

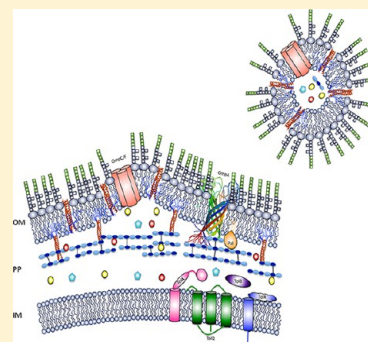
# Envelope Control of Outer Membrane Vesicle Production in Gram-Negative Bacteria

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**ABSTRACT:** All Gram-negative bacteria studied to date have been shown to produce outer membrane vesicles (OMVs), which are budded, released spheres of outer membrane with periplasmic content. OMVs have been implicated in the delivery of virulence factors in pathogenesis. However, OMVs also benefit nonpathogenic species by delivering degradative enzymes to defend an ecological niche against competing bacterial species, and they can serve as an envelope stress response. Despite these important roles, very little is known about the mechanism of production of OMVs. Here we review the advantage of vesiculation, particularly in a nonpathogenic context, as well as the hurdles that have to be overcome in Gram-negative envelope architecture before a vesicle can form and bud. Lastly, we address the question of whether OMV production is a stochastic or regulated process.



Outer membrane vesicles (OMVs) are secreted, ~20–250 nm diameter spherical structures of the Gram-negative bacterial outer membrane (OM) with periplasmic soluble components trapped in their lumen (Figures 1 and 2).<sup>1–8</sup> Vesiculation appears to be a ubiquitous physiological process. In vitro planktonic<sup>8</sup> and agar-grown laboratory cultures, bacteria living in fresh water environments,<sup>7</sup> biofilms,<sup>9,10</sup> and Gram-negative pathogens in animal hosts have been shown to produce OMVs.<sup>11,12</sup> Biochemical assays and atomic force microscopy imaging of live *Escherichia coli* show that OMV production occurs without a significant disruption in the integrity of the cell<sup>13</sup> (Figure 1B).

Why might bacteria expend so much energy (in the form of synthesized macromolecules) by secreting entire portions of their envelope? Diverse functions have been ascribed to OMVs, many of which are means by which Gram-negative bacteria interact with their environment. One important function attributed to OMVs is that they serve as virulence mediators for pathogens by carrying virulence factors, such as toxins and proteases, as well as other proinflammatory molecules and antigens, such as flagellin and peptidoglycan (PG).<sup>14–17</sup> However, OMV production occurs at a constitutive level for a wide variety of bacteria, suggesting this is a highly conserved process that is important for not only pathogens.<sup>7</sup> It has been shown that OMVs can facilitate the transfer of antibiotic resistance enzymes and exogenous DNA between strains and even between species,<sup>18,19</sup> an advantage for both pathogenic and nonpathogenic species. Furthermore, OMVs can act in a general predatory fashion that aids the parent bacterium by generating room in an ecological niche and in acquiring nutrients. By the secretion, adherence, and, in some cases, subsequent fusion of OMVs containing degradative factors, cell lysis of both Gram-negative and Gram-positive species has been observed.<sup>20,21</sup> Finally, one of the emerging functional roles of

OMVs is that they provide a general advantage in bacterial survival. An increased level of OMV production correlated with an increased rate of survival upon both antimicrobial peptide treatment and T4 bacteriophage infection.<sup>22</sup> Under these conditions, OMVs act as decoy cells absorbing the harmful agents. Survival is also improved by OMV hyperproduction in situations of OM and periplasmic stress.<sup>23</sup> We will discuss in more detail the OMV production response to envelope stress, because studies of this process have also generated mechanistic and regulatory insight into OMV production.

Numerous studies have been dedicated to determining OMV composition in various species and conditions,<sup>24–27</sup> as well as the mechanism and regulation of their biogenesis.<sup>28</sup> OMV content is likely derived from the proteins and lipids present at the site of budding; therefore, their composition could yield clues about their mechanism of production. Kulp and Kuehn recently reviewed experimental techniques and isolation of OMVs,<sup>8</sup> which are critical for evaluating the compositional analyses, so we will not address those topics here. Instead, we will focus on the architectural requirements for OMV generation and the data supporting several hypothetical models.

## GRAM-NEGATIVE ENVELOPE

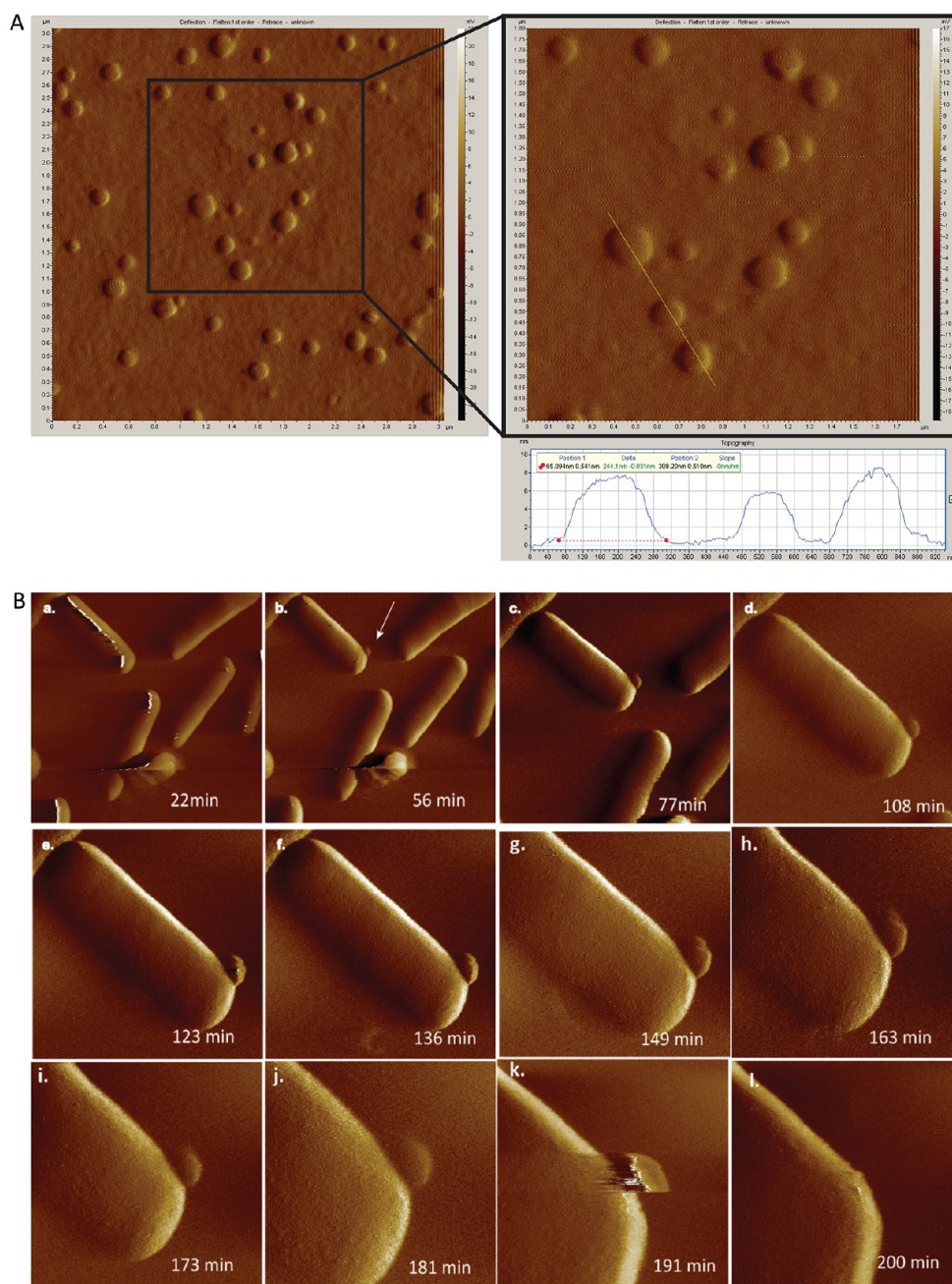
To discuss the mechanics of OMV production, we must introduce the architecture of the Gram-negative bacterial envelope from which the vesicles originate (Figure 2). The envelope consists of an OM, an inner membrane (IM), and a periplasm between the two membranes, which contains a thin layer of PG.<sup>29,30</sup> Lipoproteins are membrane-anchored via a covalently attached lipid moiety and are most frequently

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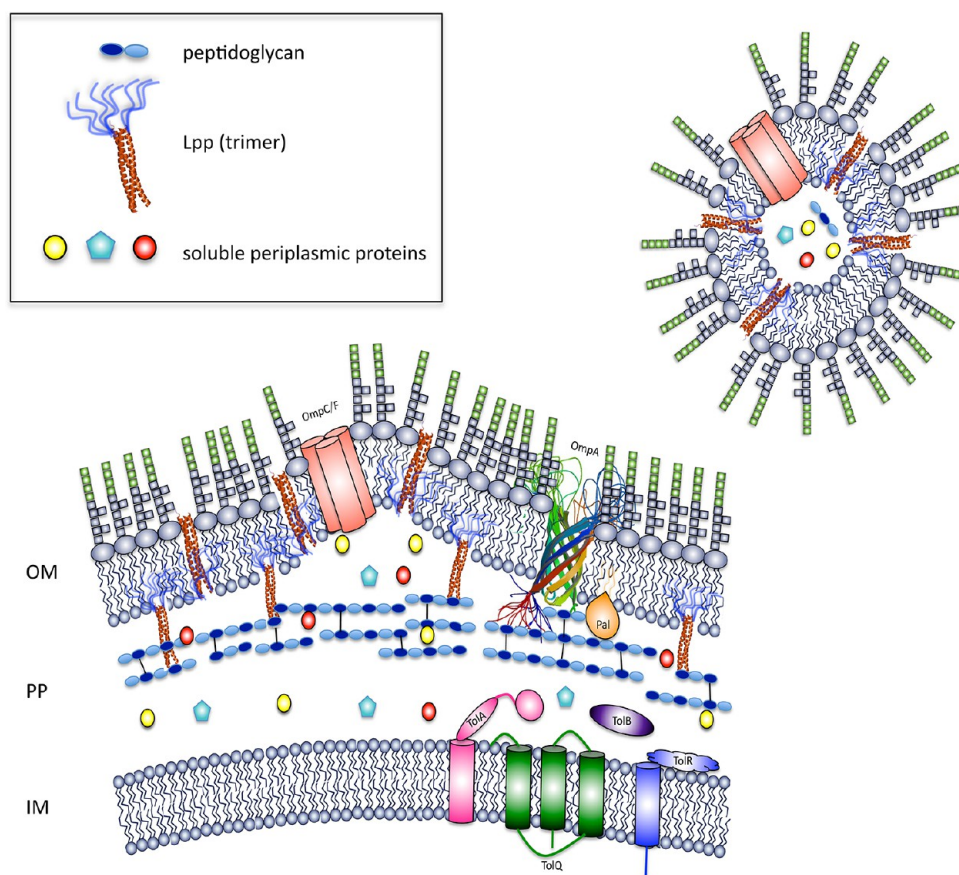
**Figure 1.** Atomic force microscopy images of pure OMVs and OMV budding by *E. coli*. (A) Purified OMVs from an *E. coli* *nlpI* transposon mutant.<sup>13</sup> OMVs were purified from an overnight culture as described previously.<sup>13</sup> A 50  $\mu$ L aliquot of the vesicle suspension was applied to gelatin-coated mica for 20 min before the sample was thoroughly rinsed with deionized water. The sample was dried under a stream of dry nitrogen and imaged in air using contact mode atomic force microscopy. The scanning speed ranged from 6 to 10  $\mu$ m/s. (B) Time course of an *E. coli* *nlpI* transposon mutant<sup>13</sup> producing an OMV. The bacteria were grown to log phase (shaking, Luria Broth, 37 °C). Cells were immobilized on gelatin-coated mica as described previously.<sup>94</sup> Continuous MacMode atomic force microscopy was performed in buffer. The images shown were collected at room temperature at the indicated times using speeds ranging from 1 to 7  $\mu$ m/s.

destined for the OM but are also found in the IM.<sup>30,31</sup> Integral OM proteins (OMPs) almost exclusively fold as  $\beta$ -barrels, whereas integral IM proteins are predominantly  $\alpha$ -helical structures.<sup>30,32</sup> Unlike the IM that has phospholipids in both leaflets, in the OM, lipopolysaccharide (LPS) predominates in the outer leaflet and phospholipids predominate in the inner leaflet.<sup>33</sup> Facing the extracellular milieu, LPS can act as a barrier against the potentially harmful environment.<sup>34</sup> LPS is also termed endotoxin, based on its highly inflammatory effects on the mammalian immune system.<sup>35</sup> OMVs contain integral OM

and OM-anchored lipoproteins, periplasmic protein, and the OM phospholipids and LPS.<sup>8</sup> As discussed in more detail below, enrichment and exclusion of several envelope components in OMVs in comparison to their abundance in the cell<sup>14–16,36–38</sup> support the concept that at least in some cases, OMV production is a regulated mechanism.

Located just underneath the OM, the PG consists of a three-dimensional meshlike network of glycan strands cross-linked by short peptides. The PG gives the bacteria their characteristic shape and also serves a protective function, preventing cell lysis





**Figure 2.** OMV production model. Overview of Gram-negative envelope architecture in the context of OMV production.

due to changes in osmolarity and mechanical stress.<sup>39</sup> It has been known for some time that PG is associated with OMVs,<sup>3</sup> and this has been further confirmed recently by the functional analysis of OMVs from the pathogens *Helicobacter pylori*, *Pseudomonas aeruginosa*, and *Neisseria gonorrhoeae*.<sup>40</sup> These were found to stimulate PG-specific immune responses in vitro as well as in a mouse model of infection.

The layers of the Gram-negative envelope are “stitched” together and stabilized via protein cross-links reaching from the IM through PG to the OM (Figure 2). Lpp, an abundant lipoprotein in *E. coli*, or its counterpart in other Gram-negative bacteria, covalently cross-links the OM and PG, providing structural envelope integrity.<sup>41,42</sup> Additionally, OmpA and the Tol–Pal system add noncovalent stability to the envelope.<sup>43,44</sup> OmpA is an OM porin containing a PG interaction motif.<sup>45,46</sup> The Tol–Pal system has been identified as part of the cell division machinery necessary for OM invagination and is highly conserved among Gram-negative bacteria.<sup>47,48</sup> The Tol–Pal system is essential in *Caulobacter crescentus*, which does not have an Lpp homologue to form the typical OM–PG covalent cross-links.<sup>49,50</sup> In other organisms, mutations and deletions in *lpp* or *pal* lead to a very fragile envelope accompanied by the leakage of periplasmic proteins, implying that membrane integrity as well as envelope stability is compromised.<sup>43,51</sup>

Like other bacterial compartments, the envelope is dynamic. Membrane biogenesis, cell division, and remodeling to adapt to new environments require that the bacterial envelope components rearrange while the cellular integrity is maintained.<sup>30,52,53</sup> Here we consolidate the data on the dynamics of

the envelope structure in light of the mechanism of OMV production.

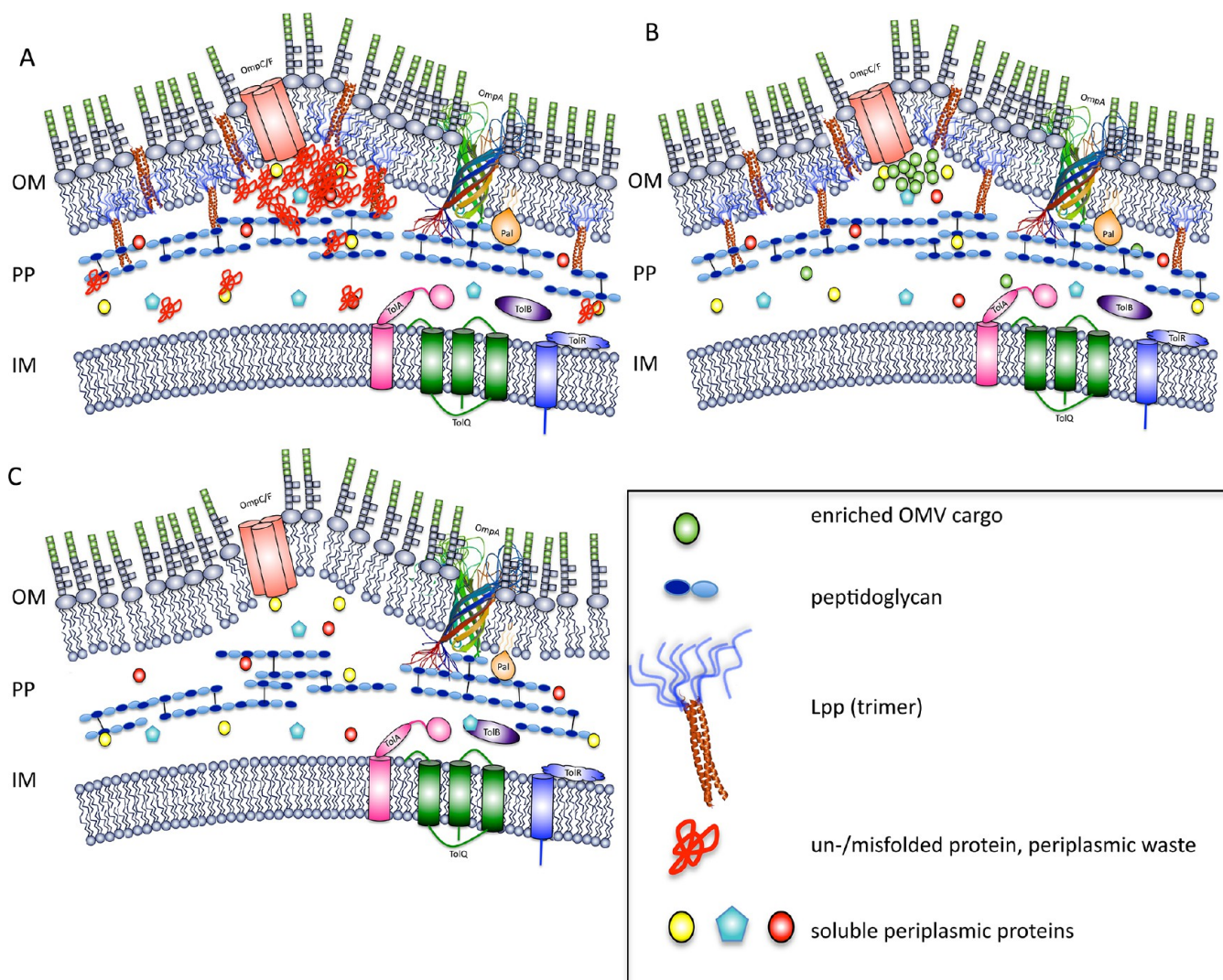
## ■ OMVS AND ENVELOPE CROSS-LINKS

Because of the cross-linked architecture of the envelope, it is very likely that an essential, initial step in generating a vesicle bud is the liberation of the OM from the covalent and noncovalent OM–PG–IM cross-links without concomitant damage and loss of membrane integrity. Historically, it was found that mutations and deletions in Lpp, the Tol–Pal system, and OmpA yield hypervesiculation phenotypes accompanied by cellular leakage as a consequence of membrane instability.<sup>43,51,54</sup> However, neither the wild-type nor numerous hypervesiculation mutants are necessarily accompanied by membrane instability,<sup>13</sup> leading us to believe that naturally occurring OMVs are produced by a more subtle process that depends on random or regulated disruptions in the density of the cross-links (Figure 3).

Before further speculating about possible mechanisms by which Gram-negative species might modulate the positioning of covalent and noncovalent envelope cross-links, we will introduce the relevant envelope players.

## ■ LPP

Lpp is the most abundant *E. coli* protein with an estimated 750000 copies per cell.<sup>55</sup> The lipid moiety of this lipoprotein anchors it to the OM, but it exists in what has been historically termed “free” and “bound” forms. The free form is solely OM-anchored, whereas the bound form refers to the Lpp that is covalently cross-linked to PG (Figure 2). The covalent cross-



**Figure 3.** Involvement of cargo and PG-OM cross-links in OMV production. (A) OMVs as cellular garbage cans. Proteinaceous waste is shed via OMVs, and the accumulation of misfolded protein may aid in vesicle formation. (B) OMV cargo enrichment. Cargo is secreted via OMVs, and its localized accumulation may aid in OMV formation by occurring in regions lacking cross-links and/or preventing cross-link formation. (C) Destabilized envelope caused by a lack of Lpp. The lack of this major OM component results in a discontinuous, leaky OM, and the absence of its ability to cross-link the OM with the PG results in a looser OM.

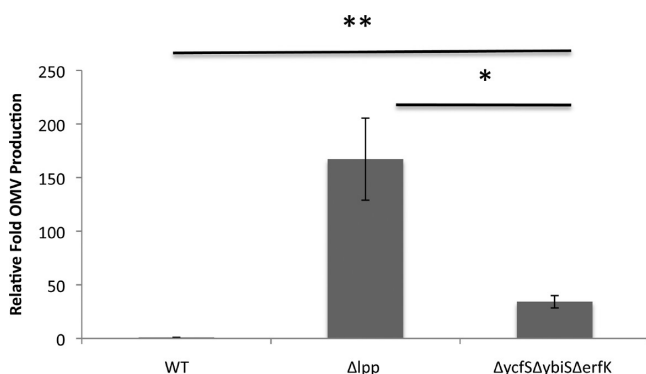
link occurs between the last residue of Lpp (Lys 58 in *E. coli*) and the diaminopimelic acid of PG. The ratio of the free form to the bound form was estimated to be 2:1.<sup>42,56–59</sup> Lpp has been shown to form a stable trimeric helical structure in solution in vitro.<sup>60,61</sup> Shu et al. suggest a model in which one of three helices is covalently attached to PG.<sup>61</sup> Free Lpp monomers most likely also associate into trimers.<sup>62</sup> Immunogold labeling has demonstrated that newly synthesized Lpp is homogeneously distributed across EDTA-permeabilized cells for the free form and across purified PG sacculi for the bound form.<sup>63</sup> It has also been suggested that the conversion of the free form to the bound form is reversible and that they are in a dynamic equilibrium.<sup>59,64</sup>

There have been studies examining if Lpp mutants behave like the full deletion strain. A lipid-anchor deficient variant of Lpp and a mutation that decreased the level of expression have been shown to have defects similar to that of the full deletion strain.<sup>65,66</sup> These results are not surprising because the absence of the lipid moiety would presumably generate a soluble protein, abolish OM targeting and consequently any structural

contribution native Lpp may give to the envelope, despite its remaining capacity to form covalent PG cross-links.

Deatherage et al. examined  $\Delta lpp$  complementation with a truncated Lpp version lacking the C-terminal lysine (Lys 58) in *Salmonella* and found that it behaved like a deletion strain in terms of OMV production and detergent sensitivity.<sup>54</sup> We decided to conduct a complementary experiment in *E. coli*. Three L,D-transpeptidases (YcfS, YbiS, and ErfK) have been shown to independently form the covalent cross-link between Lpp and PG in *E. coli*.<sup>67</sup> This triple mutant ( $\Delta ycfS \Delta ybiS \Delta erfK$ ) expresses wild-type Lpp but lacks the enzymes that allow it to catalyze the covalent cross-link. The level of OMV production of this strain was elevated ~30-fold over that of the wild-type but was significantly lower than the level of OMV production by  $\Delta lpp$ , which was ~150-fold greater than that of the wild-type (Figure 4). These results suggest that the free form of OM-localized Lpp adds to membrane stability. The reason behind the different outcomes of these data with Deatherage et al. is not completely clear, but a couple of points are noteworthy. Most obviously, the work was conducted in highly related, but





**Figure 4.** Free Lpp contributes to membrane integrity. OMVs were purified from the indicated bacterial mutants grown overnight in LB at 37 °C. OMVs were quantified by densitometry of major OMPs as described in ref 13 and compared to OMV production by the wild-type (WT).  $n = 4$ . \* $p = 0.01$ . \*\* $p = 0.001$ .

nonetheless, different bacterial species. Further, Cowles et al. have shown that the C-terminal portion of the free form of Lpp is exposed to the extracellular surface, and therefore that at least some population of Lpp adopts a transmembrane conformation.<sup>62</sup> It is possible that the Lys 58 deletion altered the charge or conformation of the protein such that the amount of Lpp in the transmembrane state was reduced or prevented along with any membrane stabilizing effect. Because free Lpp is a highly abundant protein, it is very plausible that its precise localization could substantially contribute to membrane stability.

A regulatory link is already known to exist between Lpp expression and  $\sigma^E$ , a modulator of OMV production (A. J. McBroom, I. A. MacDonald, and M. J. Kuehn, unpublished results, and ref 68). In *E. coli*,  $\sigma^E$  is a transcription factor that is activated as part of the heat shock response to the accumulation of misfolded OMPs in the envelope and is also essential under normal growth conditions.<sup>69,70</sup> A small RNA, Reg26, down-regulates cellular Lpp concentrations and is under positive control of  $\sigma^E$ .<sup>71</sup> Regulation of the network of Lpp cross-links through Reg26 upon  $\sigma^E$ -activating envelope stress allows for positive regulation of the OMV stress response,<sup>72</sup> which will be discussed in more detail later.

## PAL

Pal is an OM lipoprotein that has been shown to associate noncovalently with PG via a conserved  $\alpha$ -helical PG interaction motif.<sup>73–75</sup> It is part of the Tol–Pal system, which consists of IM proteins, TolA, TolQ, and TolR, and the periplasmic protein TolB. TolA, TolQ, and TolR interact in the membrane via transmembrane helices,<sup>76</sup> whereas TolB forms a complex with Pal.<sup>75</sup> The two complexes interact with each other via TolA and Pal in a proton motive force-dependent manner.<sup>77</sup> Additionally, it has been shown that Pal forms independent complexes with Lpp, OmpA, and TolB;<sup>78</sup> however, the Pal–Lpp interaction has not been defined in detail. It has been demonstrated that Pal is preferentially localized to the septum and the new daughter poles, which appears to be more stringent in *C. crescentus* than in *E. coli*.<sup>47,48</sup> This specificity of Pal placement, and particularly the areas of the envelope that are deficient in Tol–Pal complexes, could help explain how Pal plays a role in OMV formation.

Besides its role in envelope stability, the Tol–Pal complex has been shown to aid in OM invagination during the constriction phase of cell division.<sup>47,48</sup> There appears to be a

general correspondence of mutations in proteins involved in cell division and large circular structures emanating from the septum.<sup>54,79,80</sup> Although these structures have been labeled as “OMVs”, their size and site of budding are not what we typically see for OMVs produced by wild-type Gram-negative bacteria. Consequently, defining how Pal plays potentially separate roles in cell division and OMV production provides a challenge in the field.

## OMPA

Besides its interaction with Pal, OmpA plays additional roles in envelope stability. The carboxyl-terminal domain of *Acinetobacter baumannii* OmpA (the OmpA-like domain) has been crystallized bound to diaminopimelic acid, confirming the direct interaction with PG.<sup>46</sup> Two strictly conserved residues facilitate the binding of OmpA to PG, implying that this interaction is not species-specific but is rather a general interaction between PG and OmpA-like proteins.<sup>46</sup> Additionally, multiple groups have shown that the lack of OmpA results in hypervesiculation in *Salmonella*, *Vibrio cholerae*, and *E. coli*.<sup>54,72,81</sup> Similar to the regulation of Lpp by Reg26, a small RNA discovered in *V. cholerae*, VrrA has been shown to downregulate the expression of OmpA, which in turn increases the level of OMV production.<sup>72</sup> This mechanism is conserved across Gram-negative species.<sup>82</sup> MicA, the *E. coli* homologue, and VrrA are regulated by  $\sigma^E$ .<sup>72,83</sup> Thus, as in the case of Lpp mentioned above, this set of relationships may further explain how Gram-negative bacteria can increase the level of OMV production under  $\sigma^E$  activating conditions.<sup>8</sup>

## OMVS AND ENVELOPE STRESS

An increased level of OMV production was determined to be beneficial to bacteria challenged with stressors that lead to the periplasmic accumulation of misfolded protein waste, such as protease impairment, denaturant, or overexpressed toxic proteins.<sup>36</sup> These results lead to the hypothesis that harmful proteinaceous waste may be shed via OMVs (Figure 3A), and that OMV production is an envelope stress response.<sup>36</sup>

The accumulation of proteinaceous waste in the periplasm is generally thought to be prevented by DegP, which is a periplasmic chaperone at low temperatures and a protease at high temperatures.<sup>84</sup> The loss of this dual functional protein was found to generate hypervesiculation phenotypes in *E. coli*, *Salmonella enterica* ssp. *Typhimurium*, and *P. aeruginosa*.<sup>36,68</sup> The hypervesiculation phenotype of the *E. coli* *degP* deletion strain was temperature-dependent: at 30 °C, vesiculation levels were comparable to that of the wild-type strain, but hypervesiculation was observed at 37 °C.<sup>36</sup> Increasing temperatures lead to increasing amounts of misfolded proteins, which are expected to be the cause of the increase in the level of OMV production (Figure 3A). The addition of a hypovesiculating mutation to the *degP* mutant causes a more severe defect that is exacerbated at high temperatures, supporting the hypothesis that the high levels of OMV production by the *degP* mutant relieve toxic proteinaceous stress (C. Schwechheimer and M. J. Kuehn, unpublished results). Furthermore, the *degP* null mutation is conditionally lethal but can be suppressed by an additional mutation in *lpp*, presumably by creating a leaky cell and relieving the periplasm from the toxicity of accumulated misfolded material.<sup>85</sup> Together, these data support the idea that the accumulated misfolded periplasmic material can be toxic

and that OMV production can improve the survival of bacteria in which these toxic stressors have accumulated.

Two studies apparently contradict this hypothesis. McMahon et al. recently showed that the amounts of OMVs produced by the opportunistic pathogen *Serratia marcescens* actually increased with decreasing temperature.<sup>86</sup> However, the inverse relationship between temperature and the level of OMV production remained unexplained. This phenomenon may be specific to the envelope of *S. marcescens* as this has not been reported for other species examined thus far. Shibata and Visick examined a *Vibrio fischeri* strain from which a *degP* homologue was deleted and reported a consequent decrease in the level of OMV production.<sup>87</sup> The apparently opposite phenotype could be explained by several differences in the two experimental systems. We observed for *E. coli* that conditions in which *DegP* protease activity is required correlate with a growth defect as well as accumulation of protein in the periplasm and hypervesiculation (C. Schwechheimer and M. J. Kuehn, unpublished results). By contrast, the *degP* homologue deletion cultures of *V. fischeri* were grown at 23 °C, and the strain had no growth defect,<sup>87</sup> which suggests that this protein is not critical under the conditions tested and that there is presumably a relatively low concentration of periplasmic waste. In addition, the *degP* homologue in *V. fischeri* plays a role in biofilm formation,<sup>87</sup> and this or another function could contribute to its hypovesiculation phenotype.

## ■ POSSIBLE VESICULATION MECHANISMS

Several mechanistic scenarios have been proposed that could lead to OMV formation, taking into account the cross-linked architecture of the Gram-negative envelope. These models are not mutually exclusive as specific lipid or protein contributions are likely occurring simultaneously with cell wall remodeling to cause bulging out of the OM. Further, different mechanisms may be used during different periods of the cell cycle.

OMV production could be dictated, or at least encouraged, by localization of curvature-inducing proteins such as those containing inverted-BAR domains found in eukaryotic cells,<sup>88</sup> lipids such as cardiolipin,<sup>89</sup> or small molecules such as *Pseudomonas* quinolone signal (PQS).<sup>90</sup> PQS produced and secreted by *Pseudomonas* species aids in curvature formation in LPS liposomes,<sup>91</sup> and PQS-producing strains have increased levels of OMV production;<sup>68</sup> however, PQS homologues have not been found in all Gram-negative bacteria that have been shown to vesiculate. In addition, polysaccharide-containing components of the OM have been shown to affect OMV production. For *S. marcescens*, inactivation of the synthesis of the enterobacterial common antigen, a surface-exposed repeating sugar molecule, modulated vesiculation.<sup>86</sup> For wild-type *P. aeruginosa*, a minor species of LPS with highly charged O-antigen is enriched in OMVs.<sup>20,21</sup> Despite the appeal of such single-molecule-driven mechanisms, the connections between the envelope components cannot be ignored, and it is unlikely that stimulating membrane curvature would be sufficient to generate and release OMVs without simultaneously taking advantage of inconsistent intervals between envelope cross-links (Figure 2).

Several mechanisms by which OMV production could occur upon modulation of the covalent interactions between PG and the OM are proposed. First, the nonuniform spatial distribution of the bonds could promote particular sites to become OMV bud sites. In this scenario, there would be localized breaking of bonds or localized downregulation of cross-linking proteins,

and OM budding would occur more rapidly than diffusion of the bonds. Second, at particular sites in the envelope, OM biogenesis might occur more rapidly with respect to the underlying PG, generating a flexible OM and preventing the formation of covalent connections until the region matured. In this case, covalent bonds might be enriched in the more mature areas of the envelope. Third, regions of PG could be excised to create a localized excess of OM that could bud outward and result in an OMV.

A general prerequisite for creating a OMV seems to be the generation of space between envelope cross-links either to initiate a budding event or to stimulate progression of a protobud. Space between PG and the OM may be achieved via preferential localization of Pal,<sup>47,48</sup> downregulation of OmpA and Lpp,<sup>72</sup> and/or the absence of Lpp-PG cross-linking at a particular location. Pal forms noncovalent bonds not only with PG but also with several other proteins, so an interaction with another protein could detach a portion of the OM to form a vesicle.

Once a bud has started to emerge, cross-links could be broken during the development of the OMV, which would drive bud formation rather than resorption. As they form noncovalent bridges, it is not difficult to imagine that Pal and OmpA could dissociate from PG when the periplasmic dimension increases (discussed in more detail below). Evidence of the lability of Lpp-PG cross-links came from pulse-chase experiments in which 40% of the pulse-labeled free form of Lpp was found in the bound form after one doubling time, and the radioactivity of the bound form, as well as the relative amount of the free form, did not change after a longer chase.<sup>59,64</sup> These data showed that the Lpp-PG cross-links must be transient: If conversion to the bound form were irreversible, the ratio of the labeled free form to the labeled bound form would diminish over time, eventually leaving no pulse-labeled free form. To date, however, no enzyme has been reported to cleave the covalent Lpp-PG cross-link, and further studies are needed to determine if such an enzyme exists.

Lastly, either via an increase in the level of OM synthesis, via excision of PG, or via modulation of the rate of PG turnover with respect to OM synthesis at a location between OM-PG cross-links, a vesicle bud of the OM could emerge. This scenario suggests that OM synthesis, PG excision, and/or remodeling machinery is discretely localized and corresponds to sites of OMV production. Evidence has not yet been presented to either support or discredit this model.

## ■ OMV PRODUCTION: STOCHASTIC OR REGULATED?

The short answer to this fundamental question is that it has not yet been established whether OMV production is a stochastic or regulated process. In fact, there is evidence of both, and it may depend on the conditions. Below, we summarize and interpret the evidence supporting the two routes.

Arguing against a stochastic process is the enrichment of some proteins in OMVs in comparison to their abundance in the bacterial envelope (Figure 3B). It has been demonstrated that a chimeric protein mimicking a misfolded OMP, which is recognized by the cell as an  $\sigma^E$  envelope stress signal,<sup>92</sup> is at least 10-fold enriched in OMVs with respect to its concentration in the periplasm.<sup>36</sup> Although this cargo was an artificial chimera, such enrichment is also expected for naturally expressed unfolded proteins. In enterobacterial pathogens, Wai et al. demonstrated that the oligomeric pore-forming cytotoxin

ClyA is enriched in the secreted OMVs.<sup>15</sup> Heat-labile enterotoxin,<sup>14</sup> one of the toxins of enterotoxigenic *E. coli*, and leukotoxin from *Actinobacillus actinomcetemcomitans*<sup>16</sup> have been shown to be secreted and enriched in OMVs. Preferential sorting into OMVs has also been demonstrated for gingipains, proteases that constitute a major virulence factor of the human pathogen *Porphyromonas gingivalis*.<sup>37</sup> In this study, preferential OMV inclusion was observed along with OMV exclusion of abundant OMPs. Importantly, in this study, Haurat et al. also showed evidence that this sorting process is LPS O-antigen-dependent. Cargo enrichment in OMVs from *Myxococcus xanthus* has also been reported, as alkaline phosphatase activity was almost exclusively found in OMVs.<sup>38</sup> These data support the concept that at least in some cases, OMVs formed under native conditions are the result of a regulated process involving a cargo sorting mechanism.

Non-native conditions highlight the possibility that OMVs can also be stochastically generated because of a significant decrease in the number of envelope ties. As detailed earlier, cells lacking envelope cross-linking components exhibit unstable membranes and consequently hypervesiculate (Figure 3C). Membrane-PG dissociation was indeed observed in high-resolution cryo-electron tomography images of *C. crescentus* harboring deletions in the Tol-Pal system.<sup>80</sup> A mutation or deletion in *degP* also causes hypervesiculation without activating  $\sigma^E$ .<sup>36</sup> Accumulated misfolded envelope material could downregulate cross-linking proteins in a  $\sigma^E$ -independent manner, but it is also possible that for these non- $\sigma^E$ -inducing conditions, the periplasmic dimension increases because of the accumulation of misfolded protein waste. This may disturb the distribution of noncovalent envelope cross-links, simply because of their finite length or because periplasmic expansion distorts regions of the envelope that naturally lack cross-links (Figure 3A). Separation could become amplified further by the inability of cross-linked components to be generated or diffuse into that region because of the increased physical distance between PG and the OM. In either case, the result would be an increased level of OMV production that is regulated by the amount of bulk protein in the periplasm. Indeed, overexpression of non- $\sigma^E$ -activating periplasmic proteins causes OMV hyperproduction.<sup>23</sup> Notably, these overexpressed periplasmic proteins were not enriched in OMVs, further supporting the existence of a stochastic process.

Would periplasmic distension or changes in PG-OM cross-linking location or density ever occur naturally, without mutation or ectopic gene overexpression? Envelope stress that triggers OMP unfolding can cause  $\sigma^E$ -dependent downregulation of OmpA and Lpp by MicA and Reg26, respectively. Environmental stressors also could cause non- $\sigma^E$ -activating protein misfolding, which could overwhelm the other stress-response pathways and lead to conditions of periplasmic bloating. Accumulated material could cause the periplasmic space to expand beyond the physical distance of the PG-OM cross-links, as discussed above (Figure 3A). Thus, similar mechanisms may come into play under native conditions and result in regulated vesicle production.

In summary, changes in the envelope composition can induce regulated OMV production or accentuate a stochastic process. OMV production can be modulated by  $\sigma^E$  activation and consequent downregulation of OmpA and Lpp by MicA and Reg26, respectively, or by affecting the number or location of the ties between the PG and the OM through a physical increase in the level of periplasmic expansion.

## ■ OMVS AND PG TURNOVER

Besides being involved in the envelope cross-links, PG itself may play a role in OMV production. PG is a highly dynamic polymer that is constantly undergoing synthesis and degradation events through a highly regulated and often redundant repertoire of enzymes.<sup>39</sup> Hayashi et al. showed that a mutation in a PG amidase, an enzyme that cleaves PG amide bonds, resulted in hypervesiculation in *P. gingivalis*.<sup>93</sup> This result coincides with our data generated from *E. coli*. Strains harboring mutations in PG hydrolases, such as the endopeptidases (which cleave peptide cross-links) or the lytic transglycosylases (which cleave glycosidic bonds), also cause hypervesiculation (C. Schwechheimer and M. J. Kuehn, unpublished results). Whether these phenotypes are also due to effects on PG-OM cross-linking remains to be determined, although it is clear that not all of the mutations that cause hypervesiculation cause a decrease in the level of cross-linking and vice versa (C. Schwechheimer and M. J. Kuehn, unpublished results). These data not only reveal that altering the underlying PG can modulate vesicle formation of the OM but also support the model in which a decreasing level of PG degradation may lead to an excess of OM material that could form OMVs.

## ■ CONCLUDING REMARKS

A vast amount of understanding has been gained over the years with respect to the role OMVs play in pathogenesis as well as their more general functional properties. The regulation and mechanism of their production are still very cryptic, and the knowledge we have is fragmented; however, what seems to become clear is that there may be multiple means by which OMVs are produced. Here, we touch on envelope protein accumulation, envelope cross-links, and the biogenesis of PG and the OM. Nevertheless, major questions in the field remain. How is membrane curvature achieved? How do OMVs ultimately pinch off? How is OMV production regulated? How is cargo selectively enriched? What are the temporal characteristics of vesicle formation with respect to cell cycle? Further work will be necessary to answer these questions.

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## ■ ABBREVIATIONS

IM, inner membrane; LPS, lipopolysaccharide; OMV, outer membrane vesicle; OM, outer membrane; OMP, outer membrane protein; PG, peptidoglycan; PQS, pseudomonas quinolone signal.

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