can be viewed as ‘public goods’, as described in the main text, because they can protect penicillin-susceptible Gram-positive and Gram-negative bacteria against β-lactam antibiotics in the environment[103].

Other bacteria

The genus *Mycoplasma* contains bacterial species that lack a cell wall. It has been proposed that the membrane vesicles of pathogenic *Mycoplasma* strains have a role in antibiotic resistance[119,120]. For example, *Mycoplasma* resistance to fluoroquinolone antibiotics is thought to depend on mutations of the antibiotic target genes, such as *parC*[121]. In addition, an *Acholeplasma laidlawii* strain harbouring a mutation in *parC* that conferred ciprofloxacin resistance was shown to produce membrane vesicles that contained DNA with the mutated gene sequence, suggesting that these vesicles could help spread ciprofloxacin resistance[120].

A few studies have been conducted to gain insights into membrane vesicle biogenesis and function in *Mycobacterium tuberculosis.* As expected, these vesicles were also immunogenic, modulating the host immune response via Toll-like receptor 2 (TLR2)[122]. VirR regulates the production of these vesicles containing the TLR2 ligand and thereby controls the stimulation of the host immune system and, consequently, *M. tuberculosis*

growth in the host[123]. The production of membrane vesicles by *M. tuberculosis* is also upregulated under iron-limiting conditions, and these vesicles are enriched in the siderophore mycobactin[124]. These results further support the concept of vesicles being public goods, as iron-loaded membrane vesicles were able to support the growth of a mutant defective in iron chelation and of wild-type *M. tuberculosis* grown under iron-limiting conditions.

Box 2

Outer-membrane vesicles in biofilms

Outer-membrane vesicles (OMVs) have been shown to participate in biofilm formation[125,126], and new discoveries support the notion that OMVs have important roles in regulating biofilm-related bacterial virulence, persistence and drug resistance. OMVs are thought to promote interactions between bacteria in biofilms and thereby contribute to the structural integrity of these structures. For *Myxococcus xanthus*, for instance, in addition to the predatory aspects of its OMVs (see main text), this organism increases OMV production as well as the formation of outer-membrane extensions that seem to interconnect cells during the biofilm stage as compared with growth in aqueous culture[96]. For *Helicobacter pylori*, a unique 22 kDa protein of unknown function was present in OMVs in early biofilms but disappeared during biofilm maturation, suggesting a role for this OMV protein as a novel colonization factor[127,128]. Extracellular genomic DNA was determined to be associated with *Francisella* spp. OMVs, and electrostatic interactions between extracellular vesicle-associated DNA molecules are thought to contribute to biofilm structure[28,92,125], supporting a structural role for OMVs in *Francisella* spp. biofilms[62]. In addition, stress conditions that increase OMV production in *Pseudomonas* putida also increase cell surface hydrophobicity, contributing to biofilm formation[129]. Furthermore, a high concentration of OMVs on the surface of *Porphyromonas gingivalis* contributes to a synergistic polymicrobial biofilm between *P. gingivalis* and *Treponema denticola*, and this effect is mostly dependent on the presence of gingipains, the extracellular *P. gingivalis* proteases that are bound to lipopolysaccharides and enriched in OMVs[54,130]. Gingipains contain a non-catalytic adhesin domain that has been shown to be involved in the interaction of *P. gingivalis* with other microorganisms[131–133], and it is therefore hypothesized that the gingipain adhesins are involved in biofilm formation[130].

Box 3

Outer-membrane vesicles as tools

OMVs as vaccines

Research into the use of outer-membrane vesicles (OMVs) as vaccines has been ongoing for years (reviewed in REF. 112). Recently, studies have advanced the utility of OMVs against the human pathogens *Neisseria meningitidis, Francisella tularensis* subsp. *novicida, Burkholderia pseudomallei* and *Mannheimia haemolytica*[59,134–136]. The native contextual presentation of bacterial surface antigens in complex with an adjuvant (lipopolysaccharide (LPS)) makes OMVs an effective means to generate an adaptive immune response. However, increasing the protective responses generated by OMVs, engineering the inclusion of protective antigens in these vesicles and reducing LPS-mediated toxicity remain challenges in this field.

Beyond the presentation of protective antigens to the host's adaptive immune system, pretreatment with OMVs can further modulate the infectious process. When *Vibrio cholerae* passes through a host, it becomes hyperinfectious, and the administration of *V. cholerae* OMVs was found to protect mice after challenge with hyperinfectious *V. cholerae*[137]. In addition, the *V. cholerae*-challenged neonates of OMV-vaccinated female mice were found to harbour live *V. cholerae* that was in a hypoinfectious state, indicating that OMVs not only protect against disease, but also protect against the spread of cholera.

OMVs as a reaction scaffold

In an elegant study, *Escherichia coli* OMVs were utilized as a scaffolding platform for cascading cellulose hydrolysis reactions[138]. Many anaerobic bacteria carry a cohesin-dockerin complex on their cell surface for cellulose hydrolysis. The *E. coli* OMVs were engineered to express three cohesin domains from different organisms, along with a membrane anchor and a cellulose-binding module. The interaction of these cohesin domains with their specific dockerin molecules allowed for the assembly of three cellulases on the vesicle surface and increased sugar hydrolysis by 29-fold in comparison with soluble enzymes. Cellulose hydrolysis was chosen as a proof of principle, but OMVs could in theory be used as scaffolding platforms for any desired enzymatic reaction cascade to improve reaction rate and product yield, making OMVs natural nanoreactors. Such 'designer' OMVs were reviewed recently[139].

Vesicles as a specialized drug delivery vehicles

A promising novel approach used OMVs with low immunogenicity and carrying an antibody targeting them to cancer cells; these OMVs also carried small interfering RNA, which caused gene silencing and consequently lead to tumour regression in a mouse model[140]. If such applications of engineered OMVs for cancer therapy continue to be successful in animal models, they have the potential to be useful in the development of targeted human therapeutics.

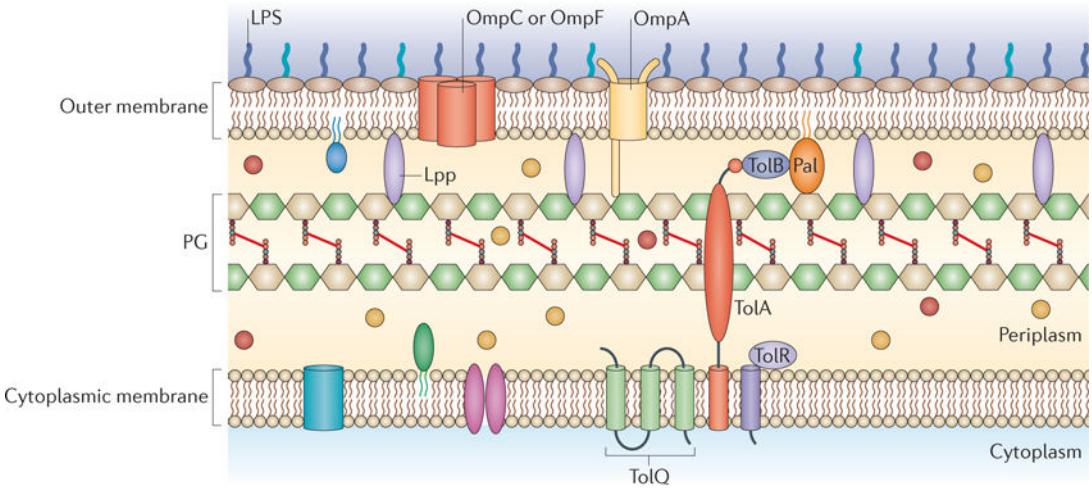

Figure 1. The composition of the Gram-negative cell envelope

The cell envelope of Gram-negative bacteria consists of two membranes, the outer membrane and the cytoplasmic membrane. The cytoplasmic membrane is composed of a phospholipid bilayer, whereas the outer membrane comprises an interior leaflet of phospholipids and an exterior leaflet of lipopolysaccharide (LPS); LPS is composed of lipid A, the core oligosaccharide and O antigen. In between the two membranes is the periplasmic space, which contains the peptidoglycan (PG) layer and periplasmic proteins. The PG layer comprises long polymers of the repeating disaccharide *N*-acetylglucosamine–*N*-acetylmuramic acid (NAG–NAM) that are linked via peptide bridges: both traditional 4–3 (D-Ala–*meso*-diaminopimelic acid (mDAP)) crosslinks and non-traditional 3–3 (mDAP–mDAP) crosslinks. Envelope proteins can be soluble (periplasmic; orange and red spheres), transmembrane proteins (pink ovals and cyan cylinder) or anchored into the leaflet of either membrane via covalently attached lipid appendages (lipoproteins; green and blue ovals). Envelope stability comes from various crosslinks: the covalent crosslinking of Braun's lipoprotein (Lpp) in the outer membrane with the PG; the non-covalent interactions between the PG and the porin outer-membrane protein A (OmpA); and the non-covalent interactions between the PG and the Tol–Pal (peptidoglycan-associated lipoprotein) complex, which is composed of TolA, TolB, TolQ, TolR and Pal, and spans the envelope from the cytoplasmic membrane across the periplasm to the outer membrane.

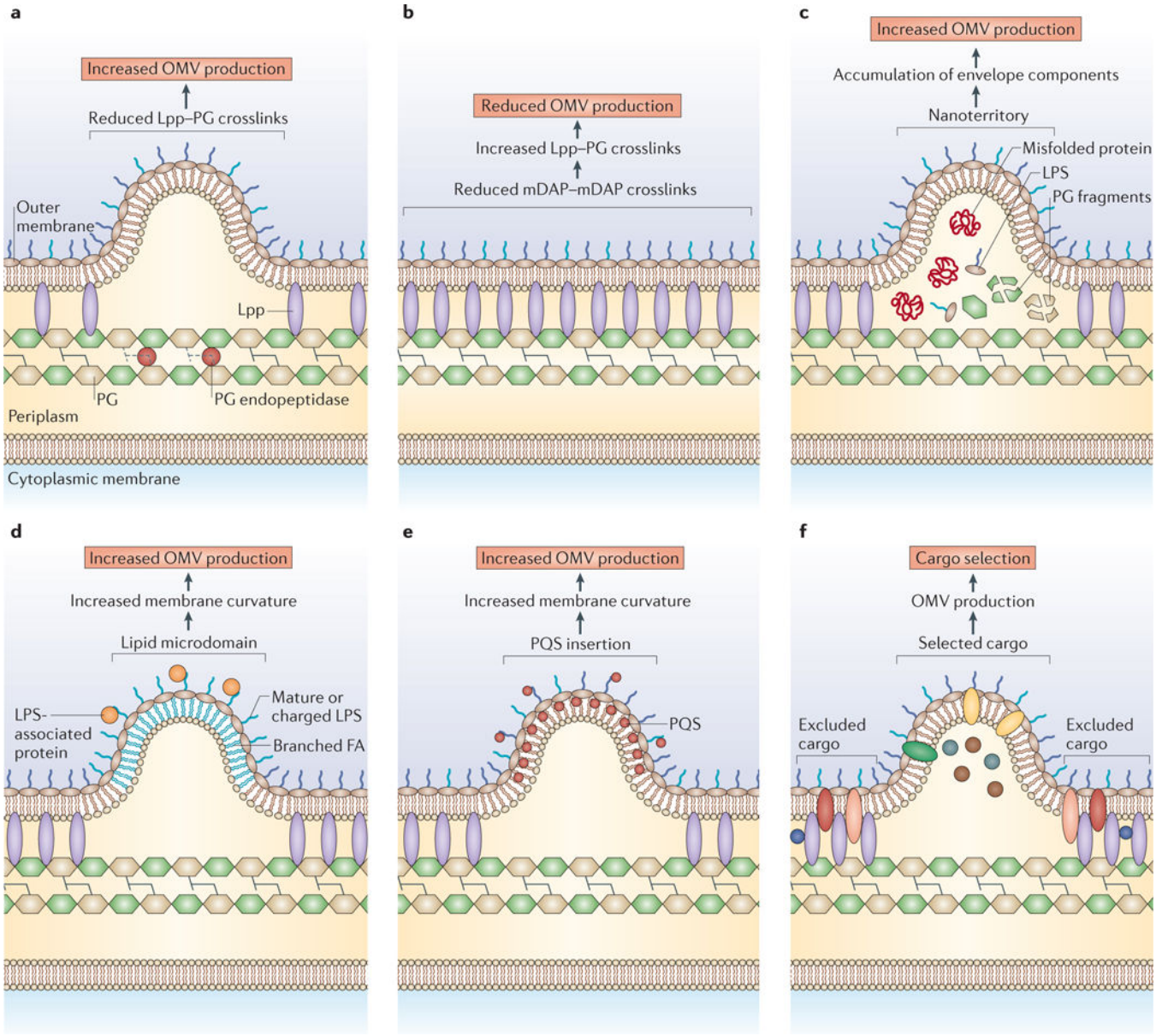

Figure 2. Outer-membrane vesicle biogenesis and cargo selection

Several factors influence the biogenesisof outer-membrane vesicles (OMVs). **a** | Peptidoglycan (PG) endopeptidases and other enzymes that are involved in regulating PG breakdown and synthesis govern the ability of the envelope to form crosslinks between Braun's lipoprotein (Lpp) and PG. Therefore, OMV production is increased in areas with reduced Lpp–PG crosslinks. **b** | *Meso*-diaminopimelic acid (mDAP)–mDAP crosslinks within the PG also control the formation of crosslinks between PG and the outer membrane. Accordingly, sites where the PG lacks mDAP–mDAP crosslinks have more Lpp–PG crosslinks, which reduces OMV production. **c** | In areas where misfolded proteins or envelope components (such as lipopolysaccharide (LPS) or PG fragments) accumulate, crosslinks are either displaced or locally depleted, promoting bulging of these outer-membrane nanoterritories and leading to increased OMV production. **d** | Some areas of the

outer membrane can become enriched in particular types of LPS, phospholipids and/or specific LPS-associated molecules. These lipid microdomains have a propensity to bulge outwards owing to their charge, their cargo or increased membrane fluidity, and this bulging results in increased OMV production. **e** | Insertion of *Pseudomonas* quinolone signal (PQS) into the outer leaflet of the outer membrane can also increase membrane curvature and lead to the formation on OMVs. **f** | Envelope components may be enriched in OMVs because of direct or indirect interactions with integral or auxiliary outer-membrane components that are prone to budding. By contrast, envelope components may be excluded from OMVs by direct or indirect interactions with integral or auxiliary envelope components that are not prone to budding. FA, fatty acid.

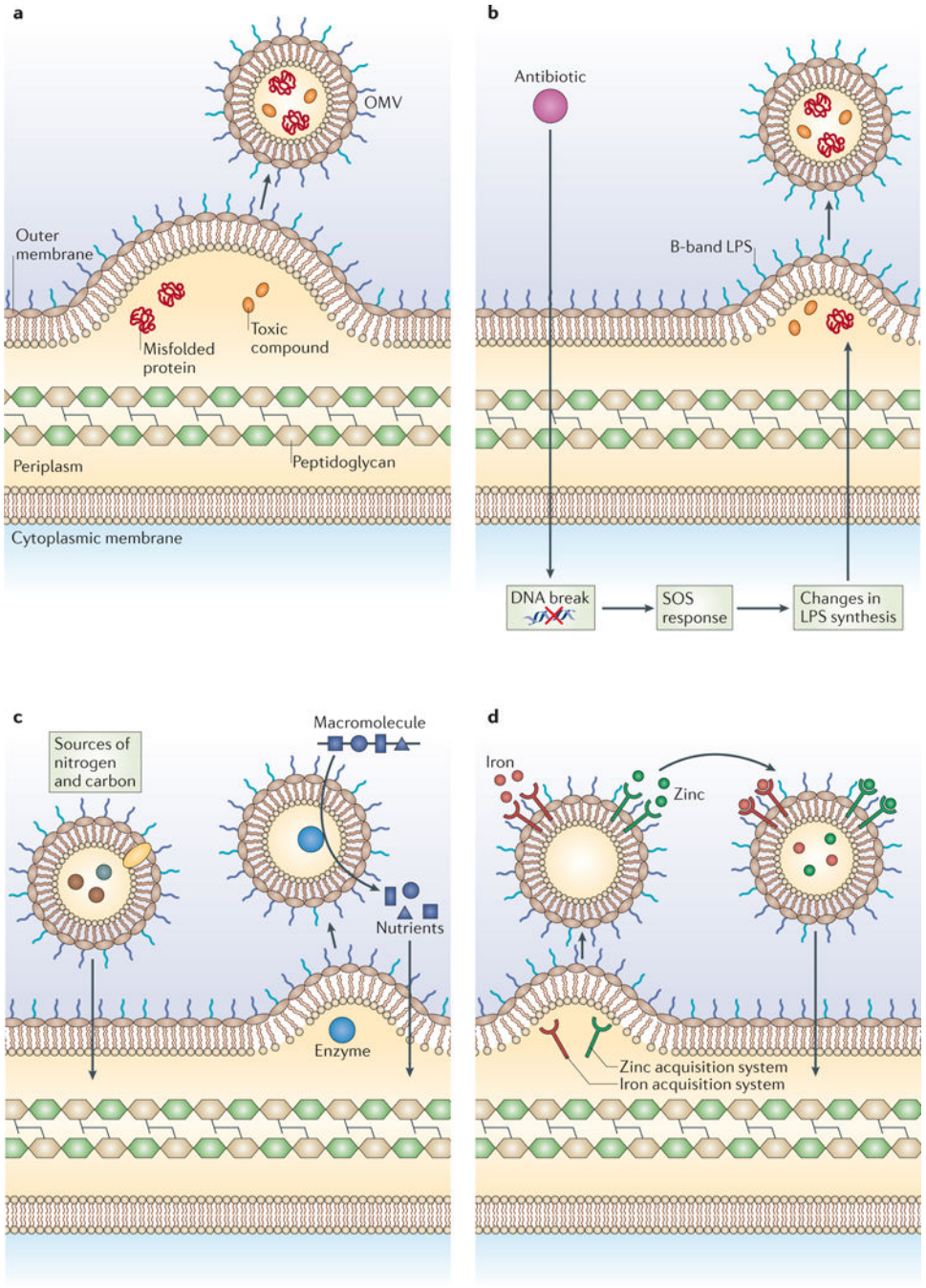

Figure 3. Functions of outer-membrane vesicles in bacterial physiology

Outer-membrane vesicles (OMVs) function in multiple pathways that promote bacterial survival. **a** | OMVs can serve as a mechanism to remove toxic compounds, such as misfolded proteins, from bacterial cells under stress conditions. **b** | Stress conditions can increase OMV production. For example, exposure to antibiotics can induce DNA breaks, which triggers an SOS response. As part of the SOS response, changes in the synthesis of lipopolysaccharide (LPS) can alter the composition of the outer membrane and increase the production of OMVs. **c** | OMVs can serve as sources of carbon and nitrogen, and can carry and

disseminate enzymes that break down complex macromolecules to provide the cell with essential nutrients. **d** | OMVs can also carry iron and zinc acquisition systems that are able to bind these metals in the environment, providing the bacteria with access to these essential compounds.

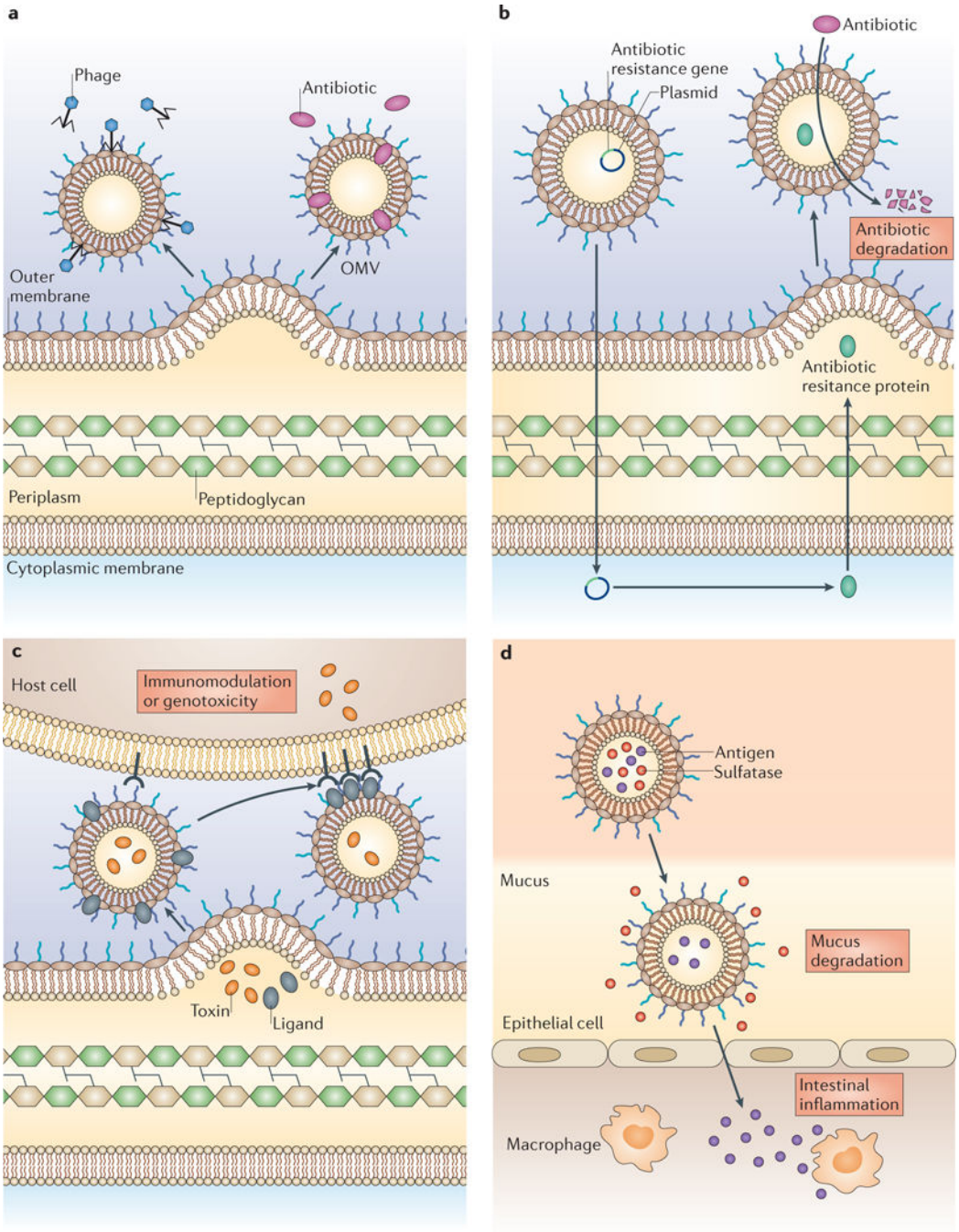

Figure 4. Functions of outer-membrane vesicles in pathogenesis

Outer-membrane vesicles (OMVs) can increase bacterial pathogenicity via multiple mechanisms. **a** | OMVs can increase bacterial resistance to antibiotics and phages by serving as decoy targets for these molecules, thus protecting the bacteria cell. **b** | OMVs can also transfer DNA between cells, including antibiotic-resistance genes, and can carry enzymes that degrade antibiotics. **c** | Pathogenic Gram-negative bacteria are thought to utilize OMVs to interact with host cells during infection. For example, bacteria can use OMVs to mediate the delivery of virulence factors, such as toxins, into host cells, including immune cells. **d** |

OMVs can also cross the mucus barrier in the gut and reach the intestinal epithelium, delivering bacterial antigens to the underlying macrophages, which triggers intestinal inflammation.