Outer Membrane Vesicle Production in *Escherichia coli* Relieves Envelope Stress and is Modulated by Changes in Peptidoglycan

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Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Biochemistry in the Graduate School of Duke University

2014
ABSTRACT

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Abstract

Bacterial outer membrane vesicles (OMVs) are spherical buds of the outer membrane (OM) containing periplasmic lumenal components. OMVs have been demonstrated to play a critical part in the transmission of virulence factors, immunologically active compounds, and bacterial survival, however vesiculation also appears to be a ubiquitous physiological process for Gram-negative bacteria. Despite their characterized biological roles, especially for pathogens, very little is known about their importance for the originating organism as well as regulation and mechanism of production. Only when we fully understand their biogenesis can we fully uncover their roles in pathogenesis and bacterial physiology. The overall goal of this research was to characterize bacterial mutants which display altered vesiculation phenotypes using genetic and biochemical techniques, and thereby begin to elucidate the mechanism of vesicle production and regulation. One part of this work elucidated a synthetic genetic growth defect for a strain with reduced OMV production (ΔnlpA, inner membrane lipoprotein with a minor role in methionine transport) and envelope stress (ΔdegP, dual function periplasmic chaperone/ protease responsible for managing proteinaceous waste). This research showed that the growth defect of ΔnlpAΔdegP correlated with reduced OMV production with respect to the hypervesiculator ΔdegP and the accumulation of protein in the periplasm and DegP substrates in the lumen of OMVs. We further demonstrated that OMVs do not solely act as a stress response pathway to rid the periplasm of otherwise damaging misfolded protein but also of accumulated peptidoglycan (PG) fragments and lipopolysaccharide (LPS), elucidating OMVs as a general stress response pathway critical for bacterial well-being. The second part of this work, focused on the role of PG structure, turnover and covalent crosslinks to the OM in vesiculation. We established a direct link
between PG degradation and vesiculation: Mutations in the gene encoding the OM lipoprotein *nlpI* had been previously established as a very strong hypervesiculation phenotype. A genetic interaction between *nlpI* and *spr*, a PG endopeptidase, had also been identified. The data presented here suggest that NlpI acts as a negative regulator of Spr activity and that the Δ*nlpI* hypervesiculation phenotype is a result of the rampant degradation of PG by Spr. Additionally, we found that changes in PG structure and turnover correlate with altered vesiculation levels. Further, non-canonical D-amino acids, which are secreted by numerous bacteria on the onset of stationary phase, are a natural factor to increase OMV production. Lastly, we discovered an inverse relationship between the concentration of Lpp-mediated, covalent crosslinks and the level of OMV production under conditions of modulated PG metabolism and structure. In contrast, the overall OM-PG crosslink concentration appears to be unchanged in situations where periplasmic accumulation (protein, PG fragments, and LPS) lead to hypervesiculation. From this work, we conclude that multiple pathways lead to OMV production: Lpp concentration-dependent and bulk driven, Lpp concentration-independent.
Dedication

To my grandfather, Hugo Georg Sponagel
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1. Introduction

1.1 The outer membrane, the barrier between the extracellular environment and the cell

Gram-negative bacteria can inhabit very different ecological niches, quite frequently even hostile environments, making the outer membrane (OM), the barrier between the extracellular milieu and the cell, essential for bacterial survival. This barrier role of the OM also makes it the site through which Gram-negative bacteria interact with their environment. Secretion processes are one route for bacteria to modify their surroundings and can have many different faces depending on the conditions. One way for bacteria to modulate their environmental behavior is to switch their life style from liquid culture into a biofilm structure by changing their cell surface. For instance, the secretion of amyloid protein structures called curli in the case of *Escherichia coli* to create an extracellular protective matrix (Barnhart and Chapman, 2006). Additionally, frequently secreted enzymes are proteases and toxins that can attack other prokaryotic or eukaryotic cells, either within polymicrobial communities or during pathogenesis creating a beneficial ecological niche for the secreting microbe (Dalbey and Kuhn, 2012). Furthermore, secretion of protein complexes can also serve to import essential components such as iron in the context of siderophores (Hider and Kong, 2010). As these examples indicate, secretion classically has been viewed as the export of soluble proteins or protein complexes (Dalbey and Kuhn, 2012), but there is also an insoluble secretion pathway, vesiculation, that has been utilized by all kingdoms of life (Deatherage and Cookson, 2012). Studying the insoluble secretion pathway of Gram-negative bacteria can provide insights into their toxicity, how bacteria manipulate host as well as other
bacterial cells on a genetic and protein level, and how bacteria cope with and survive harsh environments.

1.2 Outer Membrane Vesicles

1.2.1 Structure and Composition

All Gram-negative bacteria studied to date have been shown to secrete outer membrane vesicles (OMVs) (Berleman and Auer, 2013; Deatherage and Cookson, 2012; Kulp and Kuehn, 2010; Schwechheimer et al., 2013). OMVs are 50 to 250 nm spherical structures, as visualized by electron and atomic force microscopy (Fig. 1 A, B) (Beveridge, 1999; Schwechheimer et al., 2013), composed of OM and entrapped lumenal periplasmic content (Kulp and Kuehn, 2010). Biochemical assays and atomic force microscopy imaging of live E. coli shows that OMV production occurs without a disruption in the integrity of the cell (Fig 1B) (McBroom et al., 2006). Numerous studies have been dedicated to determine OMV composition in various species and conditions (Choi et al., 2011; Lee et al., 2007; Mullaney et al., 2009; Pierson et al., 2011). OMV content is likely derived from the proteins and lipids present at the site of budding (Kulp and Kuehn, 2010; Schwechheimer et al., 2013) but there are numerous lines of evidence suggesting enrichment and exclusion of several envelope components in OMVs in comparison to their abundance in the cell (Elhenawy et al., 2014; Evans et al., 2012; Haurat et al., 2011; Horstman and Kuehn, 2000; Kato et al., 2002; McBroom and Kuehn, 2007; Wai et al., 2003). It has been known for some time that peptidoglycan (PG), the bacterial cell wall (Vollmer and Bertsche, 2008), is also associated with OMVs (Zhou et al., 1998), and this has been confirmed more recently by the functional analysis of OMVs from the pathogens Helicobacter pylori, Pseudomonas aeruginosa and Neisseria gonorrhoeae (Kaparakis et al., 2010).
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 (McBroom et al., 2006). OMVs were purified from an overnight culture as described in chapter 8.4. A 50 µL 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 µm/s. The yellow line indicates the measurements shown below the image. (B) Time course of an *E. coli* nlpI transposon mutant 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 (Doktycz et al., 2003). 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 µm/s. Reproduced from (Schwechheimer et al., 2013).

1.2.2 Biological Function

Vesiculation appears to be a ubiquitous physiological process: *In vitro* planktonic (Kulp and Kuehn, 2010), and agar-grown laboratory cultures, bacteria living in fresh water environments (Beveridge, 1999), biofilms (Beveridge et al., 1997; Schooling and Beveridge, 2006), and Gram-negative pathogens in animal hosts have been shown to produce OMVs (Brandtzaeg et al., 1992; Hellman et al., 2000).

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 PG (Demuth et al., 2003; Ellis and Kuehn, 2010; Horstman and Kuehn, 2000; Wai et al., 2003). However, OMV secretion occurs at a constitutive level for a wide variety of bacteria, suggesting this is a highly conserved process which is not only important for pathogens (Beveridge, 1999; Schwechheimer and Kuehn, 2013). It has been shown that OMVs can facilitate the transfer of antibiotic resistance enzymes and exogenous DNA between strains and even between species (Dorward et al., 1989; Yaron et al., 2000), an advantage for both pathogenic and non-
pathogenic species. Furthermore, OMVs can act in a general predatory fashion that aids the parent bacterium by generating room in an ecological niche and to acquire 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 (Kadurugamuwa and Beveridge, 1996; Li et al., 1996). Finally, one of the emerging functional roles of OMVs is that they provide a general advantage in bacterial survival. Increased OMV production correlated with increased survival upon both antimicrobial peptide treatment and T4 bacteriophage infection (Manning and Kuehn, 2011). 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 (McBroom and Kuehn, 2007; Schwechheimer and Kuehn, 2013).

Despite their ubiquitous nature and critical biological functions, we understand very little about OMV biogenesis and regulation.

1.3 Envelope Architecture

In order to discuss the mechanics of OMV production, we must introduce the architecture of the Gram-negative bacterial envelope from which the vesicles originate (Fig. 2). The envelope consists of the OM, an inner membrane (IM) and a periplasm between the two membranes, which contains a thin layer of PG (Raetz and Dowhan, 1990; Silhavy et al., 2010). The OM is composed of the unique Gram-negative molecule lipopolysaccharide (LPS), which predominates in the outer leaflet, and phospholipids in the inner leaflet. The fatty acids of LPS are attached to a sugar backbone containing charged phosphate groups, making it a hydrophilic charged molecule. Divalent cations intercalate between LPS molecules preventing repulsion between the negatively charged entities. These lateral interactions give LPS a gel-like appearance and provide the strong
barrier function against the potentially harmful environment (Nikaido, 2003; Ruiz et al., 2006). Further indicating the importance of the LPS barrier function is the fact that bacteria can modify LPS to resist detection by the immune system as well as being killed by antimicrobial agents (Chen and Groisman, 2013). LPS is also termed endotoxin, based on its highly inflammatory effects on the mammalian immune system (Raetz and Whitfield, 2002). Lipoproteins are membrane anchored via an N-terminally covalently attached lipid moiety and are most frequently destined for the OM but are also found in the IM (Silhavy et al., 2010; Tokuda and Matsuyama, 2004). Integral OM proteins (OMPs) almost exclusively fold as β-barrels whereas integral IM proteins are predominantly α-helical structures (Luirink et al., 2005; Silhavy et al., 2010). Unlike the IM which has phospholipids in both leaflets, in the OM, LPS predominates in the outer leaflet and phospholipids in the inner leaflet (Kamio and Nikaido, 1976), as mentioned above. Located just underneath the OM, the PG consists of a three-dimensional mesh-like network of glycan strands crosslinked by short peptides. The PG gives the bacteria their characteristic shape and also serves a protective function, preventing cell lysis due to changes in osmolarity and mechanical stress (Vollmer and Bertsche, 2008).
1.3.1 Envelope Crosslinks

The layers of the Gram-negative envelope are “stitched” together and stabilized via protein crosslinks reaching from the IM through PG to the OM (Fig. 2). Lpp, an abundant lipoprotein in *E. coli*, or its counterpart in other Gram-negative bacteria, covalently crosslinks the OM and PG providing structural envelope integrity (Braun, 1975; Braun and Rehn, 1969). Additionally, OmpA and the Tol-Pal system add non-covalent stability to the envelope (Cascales et al., 2002; Wang, 2002). OmpA is an OM
porin containing a PG interaction motif (Park et al., 2011; Smith et al., 2007). 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 (Gerding et al., 2007; Yeh et al., 2010). The Tol-Pal system is essential in Caulobacter crescentus, which does not have an Lpp homologue to form the typical OM-PG covalent crosslinks (Poggio et al., 2010; Sturgis, 2001). 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 (Cascales et al., 2002; Suzuki et al., 1978).

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 maintaining cellular integrity (Egan and Vollmer, 2012; Ernst et al., 2001; Silhavy et al., 2010). One way to remodel the envelope and shed unnecessary, even harmful, components is OMV production (McBroom and Kuehn, 2007; Schwechheimer and Kuehn, 2013) (Bonnigton and Kuehn, unpublished data). This shedding of the OM with periplasmic luminal content occurs without compromising membrane stability and risking lysis, indicating a controlled severing of the OM from its crosslinks (McBroom et al., 2006; Schwechheimer and Kuehn, 2013; Wessel et al., 2013). We next view OMV production in the context of each of these envelope crosslinks.

1.3.2 Outer Membrane Vesicles and Envelope Architecture

Because of the crosslinked 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 non-covalent OM-PG-IM crosslinks without concomitant damage and loss
of membrane integrity. Previously, it was found that mutations and deletions in Lpp, Tol-Pal and OmpA yield hypervesiculation phenotypes accompanied with cellular leakage as a consequence in membrane instability (Cascales et al., 2002; Deatherage et al., 2009; Suzuki et al., 1978). However, neither wild-type nor numerous hypervesiculation mutants are necessarily accompanied by membrane instability (McBroom et al., 2006; Schwechheimer and Kuehn, 2013), leading us to suggest that naturally occurring OMVs are produced by a more subtle process that depends on random or regulated disruptions in the density of the crosslinks. Below the relevant envelope players will be introduced.

1.3.2.1 Lpp

Lpp is the most abundant E. coli protein with an estimated 750,000 copies per cell (Nikaido, 1996). The lipid moiety of this lipoprotein anchors it to the OM, but it exists in what has been historically referred to as “free” and “bound” forms. The free form is solely OM-anchored, whereas the bound form refers to the Lpp that is also covalently crosslinked to PG (Fig. 3). The covalent crosslink occurs between the last residue of Lpp (Lys 58 in E. coli) and diaminopimelic acid of PG. The ratio of the free to the bound form was estimated to be 2:1 (Braun and Bosch, 1972; Braun and Rehn, 1969; Braun and Sieglin, 1970; Braun and Wolff, 1970; Inouye et al., 1972). Lpp has been shown to form a stable trimeric helical structure in solution in vitro (Choi et al., 1986; Shu et al., 2000). Shu et al. suggest a model in which one of three helices is covalently attached to PG (Shu et al., 2000). Free Lpp monomers most likely also associate into trimers (Hiemstra et al., 1987). 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 (Inouye et al., 1974). It has also been
suggested that the conversion of free to bound form is reversible and they are in a dynamic equilibrium (Inouye et al., 1974; Inouye et al., 1972).

There have been studies examining if Lpp mutants behave like the full deletion strain. A lipid-anchor deficient variant of Lpp, as well as a mutation that decreased expression, have been shown to have similar defects as the full deletion strain (Torti and Park, 1976; Yem and Wu, 1978). These results are not surprising since 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 crosslinks.

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) (Tashiro et al., 2009). 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 and mislocalized LPS in the envelope and is also essential during normal growth conditions (Alba and Gross, 2004; De Las Penas et al., 1997; Lima et al., 2013). A small RNA, Reg26, downregulates cellular Lpp concentrations and is under positive control of $\sigma^E$ (Gogol, 2011). Regulation of the network of Lpp crosslinks through Reg26 upon $\sigma^E$-activating envelope stress allows for positive regulation of the OMV stress response (Song et al., 2008).
A Lpp close-up in the context of an overview of the Gram-negative envelope. Adapted from (Schwechheimer et al., 2013). “Bound Lpp” is lipid anchored and covalently linked to PG, whereas “free Lpp” is lipid anchored and embedded in the OM with its C-terminus externally accessible.

1.3.2.2. Pal

Pal is an OM lipoprotein that has been shown to associate non-covalently with PG via a conserved α-helical PG interaction motif (De Mot and Vanderleyden, 1994; Germon et al., 1998; Mizuno, 1979). It is part of the Tol-Pal system, which consists of IM proteins, TolA, TolQ and TolR, and the periplasmic protein TolB. TolA-Q-R interact in the membrane via transmembrane helices (Derouiche et al., 1995), whereas TolB forms a complex with Pal (Clavel et al., 1998). The two complexes interact with each other via TolA and Pal in a proton motive force-dependent manner (Cascales et al., 2000). Additionally, it has been shown that Pal forms independent complexes with Lpp, OmpA and TolB (Cascales and Lloubes, 2004), however the Pal-Lpp interaction has not been defined in detail. It has been demonstrated that Pal preferentially localizes to the
septum and the new daughter poles, which appears to be more stringent in *C. crescentus* than in *E. coli* (Gerding et al., 2007). This specificity of Pal placement and particularly the areas of the envelope which 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 (Gerding et al., 2007; Yeh et al., 2010). There appears to be a general correspondence of mutations in proteins involved in cell division and large circular structures emanating from the septum (Chakraborti et al., 1986; Deatherage et al., 2009; Goley et al., 2010). Although these structures have been labeled as “OMVs,” their size and site of budding are not what we typically see for OMVs produced by WT Gram-negative bacteria. Consequently, defining how Pal plays potentially separate roles in cell division and OMV production provides a challenge in the field.

### 1.3.2.3 OmpA

Besides its interaction with Pal, OmpA plays additional roles in envelope stability. The carboxyl-terminal domain of *Acinetobacter baumanii* OmpA (the OmpA-like domain) has been crystallized bound to diaminopimelic acid, confirming the direct interaction with PG (Park et al., 2011). 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 (Park et al., 2011). Additionally, multiple groups have shown that the lack of OmpA results in hydervesculation in *Salmonella, Vibrio cholerae* and *E. coli* (Deatherage et al., 2009; Song et al., 2008; Sonntag et al., 1978). 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 OMV production (Song et al., 2008). This mechanism is
conserved across Gram-negative species (Argaman et al., 2001). MicA, the *E. coli* homologue as well as VrrA are regulated by σE (Song et al., 2008; Udekwu and Wagner, 2007). Thus, as in the case of Lpp mentioned above, this set of relationships may further explain how Gram-negative bacteria can increase OMV production under σE-activating conditions (Kulp and Kuehn, 2010).

### 1.4 Advantage, Mechanism, and Regulation of OMV production

When we started this work, most of the research about OMVs examined their function in the context of pathogenesis (Ellis and Kuehn, 2010). However, very little was known about the benefits of OMVs for non-pathogenic Gram-negative bacteria and OMV production and regulation, which lead us to ask the following questions:

- Is OMV production critical for bacteria in a non-pathogenic context since they spent the energy to produce the secreted proteins and lipids? If so, under what conditions?
- How are OMVs produced without compromising membrane stability?
- What envelope factors regulate vesiculation and what role do they play during OMV production?

To investigate these questions, we decided to start by biochemically and genetically analyzing several of the vesiculation mutants that resulted from the transposon mutagenesis screen done by our lab (McBroom et al., 2006).

### 1.5 Acknowledgements

We thank Dr. Claretta J. Sullivan (Eastern Virginia Medical School), for collaboration with the atomic force microscopy images.
2. Synthetic genetic growth defect for conditions of increased envelope stress and reduced vesiculation

2.1 Summary

From a transposon mutant screen for OMV phenotypes, it was discovered that an *nlpA* mutant of *E. coli* produces fewer OMVs than wild type whereas a *degP* mutant produces higher levels. NlpA is an inner membrane-anchored lipoprotein that has a minor role in methionine import. DegP is a periplasmic chaperone/protease for misfolded envelope proteins that is critical when cells are heat shocked. To reveal how these proteins contribute to OMV production, the mutations were combined and the double mutant analyzed. The Δ*nlpAΔdegP* strain displayed a high temperature growth defect that corresponded to the production of fewer OMVs than Δ*degP*. This phenotype also pertained to other undervesiculation mutations in a Δ*degP* background. The periplasm of Δ*nlpAΔdegP* was determined to contain significantly more protein compared to wild type. Additionally, DegP substrates, misfolded outer membrane porins, were detected in Δ*degP*-derived OMVs. These data suggest that an accumulation of envelope proteins resulting from decreased vesiculation was toxic and contributed to the growth defect. We conclude that OMV production contributes to relieve the envelope of accumulated toxic proteins, and that NlpA plays an important role in the production of vesicles that is methionine independent.

2.2 Introduction

Members of the Kuehn laboratory previously screened random transposon mutants of *E. coli* and identified those with altered vesiculation phenotypes. One of the hypovesiculating mutants found was a nearly null-mutation in *nlpA* (McBroom et al.,
NlpA is an IM anchored lipoprotein (Yu et al., 1986) with a minor role in methionine import (Zhang et al., 2003). NlpA is positively regulated by CsgD, a transcriptional regulator important for biofilm formation (Ogasawara et al., 2011).

The screen also revealed that a nearly null mutation in degP resulted in hypervesculation, a phenotype later confirmed with the full deletion (McBroom et al., 2006; McBroom and Kuehn, 2007). DegP is a protease/chaperone that manages envelope stress caused by un- and misfolded proteins (Ortega et al., 2009; Raivio and Silhavy, 2001; Spiess et al., 1999). Transcription of degP is regulated by the σE and the cpx envelope stress pathways (Raivio and Silhavy, 2001). DegP is predominantly active as a protease at higher temperature, whereas its chaperone activity dominates at lower temperature (Spiess et al., 1999; Strauch et al., 1989). Severe stress conditions such as heat shock and growth at higher temperatures result in high levels of un- and misfolded proteins in the cell. A degP deletion becomes lethal at 42°C presumably because envelope protease activity becomes critical to relieve the envelope from undesired proteinaceous waste products (Strauch et al., 1989). The hypervesculation phenotype of ΔdegP can be significantly reduced by growing the cells at a lower temperature (~30°C), most likely due to the increase of time allowed for protein folding which reduces the amount of proteinaceous waste that accumulates in the envelope. This observation led to the hypothesis that strains lacking degP use vesiculation as a survival mechanism eliminating sub-lethal amounts of un- and misfolded proteins from the cell envelope (McBroom and Kuehn, 2007).

We reasoned that if vesiculation is critical for cells to overcome lethal envelope stress, as exists for ΔdegP at high temperature, a mutation that confers an undervesculation phenotype should be conditionally synthetically defective in
conjunction with the \textit{degP} deletion. We further hypothesized that such a defect of \(\Delta nlpA\Delta degP\) likely originated from an overloaded periplasm that was unable to both effectively degrade proteins due to the \textit{degP} defect and release accumulated, toxic proteins via OMVs because of the \textit{nlpA} defect. To further investigate the idea that reduced OMV production in a \textit{degP} deletion background causes growth arrest, we examined the synthetic effects of \(\Delta degP\) with other hypovesiculation mutations.

\subsection*{2.3 Results}

The \(\Delta nlpA\Delta degP\) growth defect correlates with reduced OMV production. To reveal whether a \(\Delta nlpA\Delta degP\) conditional synthetic defect exists, we examined growth of single and double mutant cultures after they were shifted from 37°C to 40°C. The single mutants did not exhibit significant growth defects: \(\Delta nlpA\) grew like the isogenic BW25113 background strain (WT), whereas \(\Delta degP\) had a slightly decreased level of growth in comparison to WT. However, in combination, the \(\Delta nlpA\Delta degP\) mutant had a severe growth defect upon a shift to high temperature (40°C) (Fig 4A). As expected, a more modest defect was observed when cultures were maintained at 37°C (Fig. 5B).

\textit{NlpA} and \textit{DegP} have been linked to vesiculation levels in multiple \textit{E. coli} backgrounds. Both, the under- and hypervesiculation phenotypes have been established previously in a DH5\(\alpha\) background (McBroom et al., 2006), used for further studies in an ADA600 background (McBroom and Kuehn, 2007), and confirmed here in a BW25113 background. Furthermore, the growth defect of the \(\Delta nlpA\Delta degP\) double mutant is also appreciable, although diminished in a MC4100 background (data not shown). Therefore, we could conclude that the synthetic growth defect for envelope stress and OMV production is not strain-dependent.
We reasoned that if reduced vesiculation was responsible for the growth defect, the double mutant should exhibit reduced OMV production compared to ΔdegP. OMV production was assessed in cultures grown at 40°C using an established quantitative method. This method is based on the quantitation of OMPs in the cell-free culture supernatants, normalized by optical density (OD<sub>600</sub>) to account for differences in bacterial growth (McBroom et al., 2006). As expected from earlier studies, ΔnlpA produced fewer OMVs and ΔdegP produced more OMVs compared to WT (Fig 4B). Notably, the double mutant, ΔnlpAΔdegP, produced significantly fewer OMVs than ΔdegP alone (Fig 4B). Together with the growth data, these results show that the combination of a mutation that causes hypovesiculation and a temperature-sensitive mutation that increases vesiculation results in a stress-dependent synthetic growth defect.

**Figure 4: Correlation between ΔnlpAΔdegP growth and vesiculation defects.**

(A) Indicated strains of bacteria were grown in LB overnight at 37°C, inoculated into fresh LB (t=0), and then grown at 40°C. OD<sub>600</sub> was measured hourly. Error bars
indicate standard error of the mean (SEM); n=2. (B) Relative fold OMV production in cultures of the indicated strains grown in LB overnight at 40°C was determined by quantitating OMVs, normalizing to OD$_{600}$, and dividing by OD$_{600}$-normalized OMV production in a WT culture. Error bars indicate SEM; *, p < 0.05, **, p < 0.01. WT and ΔnlpA ΔdegP, n =9; ΔnlpA and ΔdegP, n =10. Reproduced from (Schwechheimer and Kuehn, 2013).

Figure 5: Growth phenotypes at different temperatures.
Indicated strains were grown in LB overnight, inoculated into fresh media (t=0), and grown for 6 h at the indicated temperatures. OD$_{600}$ was measured hourly. Error bars indicate SEM; n=6. Reproduced from (Schwechheimer and Kuehn, 2013).

**Hypervesiculation does not correlate with membrane instability.** Since hypervesiculation has previously been associated with membrane instability (Kulp and Kuehn, 2010) we needed to consider whether the so-called “OMVs” we collected were not solely OMVs, but also included membrane fragments of compromised cells. If this were the case, the strain with the greatest growth defect should have produced the most apparent “OMVs.” Instead, we noted that ΔnlpAΔdegP, the strain with the strongest growth defect, exhibited reduced OMV production in comparison to the single degP deletion strain (compare Fig 4A and 4B). Nevertheless, to ensure that the mutations did not compromise membrane integrity, we examined cytoplasmic content in the culture supernatants, the sensitivity of the mutant strains to Actinomycin D and Sytox Green, as well as visual inspection by electron microscopy.

Cytoplasmic leakage into the supernatant was assessed by measuring adenylate kinase activity. Adenylate kinase is a cytoplasmic enzyme that is released from lysed cells and can be detected with a luminescence assay (Jacobs et al., 2013). Following the protocol of Jacobs et al. we used heat-killed cells as our positive lysis control and to ensure that the content in our samples was not above the maximum detection limit of the assay (Fig. 6A). The hypervesiculating mutant, ΔdegP, exhibited a relatively insubstantial cytoplasmic leakage defect, whereas the highest level of adenylate kinase in the supernatant was observed for ΔnlpAΔdegP, which exhibits decreased OMV production with respect to ΔdegP. These data revealed that increased cytoplasmic leakiness does not correlate with increased OMV production, consistent with previous findings from our laboratory (McBroom et al., 2006).
In other tests for membrane permeability we used Actinomycin D sensitivity and Sytox Green incorporation. Actinomycin D sensitivity reveals the presence of compromised membranes since the antibiotic normally cannot access its cytosolic substrate (Leive, 1965; Sonntag et al., 1978), mutants containing envelope defects such as Δpal and ΔmrcB show sensitivity (data not shown) and similarly, Sytox Green is a dye that exhibits fluorescence when bound to DNA but can only penetrate into cells with compromised membranes (Cowles et al., 2011). The sensitivity of the strains to these two agents was examined: Whereas ΔnlpAΔdegP had the greatest growth defect in the presence of Actinomycin D, the more severe hypervesiculating mutant, ΔdegP, was insensitive to the drug, similar to the effect on WT (Fig. 6B). Furthermore, the hypovesiculating mutant, ΔnlpA, showed greater Sytox Green fluorescence than WT, and ΔdegP and ΔnlpAΔdegP were similarly slightly permeable to Sytox Green (Fig. 6C). Thin-section transmission electron microscopy of random fields of WT, ΔdegP and ΔnlpAΔdegP bacteria further confirmed membrane integrity of the mutants (Fig. 7). We concluded that although the sensitivity of the strains to these agents differs, importantly, altered membrane integrity and permeability do not correlate with hypervesiculation.
Figure 6: Hypervesculation does not correlate with membrane instability.

(A) Luminescence of the indicated strains grown in LB for ~16 hrs at 40°C was determined by normalizing to OD$_{600}$. Heat killed cells as positive control of full lysis, numbering below indicates the dilution factor to ensure the sample values are within the assay range. Error bars indicate SEM; Heat killed 0 dilution: n=4, Heat killed all other dilutions: n=2, samples: n=4. (B) Indicated strains of bacteria were grown in LB for ~16 hrs at 40°C, and then (t=0) OD$_{600}$ was measured hourly. (+) indicates cultures containing Actinomycin D which was added at t=0. Error bars indicate SEM; n=2. (C) Relative Sytox Green fluorescence in cultures of the indicated strains grown in LB for ~16 hrs at 40°C was determined by normalizing to OD$_{600}$ and dividing by the OD$_{600}$-normalized fluorescence of the WT culture. Error bars indicate SEM; n=2. Reproduced from (Schwechheimer and Kuehn, 2013).
Figure 7: Envelope integrity of ΔdegP strains by electron microscopy.

Thin-section, negative-stained transmission electron microscopy images of cells grown ~ 16-18 h in LB at 40°C. (A-C) WT, (D,E) ΔdegP, (F-H) ΔnlpAΔdegP. Size bars (in mm): 1 (A,D,F); 0.5 (B,E,G); 0.2 (C); 0.1 (H). Reproduced from (Schwechheimer and Kuehn, 2013).
**Complementation of the ΔnlpAΔdegP growth defect.** We next tested whether the observed growth defect of the double mutant could be complemented by expression of the deleted genes on IPTG-inducible plasmids. Complementation of ΔnlpAΔdegP with degP (pDegP) was assessed upon a temperature shift (from 37°C to 40°C). Uninduced, basal expression by pDegP was sufficient to complement the double mutant to WT levels of growth (Fig. 8A). In order to distinguish whether one or both functions of DegP (protease and/or chaperone) were responsible for the complementation, we used a plasmid expressing a protease-deficient point mutant of DegP, pmDegP (Spiess et al., 1999). We found that the basal expression level of pmDegP could not complement the growth defect of ΔnlpAΔdegP under the conditions tested (Fig. 8A). Therefore, the protease activity, but not the chaperone activity, of DegP was sufficient to repair the growth defect of ΔnlpAΔdegP.

Next, we examined complementation of the ΔnlpAΔdegP temperature-sensitive growth defect by plasmid-expressed NlpA (pNlpA). Basal levels of pNlpA were not sufficient to complement the growth defect of temperature-shifted cultures (Fig. 8B). Induction of NlpA expression above basal level with temperature shift was not possible since IPTG in the presence of empty vector caused a growth defect more severe than the ΔnlpAΔdegP growth defect, although this was notably not the case for the WT or single deletion strains (data not shown). Complementation was similarly unsuccessful for the double mutant induced once the culture reached late-log/stationary phase (data not shown).

Since we could not successfully complement the ΔnlpAΔdegP growth defect using pNlpA, we needed to establish whether pNlpA actually expressed functional protein and whether the cells were capable of using the protein under these stressful
temperature shift conditions. We first tested whether the product of pNlpA could functionally complement a methionine transport defect. As shown previously (Zhang et al., 2003), NlpA can substitute for its close homologue MetQ as the periplasmic binding protein for the methionine ABC-transporter (MetD) (Dawes and Foster, 1971; Gonzalez et al., 1992). This protein becomes critical when cells lack the ability to synthesize methionine (e.g. in ΔmetE strains) and when MetH is inactive due to the lack of vitamin B12 (Dawes and Foster, 1971). Thus, to test functional pNlpA expression, we used the triple mutant ΔnlpAΔmetQΔmetE as a background strain. This strain grows poorly in D-methionine-containing minimal media because it can neither import nor synthesize D-methionine. Growth of ΔnlpAΔmetQΔmetE carrying the vector was poor, as expected, but the growth defect could be complemented by pNlpA basal level expression, even when the culture was shifted from 37°C to 40°C (Fig 8C). From these experiments, we concluded that pNlpA does express functional (methionine-binding) NlpA under temperature shift conditions.

We next investigated whether pNlpA toxicity in ΔnlpAΔdegP was due to the likely severe defects in the envelope of this temperature-shocked, periplasmic protease-deficient strain. The effect of increasing concentrations of NlpA was studied in a ΔdegP strain under temperature shift conditions and compared to the effect in a temperature-shocked WT strain. The ΔdegP strain was acutely more sensitive to increasing levels of NlpA expression (basal expression vs. IPTG induced)(Fig. 8D). We therefore concluded that strains deficient in envelope protease activity are sensitive to non-native (presumably too high) levels of NlpA.
Figure 8: Complementation of the ΔnlpAΔdegP temperature-shift growth defects by pDegP and pNlpA.

(A) Indicated strains of bacteria containing either vector (pCS19), DegP, or mDegP-encoding plasmids (pDegP, pmDegP, respectively) were grown in LB overnight at 37°C, inoculated into fresh LB (t=0), and then grown at 40°C. OD₆₀₀ was measured hourly. Error bars indicate SEM; n=4. (B) Indicated strains of bacteria containing either vector (pTrc99A) or NlpA-encoding plasmid (pNlpA) were grown in LB overnight at 37°C, inoculated into fresh LB (t=0), and then grown at 40°C. OD₆₀₀ was measured hourly. Error bars indicate SEM; n=4. (C) To verify that pNlpA expressed a functional protein, the indicated strains were grown in LB overnight at 37°C, inoculated into D-methionine and glucose-supplemented minimal media (t=0), and then grown at 40°C. OD₆₀₀ was measured hourly. Error bars indicate SEM; n=3. (D) Indicated strains of bacteria containing either vector (pTrc99A) or NlpA-encoding plasmid (pNlpA) were grown in LB overnight at 37°C, inoculated into fresh LB (t=0), and then grown at 40°C. IPTG concentrations used to induce NlpA at t=0 h are indicated, if applicable. OD₆₀₀ was measured hourly. Error bars indicate SEM; n=3. Reproduced from (Schwechheimer and Kuehn, 2013).
In light of these data, we decided to assess pNlpA complementation under somewhat less stressful conditions, by using cultures which had become adapted to high temperature growth, rather than immediately upon heat shock. First, we needed to ascertain whether ΔnlpAΔdegP exhibited a growth defect under these conditions. As compared with the temperature-shifted cultures, a reproducible, but somewhat reduced growth defect with somewhat different kinetics was observed for the heat-adapted cultures of ΔnlpAΔdegP (Figs 9A and 5C, compared with Fig 4A). To establish if the difference in growth curve shape between the shifted and adapted cultures originates solely from heat shock or the coincident heat shock and recovery from stationary phase, we conducted an experiment in which the cells were grown for two hours after inoculation into fresh media at 37°C before shifting to 40°C. The growth curve shape of the double mutant was similar as when shifted directly into fresh 40°C media, but less severe (Fig. 10A). The culture leveled out at an OD$_{600}$ of ~0.6 instead of ~0.4. This is to be expected since the culture was at a higher OD$_{600}$ when it was heat shocked. Furthermore, the growth phenotype of the double mutant became even more severe, leveling out at an OD$_{600}$ of ~0.3, when the cultures were shifted from overnight growth at 30°C into fresh 40°C media (Fig. 10B). These results suggest that the more extreme the heat shock, the lower the OD$_{600}$ when the double mutant growth arrests, and that the recovery from stationary phase has an additional exacerbating effect. The data support the hypothesis that the temperature-sensitive growth defect was related to acute protein misfolding stress.

We then assessed complementation of the heat-adapted ΔnlpAΔdegP cultures. As also seen for the shifted cultures, growth of heat-adapted ΔnlpAΔdegP cultures could be corrected by complementation with basal expression of pDegP (Fig 9B). To ascertain if
increased amounts of the DegP chaperone (pmDegP) would complement the growth defect, we induced expression with 100 μM IPTG, complementation still required the protease activity of DegP (Fig 9C). Finally, we examined complementation by pNlpA. Induced expression of NlpA from the plasmid was effective in complementing, rather than exacerbating, the growth defect for the heat-adapted cultures (compare Fig 9D with Fig 8B) and IPTG had no toxic side effects. Together these data demonstrated that plasmid-expressed NlpA could complement the ΔnlpAΔdegP growth defect under less acute denaturing conditions.

With the successful complementation using pNlpA, we could next investigate whether methionine binding by NlpA is necessary for its role in OMV production using a plasmid expressing mutant variant of NlpA (mNlpA) that cannot bind methionine. The crystal structure of the NlpA homolog, Tp32, from *Treponema pallidum*, was utilized to identify two residues, Asn 116 and Arg 119, which directly hydrogen bond methionine (Deka et al., 2004). These residues are conserved as Asn 142 and Arg 145 in NlpA. We then designed an expression plasmid for NlpA (pmNlpA) harboring alanine substitutions in these residues. We established that mNlpA could not bind methionine by the fact that basal level expression of pmNlpA could not complement the methionine transport defect of ΔnlpAΔmetQΔmetE (Fig. 9E). The ΔnlpAΔdegP growth defect, however, could be complemented by basal level expression of pmNlpA (Fig. 9D), suggesting that NlpA’s methionine binding ability is not required in OMV production.
Figure 9: Complementation of the ΔnlpAΔdegP high temperature growth defect.

(A) Indicated strains containing vector plasmid (pTrc99A) were grown in LB overnight at 40°C, inoculated into fresh media (t=0), and grown at 40°C. OD$_{600}$ was measured hourly. Error bars indicate SEM; n=2. (B) Indicated strains containing vector (pCS19) or DegP-encoding plasmids (pDegP) were grown in LB overnight at 40°C, inoculated into fresh media (t=0), and grown at 40°C. OD$_{600}$ was measured hourly. Error bars indicate SEM; n=3. (C) Indicated strains containing vector (pCS19), or DegP-encoding plasmids (pDegP, mDegP) were grown in LB overnight at 40°C, inoculated into fresh media (t=0), and grown at 40°C. IPTG concentrations used to induce DegP at t=0 h are indicated, if applicable. OD$_{600}$ was measured hourly. Error bars indicate SEM;
n=3. (D) Indicated strains containing vector (pTrc99A) or NlpA-encoding plasmids (pNlpA, pmNlpA) were grown in LB overnight at 40°C, inoculated into fresh media (t=0), and grown at 40°C. IPTG concentrations used to induce NlpA at t=0 h are indicated, if applicable. OD$_{600}$ was measured hourly. Error bars indicate SEM; n=2. (E) To verify that pmNlpA expressed a protein impaired in methionine binding, the indicated strains were grown in LB overnight at 37°C, inoculated into D-methionine and glucose-supplemented minimal media (t=0), and grown at 37°C. OD$_{600}$ was measured hourly. Error bars indicate SEM; n=3. (F) To verify the methionine independent growth defect of ΔnlpAΔdegP, the indicated strains were grown in LB overnight at 37°C, inoculated into glucose-supplemented minimal media (t=0), and grown at 40°C. OD$_{600}$ was measured hourly. Error bars indicate SEM; n=3. Growth was monitored for three days (no nights) and the graph shown here exhibits the monitored growth during these three days and eliminating the lines for the time no OD$_{600}$ was measured. Reproduced from (Schwechheimer and Kuehn, 2013).

We further substantiated the absence of a role for methionine in the nlpA phenotypes by investigating the growth of the strains in media lacking methionine. If a defect in methionine transport were critical to the growth defect of ΔnlpAΔdegP, we would expect ΔdegP to grow equally poorly as ΔnlpAΔdegP in methionine-free media. Instead, we found the growth defect of the double mutant persisted in minimal media, whereas ΔdegP grew more similar to WT (Fig. 9F). The growth of the double mutant was more comparable to WT during early-log growth, but then stopped earlier, reaching a maximum OD$_{600}$ of ~ 0.5. The modest defect observed previously for this strain in log phase growth in LB media is possibly suppressed by the overall slower growth rate in minimal media (compare log phase growth, Fig. 9F and Fig. 4A). The results from both the mutant NlpA and methionine-free media experiments are consistent with the conclusion that NlpA’s role in OMV production is methionine independent.
Figure 10: The leveling of the Δ\textit{nlpA}Δ\textit{degP} growth curve depends on the temperature shift.

(A) Indicated strains were grown in LB overnight at 37°C, inoculated into fresh media (t=0), grown for 2 h at 37°C, and then shifted to 40°C. OD\textsubscript{600} was measured hourly. Error bars indicate SEM; n=6. (B) Indicated strains were grown in LB overnight at 30°C, inoculated into fresh media (t=0), and shifted to 40°C for 7 h. OD\textsubscript{600} was measured hourly. Error bars indicate SEM; n=3. Reproduced from (Schwechheimer and Kuehn, 2013).

\textit{ΔdegP} causes accumulation of periplasmic protein and misfolded OMPs which are OMV cargo. In order to test if the \textit{ΔnlpAΔdegP} growth defect originated from an overloaded periplasm unable to both effectively degrade proteins due to the \textit{degP} deletion and release accumulated, toxic proteins via OMVs because of the \textit{nlpA} deletion,
we first needed to assess whether periplasmic protein accumulates in the ΔdegP strain. DegP is a protease/chaperone that is critical in stressful conditions, presumably to rid the periplasm of toxic product, however, it has never been shown that periplasmic protein actually accumulates in a strain lacking degP. We compared periplasmic protein densities using a Bradford assay to quantitate the amount of protein in periplasmic preparations from equivalent amounts of cells grown at 40°C. The periplasmic preparation of the ΔdegP strain contained significantly higher amounts of protein than the periplasm from the ΔdegP/pDegP strain expressing basal levels of DegP (Fig. 11A). Complementation with pmDegP, which encodes DegP lacking protease activity, did not reduce the periplasmic protein amount of ΔdegP (Fig. 11A), suggesting that the chaperone activity of DegP was insufficient to prevent periplasmic protein accumulation caused by ΔdegP.

Next, we examined if the double mutant exhibits a higher level of protein accumulation due to the hypovesiculation with respect to ΔdegP (Fig. 4B). Periplasm isolated from ΔdegP contained substantially and significantly more protein than WT, and periplasm from ΔnlpAΔdegP exhibited a slight, but significant, increase in protein than ΔdegP periplasm (Fig 11B). Periplasm purified from ΔnlpA did not exhibit an increased protein amount compared to WT (data not shown), consistent with the unaffected growth phenotype of the ΔnlpA mutant (Fig 4A), but not with its vesiculation defect (Fig 4B). This could be because there is DegP in this strain and it is able to degrade any accumulated protein. Together, these results support our model that for ΔdegP, OMVs eliminate some of the toxic protein products that would normally be degraded by DegP’s protease activity, and that a consequence of the ΔnlpA mutation in a ΔdegP background is the increased buildup of envelope proteins in the periplasm.
To ensure that the increase in periplasmic amount of ΔdegP and ΔnlpAΔdegP was not contaminated with cytoplasmic protein, we compared their protein spectrum to the WT purified periplasm. No abundant set of new bands were visible in the mutants (Fig. 12A). To further investigate this, we compared the protein profile of purified WT, ΔnlpA, ΔdegP and ΔnlpAΔdegP OMVs (Fig. 12B). No substantial differences in the protein species were detected in the OMVs either, suggesting that the periplasm of ΔdegP and ΔnlpAΔdegP contain the typical cohort of proteins, however in abnormally high amounts, as would be expected when eliminating a major periplasmic chaperone/protease. However, as a one dimensional SDS-PAGE gel is a rather crude detection method, a more sensitive assay is required to determine whether more subtle enrichment of particular proteins occurs.

To directly investigate the idea that OMVs are an outlet that can relieve periplasmic overloading, we determined whether native DegP substrates, the OMPs (Krojer et al., 2008), could be detected in the lumen of ΔdegP OMVs. Folded OMPs are integral OM proteins with surface-exposed domains, whereas we reasoned that misfolded OMPs are not surface-exposed since they would accumulate in the periplasm (and consequently in the lumen of OMVs). Freire et al. used Texas Red-X succinimidyl ester, a membrane impermeable dye that reacts with primary amines, to label the surface exposed components of OMPs (Freire et al., 2006). We anticipated that in a WT strain, nearly all of the OMPs would be folded with surface-exposed domains. By contrast, in a ΔdegP strain, the fraction of surface-exposed OMPs (folded) to total OMPs (folded and misfolded) would be lower in the OMVs. This is indeed what we found: the ratio of surface stained vs. total OMPs was reduced in a degP deletion background with respect to WT (Fig. 11C). We examined two other overvesiculation mutants (McBroom et al.,
2006; Schwechheimer et al., 2013) to ensure that this observed ratio decrease is not a general hypervesciculation phenomenon but specific to ΔdegP, and this, indeed, turned out to be the case (Fig. 13A). Additionally, we observed a ΔdegP specific increase in misfolded OMPs in a gel shift assay that allows for the preservation of the native conformation of OMPs (Fig. 13B, C) (Schweizer et al., 1978). It should be noted here that even though we could detect these misfolded OMPs in the vesicle lumen, total OMP expression in this mutant has been previously shown to be similar to WT (McBroom et al., 2006). Therefore, native DegP substrates constitute waste products eliminated in the OMVs. Together, these data support the overall concept that ΔdegP periplasm contains substantially high levels of misfolded protein which cause toxicity that can be alleviated by the production of OMVs.
Figure 11: Periplasmic protein content correlates with altered OMV production, misfolded OMPs accumulate in ΔdegP OMVs and the defects depend on DegP protease activity.

(A) Indicated strains containing vector (pCS19) or DegP-encoding plasmids (pDegP, pmDegP) were grown ~16-18 hrs in LB at 40°C. Periplasmic protein concentration in the preparations was determined by Bradford Assay. Error bars indicate SEM; * p < 0.001; n=4. (B) Strains were grown overnight in LB at 40°C, and periplasm was prepared and quantitated as in part A. Error bars indicate SEM; * p <
0.05; **, p < 0.001; n=9. (C) Surface exposed OMPs of purified OMVs (stationary phase OMVs from cells grown at 40°C) from WT and ΔdegP were labeled with the membrane impermeable dye Texas Red-X succinimidyl ester, total OMV OMPs were determined by Ruby stain with densitometry of SDS-PAGE and the ration was normalized to WT. Error indicates SEM; *, p < 0.005, n=4. Adapted from (Schwechheimer and Kuehn, 2013).

Figure 12: Protein profile of periplasmic fractions and OMVs.

Cultures were grown overnight, ~ 16 hrs, at 40°C. Purified periplasm (A) and OMV (B) samples from the indicated strains were loaded by equal protein concentration on SDS-PAGE and the gels Ruby stained. Reproduced from (Schwechheimer and Kuehn, 2013).
Figure 13: ΔdegP-specific, misfolded OMP accumulation.
(A) Surface exposed OMPs in purified OMVs from WT and hypervesiculating mutant cultures (stationary phase, 37°C) were labeled with the membrane impermeable dye, Texas Red-X succinimidyl ester. Total OMV OMPs were determined by Ruby stain densitometry of SDS-PAGE. The ratios of Texas Red:Ruby stained values were divided by the ratio for the WT. Error indicates SEM, n=2. (B) Purified OMVs from WT, ΔdegP (stationary phase, 40°C), and hypervesiculating mutant cultures (stationary phase, 37°C). (C) Purified OMVs from WT, ΔdegP (stationary phase, 40°C), were either prepared with native or denaturing sample buffer and equal protein amounts based on A_{280} were separated by SDS-PAGE, Ruby-stained bands corresponding to misfolded OMPs, present in the native preparations, indicated by the asterisk, were quantified by densitometry and normalized to the amount in native preparations of the WT. The denatured samples and samples of ΔompR and ΔompA were used to ensure quantification of the appropriate bands. Error indicates SEM; *, p < 0.05; WT, ΔdegP: n=4; ΔnlpI, ΔycfSΔybiSΔerfK: n=2. Reproduced from (Schwechheimer and Kuehn, 2013).

Other hypovesiculation mutations also have synthetic defects with ΔdegP. We further examined the synthetic effects of ΔdegP with some other hypovesiculation mutations, ΔdsbA and ΔbolA, which came out of a high throughput screen of single gene mutants with OMV phenotypes (A. Kulp, A. Manning, B. Sun, T. Ai, A. Schmidt, & M. Kuehn, unpublished data), to further investigate the hypothesis that reduced OMV production in a degP deletion background causes growth arrest. DsbA is a disulfide oxidoreductase that aids in periplasmic protein folding by inducing disulfide bonds (Bardwell et al., 1993; Kamitani et al., 1992). BolA is thought to regulate morphology changes as well as increased resistance to antibiotics and detergents in stationary phase and under conditions of stress (Freire et al., 2009; Freire et al., 2006; Santos et al., 1999; Santos et al., 2002). Both double mutants, ΔdsbAΔdegP and ΔbolAΔdegP, exhibited a synthetic growth defect comparable with ΔnlpAΔdegP under temperature shift conditions (Fig. 14A, C, compare with Fig. 4A). Additionally, the growth defect again correlated with reduced amounts of OMV production of the double mutants with respect to ΔdegP (Fig. 14B, D, compare with Fig. 4B). We also investigated these strains for cytoplasmic leakage to exclude cell lysis. As seen previously, hypervesiculation does
not correlate with increased luminescence (Fig. 14E). It should be noted that the luminescence data suggests that ΔnlpAΔdegP and ΔdsbAΔdegP have a more permeable envelope than the single mutants (Fig. 6A and Fig. 14E) and thus OMV production may be even lower than what our quantitation suggests (Fig. 4B and Fig. 14B). This is not a general trend, however, since ΔbolAΔdegP had a similar low luminescence signal as ΔdegP (Fig. 14E). These results further confirm that the combination of a mutation which reduces vesiculation and a mutation which increases vesiculation and periplasmic protein density results in a growth defect. We must stress here that this was not found to be the case for any hypovesiculation mutation combined with a degP deletion. For instance, there was no effect observed when two previously identified hypovesiculation mutations, ΔampG or ΔglnA, were combined with ΔdegP (unpublished data). Whereas this could be explained in the case of ΔampG, which we discovered produced fewer OMVs than WT at 37°C but not at 40°C, the hypovesiculation phenotype of ΔglnA persisted at 40°C (unpublished data). At this point, OMV production by these strains is not characterized well enough to understand why certain hypervesiculation mutations reduce OMV production and others do not.
Figure 14: Other undervesculation mutations have a comparable synthetic defect with ΔdegP as ΔnlpAΔdegP.
(A, C) Indicated strains of bacteria were grown in LB overnight at 37°C, inoculated into fresh LB (t=0), and then grown at 40°C. OD<sub>600</sub> was measured hourly. Error bars indicate SEM; n=6. (B, D) Relative fold OMV production in cultures of the indicated strains grown in LB overnight at 40°C was determined by quantitating OMVs, normalizing to OD<sub>600</sub> and dividing by OD<sub>600</sub>-normalized OMV production in a WT culture. Error bars indicate SEM; (B) *, p < 0.05; **, p < 0.0005; WT, ΔdsbA: n=3, ΔdegP: n=4, ΔdsbAΔdegP: n=7; (D) *, p <0.01; **, p < 0.0005; WT: n=2, ΔbolA, ΔdegP: n=3, ΔbolAΔdegP: n=4. (E) Luminescence of the indicated strains grown in LB for ~16 hrs at 40°C was determined by normalizing to OD<sub>600</sub>. Error bars indicate SEM; WT, ΔdegP, ΔdsbA, ΔdsbAΔdegP: n=4; ΔbolA, ΔbolAΔdegP: n=3. Reproduced from (Schwechheimer and Kuehn, 2013).

2.4 Discussion

The critical role OMVs play in pathogenesis has been and continues to be studied quite intensively, but besides their contribution to virulence, roles for OMVs that would benefit the originating organisms, particularly non-pathogens, have only been addressed minimally. Here, we elucidate a conditional growth advantage for bacteria that are able to produce OMVs. We have shown that mutations in both degP, which affects the bacterial response to envelope stress, and either nlpA, dsbA or bolA, which affect OMV production, lead to a synthetic genetic growth defect under conditions where envelope relief becomes critical. In the case of nlpA, the synthetic defect was shown to correlate with accumulation of protein in the periplasm. NlpA supports both OMV production and methionine import, however the methionine binding ability of NlpA was dispensable for its role in OMV production. Evidence that misfolded OMPs accumulate in ΔdegP OMVs and that reduced levels of OMV production correlates with toxicity for the double mutants suggests that OMVs are a critical outlet for accumulated toxic, envelope protein products upon exposure to a denaturing stress.

A relationship between the accumulation of protein in the periplasm and OMV production. It was previously reported that the lethality of ΔdegP at 42°C could
be rescued by an additional mutation in \textit{lpp}, which acts as an envelope “staple,” forming links between the OM to PG (Braun, 1975; Braun and Rehn, 1969; Strauch et al., 1989). This mutation caused strong hypervesciculation along with a membrane permeability defect (Cascales et al., 2002), implying that the accumulation of misfolded protein in the periplasm can cause toxicity and that relief could arise by efflux of the toxic material via OMVs as well as leaky membranes. Further, a hypervesciculating mutation was discovered to suppress the toxicity of a heterologously expressed periplasmic protein (McBroom and Kuehn, 2007). We add to these findings by demonstrating that in the absence of \textit{degP}, a substantial amount of protein accumulates in the periplasm upon heat shock, and thus that DegP is responsible for managing a significant amount of envelope protein. That misfolded OMPs accumulate in \textit{ΔdegP} OMVs and that the protease activity of DegP is sufficient to reduce the proteinaceous accumulation in the \textit{degP} mutant strain confirms that the material is composed of DegP substrates. Additionally, the accumulation of that endogenous protein becomes toxic when OMV production is also reduced. Thus, we show that OMV production acts in concert with DegP to relieve the periplasm of toxic consequences of envelope stress.

Our results also provide a more subtle assessment in comparing periplasmic overcrowding relief by DegP and OMVs. The lack of a substantial growth defect for the \textit{ΔdegP} strain during constitutive, unstressed conditions suggests OMV production is sufficient to relieve periplasmic overcrowding. The addition of the \textit{ΔnlpA} mutation modestly, albeit statistically significantly, increased the periplasmic accumulation of protein in the \textit{ΔdegP} strain in comparison to the relatively larger decrease in vesiculation (compare Figs. 4B and 11B). The lack of a fully inverse correlation of these phenotypes may be explained if the periplasmic capacity of \textit{ΔnlpAΔdegP} has reached a maximum
level due to the lack of the OMV-mediated relief. To the best of our knowledge, the periplasmic capacity as of yet has not been established, and it may be a technically challenging endeavor to do so. Regardless, the growth defect of this strain (Fig. 4A) supports the hypothesis that upon reaching this maximum periplasmic capacity, the result is toxic, signifying that the OMV stress response is a critical pathway in bacterial viability during stress.

**Why is NlpA complementation successful at constant high temperature but not upon heat shock?** NlpA complementation has been rather challenging to interpret since it was not successful under all conditions. Plasmid-expressed NlpA failed to complement under conditions where the cells underwent a rapid heat shock, but could complement the growth defect of cultures kept at constant high temperature. From these data, we concluded that there must be differences in the envelope of heat-shocked cells compared to those acclimated to high temperature. The most obvious culprit is the consequence of the $\sigma^E$ envelope heat shock response (Dartigalongue et al., 2001; Gottesman, 2004; Rhodius et al., 2006). In the shift experiment, the bacteria are undergoing a dramatic transition in the state of the envelope. The temperature shift would cause a sudden bolus of misfolded OMPs and periplasmic proteins in the envelope and, as a result of the stress response, also a burst of upregulated proteases and chaperones. By contrast, cells acclimated to growth at high temperature are accustomed to the long-term consequences of $\sigma^E$ regulon activation, with high levels of active proteases and chaperones along with down-regulated OMPs. In this scenario, the periplasm is most likely “cleaner” due to the rescue, degradation, or simply lower number of misfolded membrane and periplasmic proteins.
Consequently, we propose a scenario in which NlpA can complement during acclimation but not heat shock: The extremely crowded periplasm during heat shock may interfere with whatever function the protein fulfills (e.g. localization of budding components). This situation is plausible for several reasons. The absence of DegP causes a substantial increase in misfolded proteinaceous material in the periplasm (Fig. 11, 13B, 13C). Additionally, the chaperone activity of DegP was insufficient for complementation under these conditions (Fig. 8A, 9C and 11A). Finally, the higher the induction of NlpA, the worse the observed growth phenotype (Fig. 8D), suggesting that over-expressed NlpA is adding to the problem (e.g. accumulating proteinaceous garbage) under these conditions. By contrast, the “cleaner” periplasm of the heat-acclimated cells may allow for the observed functional complementation.

**Hypovesiculation in the context of ΔdegP.** Our results show that the detrimental effect of a hypovesiculation mutation in a degP deletion background is not solely ΔnlpA-specific, further supporting the hypothesis that reduced OMV production is the source of the defect. As of yet, we do not understand the role of BolA in OMV production but it should be noted that it is expressed at times when the maximum amount of OMV production is observed (Chatterjee and Das, 1967; Gamazo and Moriyon, 1987; Hoekstra et al., 1976). It is difficult to pinpoint the role of DsbA in OMV production due to its role as a general periplasmic folding factor (Shouldice et al., 2011). One of the DsbA downstream targets is likely to be the cause of the reduction in OMVs, since the deletion mutant of the factor responsible for DsbA recycling, DsbB (Bardwell et al., 1993), also acts as a hypovesiculator (A. Kulp, A. Manning, B. Sun, T. Ai, A. Schmidt, & M. Kuehn, unpublished data). We hypothesize that when a mutation is dominant over the ΔdegP hypovesiculation, the gene product may be in one way or another
mechanically involved in OMV production. These genes will be of particular interest to fully understand the mechanism of vesiculation.

In sum, these experiments have exposed a critical function for OMV production in general bacterial viability. Further experiments are necessary to determine how stress-induced periplasmic protein accumulation is sensed, in order to more fully understand how these factors contribute to optimal bacterial growth.

2.5 Acknowledgements

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3. Characterization of the role of the inner membrane lipoprotein NlpA in vesiculation

3.1 Summary

The data described in Chapter 2 suggest that NlpA may act as a mechanistic or regulatory component of OMV production. When we further examined the role of NlpA in this process, we discovered it acts at the end of the logarithmic growth phase and at the beginning of stationary phase in a WT as well as a degP deletion background. Notably, this is also the time when OMV production is most prevalent. Protein accumulation in the periplasm is not detectably different for ΔdegP and ΔnlpAΔdegP in log phase, further indicting that the reduction in OMV production leads to envelope protein waste build-up under conditions of misfolded protein waste. NlpA has very strong homology (81%) to the periplasmic methionine binding protein MetQ, which led us to investigate if MetQ also plays a role in vesiculation. The data presented here demonstrate that NlpA and MetQ can both fulfill the same roles in methionine import and OMV production, but NlpA appears more specialized in OMV production whereas MetQ functions primarily in methionine import. Lastly, we present evidence suggesting that both NlpA and MetQ add to the structural integrity of the envelope.

3.2 Introduction

Of all the vesiculation mutants identified by the transposon mutagenesis screen done by our lab (McBroom et al., 2006), we chose to further characterize the hypovesiculation mutant nlpA more closely for several reasons. Besides its minor role in methionine import (Fig. 8C, 9E) (Zhang et al., 2003), NlpA is a protein of unknown function. Transposon insertion or full deletion mutants of nlpA show no global defects,
except under conditions when vesiculation becomes critical in envelope accumulation relief to enable cellular growth as demonstrated in Chapter 2. These data indicate that the reduced level of OMV production is not a result of indirect effects but rather the result of a missing regulatory or mechanistic component of the vesiculation process. Finally, based on its location in the envelope as an inner membrane lipoprotein (Yu et al., 1986), NlpA may act as a functional component in OMV production.

MetQ is a very close relative of NlpA, with 81% homology (Uniprot, 2011). MetQ is the periplasmic binding protein of the methionine ABC transporter (Gal et al., 2002; Merlin et al., 2002). NlpA can support methionine transport in the absence of metQ (Zhang et al., 2003). However, the homologues differ in their transcriptional regulation. NlpA is positively regulated by CsgD (Ogasawara et al., 2011), a transcriptional regulator important for biofilm formation, a stationary phase phenomenon, which is induced during mid-log phase (Brombacher et al., 2003; Hammar et al., 1995). MetQ is controlled by MetJ. MetJ also controls the transcription of the other two units of the corresponding ABC-transporter and is regulated by the cellular methionine pool, implicating a critical role for this operon in methionine import (Ecocyc, 2011; Merlin et al., 2002). We investigated whether these highly homologous genes, nlpA and metQ, could fulfill the same role in OMV production.

3.3 Results

The ΔnlpA defect depends on growth phase. We noticed that the growth defect of the double mutant becomes reproducibly more severe after about 3 hours of growth, when the cells reach an OD600 of ~0.4 (Fig. 4A), or in minimal media when the cells reach an OD600 of ~0.5 (Fig. 9F), furthermore ΔnlpA exhibits a growth defect in the WT and ΔdegP background after 4 hours at 30°C, when the cells also reach an OD600 of ~0.4
(Fig. 5A). These data suggested that the ΔnlpA defect may be growth phase-dependent. To investigate this hypothesis, we first quantified OMVs produced only during log phase growth. ΔnlpA and ΔnlpAΔdegP produced comparable amounts of OMVs during log phase as WT and ΔdegP, respectively (Fig. 15A). Thus, there is no observable OMV production defect with respect to ΔnlpA during log phase growth. However, when we quantified OMVs produced only in stationary phase, we observed the previously described, significantly reduced vesiculation phenotypes for ΔnlpA and ΔnlpAΔdegP compared with WT and ΔdegP, respectively (Fig. 15B). These data suggest that the loss of nlpA has growth phase-dependent consequences, and that these are most severe during late-log and stationary phase.

We again wanted to ensure that membrane integrity was not compromised in the mutants we were studying. Log phase WT was insensitive to Actinomycin D and Sytox Green, and ΔnlpA exhibited WT behavior during Sytox Green treatment (Fig 15C and D). However, despite the fact that ΔdegP and ΔnlpAΔdegP had comparable and significant log-phase vesiculation phenotypes (Fig. 15A), their sensitivity to Actinomycin D and Sytox Green was not similar (ΔdegP and ΔnlpAΔdegP were minimally Actinomycin D sensitive; but only ΔdegP, not ΔnlpAΔdegP, was affected by Sytox Green (Fig. 15C and D)).

Next, we further examined the growth phase-dependent effect of the ΔnlpA mutation on periplasmic protein amount with the periplasmic protein accumulation assay described above. As expected from their comparable levels of log phase OMV production (Fig. 15A), no difference in the amount of periplasmic protein was observed for ΔdegP and the double mutant strain for log phase cultures (Fig. 15E). (The periplasmic protein content of WT and ΔnlpA during log phase could not be evaluated.
as these were below the detection level of this assay). This result contrasted with the significant difference in periplasmic protein observed in these cultures when grown to stationary phase (Fig 11B). These data support our hypothesis that the loss of *nlpA* has growth phase-dependent consequences, and that these are most severe during late-log and stationary phase.
Figure 15: The $\Delta nlpA$ defect is growth phase dependent.
(A) Relative fold OMV production in log phase cultures of the indicated strains, grown in LB to an OD$_{600}$ of ~ 0.4 at 40°C, was determined by quantitating OMVs, normalizing to OD$_{600}$ and dividing by OD$_{600}$-normalized OMV production in a WT culture. Error bars indicate SEM; WT: n=3; ΔnlpA: n=4; ΔdegP: n=5; ΔnlpAΔdegP: n=6. (B) Relative fold OMV production in stationary phase cultures of the indicated strains grown in LB overnight at 40°C, after discarding log phase-produced OMVs at ~24 hours, was determined as in part A. Error bars indicate SEM; *, p < 0.005; WT: n=3; ΔnlpA, ΔdegP, ΔnlpAΔdegP: n=5. (C) Indicated strains of bacteria were grown in LB overnight, inoculated into fresh LB (t=0), and grown at 40°C. OD$_{600}$ was measured hourly. (+) indicates the cultures containing Actinomycin D, which was added at t=0. Error bars indicate SEM; n=2. (D) Relative Sytox Green fluorescence in cultures of the indicated strains grown in LB to an OD$_{600}$ of ~ 0.4 at 40°C was determined by normalizing to OD$_{600}$ and dividing by the OD$_{600}$-normalized fluorescence of the WT culture. Error bars indicate SEM; n=2. (E) Strains were grown to an OD$_{600}$ of ~ 0.4, in LB at 40°C, and periplasmic protein concentration in the preparations was determined by Bradford Assay. Error bars indicate SEM; ΔdegP: n= 8; ΔnlpAΔdegP: n=6. Adapted from (Schwechheimer and Kuehn, 2013).

NlpA and MetQ can function redundantly, but perform distinct tasks when both are present. A mutation in the gene encoding MetQ, a close homologue of NlpA, was not identified in the original transposon screen (McBroom et al., 2006), consequently, we expected that MetQ may not behave similarly to NlpA in terms of OMV production (although there was a possibility it was missed because it was not a saturating screen). In order to investigate MetQ functions in OMV production, we first tested the OMV phenotype of ΔmetQ. For cultures grown at 37°C, ΔmetQ actually slightly hypervesiculated, but when the cultures were grown at 40°C, ΔmetQ hypovesiculated, like ΔnlpA (Fig 16A). We also tested the double mutant, ΔnlpAΔmetQ, for a synthetic effect and did not find one at either temperature, demonstrating that these homologues are not epistatic for vesiculation (Fig. 16A). Thus, the lack of metQ results in a vesiculation defect similar to that of cells without nlpA only when the bacteria are somewhat stressed (growth at 40°C), however the gene functions for metQ
and \textit{nlpA} do not completely overlap since they exhibit different phenotypes when grown at 37°C.

We next investigated whether \textit{metQ} would behave like \textit{nlpA} in genetic interactions with \textit{ΔdegP}. We constructed a \textit{ΔmetQΔdegP} strain and conducted the 37°C to 40°C temperature shift experiment to check growth and quantitate OMV production. We found that \textit{ΔmetQΔdegP}, indeed, had a growth defect compared with either single mutant, although it is noteworthy that the growth defect of \textit{ΔnlpAΔdegP} was more severe (Fig 16B). This difference was exacerbated when the double mutant cultures experienced a 30°C to 40°C temperature shift (Fig. 16C), similar to what we observed in Chapter 2 (Fig. 10B). Furthermore, similar to \textit{ΔnlpAΔdegP}, \textit{ΔmetQΔdegP} also exhibited significantly reduced levels of vesiculation, in comparison to \textit{ΔdegP} (Fig. 16D). Lastly, we investigated whether \textit{ΔmetQ} phenotypes would also be growth phase dependent as was the case for \textit{ΔnlpA} (Fig. 15) (Schwechheimer and Kuehn, 2013). NlpA and MetQ behave differently during log phase OMV production, where \textit{ΔnlpA} showed no OMV defects (Fig. 15A), \textit{ΔmetQ} is dominant over \textit{ΔdegP} (Fig. 16E). Together, these data suggested that NlpA and Met Q both play roles in vesiculation, but that these are somewhat different roles.
Figure 16: NlpA and MetQ have conserved as well as specialized functions.

(A) Relative fold OMV production in cultures of the indicated strains grown in LB overnight at 37°C or 40°C was determined by quantitating OMVs, normalizing to
OD$_{600}$, and dividing by OMV production in a WT culture. Error bars indicate SEM. 37°C, n=3; 40°C, n=4. (B) Indicated strains of bacteria were grown in LB overnight at 37°C, inoculated into fresh LB, and then grown at 40°C. OD$_{600}$ was measured hourly. Error bars indicate SEM; n=3. (C) Indicated strains of bacteria were grown in LB overnight at 30°C, inoculated into fresh LB, and then grown at 40°C. OD$_{600}$ was measured hourly. Error bars indicate SEM; n=3. (D) Relative fold OMV production in cultures of the indicated strains grown in LB overnight at 40°C was determined by quantitating OMVs, normalizing to OD$_{600}$ and dividing by OMV production in a WT culture. Error bars indicate SEM. *, p < 0.05; **, p < 0.01; WT, ΔdegP; ΔnlpAΔdegP, n=10; ΔmetQΔdegP, n=5. (E) Relative fold OMV production in log phase cultures of the indicated strains, grown in LB to an OD$_{600}$ of ~ 0.4 at 40°C, was determined by quantitating OMVs, normalizing to OD$_{600}$ and dividing by OD$_{600}$-normalized OMV production in a WT culture. Error bars indicate SEM; *, p < 0.05; WT, ΔmetQΔdegP: n=6; ΔmetQ, ΔdegP: n=3.

**NlpA and MetQ appear to function as putative scaffolding elements within the envelope.** Genetic evidence suggested an important role for NlpA in OMV production, as ΔnlpA had a dominant effect on a diverse set of hypervesiculating mutants, but how this IM protein functions in an OM budding event remained unclear. To investigate a potential structural role of NlpA in OMV budding, we tested the effect of ΔnlpA on OMV production by a mutant that contains only “free Lpp” (ΔubiSΔycfSΔerf) (Magnet et al., 2007). This triple mutant is impaired in the formation of covalent PG-OM Lpp crosslinks (ΔubiSΔycfSΔerfK) and has a hypervesiculation phenotype, as expected from a strain lacking these connections (Fig.18A) (Schwechheimer et al., 2013). Notably, the deletion of nlpA led to an increase in OMV production in that background, the first time we ever observed such an effect by ΔnlpA (Fig.17A). Since we previously demonstrated ΔnlpA’s role in stationary phase (Fig. 15B) (Schwechheimer and Kuehn, 2013), we were curious to determine if this is also the case in the ΔubiSΔycfSΔerfK background. To test this, we quantified OMV production in log phase only and were surprised to find that ΔnlpA did increase vesiculation even in log phase (Fig. 17B). These results led us to hypothesize a structural role of NlpA within the envelope. We further
tested if the deletion of \textit{nlpA} is also dominant over \textit{ΔompA}, as it was previously shown with genetic and crosslinking studies that there is an interaction between Lpp and OmpA, an OM β-barrel protein with a periplasmic domain (Choi et al., 1986; Pautsch and Schulz, 1998; Sonntag et al., 1978). Indeed, \textit{ΔnlpA} also exacerbated the \textit{ΔompA} hypervesiculation phenotype in overnight growth (Fig. 17C) and log phase cultures (Fig. 17D). These results led us to hypothesize a structural role of the IM lipid-anchored protein, NlpA, within the envelope: NlpA could provide an IM-based scaffolding site to stabilize the sites of Lpp- and OmpA-based envelope crosslinks. Further, we investigated and confirmed that \textit{ΔmetQ} also led to hypervesiculation in a free Lpp only mutant (\textit{ΔybiSΔycfSΔerfK}) (Fig. 17E), as well as in the \textit{ΔompA} deletion strain (Fig. 17F) in overnight, but not log phase cultures (Fig. 17F and 17H) suggesting that the two homologues play similar roles in stabilizing the envelope in stationary phase, but the loss of \textit{nlpA} causes a stronger effect in log phase.
Figure 17: ΔnlpA and ΔmetQ exacerbate hypervesculation in envelope crosslink mutants.
(A,C,E, and G) Relative fold OMV production in cultures of the indicated strains grown in LB overnight at 37°C was determined by quantitating OMVs, normalizing to OD_{600} and dividing by OD_{600}-normalized OMV production in a WT culture. Error bars indicate SEM. WT: n=2, ΔycfSΔybiSΔerfK: n=10, ΔycfSΔybiSΔerfKΔnlpA: n=5; *, p < 0.05; WT, ΔompA, ΔnlpAΔompA: n=4; *, p < 0.05; WT: n=12, ΔycfSΔybiSΔerfK: n=13, ΔycfSΔybiSΔerfKΔmetQ: n=16; *, p < 0.05; WT, ΔompA, ΔmetQΔompA: n=4; *, p=0.01. (B, D, F, and H) Relative fold OMV production in log phase cultures of the indicated strains, grown in LB to an OD_{600} of ~0.4 at 37°C, was determined by quantitating OMVs, normalizing to OD_{600} and dividing by OD_{600}-normalized OMV production in a ΔycfSΔybiSΔerfK or ΔompA culture. Error bars indicate SEM; ΔycfSΔybiSΔerfK, ΔycfSΔybiSΔerfKΔnlpA: n=4; *, p < 0.05; ΔompA, ΔnlpAΔompA: n=10; *, p < 0.05; ΔycfSΔybiSΔerfK, ΔycfSΔybiSΔerfKΔmetQ: n=8; ΔompA, ΔmetQΔompA: n=6. Reproduced from Schwechheimer, Kulp, and Kuehn, submitted.

**Lpp contributes more to OM integrity than covalent PG-OM crosslinks.** The results presented in Fig. 17A lead us to expect that ΔnlpA also would be dominant over the full lpp deletion mutant, but this turned out not to be the case (Fig. 18A), suggesting a difference in the architecture of the envelopes of the Lpp null (Δlpp) and the free Lpp (ΔybiSΔycfSΔerfK) mutants. These ΔnlpA-dependent differences lead us to quantify and compare OMV production by these lpp mutants directly. We found that OMV production by the mutant expressing free Lpp was elevated ~30-fold over that of the WT but was significantly lower than that of the full null mutant, Δlpp (Fig. 18B). These results suggest that the free form of OM- localized Lpp adds to membrane stability. We hypothesize that ΔnlpA had no effect on the full lpp deletion mutant because the envelope is so fragile that other envelope defects are negligible.
3.4 Discussion

Despite the critical biological role OMVs play, very little is understood about their production and regulation. By following up on the work presented in Chapter 2, we elucidate here the ΔnlpA stationary phase-specific hypovesiculation phenotype in a
WT and ΔdegP background. This is, rather intriguingly, a time in the bacterial life cycle where OMV production is highest (Chatterjee and Das, 1967; Gamazo and Moriyon, 1987; Hoekstra et al., 1976). Additionally, the protein accumulation defect of ΔnlpAΔdegP with respect to ΔdegP solely occurs in stationary phase, correlating with the time of OMV reduction. Furthermore, we identified the NlpA homologue, MetQ, to play a role in vesiculation under conditions of envelope stress such as high temperature and protease impairment. The data presented here suggest that NlpA as well as MetQ support both OMV production and methionine import, however MetQ is specialized for the import of the amino acid, whereas NlpA is more critical for vesiculation. The data further support a model in which both lipoproteins support vesiculation through interactions with envelope stabilizing factors. This work contributes to our overall understanding in OMV production by elucidating a growth phase-specific OMV production defect, as well as introducing new components and their putative roles as envelope scaffolding units in the vesiculation process, NlpA and MetQ.

**Growth phase dependence of NlpA.** It has been well-established that vesicle production varies with the bacterial life cycle (Kuehn and Kesty, 2005), but to the best of our knowledge, no growth phase dependent OMV production mutant has yet been identified, which makes ΔnlpA unique. Interestingly, it has been demonstrated for numerous species that the maximum rate of OMV production occurs at the end of log phase (Chatterjee and Das, 1967; Gamazo and Moriyon, 1987; Hoekstra et al., 1976), which is exactly where we found NlpA’s role begins to be critical. The growth phase-dependent phenotypes we observed here are consistent with NlpA’s transcription pattern: NlpA is positively regulated by CsgD (Ogasawara et al., 2011), a transcriptional regulator important for biofilm formation, a stationary phase phenomenon which is
induced during mid-log phase (Brombacher et al., 2003; Hammar et al., 1995).
Furthermore, it has been shown that CsgD itself it regulated by σ^{S} (Bougdour et al., 2006; Gualdi et al., 2007), the stationary phase σ factor, and that it can respond to cell density (Brombacher et al., 2003). It should be noted that the csg operons are usually induced during growth in minimal media and lower temperature but it has been shown that σ^{S} is induced during heat shock (Muffler et al., 1997), which would consequently lead to CsgD and NlpA expression. This supports the need for NlpA and in turn OMV production, in addition to DegP, as critical responses to heat shock.

**Potential roles for NlpA in OMV production.** NlpA’s location in the envelope suggests it could provide either a mechanistic or signaling role in OMV production. Most *E. coli* lipoproteins are anchored to the OM (Narita et al., 2004; Tokuda and Matsuyama, 2004). NlpA is unusual in that it is one of the minority of lipoproteins anchored to the IM, as well as one of the very few in the family of periplasmic binding proteins that appear to be membrane anchored (Tam and Saier, 1993). The atypical IM anchoring of NlpA indicates that location is likely to play a critical biological role.

NlpA’s restricted mobility in the membrane could contribute mechanistically to vesiculation. For example, NlpA could initiate the process at a particularly favorable location of the cellular envelope environment on the onset of stationary phase. As such, NlpA would serve as a membrane marker where other, as yet unidentified envelope proteins, assemble to form a complex that results in OMV budding and release. This model is consistent with the remaining levels of OMV production observed in the strains harboring the *nlpA* null mutation. Without this recruiting factor and/or location marker, the OMV production machinery could still assemble, but would produce OMVs less efficiently. NlpA may also function as a scaffolding element within the envelope. For
example, NlpA could initiate OMV formation by reducing its interactions with other envelope crosslinking components (e.g. bound Lpp, OmpA) and this dynamic interaction could be critical at particular times. Indeed, periplasmic expansion has been noted in the literature in stationary phase (Ishihama, 1997; Reeve et al., 1984; Siegele and Kolter, 1992), and the inability to expand (e.g. in a cell with overexpressed nlpA) would cause severe problems at this time, or for cells with impaired proteases and accumulated envelope protein (e.g. in a degP mutant). The observed undervesiculation phenotype for ΔnlpA may result from a compensatory tighter envelope linkage by an alternative factor.

Alternatively, the traditional functions of periplasmic binding proteins, substrate transport and chemotaxis (Tam and Saier, 1993), may play a role in vesiculation. As such, NlpA could act as a sensor, transmitting some kind of input signal to the machinery that stimulates OMV production in a growth phase specific manner, again by reducing envelope crosslinks. Complementation results using mNlpA suggests that this activity would be independent of its ability to bind methionine, but it could respond to binding of other substrates or polypeptides. In this scenario, residual levels of OMVs produced in the absence of nlpA may originate from a basal level of OMV release that maintains a continuous state of “housecleaning” of the envelope.

**Hypothesized functional specialization of NlpA and MetQ.** The NlpA and MetQ homologues are very similar in that they both help import methionine and both null mutants exhibit a growth defect in conjunction with ΔdegP, yet the mutants do exhibit sufficiently different phenotypes, supporting a model in which they specialize in distinct functions. One of the differences between the two genes is that metQ, not nlpA, is controlled by MetJ. MetJ also controls the transcription of the other two units of the corresponding ABC-transporter and is regulated by the cellular methionine pool,
implying a critical role for this operon in methionine import (Ecocyc, 2011; Merlin et al., 2002). By contrast, nlpA is monocistronic and not under the control of the MetJ repressor, suggesting merely a secondary role in nutrient acquisition (Ecocyc, 2011) but as mentioned earlier under the control of CsgD, a biofilm transcriptional regulator (Ogasawara et al., 2011). The role of MetQ as the primary periplasmic binding protein for methionine import is also supported by the greater defect under methionine-requiring conditions caused by ΔmetQ in comparison to ΔnlpA (Zhang et al., 2003) and (data not shown). Conversely, the data suggests that NlpA plays a more prominent role in OMV production than MetQ, as the growth defect caused of ΔnlpAΔdegP is more substantial than that exhibited by ΔmetQΔdegP (Fig. 16B, 16C).

The lack of a hypovesiculation phenotype and, in fact, the observation of a slight hypervesiculation phenotype of ΔmetQ at 37°C suggests that NlpA can be sufficient for OMV production (Fig. 16A). However, for cells in conditions where maintaining a “healthy envelope state” are more critical, NlpA activity is insufficient. In cases of elevated growth temperature or when the major protease/chaperone DegP is missing, we observed phenotypes associated with the loss of metQ, such as a growth defect (Fig. 16B) and reduced levels of OMV production (Fig. 16A, D). Therefore, we hypothesize that the effect we observe in the ΔmetQ and ΔmetQΔdegP mutants is predominantly due to an insufficient compensatory concentration of NlpA. This reasoning would also explain why we did not find a synthetic undervesiculation defect for the ΔnlpAΔmetQ double mutant compared with the ΔnlpA mutant (Fig 16A). Additionally, it should be noted that subtle contributions to OMV production by MetQ would be beyond the sensitivity of our assay.
We hypothesize that methionine binding of NlpA and potentially MetQ is not only unnecessary for, but, in fact, actually precludes their ability to participate in OMV production. This model is derived from the results showing that, compared with pNlpA, a lower level of induction of pmNlpA was required for complementation of ΔnlpAΔdegP (Fig. 9D). Less mNlpA may be necessary under these OMV-inducing conditions, since all of this form of the protein is available to aid in OMV production, whereas the ability of WT NlpA to bind methionine might prevent some of the protein from participating in OMV production.

When searching for homologs in other bacterial species we found that both NlpA and MetQ belong to the NlpA lipoprotein family (Uniprot, 2011) along with 13 other members. Interestingly, different organisms have different numbers of NlpA and MetQ homologous. A number of organisms have only one copy whereas Pasteurella haemolytica, for instance, carries three. We hypothesize that when only one copy is present, the same protein carries out both the role of NlpA and MetQ, whereas in P. haemolytica, perhaps even further specialization has taken place.

In sum, these experiments have exposed a critical function for OMV production in general bacterial viability. We propose a model in which MetQ, having a higher affinity for methionine (Zhang et al., 2003), is involved in methionine import, whereas the methionine-free form of NlpA is involved in OMV production. If the concentration of MetQ is insufficient (e.g. in a metQ null mutant) then NlpA is recruited to transport methionine and diverted from its role in OMV production. The unbound state of MetQ can participate in OMV production (e.g. in a nlpA null mutant) but in a WT cell, its role in OMV production may be negligible, especially since MetQ transcription is suppressed by MetJ when the cellular methionine concentration is adequate (Ecocyc, 2011; Merlin et
More experiments are necessary to gain further support for this model, as well as to identify binding partners of NlpA.

**Free Lpp contributes to membrane integrity.** The result that the free Lpp triple mutant and the lpp null mutant exhibited significantly different OMV phenotypes was surprising since Deatherage et al. examined Δlpp 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 (Deatherage et al., 2009). The reason behind these different outcomes is not completely clear, but a couple of points are noteworthy. Most obviously, the work was done in highly-related, but nonetheless, different bacterial species. Further, Cowles et al. has 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 (Cowles et al., 2011). 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 and any membrane stabilizing effect with it. Since free Lpp is a highly abundant protein, it is very plausible that its precise localization could substantially contribute to membrane stability.

### 3.5 Acknowledgements

We thank the National BioResource Project (NIG, Japan)::*E. coli* for the Keio Collection. This work was supported by NIH grants R01AI079068, R01GM099471, and Duke University Medical Center.
4. NlpI and Spr Modulate Outer Membrane Vesicle Production by Regulation of Peptidoglycan Remodeling

4.1 Summary

Previously, it was discovered that a transposon insertion in *nlpI* of *Escherichia coli* caused a hypervesiculation phenotype. Also, a genetic interaction between *nlpI* and *spr*, a peptidoglycan (PG) endopeptidase, had been previously identified. In this chapter, we present data suggesting that the hypervesiculation phenotype of the *nlpI* mutant is a result of increased PG turnover, which is accompanied by a significant decrease in covalent OM-PG envelope stabilizing crosslinks. Our data indicate that the increased PG metabolism is a consequence of rampant Spr activity, which in WT cells is directly or indirectly negatively regulated by NlpI. This work provides new insights into the mechanism and regulation of vesiculation and suggests that WT bacteria can control OMV production via PG metabolism and OM-PG crosslinking.

4.2 Introduction

Previously, our lab screened for and identified numerous transposon insertions with altered vesiculation phenotypes in *Escherichia coli* (McBroom et al., 2006). One very strong hypervesiculation mutant identified in this work was an effective deletion of *nlpI*. NlpI is an OM-anchored lipoprotein that has been associated with cell division but its actual function has not yet been identified (Ohara et al., 1999; Pierce et al., 2011). In this chapter, we were interested in understanding the reason behind the strong hypervesiculation phenotype of the *nlpI* mutant. In prior work, Tadokoro et al. found that temperature-sensitivity of a mutation in the putative peptidoglycan (PG) endopeptidase gene *spr* could be suppressed by an additional mutation in *nlpI*.
Spr is most likely also an OM lipoprotein based on sequence structure, but its lipidation has not been experimentally demonstrated (Uniprot, 2011). More recently, Singh et al. characterized Spr as a murein DD-endopeptidase (Singh et al., 2012).

The PG (or murein) net-like structure within the Gram-negative envelope is composed of glycan chains crosslinked by short peptides in the periplasm, encapsulating the hypertonic cytoplasm and protecting Gram-negative cells from lysis due to osmotic changes and mechanical stress (Vollmer and Bertsche, 2008). For envelope stability, PG is covalently crosslinked to the OM via the short OM-anchored lipoprotein Lpp (Braun and Rehn, 1969; Braun and Wolff, 1975). A deletion of lpp results in a fragile envelope structure associated with membrane shedding and cellular leakage (Braun and Wolff, 1975; Cascales et al., 2002; Deatherage et al., 2009).

With these genetic and biochemical connections in mind, we investigated OMV production of the ΔnlpIΔspr double mutant strain and found Δspr suppressed the hyperversiculation phenotype of the nlpI mutation. Furthermore, we present data indicating a high level of PG turnover in the absence of NlpI, suggesting that NlpI negatively regulates Spr activity. Additionally, we find a reduction in covalent Lpp-OM crosslinking in the ΔnlpI strain, which is also restored to a wild-type level by the deletion of spr. The data presented here suggest a model in which increased PG turnover results in a decrease in Lpp-OM crosslinking, leading to hypervesiculation.

### 4.3 Results

A deletion in spr specifically suppresses the hypervesiculation phenotype of the ΔnlpI mutant. First, we ensured that, like the nearly full deletion transposon mutant discovered earlier, the E. coli full deletion mutant of nlpI (ΔnlpI) hypervesiculates (Fig.
19A) and could be complemented with a high copy expression plasmid (pTrc99A) carrying \textit{nlpI} (pNlpI). Uninduced expression of NlpI was sufficient to nearly completely reduce OMV production to the amount produced by the isogenic wild-type strain (WT) (Fig. 20).

A genetic association between \textit{nlpI} and \textit{spr} had been reported previously (Tadokoro et al., 2004), therefore we investigated whether the loss of \textit{spr} had an effect on the strong hypervesiculation phenotype of the \textit{ΔnlpI} mutant. We found a modest hypervesiculation phenotype for the \textit{Δspr} mutant and a similar modest phenotype for the \textit{ΔnlpIΔspr} double mutant (Fig. 19A). Thus, the deletion of \textit{spr} in an \textit{ΔnlpI} strain background reduced OMV production by ~ 30-fold. It should be noted that all strains and treatments assayed here and in the subsequently-described experiments were assessed for growth and membrane integrity (Table 1), as described in chapter 2 (Schwechheimer and Kuehn, 2013), so that we could be confident that changes in OMV production levels were not due to substantial differences in cell density or lysis.

To examine the specificity of the \textit{spr} deletion on the suppression of the \textit{ΔnlpI}-associated hypervesiculation phenotype, deletion mutations in \textit{nlpA}, \textit{dsbA}, and \textit{bolA} were introduced into the \textit{ΔnlpI} strain. Individually, the deletion of \textit{nlpA}, \textit{dsbA}, and \textit{bolA} result in a hypovesiculation phenotype, and each deletion partially suppresses another hypervesiculating mutant, \textit{ΔdegP} (Fig. 14B, 14D; Chapter 2) (Schwechheimer and Kuehn, 2013). DegP is a periplasmic chaperone and protease that manages misfolded periplasmic protein stress (Ortega et al., 2009; Raivio and Silhavy, 2001; Spiess et al., 1999). In \textit{degP} deletion mutants, misfolded proteins that accumulate are shed via OMVs, and the ability of the \textit{ΔdegP} strain to hypervesiculate prevents the otherwise negative effects of toxic periplasmic protein accumulation on bacterial growth (Chapter 2)
(Schwechheimer and Kuehn, 2013). (Why mutations in \textit{nlpA}, \textit{dsbA}, and \textit{bolA} reduce vesiculation levels is not yet understood: NlpA, DsbA and BolA play a role in methionine import, periplasmic disulfide bond formation, and stationary phase transition, respectively (Fig. 8C, 9E; Chapter 2) (Bardwell et al., 1993; Freire et al., 2009; Freire et al., 2006; Kamitani et al., 1992; Schwechheimer and Kuehn, 2013; Zhang et al., 2003).) Unlike the deletion in \textit{spr}, none of these deletions had an effect on the hypervesiculation phenotype of the $\Delta nlpI$ mutant (Fig. 19A). To further examine the specificity of the interaction of \textit{spr} and \textit{nlpI}, we determined if deletions in genes encoding the three structural and/or functional Spr homologues, \textit{ydhO}, \textit{yafL}, and \textit{yebA} (Singh et al., 2012), could also suppress the hypervesiculation phenotype of the $\Delta nlpI$ mutation. Among this family, only the \textit{spr} deletion reduced OMV production of the $\Delta nlpI$ mutant (Fig. 19A, 19B).

It was previously not only shown that the thermosensitive growth of \textit{spr} mutants could be suppressed by the loss of \textit{nlpI}, but also by the overexpression of penicillin binding protein (PBP) 7, another murein DD-endopeptidase which is encoded by \textit{pbpG} (Romeis and Holtje, 1994; Tadokoro et al., 2004). These phenotypes suggested to us that NlpI may play a role in the negative regulation of PBP7 activity. We examined whether the deletion of \textit{pbpG} would reduce OMV production of the $\Delta nlpI$ mutant and found that this was not the case (Fig. 19C). Together, these data demonstrate the specific role in OMV production that Spr plays with respect to NlpI.
Figure 19: Deletion of spr specifically suppresses the hypervesiculation phenotype of the Δnlpl mutant.
(A-C) Relative fold OMV production in cultures of the indicated strains grown in LB overnight at 37°C was determined by quantitating OMVs, normalizing to OD_{600}, and dividing by OD_{600}-normalized OMV production in a WT culture. Statistical comparisons are with WT levels unless denoted by a bracket. *, p \leq 0.05; n>3. Error bars indicate standard error of the mean (SEM). Reproduced from Schwechheimer, Rodriguez, and Kuehn, submitted.

![Graph showing relative fold OMV production](image)

**Figure 20: NlpI complementation.**

Relative fold OMV production in cultures of the indicated strains grown in LB overnight at 37°C was determined by quantitating OMVs, normalizing to OD_{600}, and dividing by OD_{600}-normalized OMV production in a WT culture. *, p \leq 0.05; n=3. Reproduced from Schwechheimer, Rodriguez, and Kuehn, submitted.
Table 1: Growth and membrane integrity phenotypes of mutant cultures.

<table>
<thead>
<tr>
<th>Strains/Treatments¹</th>
<th>OMV Production²</th>
<th>Growth³</th>
<th>Growth w/ Actinomycin D⁴</th>
<th>Sytox Green⁵</th>
<th>Adenylate Kinase⁶</th>
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<td>WT</td>
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¹ Sets of assays performed in the same experimental group are separated by a space in the table; heat-killed cells were used as a positive control for Sytox Green and adenylate kinase activity; each assay was repeated at least twice.

² Fold-change in OMV production relative to WT or control. See Figures for statistical evaluations.

³ Growth in LB: WT, like WT or untreated; <WT, below WT or control.

⁴ Growth in LB with 5 µg/mL Actinomycin D: WT, like WT or control; <WT, below WT or control.

⁵ Sytox Green entry: +, no significant difference from WT or control; (l) significantly less than WT; ++, significantly higher compared to WT or control.

⁶ Adenylate kinase in cell-free supernatant: +, ~100-999 LU/OD; ++, ~1000-9999 LU/OD. For comparison, heat killed cells yielded >40,000 LU/OD for log phase cells and >20,000 LU/OD for stationary phase; BD, below detection, refers to samples with a negative value.

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The ΔnlpIΔspr double mutant remains capable of hyperproducing OMVs. We next investigated the converse question: whether the deletion of spr reduced OMV production for any other hypervesiculating mutant. We examined OMV production for the ΔsprΔdegP double mutant and found that the loss of spr had no effect on hypervesiculation caused by the deletion of degP (Fig. 21), further supporting the specific relationship between nlpl and spr. However, in order to interpret this data, we needed to consider that the ΔnlpIΔspr double mutant may be impaired completely in its ability to overproduce OMVs, perhaps because it lacks some functional components that are necessary for inducing OMVs. To address this, we tested whether OMV production by the ΔnlpIΔspr double mutant could be increased by the deletion of degP. The data demonstrate that the triple mutant could, in fact, hypervesiculate (Fig. 21), revealing that the loss of spr did not generally prevent OMV hyperproduction. These results further suggest that there are multiple, independent routes to induce OMV production.

Figure 21: Deletion of degP causes hypervesiculation in the ΔnlpIΔspr mutant.

Relative fold OMV production in cultures of the indicated strains was determined as in Fig 19. *, p ≤ 0.05; WT, n≥5. Error bars indicate SEM. Reproduced from Schwechheimer, Rodriguez, and Kuehn, submitted.
NlpI plays a role in the inhibition of Spr activity. Since Spr had recently been identified as a PG DD-endopeptidase (Singh et al., 2012), and the loss of spr suppressed hypervesiculation associated with the ΔnlpI mutation, we hypothesized that the increased OMV production of the ΔnlpI mutant may result from rampant PG degradation by uncontrolled Spr in the mutant. Consequently, we predicted that survival of the ΔnlpI mutant might depend on continuous PG synthesis, and that this would be particularly visible in stationary phase, when PG synthesis is typically negligible in a WT cell. To investigate this hypothesis, we compared the sensitivity of stationary phase cultures to treatment with 10 mM D-Methionine (D-Met), which has an inhibitory effect on PG synthesis (Caparros et al., 1992; Lam et al., 2009). In “pseudo-stationary phase” cultures (for this analysis, the cultures had to be resuspended in fresh media), 10 mM D-Met would be expected to have minimal effects on the WT strain because of the low rate of PG turnover, whereas mutants with overactive PG turnover would be sensitive and exhibit growth defects. We assessed the sensitivity of pseudo-stationary phase cultures of ΔnlpI, Δspr, ΔnlpIΔspr, and WT to treatment with 10 mM D-Met by monitoring OD$_{600}$. As suspected, the nlpI deletion strain was hypersensitive to 10 mM D-Met: after 2 h, the OD$_{600}$ dropped off dramatically, whereas the WT strain was unaffected (Fig. 22A). To ensure that it was indeed the loss of nlpI that caused D-Met hypersensitivity, we complemented the deletion strain with pNlpI and found that uninduced expression of NlpI was sufficient to reduce D-Met sensitivity of the ΔnlpI mutant to a WT level (Fig. 22B). These data suggest that the ΔnlpI mutant has a hyperactive PG metabolism in pseudo-stationary phase.

The ΔnlpIΔspr mutant showed only a modest growth defect with 10 mM D-Met, indicating that the PG turnover in the double mutant is more like that in a WT strain.
The suppression of ΔnlpI-dependent D-Met sensitivity by a deletion in spr supports our hypothesis that NlpI negatively regulates Spr activity. Since PG is essential (Vollmer and Bertsche, 2008; Weidel and Pelzer, 1964), it follows that the continuous PG degradation by overactive Spr would be counteracted by upregulation of PG synthesis. Thus, in the absence of NlpI, continuous synthesis of new PG, even in stationary phase, likely allows the cell to survive uncontrolled Spr-mediated PG degradation.

To examine whether expression of Spr or NlpI were sufficient to recover D-Met hypersensitivity of the ΔnlpIΔspr strain, we analyzed growth of the ΔnlpIΔspr mutant transformed with pSpr (spr in the pTrc99A high-copy expression plasmid) and pNlpI. As expected, expression of Spr increased D-Met hypersensitivity of the double mutant (Fig. 22C), a phenotype similar to that of the ΔnlpI strain (Fig. 22A), whereas expression of NlpI did not have much of an effect on D-Met hypersensitivity (Fig. 22C), a phenotype similar to that of the Δspr strain (Fig 22A). These data support the hypothesis that it is increased Spr activity that causes the ΔnlpI mutant to be sensitive to D-Met in pseudo-stationary phase.
Figure 22: Sensitivity to D-Met during non-exponential phase growth indicates high PG turnover.
(A) Cultures of the indicated strains were grown in LB overnight at 37°C, cells were pelleted and resuspended into fresh LB (t=0) +/- 10 mM D-Met, and then grown at 37°C. OD$_{600}$ was measured hourly. Data represents the average of two independent experiments. (B,C) Cultures of the indicated strains carrying the vector, or NlpI- or Spr-encoding plasmids, were grown in LB overnight at 37°C, cells were pelleted and resuspended into fresh LB (t=0) +/- 10 mM D-Met, and then grown at 37°C. OD$_{600}$ was measured hourly. Data represents the average of two independent experiments. Reproduced from Schwechheimer, Rodriguez, and Kuehn, submitted.

Other PG endopeptidases do not rescue D-Met sensitivity of the ΔnlpI mutant.

Next, to again test specificity, we investigated if any of Spr’s structural or functional homologues could suppress the D-Met sensitivity of the ΔnlpI mutant. Consistent with the lack of suppression seen for the hypervesiculation phenotype in Fig. 19B, none of the spr homolog deletions could rescue D-Met hypersensitivity of the ΔnlpI mutant (Fig. 23A). Further, we investigated if ΔpbpG could suppress D-Met hypersensitivity of the ΔnlpI mutant. Again, as seen for OMV production (Fig. 19C), no improvement in growth with D-Met was observed for the ΔnlpIΔpbpG double mutant (Fig. 23B). Together, the D-Met sensitivity data (Fig. 22 and 23) and the vesiculation data (Fig. 19) suggest that the hypervesiculation phenotype caused by the deletion of nlpI is linked to the uncontrolled PG degradation of hyperactive Spr.
Loss of *dacB*, not *spr*, rescues D-Met hypersensitivity of the Δ*nlpI* mutant in log phase. Singh et al. determined that Spr activity is most critical during active growth and cell division in log phase. Thus, we hypothesized that Spr activity suppression by
NlpI would be decreased or even non-existent during log phase, when maximum Spr activity is required by the cell. To test this, we first examined whether the ΔnlpI mutant was sensitive to D-Met in log phase and found that this was the case (Fig. 24A). However, under these conditions, the additional deletion of spr did not provide rescue (Fig. 24A). Similarly, none of the spr homologue mutants reduced the nlpI deletion D-Met sensitivity (Fig. 24B). These data suggested that NlpI may negatively regulate another PG hydrolase. As mentioned previously, it had been noted that spr mutants could be rescued by additionally mutating nlpI (Tadokoro et al., 2004), indicating that the relief came from increased activity of an enzyme with a similar function as Spr that is also negatively regulated by NlpI. To test whether we could identify the gene responsible for this activity, we constructed double deletion mutants of nlpI with each of E. coli’s additional three endopeptidases: pbpG, mepA or dacB. We found that the loss of dacB, the gene that encodes PBP4, suppressed D-Met hypersensitivity of the ΔnlpI mutant in log phase (Fig. 24C). These data suggest that NlpI can negatively regulate PBP4 activity during active growth.
Figure 24: In log phase, deletion of *dacB* suppresses D-Met sensitivity of the Δ*nlpI* strain.
(A-C) Indicated strains of bacteria were grown in LB overnight at 37°C, inoculated into fresh LB (t=0), and then grown at 37°C. OD<sub>600</sub> was measured hourly. Data represents the average of two independent experiments. Reproduced from Schwechheimer, Rodriguez, and Kuehn, submitted.

Similar phenotypes result from overexpression of functional Spr and the deletion of <i>nlpI</i>. If our hypothesis is correct, that NlpI acts as a negative regulator of Spr late in the bacterial life cycle, then the overexpression of Spr should yield similar phenotypes as the deletion of <i>nlpI</i>. To test this hypothesis, we studied stationary phase cultures of a highly-induced (500 µM IPTG) Δspr pSpr strain. Even without the addition of D-Met, strongly induced Spr lead to a growth defect with respect to the WT strain, mimicking the sensitivity of the ΔnlpI mutant (Fig. 25A). As expected, the addition of 10 mM D-Met had a further detrimental effect on cells overexpressing Spr (Fig. 25A).

We next wanted to verify that it was the PG endopeptidase activity of Spr that caused these phenotypes. To examine this hypothesis, we constructed an expression plasmid encoding Spr with a point mutation in the catalytic triad (C68A)(pmSpr), which has been previously shown to cause impaired PG endopeptidase activity (Aramini et al., 2008; Singh et al., 2012). To ensure that the mutation did not interfere with expression and OM localization, we constructed a C-terminal FLAG-tagged version of Spr (pSpr-F) and mutant Spr (pmSpr-F), in order to be able to monitor Spr and mSpr location and expression levels. The FLAG-tag did not interfere with Spr function, since basal and induced expression (with 500 µM IPTG) of Δspr pSpr-F yielded results very similar to those using the untagged version (Fig. 26, Fig. 25C), although OMV production was observed to be slightly higher. Both pSpr-F and pmSpr-F localized to the OM and were expressed at similar levels (if anything, there was a slight increase in pmSpr-F compared with pSpr-F) (Fig. 27), indicating that the C68A mutation did not disturb localization or
stability. Therefore, we could anticipate that any phenotypes observed for the mutant would be a result of the loss of activity, rather than a decrease in expression or mislocalization. When we compared the effect of 10 mM D-Met treatment of strains expressing mSpr and Spr, we observed that increased expression of mSpr resulted in a moderate growth defect, but nowhere near as detrimental as the overexpression of functional Spr (Fig. 25B), supporting the hypothesis that it is the endopeptidase activity of Spr that is negatively regulated by NlpI.

Next, we examined if overexpression of the functional form of Spr could significantly increase OMV production compared to overexpression of the mutant Spr. A slight elevation in OMV levels was also expected for the mutant, since we have shown previously that increased envelope protein expression in general can lead to hypervesiculation (McBroom and Kuehn, 2007; Schwechheimer and Kuehn, 2013). We found that uninduced expression of functional Spr in the Δspr background reduced the slight OMV overproduction phenotype of the Δspr strain to a WT level, whereas vesiculation increased in an IPTG concentration-dependent manner (Fig 25C). When the experiment was repeated with mSpr induced with 500 μM IPTG, we found that OMV production was slightly elevated with respect to WT levels (as anticipated), but significantly less than functional Spr. In summary, these data strengthen the hypothesis that NlpI negatively and specifically regulates the DD-endopeptidase activity of Spr, impacting OMV production.
Figure 25: Similar phenotypes result from Spr overexpression and the deletion of \textit{nlpI}. 

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(A, B) Indicated strains of bacteria carrying the pTrc99A plasmid, Spr-encoding or mSpr-encoding, were grown in LB overnight at 37°C (induced at inoculation if indicated), cells were pelleted and resuspended into fresh LB (t=0) +/- 10 mM D-Met, and then grown at 37°C. OD$_{600}$ was measured hourly. Data represents the average of two independent experiments. (C) Relative fold OMV production in cultures of the indicated strains grown in LB overnight at 37°C (induced at inoculation if indicated) was determined as described in Fig 1. *, p ≤ 0.05; WT: n≥3. Error bars indicate SEM; p values refer to comparisons with WT unless denoted by a bracket. Reproduced from Schwechheimer, Rodriguez, and Kuehn, submitted.

**Figure 26: Spr-F can induce OMV Production.**

Relative fold OMV production in cultures of the indicated strains grown in LB overnight at 37°C was determined by quantitating OMVs, normalizing to OD$_{600}$, and dividing by OD$_{600}$-normalized OMV production in a WT culture. *, p ≤ 0.05; n=3. Reproduced from Schwechheimer, Rodriguez, and Kuehn, submitted.
Figure 27: Localization and expression of Spr-F and mSpr-F.

Anti-FLAG immunoblots of whole cell (WC) and purified outer membrane (OM). The quantified data represents densitometry of the OM FLAG signal divided by the densitometry of a blot intensity control, which was finally divided by WC protein to account for different cell density and normalized to pSpr-F. Reproduced from Schwechheimer, Rodriguez, and Kuehn, submitted.

PG-crosslinked Lpp levels negatively correlate with OMV production levels in the ΔnlpI and ΔnlpIΔspr mutants. Our discovery of the relationship between OMV production and PG turnover led us to consider the effect of PG turnover on the number of covalent crosslinks between the OM and PG and the potential downstream effect on OMV production. We hypothesized that there may be an inverse relationship between the degree of OMV production and the concentration of covalent Lpp-PG crosslinks,
especially since it was previously demonstrated that OMVs from normally growing *E. coli* contained very little Lpp in comparison to the OM (Wensink and Witholt, 1981). In the case of the ΔnlpI mutant, the high rate of PG turnover might lead to low numbers of crosslinks, causing a loosely associated OM and consequent hypervesiculation.

To investigate this hypothesis, we developed an immunoblotting assay that takes advantage of the fact that Lpp isolated with purified PG sacculi is solely in its crosslinked form. This form has been historically referred to as the “bound” form due to its covalent linkage to PG. The “free” form of Lpp is considered to be the form that is OM-anchored via the protein’s lipid moiety, but not covalently crosslinked to PG (Chapter 1, Fig. 3). After sacculi isolation, lysozyme digestion, and SDS-PAGE, bound Lpp was quantified by immunoblotting. Whole cell extract of the *lpp* deletion strain, Δlpp, was used as a negative control for immunoblotting (Fig 28A). To establish that we could distinguish between the bound and free forms of Lpp, we utilized a triple mutant strain, ΔycfSΔybiSΔerfK, that expresses only free Lpp because it lacks the enzymes that form the covalent bond (Magnet et al., 2007). We first verified that similar levels of free Lpp were present in whole cell preparations of the WT strain and the triple mutant strain (Fig 28A). The concentration of free Lpp in the samples was determined by boiling whole cell samples in Laemelli buffer and 1% SDS in PBS without lysozyme prior to SDS-PAGE (Cowles et al., 2011) Next, we isolated and analyzed purified PG sacculi from these strains. Lpp could be detected in PG sacculi from the WT strain, whereas PG sacculi from the triple mutant contained no detectable Lpp (Fig 28A). These data showed that this assay allows selective quantitation of bound Lpp.

The levels of covalent Lpp crosslinking in the WT, ΔnlpI, and ΔnlpIΔspr strains were compared using our bound Lpp assay. We found significantly reduced levels
(~40%) of covalently crosslinked Lpp for the Δnlpl strain with respect to WT levels, however we found WT levels of crosslinked Lpp for the ΔnlplΔspr double mutant (Fig. 28B). To establish that the observed decrease of bound Lpp in the Δnlpl mutant was not a reflection of overall decreased Lpp expression, we evaluated whether there was a corresponding increase in the level of free Lpp in this strain. We found that, indeed, levels of free Lpp in the Δnlpl strain were higher than in the WT strain by approximately two-fold (Fig. 28C). To ensure that the deletion in nlpl did not affect Lpp localization, we examined whether Lpp fractionated with the PG in the ΔycfSΔybiSΔerfKΔnlpl mutant. Lpp fractionated similarly for this mutant and the triple mutant expressing only free Lpp (Compare Fig. 28A and Fig. 29). These data support our hypothesis that NlpI regulation of Spr-dependent PG hydrolysis affects Lpp crosslinking, which in turn affects OMV production. We propose that the increase in PG turnover prevents establishment of WT levels of PG crosslinking and that this at least partially contributes to the increase in vesiculation.
Figure 28: PG crosslinked Lpp negatively correlates with OMV production in the ΔnlpI mutant, and levels are restored with spr deletion.

(A) Anti-Lpp immunoblot of whole cell and copurified PG of the indicated strains. Composite figure created from the rearrangement of lanes from a single immunoblot. (B) Relative fold crosslinked Lpp in cultures of the indicated strains grown in LB to an OD$_{600}$ of ~ 0.4 at 37°C was determined by quantitative immunoblotting of Lpp copurified with PG, normalizing to OD$_{600}$ and dividing by OD$_{600}$-normalized crosslinked Lpp in a WT culture. *, p ≤ 0.05; n>3. Error bars indicate SEM; p value refers to comparison with WT. (C) Relative fold of free Lpp in cultures of the indicated strains grown overnight in LB. *, p ≤ 0.05; n=3. Reproduced from Schwechheimer, Rodriguez, and Kuehn, submitted.
4.4 Discussion

The mechanism of OMV production is still rather cryptic however insight regarding the relationship between the envelope architecture and vesiculation phenotypes can help shed light on molecular properties involved in this process. Here, we investigated the cause of the strong hypervesiculation phenotype of the $\Delta nlpI$ mutant strain and found that multiple components of the envelope of this mutant were affected; the data suggest high levels of PG turnover and decreased levels of covalent Lpp-OM crosslinks for the $\Delta nlpI$ mutant compared to the WT. The data are consistent with a model in which NlpI is a negative regulator of the PG hydrolases Spr and PBP4. Although deletions in Lpp have been previously shown to alter OMV production (Cascales et al., 2002; Deatherage et al., 2009; Suzuki et al., 1978), the fact that vesiculation can be modulated indirectly through changes in PG and consequent detectable differences in bound Lpp levels had not previously been reported. These data
provide new insights into OMV biogenesis, and suggest that WT Gram-negative bacteria can modulate vesiculation by altering PG-OM crosslinking via localized modulation of PG turnover.

**Model of NlpI's effect on the bacterial envelope.** The pseudo-stationary phase D-Met sensitivity phenotype for the *nlpI* deletion strain is consistent with either NlpI repressing a PG endopeptidase or increasing activity of a PG synthase, since either upregulated PG turnover or a reduction in PG synthesis would render cells sensitive to a PG synthesis inhibitor. Also consistent with either scenario, the deletion of *nlpI* would lead to an alteration in PG remodeling, and, via changes in Lpp crosslinking levels, this would lead to hyperproduction of OMVs. With the likely high PG turnover in the mutant cells, there may simply not be enough time to establish a WT concentration of covalently-crosslinked Lpp. The result is a substantial reduction in bound Lpp (Fig. 29B) and a dramatic increase in OMV production (Fig. 19A).

In order to distinguish which of these models of NlpI activity is correct, or if NlpI actually regulates both activities, we relied on the other genetic interaction data described in this study. We found that *nlpI* and *spr* were phenotypically linked with regards to D-Met sensitivity, Lpp crosslinking, and OMV production. Incorporating all of the phenotypic observations, we generated several models consistent with the phenotypic characterization of the WT and mutant strains (See Fig 30 and Fig. 31). We conclude that NlpI regulates PG remodeling through Spr, either directly or indirectly, and that this is particularly pertinent later on in the bacterial life cycle. Although our data demonstrate that NlpI modulates the endopeptidase activity of Spr, as of yet, we do not know if this regulation occurs via direct interactions between NlpI and Spr or if their interaction is indirect. We are working to elucidate the molecular details of this
interaction. If NlpI activates another factor (such as a PG synthase as shown in Fig 30 and Fig. 31), this factor must somehow repress Spr activity, since a deletion in spr suppresses the nlpI deletion mutant D-Met sensitivity, Lpp crosslinking, and OMV phenotypes. For log-phase cells, NlpI acts through dacB to modulate PG remodeling. The model is further supported by the data demonstrating that strong overexpression of Spr, but not the mutant Spr lacking endopeptidase activity, leads to similar phenotypes as the ΔnlpI mutant. For a more detailed examination of the modulation of the processes occurring in the various mutant backgrounds based on our working models see Fig. 31.

Interestingly, overexpression of both Spr and mSpr created a growth defect in the absence of D-Met (Fig. 25 A and B). It is not surprising that increased levels of a PG degradation enzyme have harmful consequences, since PG is essential for viability (Vollmer and Bertsche, 2008), but we were rather surprised that the inactive mutant form showed the same phenotype. Considering this result, we propose that mSpr might bind well to PG as a substrate but then interferes with PG homeostasis because of its inability to cleave and therefore dissociate from the PG. Further work is necessary to elucidate the molecular mechanism for the observed phenotypes.
Figure 30: Model of NlpI’s regulatory interactions.

(A) NlpI directly modulating the endopeptidase activity of PBP4 and Spr. (B) NlpI indirectly modulating the endopeptidase activity of Pbp4 and Spr through an unidentified PG synthase. Reproduced from Schwechheimer, Rodriguez, and Kuehn, submitted.
Figure 31: Model of NlpI’s regulatory interactions in the various mutant backgrounds used in this study

The left column indicates the direct interaction, whereas the right column indicates the indirect interaction through a yet unidentified PG synthase. Reproduced from Schwechheimer, Rodriguez, and Kuehn, submitted.

Insight into OMV production and regulation by WT cells. How might this data on mutant phenotypes and suppressors elucidate the mechanism and modulation of
OMV production by WT cells? The data presented here indicate that one route to alter vesiculation is through modulation of PG structure and consequently bound Lpp. Our current hypothesis is that the equilibrium between PG synthesis and degradation determines the concentration of covalently crosslinked Lpp, and that bound Lpp levels inversely affect OMV production. Hence, a perturbation of the PG remodeling equilibrium would alter bound Lpp levels, and, consequently, levels of OMV production.

For a WT cell, we propose that at least part of the PG synthesis and degradation equilibrium is achieved via direct or indirect modulation by NlpI of PBP4 (encoded by \textit{dacB}) in log phase, and Spr in stationary phase. The loss of \textit{nlpI} leads to D-Met sensitivity in log phase due to increased PG degradation due to rampant PBP4 activity (which can be rescued by deleting \textit{dacB}). The loss of \textit{nlpI} leads to D-Met sensitivity in stationary phase due to rampant Spr activity (which can be rescued by the deletion of \textit{spr}). In addition, for the \textit{ΔnlpI} mutant, high PG turnover is associated with hypervesiculation and a decrease in bound Lpp (which could both be rescued by the deletion of \textit{spr}).

We propose that these same events occur in WT bacteria at a less ubiquitous level. There are likely localized patches of PG that are actively being remodeled and that these have an increased likelihood to be sites of OMV budding due to the lack of Lpp-PG crosslinks. The number and location of these patches may vary depending on growth phase. In addition, WT cells are able to regulate vesiculation levels (e.g. in times of environmental stress or to promote virulence factor delivery) by altering PG-OM crosslinking via modulation of PG turnover.
4.5 Acknowledgements

We thank Daniel Rodriguez for the construction of pSpr, pmSpr, pSpr-F, and pmSpr-F and Adam Kulp for the construction of pNlpI. We are grateful for the generous contribution of Lpp antibody by Tom Silhavy (Princeton University). We also thank the National BioResource Project (NIG, Japan)::E. coli for the Keio Collection. This work was supported by NIH grant 5R01GM099471.
5. Interspecies D-Amino Acid-Regulated Signaling of Outer Membrane Vesicle Production in Gram-negative Bacteria

5.1 Summary

Numerous bacteria secrete D-amino acids at the onset of stationary phase, which, we noted, is coincident with the previously observed peak of OMV production. In this work, we found that D-amino acids (D-AAs) are natural factors that modulate OMV production. Secreted non-canonical D-AAs are known to be incorporated into peptidoglycan (PG) and signal the downregulation of stationary phase PG for *Vibrio cholerae*. For *Escherichia coli*, D-Alanine is a PG structural component which is cleaved from the PG during the substantial remodeling of the PG sacculus that occurs during the transition from log to stationary phase. We recently determined that an inverse relationship exists between OMV production and covalent OM-PG crosslinking for the hypervesiculating mutant, Δ*nlpI*. Using envelope structure analysis, we found that this relationship also holds for D-AA-induced hypervesiculation phenotypes. From this work, we conclude that the OMV biogenesis pathway in Gram-negative bacteria can be regulated by both internally-generated and environmentally-available secreted bacterial components. To our knowledge, this is the first time D-AAs have been implicated as a mechanism of interspecies communication.

5.2 Introduction

Our lab has utilized a genetic approach to study the process of OMV production. By analyzing the effect of genetic mutations on OMV production, we have begun to identify bacterial components – both mechanistic and regulatory – that are key players in this process (Chapers 2, 3, and 4) (Macdonald and Kuehn, 2013; McBroom et al., 2006;
McBroom and Kuehn, 2007; Schwechheimer and Kuehn, 2013). Further contributions to a general understanding of envelope dynamics involved in OMV budding have been made by other groups who studied mutants lacking specific envelope components or containing modified envelope components and investigated interactions of the cell surface components with extracellular factors (Cascales et al., 2002; Deatherage et al., 2009; Mashburn and Whiteley, 2005; McMahon et al., 2012; Suzuki et al., 1978; Tashiro et al., 2009; Wessel et al., 2013).

With the Gram-negative envelope architecture in mind (Chapter 1), presumably one of the first steps in the production of an OMV is the liberation of the OM from the underlying PG without compromising cellular integrity (Kulp and Kuehn, 2010; Mashburn-Warren and Whiteley, 2006; Schwechheimer et al., 2013). Our recent findings suggest an inverse relationship between vesicle production and covalent OM-PG crosslinking for the strong hypervesiculating mutant strain, ΔnlpI (Chaper 4) (Schwechheimer, Rodriguez, and Kuehn, submitted). Our data indicate that NlpI negatively regulates the activity of the Spr endopeptidase, and thus in the ΔnlpI mutant, PG is constantly undergoing remodeling, reducing the ability of the mutant to make a normal level of Lpp crosslinks, which in turn promotes OMV production. As a consequence of these studies, we became interested in identifying native factors that regulate OMV production and determine whether the mechanism for induced OMV production is based on the same putative molecular mechanism revealed by the hypervesiculating mutant.

In 2009 Lam and coworkers discovered that during the transition from log to stationary phase numerous Gram-negative bacteria secrete and incorporate non-canonical D-amino acids (D-AAs) into PG, thereby reducing and strengthening the
stationary phase PG (Lam et al., 2009). Their research also identified the periplasmic broad-spectrum *Vibrio cholerae* racemase, BsrV, which converts L-amino acids into their D-enantiomer counterparts. Another means by which D-amino acids are secreted by Gram-negative bacteria is during the PG remodeling step during the transition from log to stationary phase. This step involves the cleavage from tetra- to tripeptides, releasing D-Alanine (D-Ala), a permanent PG component, from the PG sacculus (Vollmer and Bertsche, 2008). Considering the timing of D-AA secretion, the peak time of vesiculation (Chatterjee and Das, 1967; Chowdhury and Jagannadham, 2013; Gamazo and Moriyon, 1987; Hoekstra et al., 1976), and our recent data implicating the modulation of PG as a potential means to alter vesiculation (Chapter 4, Fig. 28) (Schwechheimer, Rodriguez, and Kuehn, submitted), we wondered whether non-canonical and canonical D-AAs might play a role in the regulation and/or biogenesis of OMVs.

In this chapter, we demonstrate that D-AAs affect levels of vesiculation in the natural D-AA producing species, *V. cholerae*, as well as in the model organism, *Escherichia coli*. When we analyzed the effect of D-AAs on OMV production, we found that these lead to alterations in PG-OM crosslinking. Lpp crosslinking levels were inversely correlated with OMV levels, as seen previously for the strong hypervesiculation mutant Δ*nlpI* (Chapter 4, Fig. 28). These data confirm the molecular basis for the natural regulation of OMV production by Gram-negative bacteria in the environment.

### 5.3 Results

Non-canonical D-amino acids stimulate OMV production in the natural producer *Vibrio cholerae*. We first examined whether a defect in the ability to produce non-canonical D-AAs affected the levels of OMVs produced in cultures of *V. cholerae*. 

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We compared OMV production of WT *V. cholerae* and an isogenic ΔbsrV mutant. *bsrV* encodes the periplasmic racemase which converts L-AAs into their D-enantiomer counterparts, and thus the ΔbsrV mutant does not produce or secrete D-AAs (Lam et al., 2009). OMVs produced in stationary phase were quantified, and we determined that the loss of non-canonical D-AA production caused a ~20% decrease in OMV production (Fig. 32A), suggesting that non-canonical D-AAs naturally produced in a culture of *V. cholerae* stimulate OMV production. It should be noted that each strain we used in this study for OMV quantification was further characterized for membrane integrity defects to ensure quantification of true OMVs, not shed membrane fragments due to fragile cells (Table 2). We then tested if a mixture of 0.5 mM D-Methionine (D-Met) and 0.5 mM D-leucine (D-Leu) could complement the hypovesiculation phenotype of ΔbsrV. These concentrations of D-AAs had been shown previously to complement other ΔbsrV phenotypes (Lam et al., 2009). We found that the exogenous addition of D-AAs increased OMV production of the non-D-AA producing mutant to a WT level (Fig 32B). These results indicated that mutant cells were still capable of WT levels of OMV production, and that secreted D-AAs were able to access and trigger vesicle production. In addition, it suggested that in a WT culture, secreted D-AAs could be involved in triggering OMV production.

In order to test these hypotheses, we examined whether spent supernatant from an WT *V. cholerae* culture, which would include naturally secreted levels of non-canonical D-AAs, could increase vesiculation by the ΔbsrV mutant to a WT level. When pelleted WT and ΔbsrV mutant cells were resuspended in WT spent supernatant and incubated for 3h, we found an increase in OMV production in the ΔbsrV culture up to the WT level (Fig. 32C). A comparison of OMV production by WT and ΔbsrV cells
treated with spent ΔbsrV culture supernatant, yielded the expected result that ΔbsrV supernatant did not increase vesiculation (Fig. 32D). Therefore, a component in the WT supernatant which was not present in the ΔbsrV supernatant was able to stimulate OMV production in the ΔbsrV culture.

It should be noted that this experiment was performed with log phase cells. Overnight treatment of pelleted stationary phase cells with spent WT V. cholerae supernatant not only complemented OMV production in the ΔbsrV culture up to WT levels, it actually increased OMV production above WT levels (Fig. 32E). The increase beyond WT levels suggested that the mutant strain may have upregulated a non-canonical D-AA-independent mechanism for vesiculation in order to compensate for the underproduction phenotype. This compensation appears to be present after mid-log phase, when OMV production peaks, possibly because it is critical for the cells at this time during the transition from log to stationary phase (Chatterjee and Das, 1967; Chowdhury and Jagannadham, 2013; Gamazo and Moriyon, 1987; Hoekstra et al., 1976). In addition, log phase WT cells showed a higher level of OMV production in the ΔbsrV spent supernatant experiment (Fig 32D), whereas stationary phase WT cells did not increase vesicle production above the level produced by ΔbsrV cells (Fig 32F), suggesting mature bacteria may be past the point of producing the D-AAs in the culture.

Together, these data indicate that native concentrations of naturally-produced, secreted non-canonical D-AAs can induce OMV production in V. cholerae.
Figure 32: Non-canonical D-Amino acids present in culture supernatants stimulate OMV production in *V. cholerae*

(A) Relative fold OMV production in stationary phase cultures of the indicated *V. cholerae* strains grown in LB overnight at 37°C was determined by quantitating OMVs, normalizing to OD$_{600}$ and dividing by OD$_{600}$-normalized OMV production by the WT.

(B) Relative fold OMV production was determined as in part A. When the stationary
phase cells were resuspended in fresh media, the 0.5 mM D-AAs were added to the ΔbsrV culture. (C, D) Pelleted log- phase (OD$_{600}$ 0.3) WT and ΔbsrV V. cholerae cells were resuspended in and incubated (37°, 3h) with spent OMV-free overnight supernatant from WT and ΔbsrV V. cholerae cultures. OD-normalized relative fold OMV production was determined for each pair of conditions as in Part 1A. (E, F) Pelleted stationary phase V. cholerae cells were resuspended in and incubated with (37°C, overnight) spent OMV-free overnight WT or ΔbsrV V. cholerae culture supernatant. OD-normalized relative fold OMV production was determined for each pair of conditions as in Part 1A. Error bars indicate SEM; p values refer to comparisons with WT. *, p ≤ 0.05; n=3. Reproduced from Schwechheimer and Kuehn, submitted.
Table 2: Growth and membrane integrity phenotypes of mutant and treated cultures.

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<th>OMV Production</th>
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<th>Growth w/ Actinomycin D&lt;sup&gt;4&lt;/sup&gt;</th>
<th>Sytox Green&lt;sup&gt;5&lt;/sup&gt;</th>
<th>Adenylate Kinase&lt;sup&gt;6&lt;/sup&gt;</th>
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<sup>1</sup> Strains are WT and mutant *E. coli* unless otherwise noted. Sets of assays performed in the same experimental group are separated by a space in the table; heat-killed cells were used as a positive control for Sytox Green and adenylate kinase activity; sup, treatment with OMV-free spent culture supernatant; each assay was repeated at least twice.

<sup>2</sup> Fold-change in OMV production relative to untreated or control treatment in each group. See Figures for statistical evaluations.

<sup>3</sup> Growth in LB: UT, like untreated or control treatment in each group.

<sup>4</sup> Growth in LB with 5 μg/mL Actinomycin D: UT, like untreated or control treatment in each group.

<sup>5</sup> Sytox Green entry: UT, no significant difference from untreated or control treatment in each group; <UT, significantly less than untreated or control treatment in each group; >UT, significantly higher compared to untreated or control treatment in each group.

<sup>6</sup> Adenylate kinase in supernatant: +, ~100-999 LU/OD; ++, ~1000-9999 LU/OD; For comparison, heat killed cells yielded >40,000 LU/OD for log phase cells and >20,000 LU/OD for stationary phase; BD, below detection, refers to samples with a negative value.
<table>
<thead>
<tr>
<th>Strains/Treatments</th>
<th>OMV Production</th>
<th>Growth</th>
<th>Growth with Actinomycin D</th>
<th>Sytox Green</th>
<th>Adenylate Kinase</th>
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<tr>
<td>V. cholerae WT cells / V. cholerae WT sup</td>
<td>1</td>
<td>UT</td>
<td>UT</td>
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<tr>
<td>V. cholerae ΔbsrV cells / V. cholerae WT sup</td>
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<tr>
<td>V. cholerae WT cells / V. cholerae ΔbsrV sup</td>
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<tr>
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<tr>
<td>E. coli WT cells / E. coli WT sup</td>
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<tr>
<td>E. coli WT cells / V. cholerae WT sup</td>
<td>4</td>
<td>UT</td>
<td>UT</td>
<td>&lt;UT</td>
<td>BD</td>
</tr>
<tr>
<td>E. coli WT cells / V. cholerae ΔbsrV sup</td>
<td>2</td>
<td>UT</td>
<td>UT</td>
<td>&lt;UT</td>
<td>BD</td>
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</table>

1 Strains are WT and mutant E. coli unless otherwise noted. Sets of assays performed in the same experimental group are separated by a space in the table; heat-killed cells were used as a positive control for Sytox Green and adenylate kinase activity; sup, treatment with OMV-free spent culture supernatant; each assay was repeated at least twice.
2 Fold-change in OMV production relative to untreated or control treatment in each group. See Figures for statistical evaluations.
3 Growth in LB: UT, like untreated or control treatment in each group.
4 Growth in LB with 5 µg/mL Actinomycin D: UT, like untreated or control treatment in each group.
5 Sytox Green entry: UT, no significant difference from untreated or control treatment in each group; <UT, significantly less than untreated or control treatment in each group; >UT, significantly higher compared to untreated or control treatment in each group.
6 Adenylate kinase in supernatant: +, ~100-999 LU/OD; ++, ~1000-9999 LU/OD; For comparison, heat killed cells yielded >40,000 LU/OD for log phase cells and >20,000 LU/OD for stationary phase; BD, below detection, refers to samples with a negative value.
Non-canonical D-amino acids secreted by *V. cholerae* stimulate OMV production in *E. coli*. Considering that bacteria in nature are known to encounter and respond to signaling products secreted from diverse other species present in the environment, and that exogenous addition of D-AAs could induce OMV production in the *V. cholerae* culture, we hypothesized that D-AAs naturally produced from *V. cholerae* could also stimulate OMV production in a completely different bacterial species. In order to test this, we examined whether the model bacterial species *E. coli* could be the “receiver” of the non-canonical D-AA “signal” secreted by *V. cholerae*. *E. coli* does not naturally produce non-canonical D-AAs, so a WT strain of *E. coli* could be used for these experiments (Lam et al., 2009). OMV production by *E. coli* cells grown in spent WT *E. coli*, WT *V. cholerae*, and ΔbsrV *V. cholerae* supernatants from overnight cultures was compared. We found that WT *E. coli* OMV production increased significantly with the addition of WT *V. cholerae* supernatant (~4-fold), compared with the control treatment with *E. coli* supernatant (Fig. 33). WT *E. coli* cultured in ΔbsrV spent supernatant (lacking the non-canonical D-AAs) exhibited significantly lower OMV production levels than when grown in WT *V. cholerae* supernatant (Fig. 33), consistent with the results seen for *V. cholerae* OMV production (Fig 32A). We noted slightly elevated vesiculation levels for *E. coli* grown with ΔbsrV supernatant compared to the control *E. coli* supernatant (Fig. 33). This result echoes the previous indication that the ΔbsrV mutant cells have compensated for their inability to generate and utilize non-canonical D-AAs in order to stimulate OMV secretion, and additionally suggests that the compensatory factor may be in the form of a secreted, but as yet unidentified product. In summary, these data suggest that, at their naturally-produced amounts, non-canonical D-AAs from one
species can contribute to OMV production by another species, even if the receiving species cannot produce the non-canonical D-AAs itself. Since we have shown previously that increased OMV production is beneficial to bacterial well-being under numerous conditions (Manning and Kuehn, 2011; McBroom and Kuehn, 2007; Schwechheimer and Kuehn, 2013), as far as we know, this is the first time D-AA’s have been implicated as a “public good” mechanism of interspecies communication.

Figure 33: Non-canonical D-Amino acids secreted naturally by V. cholerae stimulate E. coli OMV production.

Pelleted stationary phase E. coli cells were resuspended in and incubated overnight in spent OMV-free overnight culture supernatant of WT E. coli (Ec), and WT or DbsrV mutant V. cholerae (Vc) cultures, as indicated. Relative fold OMV production was determined by quantitating OMVs, normalizing to OD$_{600}$, and dividing by OD$_{600}$-normalized OMV production in the supernatant-treated WT culture. Error bars indicate SEM; p values refer to comparisons with treatment with WT E. coli supernatant unless denoted by a bracket. *, p ≤ 0.05; n≥3. Reproduced from Schwechheimer and Kuehn, submitted.

D-Met treatment increases OMV production and decreases covalent Lpp crosslinking

Considering the fact that non-canonical D-AAs stimulated vesiculation in V. cholerae and V. cholerae spent supernatant containing D-AAs induced OMV production
in *E. coli*, we proceeded to investigate whether treatment with D-Met was sufficient to increase OMV production in *E. coli*. We found that a one hour treatment with 5 mM D-Met increased OMV production by approximately four-fold, comparable to the spent WT *V. cholerae* supernatant (Fig. 34A). It should be noted that the L-enantiomer counterpart had no effect on the level of vesiculation (data not shown). These results supported the model that it is the secreted D-AAs from *V. cholerae* that are critical in the induction by spent supernatant of OMV promotion in *E. coli*.

Since we were able to use *E. coli* as a “receiver” species, we could take advantage of the comprehensive Keio library of individual gene knockout mutant strains to understand mechanistic details of OMV induction by D-Met (Baba et al., 2006). Lam et al. demonstrated that D-Met was incorporated into PG (Lam et al., 2009), which led us to wonder if D-Met incorporation into the PG was necessary for the increase in OMV production. The ΔynhGΔycbB strain was used to examine this question, since this mutant is nearly completely defective in its ability to incorporate D-Met into its PG (Cava et al., 2011a). We did not detect a difference in OMV production between the D-Met-treated WT and double mutant cultures (Fig. 34A). Therefore, D-Met incorporation into PG does not seem necessary for the increase in vesiculation observed upon exogenous D-Met treatment.

Exogenously-added D-Met is also known to be able to access the cytoplasm in *E. coli*. D-Met is imported into the cytoplasm by a MetI-dependent import machinery (Kadner, 1977; Merlin et al., 2002). Therefore, to further elucidate the location of D-Met-mediated OMV-stimulating activity, we used a metI deletion strain (ΔmetI) which lacks MetI and the ability to import D-Met into the cytoplasm (Kadner, 1977; Merlin et al., 2002). D-Met treatment stimulated OMV production by the ΔmetI strain, similar to WT
Therefore, OMV induction by D-Met in *E. coli* does not require the cytoplasmic localization of the D-AA.

We next considered the potential impact of the D-AAs on the cell envelope structure. In chapter 4, we have described an inverse relationship between OMV production and covalent PG-OM Lpp crosslinking for the strong hypervesiculating mutant, *ΔnlpI* (Schwechheimer, Rodriguez, and Kuehn, submitted) and were interested if this mechanism was also relevant to this natural mechanism of upregulation of OMV production by D-AAs in WT cells. Consequently, we examined the levels of covalently-crosslinked Lpp in the bacteria after the 5 mM D-Met treatment using our previously-established protocol (Chapter 4) (Schwechheimer, Rodriguez, and Kuehn, submitted). We detected a ~30% decrease in crosslinking for the D-AA-treated WT cells compared with untreated control, and this was true for the *ΔmetI* mutant as well (Fig. 34B). To ensure that the decrease in bound Lpp was not just a consequence of an overall decrease in cellular Lpp, we quantitated the amount of free Lpp using a previously published protocol (Cowles et al., 2011). The amount of free Lpp in both strains did not decrease upon D-Met treatment (Fig. 34C). Together these data suggest that exogenous D-Met causes an increase in OMV production by acting in the envelope, and that the increase is driven by a decrease in crosslinked Lpp.
Figure 34: D-Met treatment increases OMV production and decreases covalent Lpp crosslinking.
(A) Relative fold OMV production in cultures of the indicated D-Met-treated strains grown in LB at 40°C was determined by quantitating OMVs, normalizing to OD \textsubscript{600}, and dividing by OD \textsubscript{600}-normalized OMV production in an untreated culture of the same strain. (B) Relative fold crosslinked Lpp in cultures of the indicated D-Met-treated strains grown in LB at 40°C was determined by quantitative immunoblotting of Lpp copurified with PG, normalizing to OD \textsubscript{600}, and dividing by OD \textsubscript{600}-normalized crosslinked Lpp in untreated cultures. (C) Relative fold free Lpp in cultures of the indicated D-Met treated strains grown in LB at 40°C was determined by quantitative immunoblotting of Lpp in whole cell preparations, normalizing to OD \textsubscript{600}, and dividing by OD \textsubscript{600}-normalized Lpp in untreated cultures. Error bars indicate SEM; p values refer to comparisons with untreated samples of the same strain unless denoted by a bracket. *, p ≤ 0.05; n≥3. Reproduced from Schwechheimer and Kuehn, submitted.

In order to investigate the role of crosslinked Lpp in D-AA-stimulated OMV production, we considered using the full deletion mutant \(\Delta lpp\) which lacks Lpp. This mutant has a strong hypervescication phenotype, ~167-fold over WT (Chapter 3, Fig. 18), however upon further characterization, we concluded that the increase in apparent “OMVs” by the \(\Delta lpp\) mutant was actually due to loss of envelope integrity (Schwechheimer et al., 2013; Sonntag et al., 1978; Strauch et al., 1989). Therefore, this mutant was not ideal for these studies. Sanders and Pavelka demonstrated that the \(\Delta ycfS\Delta ybiS\Delta erfK\) triple mutant lacks the three LD-transpeptidases required to form the covalent crosslink between Lpp and PG (Magnet et al., 2007). We determined that the basal level of OMV production by the \(\Delta ycfS\Delta ybiS\Delta erfK\) mutant strain is ~34-fold higher than that of the isogenic WT strain (Chapter 3, Fig. 18B) (Schwechheimer et al., 2013) and that the mutant exhibits near-WT membrane integrity (Table 2) indicating that free Lpp must somehow contribute to membrane integrity. Recently, it was found that the triple \(\Delta ycfS\Delta ybiS\Delta erfK\) mutant strain was insensitive to high concentrations of D-Met which typically cause a 50% reduction in viability of the WT strain (Sanders and Pavelka, 2013). We were curious to determine if the mutant also would be insensitive to D-Met with regards to OMV production. Note that we
were using a lower concentration of D-Met than the Sanders and Pavelka study since we were interested in OMV induction conditions, not viability (Table 2). Whereas the WT strain responded to 5 mM D-Met, OMV production did not increase for the ΔycfSΔybiSΔerfK mutant (Fig. 35A), revealing that this strain was not only insensitive to D-Met in terms of viability, but also in terms of OMV production. To further characterize this strain, we tested if the ΔycfSΔybiSΔerfK mutant strain would still respond to spent V. cholerae supernatant. To our surprise, this triple deletion strain responded more strongly than the isogenic E. coli WT strain, increasing vesiculation by an additional 1.7-fold over WT (Fig. 35B). This suggests that a) there are products besides D-Met present in the spent supernatant that can stimulate OMV production (which could be any of the other D-AAs or other factor) and b) that the loss of bound Lpp and/or the loss of these three LD-transpeptidases somehow render the cells unresponsive to D-Met but more responsive to the other factors present in the spent supernatant.
Figure 35: ΔycfSΔybiSΔerfK responds to spent V. cholerae supernatant but not D-Met.

(A) Relative fold OMV production by untreated and 5 mM D-Met treated WT and ΔycfSΔybiSΔerfK mutant E. coli strains was determined as in Fig 3A. (B) Relative fold OMV production was determined as in Fig 1E for the indicated pelleted WT and mutant stationary phase E. coli strains resuspended and incubated overnight in spent OMV-free overnight WT V. cholerae (Vc) culture supernatant. Error bars indicate SEM; p values refer to comparisons with untreated samples of the same strain unless denoted by a bracket. *, p ≤ 0.05; n≥3. Reproduced from Schwechheimer and Kuehn, submitted.
**D-Ala treatment leads to an increase in OMV production and a decrease in covalent Lpp crosslinking.** In considering the regulation of OMV production by D-AAs, we needed to also consider the possible role of canonical D-AAs, such as D-Ala, which is a permanent component of PG (Vollmer and Bertsche, 2008). D-Ala is a PG precursor synthesized in the cytoplasm (Zawadzke et al., 1991) but is also present in the periplasm as a cleaved product of PG remodeling from tetrapeptides to tripeptides during the transition from log to stationary phase (Vollmer and Bertsche, 2008). It is noteworthy that these PG cleavage events, which lead to free periplasmic D-Ala, are coincident with the peak time of OMV production discussed earlier.

By comparing OMV production in D-Ala treated and untreated cultures of *E. coli*, we found that D-Ala lead to increased vesiculation in a concentration-dependent manner (Fig. 36A). Again, the addition of L-AAs counterpart had no affect on OMV production (data not shown). Periplasmic D-Ala is able to enter the cytoplasm via the CyaA importer (Robbins and Oxender, 1973), thus to determine whether D-Ala acts in the cytoplasm or the periplasm to increase OMV production, we repeated the D-Ala experiments using the ΔcycA mutant strain, which was shown to be impaired for D-Ala import (Robbins and Oxender, 1973). We still observed an increase in OMV production (using two concentrations of D-Ala) over the untreated control in the ΔcycA mutant, although the increase was lower than that seen for the WT strain (Fig. 36B). These results indicated that, unlike the case for D-Met, the full OMV-inducing activity of exogenously-added D-Ala was determined by both cytoplasmic and envelope locations of this D-AA.

When we quantified covalent Lpp crosslinking, we found an approximate 20% decrease for both WT and ΔcycA mutant D-Ala-treated strains in comparison to the
untreated controls (Fig. 36C). In control experiments we examined free Lpp levels and found that these did not decrease upon D-Ala treatment for either strain, in fact, they increased slightly in treated cultures over untreated (Fig. 36D). This was consistent with the decreased levels of the crosslinked form in the treated cultures. These data indicate that a decreased amount of covalent Lpp contributes to the increase in D-Ala-induced OMV production in both WT and ΔcycA strains.
Figure 36: D-Ala treatment increases OMV production and decreases covalent Lpp crosslinking
(A, B) Relative fold OMV production in cultures of the indicated D-Ala-treated E. coli WT and DcycA mutant strains grown in LB at 40°C was determined by quantitating OMVs, normalizing to OD\textsubscript{600}, and dividing by OD\textsubscript{600}-normalized OMV production in a untreated culture. (C) Relative fold crosslinked Lpp in cultures of the indicated D-Ala-treated strains grown in LB at 40°C was determined as in Fig 3B. (D) Relative fold free Lpp in cultures of the indicated D-Ala treated strains grown in LB at 40°C was determined as in Fig 3C. Error bars indicate SEM; p values refer to comparisons with untreated samples of the same strain. *, p ≤ 0.05; n\geq3. Reproduced from Schwechheimer and Kuehn, submitted.

5.4 Discussion

Currently, our understanding of the native mechanism of WT OMV production is rather cryptic and fragmented, but understanding this is especially critical due to the strong association of OMVs with pathogenesis. Furthermore, it is thought that such a mechanism might be conserved - after all, OMVs are produced by a wide-range and large number of diverse bacterial species. Insights pertaining to one organism could lead to understanding of similar regulatory and mechanistic processes in other, less well-studied, organisms. Until now, mechanistic insights into which bacterial factors are involved in the process and modulation of vesiculation have come from studies of mutants (Kulp and Kuehn, 2010). It is now critical to test the hypotheses generated from the study of mutants to further our understanding of what occurs for WT cells in natural surroundings.

Mechanistic basis for D-AA-modulated OMV production. Considering OMV production by WT cells peaks during the transition from late log to stationary phase (Chatterjee and Das, 1967; Chowdhury and Jagannadham, 2013; Gamazo and Moriyon, 1987; Hoekstra et al., 1976), and that PG is remodeled very actively during this period (Vollmer and Bertsche, 2008), along with our recent characterization of the hypervesisculating ΔnlpI mutant suggesting that PG remodeling is a key step to
modulating bound Lpp and OMV levels (Chapter 4) (Schwechheimer, Rodriguez, and Kuehn, submitted), we suspected this period to be critical to OMV biogenesis. It was also known that during this transition period, actively growing cells release D-AAs, which we now understand also can promote interspecies signaling. Consequently, we tested whether D-AAs could promote OMV production and found that spent supernatants from D-AA-producing \textit{V. cholerae} induce OMV production in \textit{V. cholerae}, as well as in a non-producer of non-canonical D-AAs, \textit{E. coli}. Further investigations showed that treatment with D-AAs was sufficient to induce vesiculation in both \textit{V. cholerae} as well as \textit{E. coli}. Furthermore, \textit{E. coli} could also respond to D-Ala, which it produces itself and is cleaved from the PG sacculus during PG remodeling. The ability of diverse species to use the same inducer suggests that there are likely molecular similarities in the mechanisms that lead to OMV production in Gram-negative bacteria.

To gain mechanistic insight into the regulation of OMV production by D-AAs, we compared the phenotypes and envelope characteristics of D-AA-treated WT and mutant strains of \textit{E. coli}. The results revealed that D-AA-induced OMV production correlates with a reduction in bound Lpp levels. It is noteworthy that these data pertaining to the mechanism by which OMV production in WT cells is induced under native induction conditions, support the Lpp-based mechanistic model for OMV production, which was conceived of by the analysis of hypervesiculation mutants.

How do D-AAs modulate OMV production? We considered that D-AAs could indirectly affect OMVs by altering protein biosynthesis, since it was recently demonstrated that D-AAs interfere with protein biosynthesis in \textit{Bacillus subtilis} (Leiman et al., 2013) In that study, it was determined that \textit{B. subtilis} lacks \textit{dtd}, a gene encoding a D-tyrosyl-tRNA deacetylase which prevents misincorporation of D-AAs into proteins.
Complementation prevented D-AA-dependent biofilm dissociation in *B. subtilis*. However, the *E. coli* strain used in our work (BW25113) carries the gene for this D-tyrosyl-tRNA deacetylase (Ecogene), suggesting that the D-AA-dependent modulation of OMV production that we observed is unlikely to be a result of impaired protein biosynthesis. In addition, when we investigated the location of the D-AA activity using mutants that lacked specific D-AA importers, we found these retained most, if not all, of their D-AA-stimulating activity, and thus the mechanism was not dependent on the ability of the D-AAs to access the cytoplasm. We did notice decreased hypervesiculation in D-Ala-treated Δ*cycA* cells compared to treated WT cells, and we entertained the idea that this could be due to residual amounts of D-Ala entering the cytoplasm. However, since we observed similar levels of decreased Lpp crosslinking in the WT and Δ*cycA* background, we do not think that is the case. We conclude that in the case of WT cells, the strong induction of OMV production by D-Ala relies in part on the decreased levels of Lpp crosslinking, as well as on some other, *cycA*-dependent factor.

Our data indicate that D-Met incorporation into PG is not necessary for OMV biogenesis stimulation (Fig. 3A). However, it should be mentioned that the mutant strain used for this experiment (Δ*ynhGΔycbB*) exhibits a residual amount of D-Met PG incorporation (Cava et al., 2011b). To fully eliminate D-Met PG incorporation, quintuple mutations would be necessary (Δ*ynhGΔycbBΔycfSΔybiSΔerfK*) (Cava et al., 2011b). Nevertheless, we do think our data supports the concept that D-Met incorporation into PG is not critical for D-Met-dependent OMV production stimulation, since the Δ*ynhGΔycbB* double mutant strain showed an approximate 75% decrease in incorporation (Cava et al., 2011b) and we expect we would have at least seen a reduction in vesiculation if incorporation were required for D-Met-dependent vesiculation.
We considered whether D-AAAs could regulate OMV production by directly interfering with PG biosynthesis enzymes. There is evidence that non-canonical D-AAAs compete for the penicillin binding proteins (Cava et al., 2011b), including the main PG synthases (Vollmer and Bertsche, 2008): The presence of non-canonical D-AAAs reduces PG synthesis, disturbing the PG-synthesis-degradation-equilibrium. We propose that disturbing of this PG remodeling equilibrium by non-canonical D-AAAs and D-Ala could thus indirectly alter the ability of the cell to establish of Lpp-PG crosslinks.

Alternatively, they could interfere directly with the LD-transpeptidases that form the covalent Lpp crosslinks, thus altering OMV levels. Consistent with this model (Fig 37), we observed decreased Lpp crosslinking (Fig. 34B, 36C) and inversely upregulated OMV production (Fig. 34A, 36A and B) for D-Met and D-Ala treated cultures. The effect of the canonical D-AA, D-Ala, is likely a special case, since it is a permanent component of PG (Vollmer and Bertsche, 2008). It is possible that periplasmic D-Ala released during the log-stationary phase transition can compete for sites in the penicillin binding proteins and thereby contribute to the concurrent observed increase in OMV production. More work is necessary to elucidate its mode of action in terms of increasing OMV production during the cell cycle.

The result that an E. coli strain containing only free Lpp (ΔycfSΔybiSΔerfK) is non-responsive to D-Met, but more sensitive to spent WT V. cholerae supernatant than WT (Fig 35) was quite surprising to us. This difference could be due to the variety of non-canonical D-AAAs, or the as of yet unidentified factor that stimulates OMV biogenesis, secreted by V. cholerae. Currently, we have two working models why the ΔycfSΔybiSΔerfK mutant strain may be unresponsive to D-Met. First, the membrane permeability of this strain is altered in comparison to WT, which is plausible, since free
Lpp contributes to membrane stability, as shown previously (Schwechheimer et al., 2013). Second, the interaction of non-canonical D-AA with the extracellular surface is important and affected in the mutant, since the increased amount of free Lpp could alter the external membrane characteristics substantially. The C-terminal end of free Lpp is extracellularly exposed (Cowles et al., 2011) and Lpp is extremely abundant (Braun, 1975; Silhavy et al., 2010), so a change in its membrane orientation would be expected to make a large impact on the properties of the cell surface.

**Figure 37:** Working model of D-AAs modulating OMV production.
The data suggest that D-AAs affect PG remodeling, which in turn decreases Lpp crosslinking and consequently upregulates vesiculation. Reproduced from Schwechheimer and Kuehn, submitted.

**Implications for native OMV production and induction.** In summary, these data indicate that the free form of a native structural component of the *E. coli* cell wall, D-Ala, as well as natively-secreted non-canonical D-AAs at native concentrations can increase OMV production, and that this is partially achieved by a decrease in Lpp-mediated covalent crosslinking between the PG and the OM. It should be noted that Lam and coworkers determined the total concentration of secreted non-canonical D-AAs to be ~ 1 mM by *V. cholerae* (Lam et al., 2009), whereas we noticed that treatment with 5 mM D-Met yielded similar induction of OMV production by *E. coli* as treatment with spent *V. cholerae* supernatant (compare Fig. 33 and 34A). This result lead us to hypothesize that the potency of a single non-canonical D-AA is lower than that of a natural mixture of D-AAs and other factors secreted by *V. cholerae*.

Previous studies have demonstrated the presence of OMVs in biofilms and have begun to reveal their importance in the development and stability of these natural multibacterial structures (Duperthuy et al., 2013; Schooling and Beveridge, 2006; Schooling et al., 2009; Yonezawa et al., 2009). In light of their coincident timing, the fact that D-AAs are secreted into the extracellular milieu (Lam et al., 2009), and the data regarding D-AA-stimulated and cross-species induction of vesiculation presented here, we hypothesize that the D-AA-regulated vesiculation may impact biofilm formation. Additionally, beyond biofilm communities, D-AA stimulated OMV production can be important in many other types of multifactorial bacterial systems, since diverse species have been identified to produce D-AAs (Lam et al., 2009).
5.5 Acknowledgements

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6. Modulation of Outer Membrane Vesicle Production by Peptidoglycan Remodeling and Periplasmic Accumulation

6.1 Summary

In Chapters 4 and 5, we determined an inverse relationship between OMV production and covalent OM-peptidoglycan (PG) crosslinking for the hypervesiculating mutant, ΔnlpI, and for induction of OMV production by environmental D-amino acids. In this chapter, we found that more subtle modifications of PG remodeling and crosslinking similarly modulate higher or lower OMV production, inversely correlating with bound Lpp levels. Finally, we report that exceptions exist to the inverse relationship of overall bound Lpp levels and OMV production. The exceptions include strains in which OMV production is driven by an increase in “periplasmic pressure” resulting from the accumulation of protein, PG fragments, or lipopolysaccharide. From this work, we conclude that OMV production can be driven by distinct Lpp concentration-dependent and Lpp concentration-independent pathways.

6.2 Introduction

The hypothesis that the OM has to be dissociated from the underlying PG for an OMV bud to form has been long-appreciated (Hoekstra et al., 1976; Mashburn-Warren and Whiteley, 2006). Indeed, the complete loss of envelope stabilizing factors leads to extremely high OMV production although this is accompanied by cellular leakage (Deatherage et al., 2009; Schwechheimer et al., 2013). It is critical to remember that WT bacteria, in normal and in inducing conditions, along with numerous hypervesiculation mutants, produce OMVs without compromising the envelope stability (Table 1 and 2) (Fulsundar et al., 2014; Henry et al., 2013; Macdonald and Kuehn, 2013; McBroom et al.,...
2006; McBroom and Kuehn, 2007; Schwechheimer and Kuehn, 2013), suggesting that under these conditions the envelope structure is modulated in a more moderate and regulated fashion.

In Chapter 4, we studied the envelope architecture of the ΔnlpI mutant strain of *E. coli* to understand its strong hypervesiculation phenotype and discovered that covalent OM-PG crosslinking is reduced in this strain, likely as a consequence of increased PG turnover (Schwechheimer, Rodriguez, and Kuehn, submitted). We further confirmed this model in experiments using WT cells: WT increased OMV production by *E. coli* exposed to environmental D-AAs was concomitant with decreased levels of OM stabilizing crosslinks (Chapter 5) (Schwechheimer and Kuehn, submitted).

In this chapter, we were interested in further investigating the inverse relationship between vesiculation and OM-PG crosslinking. We analyzed the effect on OMV production of mutations that alter PG degradation and crosslinking and found that subtle alterations in PG and envelope structure lead to differences in vesiculation. Modulation of PG remodeling correlated again with an inverse relationship between the level of OMV production and Lpp covalent crosslinking further supporting this as a mechanism for OMV biogenesis. We were then curious whether this model extended to other hypervesiculating conditions, particularly those involving build-up of material in the periplasm, since some previously identified genetic interactions suggested otherwise. We investigated the levels for a mutant in which periplasmic misfolded protein accumulation led to upregulated OMV production (McBroom and Kuehn, 2007; Schwechheimer and Kuehn, 2013). Interestingly, Lpp crosslinking levels were not inversely correlated with OMV levels when OMV production was driven by periplasmic accumulation of protein. Further analysis of mutants demonstrated that accumulation
of PG fragments or lipopolysaccharide (LPS) behaved similarly. Together, these data are consistent with two mechanisms as a basis for OMV production by Gram-negative bacteria, one dependent on and the other independent of levels of bound Lpp.

### 6.3 Results

**OMV production and Lpp crosslinking changes inversely with altered PG structure.** PG is a highly dynamic polymer, especially during cell growth and growth phase transitions (Vollmer and Bertsche, 2008). We hypothesized that alterations in the PG structure underlying the OM could be a means by which cells may modulate OMV production. This idea is strengthened by previous results demonstrating that the deletion of the amidase autolysin in Porphyromonas gingivalis, an enzyme that cleaves PG amide bonds, led to an increase in OMV production (Hayashi et al., 2002). Our recent discoveries implicating increased PG turnover as the likely cause for reduced covalent OM-PG crosslinking, and consequently hypervesiculation of the nlpI deletion mutant as well as D-AA-induced hypervesiculation, lend further support to this hypothesis (Chapter 4 and 5) (Schwechheimer, Rodriguez, and Kuehn, submitted; Schwechheimer and Kuehn, submitted).

To further examine the relationship between modulation of PG structure and levels of OMV production, we examined another PG hydrolase mutant, ΔmepAΔdacBΔpbpG, which lacks three of the endopeptidases that cleave the PG peptide bonds. We observed that OMV production increased in this mutant strain, similar to the data for P. gingivalis (Hayashi et al., 2002)(Fig. 38A). The increase in OMV production was consistent with the level observed for the deletion of the endopeptidase Spr (Chapter 4, Fig. 19A and 25C) (Schwechheimer, Rodriguez, and Kuehn, submitted). Note that we tested this strain along with all other strains used in this work for their
membrane integrity with previously published assays (Chapters 2, 4 and 5) (Schwechheimer and Kuehn, 2013), so we could be sure that we are quantifying true vesiculation, not the presence of membrane fragments as a consequence of membrane instability (Table 3).

Next, we examined the Lpp crosslinking levels of the ΔmepAΔdacBΔpbpG strain. We used our previously established immunoblotting assay that allows us to distinguish between the PG crosslinked form of Lpp (historically referred to as ‘bound’ form) and the ‘free’ form, which is solely OM lipid-anchored but not PG crosslinked (Chapter 4) (Schwechheimer, Rodriguez, and Kuehn, submitted). As expected, we again found an inverse relationship between OMV production and bound Lpp (Fig. 38B). The amount of free Lpp was comparable to WT (Fig. 38C), suggesting that the observed decrease is not a result of an overall decrease in Lpp.

We also investigated the LD-transpeptidase ΔynhGΔycbB double mutant, which contains the common D-Alanine (D-Ala)-Diaminopimelic acid (DAP) peptide crosslink but lacks the minor DAP-DAP crosslink (Magnet et al., 2008). We were especially interested in this strain in light of its relationship to Lpp crosslinking, since it had been shown that DAP-DAP muropeptides are enriched in covalently crosslinked Lpp (Glauner et al., 1988). The loss of DAP-DAP crosslinks correlates with a strong hypovesiculation phenotype, ~ 60% less than WT (Fig. 38D). When examining the Lpp crosslinking levels of this mutant we found a significant increase in covalently attached Lpp (by ~ 2.6 fold, Fig. 38E), whereas the concentration of free Lpp resembled WT levels (Fig. 38C). It should be noted that this was the first time we observed an increase in bound Lpp, demonstrating that bound Lpp levels can have a dynamic range in both directions. Taken together, these data suggest that the modulation of PG structure can
alter the levels of OMV production in either direction via an inverse relationship to PG-Lpp crosslinking.

Figure 38: OMV production and Lpp crosslinking changes inversely with altered PG structure.
(A) Relative fold OMV production in cultures of the indicated strains grown in LB overnight at 37°C was determined by quantitating OMVs, normalizing to OD$_{600}$, and dividing by OD$_{600}$-normalized OMV production in a WT culture. Error bars indicate standard error of the mean (SEM). *, p ≤ 0.05; n=4. (B) Relative fold crosslinked Lpp in cultures of the indicated strains grown in LB to an OD$_{600}$ of ~ 0.4 at 37°C was determined by immunoblotting of PG copurified Lpp, normalizing to OD$_{600}$ and dividing by OD$_{600}$-normalized crosslinked Lpp in a WT culture. *, p ≤ 0.05; n=3. (C) Relative fold of free Lpp in cultures of the indicated strains grown overnight in LB. n=3. (D) Relative fold OMV production in cultures of the indicated strains grown in LB overnight at 37°C was determined as in part A. Error bars indicate SEM. *, p ≤ 0.05; n=5. (E) Relative fold crosslinked Lpp in cultures of the indicated strains grown in LB to an OD$_{600}$ of ~ 0.4 at 37°C was determined as in part B. *, p ≤ 0.05; n=3. Error bars indicate SEM; p values refer to comparisons with WT. Reproduced from Schwechheimer, Kulp, and Kuehn, submitted.
Table 3: Growth and membrane integrity phenotypes of mutant cultures.

<table>
<thead>
<tr>
<th>Strains/ Treatments</th>
<th>OMV Production</th>
<th>Growth</th>
<th>Growth w/ Actinomycin D&lt;sup&gt;4&lt;/sup&gt;</th>
<th>Sytox Green&lt;sup&gt;5&lt;/sup&gt;</th>
<th>Adenylate Kinase&lt;sup&gt;6&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Log phase cultures:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔycfSAybISerfK</td>
<td>1</td>
<td>WT</td>
<td>WT</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ΔycfSAybISerfKΔnlpA</td>
<td>3</td>
<td>WT</td>
<td>WT</td>
<td>(l)</td>
<td>+</td>
</tr>
<tr>
<td>ΔompA</td>
<td>1</td>
<td>WT</td>
<td>WT</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ΔnlpAΔompA</td>
<td>2</td>
<td>WT</td>
<td>WT</td>
<td>+</td>
<td>BD</td>
</tr>
<tr>
<td><strong>Overnight cultures:</strong></td>
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<td></td>
<td></td>
</tr>
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<td>+</td>
<td>++</td>
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<td>+</td>
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<tr>
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<td>63</td>
<td>WT</td>
<td>WT</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ΔompA</td>
<td>26</td>
<td>WT</td>
<td>WT</td>
<td>++</td>
<td>+</td>
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<tr>
<td>ΔnlpAΔompA</td>
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<td>WT</td>
<td>WT</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
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<td>WT</td>
<td>WT</td>
<td>+</td>
<td>++</td>
</tr>
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<td>WT</td>
<td>WT</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
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<td>3</td>
<td>WT</td>
<td>WT</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
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<td>WT</td>
<td>WT</td>
<td>(l)</td>
<td>+</td>
</tr>
<tr>
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<td>15</td>
<td>&lt;WT</td>
<td>WT</td>
<td>+</td>
<td>BD</td>
</tr>
</tbody>
</table>

1 Sets of assays performed in the same experimental group are separated by a space in the table; heat-killed cells were used as a positive control for Sytox Green and adenylate kinase activity; each assay was repeated at least twice.

2 Fold-change in OMV production relative to WT or untreated. See Figures for statistical evaluations.

3 Growth in LB: +, like WT or control; -, below WT or control.

4 Growth in LB with 5 μg/mL Actinomycin D: +, like WT or control; -, below WT or control.

5 Sytox Green entry: +, no significant difference from WT or control; (l) significantly less than WT or control; ++, significantly higher compared to WT or control.

6 Adenylate kinase in supernatant: +, ~100-999 LU/OD; ++, ~1000-9999 LU/OD; For comparison, heat killed cells yielded >40,000 LU/OD for log phase cells and >20,000 LU/OD for stationary phase; BD, below detection, refers to samples with a negative value.
The inverse relationship between Lpp crosslinks and OMV production does not hold for mutants that accumulate periplasmic protein. We were very curious to determine if the protein accumulation-driven increased OMV production of the ΔdegP mutant also exhibited a decrease in covalent Lpp crosslinks. We quantified covalent Lpp crosslinks of the ΔdegP strain and found that Lpp crosslinking levels were not different from the WT (Fig. 39A). Free Lpp was slightly elevated, but not statistically significance (Fig. 39B). These data support a hypothesis in which accumulation of periplasmic protein creates an increase in periplasmic pressure, which in turn can lead to hypervesiculation but that this occurs without altering the total numbers of Lpp crosslinks.

Accumulation of PG fragments correlates with increased OMV production without altering Lpp crosslinking. In light of the data presented for the protein accumulation mutant, we wondered whether periplasmic PG fragment accumulation could also lead to hypervesiculation, and if so, whether it would depend on Lpp crosslink levels. The ΔampG mutant lacks the inner membrane (IM) permease AmpG, and this mutant is impaired in transporting muropeptides from the periplasm to the cytoplasm for PG recycling (Jacobs et al., 1994). Uehara and Park demonstrate that in the absence of ampG, PG fragments accumulate in the periplasm and are consequently cleaved by the amidase, AmiD. We utilized the ΔampGΔamiD double mutant which also lacks AmiD and therefore cannot degrade the larger PG fragments into smaller ones and accumulates them in the periplasm (Uehara and Park, 2007). The ΔampGΔamiD mutant strain was shown to release large PG fragments into the cell-free medium although these were too large to diffuse through the OM porins (Uehara and Park, 2007), suggesting they were likely to be in OMVs. These data were consistent with the notion that
periplasmic accumulation of PG fragments caused their subsequent shedding into the medium via OMVs, similar to hypervesiculation caused by protein accumulation in a degP mutant (Chapter 2) (Schwechheimer and Kuehn, 2013).

When we examined OMV production, we determined that the ΔampGΔamiD mutant exhibited ~14 fold increased OMV production with respect to WT (Fig. 39C). To investigate whether this PG fragment-induced hypervesiculation mechanism was consistent with periplasmic protein-induced OMV induction (Chapter 2) (Schwechheimer and Kuehn, 2013), we tested whether deletion of nlpA affected the ΔampGΔamiD OMV phenotype. Again, unlike for ΔdegP, ΔnlpA was not epistatic to ΔampGΔamiD (Fig 39C), supporting the model that there are multiple routes to vesiculation. Next, we were curious to determine if the route of OMV production that is driven by accumulation of periplasmic PG or protein content is also dependent on decreased Lpp crosslinking. The data in Fig. 39A and 39B show that the Lpp crosslinking levels as well as the amount of free Lpp of the ΔampGΔamiD mutant matched that of the WT strain. In sum, a mutant in which PG fragments likely accumulate in the periplasm hypervesiculated without exhibiting altered levels of Lpp crosslinking.
Figure 39: Accumulation periplasmic PG fragments correlates with increased OMV production without alteration of Lpp crosslinking.

(A) Relative fold crosslinked Lpp in cultures of the indicated strains grown in LB to an OD<sub>600</sub> of ~ 0.4 at 37°C was determined by quantitative immunoblotting of Lpp copurified with PG, normalizing to OD<sub>600</sub> and dividing by OD<sub>600</sub>-normalized crosslinked Lpp in a WT culture. n=3. (B) Relative fold of free Lpp in cultures of the indicated strains grown overnight in LB at 37°C. n=3. (C) Relative fold OMV production in cultures of the indicated strains grown in LB overnight at 37°C was determined by quantitating OMVs, normalizing to OD<sub>600</sub> and dividing by OD<sub>600</sub>-normalized OMV production in a WT culture. *, p ≤ 0.05; NS, p > 0.05; n=4. Reproduced from Schwechheimer, Kulp, and Kuehn, submitted.

LPS accumulation also leads to hypervesiculation without modulating bound Lpp concentration. We reasoned that accumulation of LPS fragments could generate a
similar effect regarding OMV production as either the accumulation of PG fragments described above (Fig. 39C) or periplasmic protein as described previously (Chapter 2) (McBroom and Kuehn, 2007; Schwechheimer and Kuehn, 2013). The OM of Gram-negative bacteria is asymmetric with the inner leaflet composed of phospholipids and the outer leaflet composed of LPS, a unique molecule to Gram-negative bacteria of which at least the minimal lipid A backbone structure is essential in most species (Galloway and Raetz, 1990; Raetz, 1990; Raetz and Whitfield, 2002). Data published recently indicate that individual mutations (ΔrfaC, ΔrfaG, and ΔrfaP) that alter the sugar core structure of LPS lead to periplasmic LPS accumulation due to the disruption of LPS maturation in the envelope of the cell (Lima et al., 2013). Additional evidence further supports the concept of periplasmic LPS accumulation: rfaC and rfaG mutant strains contain an increased amount of LPS in comparison to WT (Gmeiner and Schlecht, 1979; Klein et al., 2014), and increasing LPS production of ΔrfaC leads to abnormal structures in the periplasm, implying that LPS overproduction results in a reduction of proper, OM-localized LPS, but not a reduction in the overall amount of LPS in the envelope (Ogura et al., 1999).

As expected, all LPS core mutants exhibited hypervesiculation phenotypes (Fig. 40A). However, mutant strains ΔrfaC, ΔrfaG, and ΔrfaP activate the σE envelope heat shock response, a process discovered to require both mislocalized, periplasmic LPS as well as a misfolded outer membrane protein (OMP) component for activation (Lima et al., 2013). Since σE activation implicated the presence of misfolded OMPs, and previous work from our lab showed that periplasmic protein accumulation leads to hypervesiculation, we needed to determine if the reason for hypervesiculation in the ΔrfaC, ΔrfaG and ΔrfaP mutants was actually solely due to increased periplasmic protein.
levels. We measured the amount of periplasmic protein in the mutants and found that the $\Delta_{rfaC}$ and $\Delta_{rfaP}$ mutant contained WT levels (Fig. 40B), supporting the hypothesis that it is the periplasmic LPS, not the increase in protein, that leads to hypervesiculation. Interestingly, the periplasmic protein concentration in the $\Delta_{rfaP}$ strain was significantly higher than that of the WT (Fig. 40B), thus we could not distinguish whether the hypervesiculation phenotype of this mutant resulted from accumulation of periplasmic protein or LPS, or a combination of these.

Next, we determined the amount of covalently crosslinked Lpp in these LPS mutants in order to see if these inversely correlated with the OMV phenotypes. Covalent Lpp crosslinking was unchanged with respect to WT for $\Delta_{rfaC}$ and $\Delta_{rfaG}$; $\Delta_{rfaP}$ exhibited a slight reduction, albeit not statistically significant (Fig. 40C). In control experiments, the amount of free Lpp in the strains was also not significantly different from WT (Fig. 40D). These results support the hypothesis that OMV production in these LPS mutants is predominantly driven by accumulated material rather than a decrease in overall covalent Lpp crosslinking.

Overexpression of a $\sigma^E$-stimulating model misfolded polypeptide lead to hypervesiculation as well as enrichment of the polypeptide in the OMVs (McBroom and Kuehn, 2007). Therefore, to further our model that the accumulation of any mislocalized or misfolded envelope material leads to hypervesiculation, we tested whether OMVs produced by a mutant strain which mislocalized LPS were enriched in lipid. We quantified the lipids in OMVs produced by WT, $\Delta_{rfaC}$, $\Delta_{rfaG}$, and $\Delta_{rfaP}$ strains using a lipophilic dye (FM4-64) that becomes fluorescent upon membrane intercalation. Then, we divided this number by the quantity of OMPs in the OMVs from each of the strains. The results show a four-fold increase in the lipid to OMP ratio for $\Delta_{rfaC}$ OMVs, a 15-fold
increase for Δ*rfα*G and a 29-fold increase for Δ*rfα*P OMVs, with respect to the WT OMV control, confirming lipid accumulation in the OMVs of Δ*rfα*C, Δ*rfα*G, and Δ*rfα*P (Fig. 40E). These data strongly support accumulated lipid, LPS, was found in the secreted OMVs.
Figure 40: Accumulation of periplasmic LPS correlates with increased OMV production without alteration of Lpp crosslinking.

(A) Relative fold OMV production in cultures of the indicated strains grown in LB overnight at 37°C was determined by quantitating OMVs, normalizing to OD$_{600}$, and dividing by OD$_{600}$-normalized OMV production in a WT culture. *, p ≤ 0.05; n≥3. Error
bars indicate SEM. (B) Protein concentrations in periplasm preparations of the indicated strains grown ~16-18 hrs in LB at 37°C were determined by Bradford Assay. *, p \leq 0.05; WT: n=9; ΔrfuC: n=8; ΔrfuP: n=7. Error bars indicate SEM. (C) Relative fold crosslinked Lpp in cultures of the indicated strains grown in LB to an OD\textsubscript{600} of ~ 0.4 at 37°C was determined by quantitative immunoblotting of Lpp copurified with PG, normalizing to OD\textsubscript{600} and dividing by OD\textsubscript{600}-normalized crosslinked Lpp in a WT culture. n=3. (D) Relative fold of free Lpp in cultures of the indicated strains grown in LB at 37°C. (F) Protein concentration was determined by quantitating OMPs via densitometry, lipids were quantitated by the lipophilic dye FM4-64 of OMVs purified from cultures of the indicated strains grown in LB overnight at 37°C. *, p \leq 0.05; n=3. Error bars indicate SEM. Reproduced from Schwechheimer, Kulp, and Kuehn, submitted.

### 6.4 Discussion

Despite investigations revealing that OMVs function in critical areas such as pathogenesis, bacterial survival, and envelope stress, our knowledge of the mechanism and regulation of OMV production remains quite cryptic. To gain mechanistic insight into OMV production, we analyzed the phenotypes and composition of the effect of mutants. The results reveal a specific relationship between PG dynamics, periplasmic content, and OMV production. We have shown here that multiple routes modulate vesiculation: one that is bound Lpp-dependent and another that is bound Lpp-independent. Both of these pathways appear to be stimulated by multiple factors: covalent Lpp crosslinking can be altered by changes in PG structure, and increased PG degradation, as shown here and previously (Chapters 4 and 5) (Schwechheimer, Rodriguez, and Kuehn, submitted; Schwechheimer, Kulp, and Kuehn, submitted); whereas envelope accumulation of material (protein, PG fragments and LPS, or a combination of these) result in hypervesiculation with minimal or no contribution from changes in bound Lpp levels.

**Fine-tuning of OMV production through PG biosynthesis and structure.** Our earlier reports proposed a relationship between PG remodeling, Lpp crosslinking and
OMV production. Form this work the questions arose, if this relation would continue to hold up for other mutations and conditions that modulate vesiculation, and if WT bacteria could affect OMV production via similar PG affecting routes (D-AA-independent). The fact that the triple endopeptidase deletion mutant hypervesiculates and also exhibits a decrease in covalent Lpp crosslinking (Fig 38B) means that the equilibrium of PG turnover may dictate the number of covalent envelope crosslinks and thus indirectly modulate OMV production.

An interesting reverse situation was found with the loss of the genes responsible for the minor DAP-DAP PG crosslinks (ΔynhGΔycbB). In this mutant, OMV production is downregulated (Fig. 38D) with a concomitant increase in bound Lpp (Fig. 38E). To explain this result, we hypothesize that DAP-DAP crosslinks may serve as location markers for bound Lpp. When these markers are missing (e.g. in ΔynhGΔycbB), bound Lpp could be more randomly distributed across the PG sacculus and at a higher concentration. This model is supported by the observation that PG-Lpp crosslinks were previously shown to be enriched at sites of DAP-DAP crosslinks (Glauner et al., 1988).

OMV production relieves stress caused by the accumulation of diverse, potentially harmful products in the envelope. We and others have shown that hypervesiculation can aid in the release of otherwise harmful periplasmic accumulation of protein in mutant as well as WT cells (Chapter 2) (Schwechheimer and Kuehn, 2013; Strauch et al., 1989). Here we demonstrate that not just the accumulation of periplasmic protein, but also of PG fragments and LPS, leads to an increase in OMV production, indicating that OMV production is a general mechanism by which Gram-negative bacteria cope with build-up of material in the envelope. These results further establish the general and important role OMV production plays in bacterial well-being.
We considered that OM shedding may actually be a harmful phenomenon for the bacteria, expending a great deal of “macromolecular synthetic energy”, and that expanding the periplasm to accumulate the excess material could be a more sensible solution for the cell. It has been shown that the eukaryotic endoplasmic reticulum deals with an increase in misfolded protein load by expanding its membranes (Sriburi et al., 2004), so why is this not a possibility for Gram-negative bacteria? The answer is straightforward when considering the envelope architecture, the envelope has limited room to expand, since it is connected by Lpp, a finite covalent crosslink between the OM and PG. With such volume limitations, the alternative would be to either increase the concentration of misfolded / mislocalized envelope material, which would become toxic to the proper function of the envelope, or to bulge out membrane in between the crosslinks, and indeed these might be what leads to spontaneous membrane fusion events, seen as OMV budding.

Apparently, the trapped periplasmic material cannot prevent the formation of bound Lpp, but possibly it pushes the OM outward, either by taking advantage of “nanoterritories” of OM containing locally decreased levels of bound Lpp, or by displacing bound Lpp to sites on the periphery of the outwardly bulging OM.

Very recently, YciM was identified as a negative regulator of LPS biosynthesis, and an excess of LPS was confirmed to be responsible for the death of yciM mutants (Mahalakshmi et al., 2013). Interestingly, they report that suppressor mutations include those that either downregulate LPS biosynthesis via other routes, or they are part of a group of genes that is involved in OM assembly or organization (lpp, rfaP, ybcN, galU). Notably, all the mutants from the second group hypervesiculate (A. Kulp, A. Manning, B. Sun, T. Ai, A. Schmidt, and M. Kuehn, unpublished data)(Deatherage et al., 2009;
Schwechheimer et al., 2013). This observation further supports our hypothesis that the accumulation of large quantities of envelope components is lethal and shedding OMVs and eliminating the accumulated material from the periplasm can relieve and rescue this situation.

**The relationship between bound Lpp and OMV budding.** In sum, our data indicate that there are at least two mechanisms that lead to OMV budding, one that relies on overall decreased levels of Lpp crosslinking, and one that is independent of Lpp crosslinking levels. Regarding the mechanism of budding under conditions of protein, PG fragment, or LPS accumulation, where OMV levels are independent of overall bound Lpp levels, it seems reasonable that the accumulated material might preferentially localize to “nanoterritories” containing locally-reduced amounts of bound Lpp, or by displacing bound Lpp to sites on the periphery of the outwardly bulging OM.

To extrapolate our results to understand how WT bacteria might modulate OMV levels, we propose that bacteria may be able to modify OMV production by localized stimulated modulation of the PG structure (by modulating the equilibrium between its synthesis and degradation), or by bulk deposits of envelope material, either of which has consequent effects on either localized or overall bound Lpp concentrations.

### 6.5 Acknowledgements

This work was supported by NIH grant R01GM099471. We are grateful for the generous contributions of Tom Silhavy (Lpp antibody), the National BioResource Project (NIG, Japan) for the *E.coli* Keio Collection, and Adam Kulp for quantifying vesiculation of the LPS mutants.
7. Concluding Remarks

OMVs are produced by all Gram-negative bacteria studied to date, indicating a crucial role for these secretory units in bacterial physiology. They are also a way by which bacteria can interact with their environment during pathogenesis, in multi-microbe communities such as biofilms, and during nutrient acquisition. Despite these critical biological functions of OMVs, very little is still understood about their biogenesis and regulation. Here, we have analyzed vesiculation mutants that came from a transposon screen done by our lab to gain insight into the native mechanism and regulation of OMV biogenesis.

We have demonstrated in these studies how the powerful combination of genetics and biochemical analysis could throw open the field to mechanistic understanding of OMV biogenesis, an investigation strategy that had not been applied to the field.

Our lab recently screened the E. coli Keio Collection, a collection of all non-essential gene deletion strains (Baba et al., 2006), for their vesiculation phenotypes and identified a plethora of mutants for which we do not have an explanation, as of yet. It will be important to see how these mutants fit into the models we have built in these studies or if they present other paradigms, in this clearly dynamic system.

We are very excited about our novel finding of a natural factor, D-amino acids, to modulate vesiculation, including as a form of interspecies communication. This discovery is of great interest, since we have already shown here that two species are responsive to non-canonical D-AAs suggesting we have discovered a vesiculation induction mechanism conserved in other Gram-negative bacteria. This hypothesis is
further supported by the finding of Lam et al. that numerous Gram-negative species produce these non-canonical D-AAs (Lam et al., 2009).

In the following sections of this chapter, current working models are presented which help summarize the data from this thesis research.

7.1 Mechanistic vesiculation models based on the data presented here

Our data indicate that there are at least two routes to OMV production: bound Lpp concentration-independent (Fig. 41) and bound Lpp concentration-dependent (Fig. 42). Under conditions when periplasmic material accumulates (protein waste, PG fragments, and mislocalized LPS), the increase in periplasmic pressure may push out the OM creating an emerging vesicle bud (Fig. 41). The cause and effect remain to be determined. In other words, the periplasmic build-up may preferentially occur at sites of a local decrease in bound Lpp concentration, or, alternatively the periplasmic build-up may cause Lpp or other membrane crosslink components to be rearranged.

![Figure 41: Bound Lpp-independent OMV production.](image)

Periplasmic bulk accumulation creates pressure, pushing the OM outwards. This may preferentially occur between Lpp crosslinks, or push the crosslinks away from the site.
Changes in PG structure and turnover modulate OMV production in a bound Lpp-dependent manner (Fig. 42). Altering the PG metabolism or changing the PG structure by decreasing its breakdown can result in a decrease in covalently bound Lpp leading to hypervesiculation (Fig. 42A). *Vice versa*, if the cell is missing the minor PG DAP-DAP crosslinks, which have been shown to be enriched for bound Lpp (Glauner et al., 1988), the bound Lpp concentration increased creating a tighter envelope that resulted in hypovesiculation (Fig. 42B).

Figure 42: Bound Lpp-dependent OMV production.

(A) Equilibrium for PG synthesis and breakdown, which determines the amount of covalently crosslinked Lpp. (B) Minor DAP-DAP PG crosslinks have been shown to be enriched in covalently crosslinked Lpp, these crosslinks could serve as Lpp localization markers.
The data shown here support a model in which NlpA (to a lesser extent MetQ) serves as an envelope crosslink with bound Lpp and/or OmpA (Fig. 43). The lack, as in the data presented here, or potentially the reduction in a native setting of \textit{nlpA} (\textit{metQ}) may be a means by which cells can regulate vesiculation levels. Furthermore, as in the model presented in Fig. 43, NlpA (MetQ) could serve as a boundary marker to determine the size of the vesicle.

\textbf{Figure 43: NlpA scaffolding model.}

Model of NlpA as a scaffolding protein marking the boundaries of a vesicle bud and aiding in envelope crosslinking.

In sum, we have demonstrated here that vesiculation can serve as a critical envelope stress response when the cells have to eliminate undesirable and potentially harmful components from the envelope. Furthermore, our data indicate that Gram-negative bacteria can modulate OMV production in a dynamic range. We hypothesize that cells can use either one or a combination of mechanisms presented here to change the levels of vesiculation creating the best outcome for bacterial well-being.
7.2 Future directions

The data presented here provided insights to establish these new models for OMV biogenesis, but numerous mechanistic details are still unexplored, which we address and summarize in this section.

A question that arises from the data showing periplasmic waste accumulation and consequent shedding by OMVs is the following: Is cargo sorted into OMVs by an active process or is it solely a result from the overabundance in the periplasm? To gain insights into this question of active sorting the waste products could be tagged and pulled down after crosslinking to identify components they come into contact with, which would include sorting factors.

Additionally, we gained much information by starting to examine the role of Lpp in OMV production but there are still unanswered questions that are expected to provide great mechanistic insights such as: If there is a rearrangement of bound Lpp especially under conditions of periplasmic accumulation? In order to study this, tagging and visualizing very small changes in the localization and binding status of Lpp, which is currently unavailable, would be of great use.

Furthermore, here we have solely examined Lpp, but it is to be expected that Pal and OmpA will also play a role in the vesiculation process. Therefore, a natural follow-up to my thesis research is to investigate the role of these other non-covalent envelope crosslinks in OMV production. We started analyzing the role of Lpp in OMV production in part because it was the most straightforward crosslink to study due to its covalent PG crosslink and the consequent purification with PG. Since OmpA and Pal only have noncovalent PG association the help of a chemical crosslinker will be necessary.
7.3 Remaining questions for the field

As mentioned throughout my thesis a vast amount of understanding has been gained over the years as to the role OMVs play in pathogenesis as well as their more general functional properties. The regulation and mechanism of their production is still not fully elucidated, but what seems to become clear is that there may be multiple means by which OMVs are produced. Nevertheless, major questions in the field remain unanswered: How is membrane curvature achieved? How do OMVs ultimately pinch off? How is cargo selectively enriched? What are the temporal characteristics of vesicle formation with respect to cell cycle? Is this process conserved in other Gram-negative organisms? Further work will be necessary to answer these questions.
8. Materials and Methods

8.1 Growth conditions and reagents

Strains and plasmids used in this work are summarized in Table 3. Bacteria were grown in liquid culture in Luria–Bertani (LB) broth (EM Science) or on plates of solid LB agar supplemented with 50 mg/ mL kanamycin or 100 mg/ mL ampicillin (Sigma). Isopropyl β-D-1-thiogalactopyranoside (IPTG) (VWR) was added to induce protein expression if indicated. The single gene mutants originate from the Keio Collection (Baba et al., 2006). To create mutants with multiple deletions, the kanamycin resistance marker was removed from the single mutant (Cherepanov and Wackernagel, 1995). The additional mutation was then added by transduction of the marked gene deletion using P1 phage (Silhavy et al., 1984) from the donor single Keio mutant strain into the unmarked Keio recipient mutant strain. The mutants constructed for this work were either sequenced with a primer upstream and downstream of the deleted gene or PCR amplified with primers upstream/ downstream of the deleted gene and the kanamycin cassette to confirm the genotypes.

For the D-Met stationary phase growth curves, 25 ml cultures were inoculated (1:250 dilution), cultured overnight (~16 h) at 37°C after which the cells were pelleted with the Beckman Avanti J-25 centrifuge (JLA-16.250 rotor, 10 000 g, 10 min, 4°C), and resuspended in fresh media treated with the indicated concentration of D-AA or the same volume of LB (untreated controls), and incubated for 6 h at 37°C with hourly optical density measurements at 600 nm (OD₆₀₀).

Cultures (5 mL) were inoculated to an OD₆₀₀ ~0.03, D-AAs were added at t=30 min (if applicable), cultures were incubated at 37°C for 6 h, and OD₆₀₀ was measured
hourly to assess growth for growth curves. Better, similar, or worse growth of the mutant or D-AA treated cultures were determined by comparing OD₆₀₀ for 6 h with that of the WT or untreated control culture. We considered a difference in 0,1 OD₆₀₀ to the control culture when at least 3 time points differed and are summarized the data in Table 1-3.

**Table 4: Strains and Plasmids.**

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<th>Strains</th>
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<tr>
<td><strong>MKW620</strong></td>
<td>N16961 ΔbsrV</td>
<td>(Lam et al., 2009)</td>
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</table>

### Plasmids

- **pCS19**: pQE60-derived vector with lacIq, AmpR, IPTG-inducible
  - Spiess et al. (1999)
- **pCS20 (pDegP)**: degP in pCS19, AmpR, IPTG-inducible
  - Spiess et al. (1999)
- **pCS21 (pmDegP)**: degP[S210A] in pCS19, AmpR, IPTG-inducible
  - Spiess et al. (1999)
- **pTrc99A**: trc promoter, AmpR, pBR322 ori
- **pNlpA**: nlpA in pTrc99a, AmpR, IPTG-inducible
  - (Schwechheimer and Kuehn, 2013)
  - (Schwechheimer and Kuehn, 2013)
- **pNlpI**: nlpI in pTrc99A, AmpR, IPTG-inducible
  - (Schwechheimer, Rodriguez, and Kuehn, submitted)
- **pSpr**: spr in pTrc99A, AmpR, IPTG-inducible
  - (Schwechheimer, Rodriguez, and Kuehn, submitted)
- **pmSpr**: spr with C68A point mutation
  - (Schwechheimer, Rodriguez, and Kuehn, submitted)
- **pSpr-F**: spr-FLAG in pTrc99A, AmpR, IPTG-inducible
  - (Schwechheimer, Rodriguez, and Kuehn, submitted)
- **pmSpr-F**: spr-FLAG with C68A point mutation
  - (Schwechheimer, Rodriguez, and Kuehn, submitted)
8.2 Construction of expression vectors

pNlpA, a pTrc99A derivative was constructed in order to express nlpA under control of the trc promoter. nlpA was amplified from genomic DNA using the primers 5’-GATATCTAGACCGCTTGGTGTCAG-3’ containing the XbaI restriction site and 5’-GATTAAGCTTTTACCCAGGCACCAGC-3’ containing the HindIII restriction site. The nlpA-containing XbaI-HindIII fragment was then ligated with the XbaI-HindIII cleaved pTrc99A fragment. The restriction enzymes were purchased from New England Bio Labs. pmNlpA, a pNlpA derivative, was created in order to express mnlpA under trc promoter control. The point mutations were introduced by site directed mutagenesis of pNlpA using the primers 5’-CAACGACCACCCGCGCTTGGTCGACACTTTTACTG-3’ and 5’-CAGTAAAAGTGCCGACCAAGCGCGTCGGGTCGTTG-3’. To confirm the constructs, the plasmids were sequenced using a primer upstream and downstream of the cloned gene.

In order to express nlpI and spr under control of the trc promoter, pNlpI and pSpr were constructed using the pTrc99A vector. nlpI was amplified from genomic DNA using the primers 5’-GATATCTAGACCGCTTGGTGTCAG-3’ containing the XbaI restriction site and 5’-GATTAAGCTTTTACCCAGGCACCAGC-3’ containing the HindIII restriction site. The nlpI-containing XbaI-HindIII fragment was then ligated with the XbaI-HindIII cleaved pTrc99A fragment. spr was amplified from genomic DNA using the primers 5’-AAAAGGATCCGTCTCGTGCTTGGC-3’ containing the BamHI restriction site and 5’-AAAACCTGCAGCTCGTCAGGATAGCCCAAGG-3’ containing the PstI restriction site. The spr-containing BamHI-PstI fragment was then ligated with the BamHI-PstI cleaved pTrc99A fragment. The restriction enzymes were
purchased from New England Bio Labs. A pSpr derivative, pmSpr, was created to express mutant Spr using the inducible trc promoter. The point mutation was introduced by site directed mutagenesis of pSpr using the primers 5’-GGTATCGATGCGTCTGGTTTCGTACAGCG-3’ and 5’-CGCTGTACGAAACCAGACGCATCGATACC-3’. Spr and mSpr C-terminally FLAG-tagged were made from the Spr and mSpr constructs in pTrc99A using the same forward primer as above 5’-AAAAGGATCCGTCTCGTGTCCTGGGC-3’ and the reverse primer containing the FLAG-tag 5’-AACTGCAGTCATTTGTCATCGTCATCTTTATAATCTCTAGAGCTGGCGCTGAGAA CCCG-3’. The plasmid constructions were confirmed by sequencing using a primer upstream and downstream of the cloned gene.

8.3 **Outer membrane purification**

OM was prepared as described previously in (Kesty and Kuehn, 2004).

8.4 **OMV purification and quantitation**

Unless indicated, media (250 mL) was inoculated (1:250 dilution) from bacterial cultures grown overnight at 37°C and cells were grown overnight again at 37°C (~16 h). For the D-AA treatment, 250 mL cultures were inoculated (1:125 dilution) and incubated for 2 h at 40°C to an OD$_{600}$ of ~0.4, either the indicated concentration of D-AA or LB of the same volume was added to the untreated controls. Cultures were then incubated for an additional hour in the presence of the D-AA at 40°C. To assess stationary phase OMV production of cells cultured in spent supernatant (see preparation below), the 250 mL overnight or OD$_{600}$ 0.3 cultures were pelleted with the Beckman Avanti J-25 centrifuge (JLA-10.500 rotor, 10 000 g, 10 min, 4°C) and the cells were resuspended in fresh 250 mL
media or spent supernatant and they were again grown overnight or 3h at 37°C. OMVs were isolated from broth cultures as follows. Cells were pelleted with the Beckman Avanti J-25 centrifuge (JLA-10.500 rotor, 10 000 g, 10 min, 4°C) and the resulting supernatants filtered [low protein binding Durapore membrane, 0.45 µm (0.22 µm for V. cholerae) polyvinylidene fluoride, Millipore]. Filtrates were centrifuged again with the Beckman Avanti J-25 centrifuge (JLA-16.250 rotor, 38 400 g, 3 h, 4°C) followed by another step of centrifugation with the Beckman Optima TLX Ultracentrifuge if the pellets were not visible. In these cases, most of the supernatant was poured off, and the region where pelleted material should be was “resuspended” in the residual supernatant and re-pelleted (TLA 100.3 rotor, 41 000 g, 1 h, 4°C). Pellets were resuspended in Dulbecco’s phosphate buffered saline with added salt (0.2 M NaCl) (DPBSS), and filter-sterilized through 0.45 µm (0.22 µm for V. cholerae) Ultra-free spin filters (Millipore). A portion of the filtrate was plated on LB agar and incubated at 37°C overnight to verify that the suspensions were free of bacteria.

To quantitate OMV yield, OMV preparations were boiled for 6 min in 2X Laemelli buffer, separated by 15 % SDS-PAGE, and stained with SYPRO Ruby Red (Molecular Probes) overnight in the dark. Prior to and after staining, the gel was fixed for 1 h in a solution of 10% MeOH and 7% acetic acid. Ruby-stained proteins were detected under UV light. E. coli Omps F/C and A were quantified by densitometry (NIH Image J software). The Omp density values were divided by the OD_{600} of the original culture to calculate OMV production and this value was divided by the OMV production of the WT or untreated control strain to determine relative fold OMV production.
8.5 OMV-free supernatant

Cultures were grown overnight at 37°C (~16 h), cells pelleted with the Beckman Avanti J-25 centrifuge (JLA-10.500 rotor, 10 000 g, 10 min, 4°C), and the OMVs removed from the resulting supernatants by a Pall tangential flow system with a 100kD filter run by a Masterflex console drive. To ensure sterility the supernatant was filtered once more (low protein binding Durapore membrane, 0.45 µm polyvinylidene fluoride, Millipore).

8.6 Membrane integrity assays

8.6.1 Adenylate Kinase Leakage Assay

A ToxiLight bioassay kit (Lonza) was used to assess membrane integrity by detecting the amount of adenylate kinase in the culture supernatant (Jacobs et al., 2013; Schwechheimer and Kuehn, 2013).

Cultures (5 ml) were grown overnight (37 or 40°C, ~16 hrs, depending on the OMV purification conditions, diluted 10-fold with LB, and 100 µl was placed in a white 96 well plate (in duplicate). To prepare heat killed cells for a positive control, 5 ml of bacterial culture was pelleted (10 000 g, 5 min, room temperature), resuspended in 1 ml sterile deionized water, and boiled for 3 min followed by sterile filtering (0.45 µm Ultra-free spin column filters, Millipore); lysates were diluted 10-1000-fold as indicated in Fig. 6A. For D-AA treated cultures, 5 mL cultures were inoculated (1:100 dilution), incubated (2 h, 40°C) until they reached OD₆₀₀ ~0.4, treated with the indicated concentration of D-AA or the same volume of LB (for the untreated controls), and incubated for an additional hour at 40°C. For cells that were cultured in spent supernatant (see preparation 6.4), the 25 mL overnight cultures were pelleted with the Beckman Avanti J-25 centrifuge (JLA-10.500 rotor, 10 000 g, 10 min, 4°C), resuspended in spent supernatant
of the indicated strain, and incubated overnight at 37°C, unless indicated otherwise. To prepare heat killed cells for a positive control, a 5 mL culture was grown overnight (stationary phase) or to an OD<sub>600</sub> of ~0.4 (log phase), pelleted in a microfuge (10 000 g, 5 min, room temperature), resuspended in 1 mL sterile deionized water, and boiled for 3 min followed by sterile filtering (0.45 µm Ultra-free spin column filters, Millipore); lysates were diluted 100-fold and 100 µl.

All samples were diluted 10-fold with LB, and 100 µl was placed in a white 96 well plate (in duplicate). To all sample wells, 100 µl of ToxiLight reagent was added, and the mixture incubated at room temperature for 30 min. Luminescence was measured with a Molecular Devices SpectraMAX GeminiXS spectrometer. The average of the duplicate sample measurement was multiplied by the dilution factor, the absolute value was used when the measurement was negative (below detection), and divided by the OD<sub>600</sub> value of the original culture to account for differences in culture density.

The data was summarized in Table 1-3.

8.6.2 Actinomycin D Sensitivity

The sensitivity of cells to Actinomycin D was also used to assess membrane integrity defects (Leive, 1965; Schwechheimer and Kuehn, 2013).

To assess the integrity of stationary-phase bacteria, the 25 mL cultures were grown overnight (16-18 h), and then Actinomycin D was added (t=0, the start of the growth curve). Cultures were grown at 37 °C or 40°C depending on the OMV purification conditions. To assess the integrity of log-phase bacteria, cultures were inoculated to an OD<sub>600</sub> of ~0.02 (t=0), grown to an OD<sub>600</sub> of ~0.4, and then Actinomycin D was added. For D-AA treated cultures, 25 mL cultures were inoculated (1:125 dilution), incubated (2 h, 40°C) until they reached OD<sub>600</sub> ~0.4, treated with the indicated
concentration of D-AA or the same volume of LB (untreated controls), and incubated for an additional hour at 40°C. For stationary-phase bacteria, 25 mL cultures were grown overnight (37°C 16-18 h). For cells that were cultured in spent supernatant (see preparation 6.4), the 25 mL overnight cultures (37°C) were pelleted with the Beckman Avanti J-25 centrifuge (JLA-10.500 rotor, 10 000 g, 10 min, 4°C), resuspended in spent supernatant of the indicated strain, and incubated again overnight at 37°C, unless indicated otherwise.

Actinomycin D (Sigma) dissolved in ethanol was added to untreated or treated 25 mL cultures to a final concentration of 5 µg/mL, cultures were incubated at the above indicated growth conditions and OD$_{600}$ was measured. Cultures were kept in the dark since Actinomycin D is light sensitive.

Better, similar, or worse growth of the mutant or D-AA treated cultures were determined by comparing OD$_{600}$ with that of the WT (6 h) or untreated (3 h) control culture. We considered a difference in 0.1 OD$_{600}$ to the control culture when at least 3 time points differed and are summarized the data in Table 1-3.

**8.6.3 Sytox Green Sensitivity**

The ability of Sytox Green to enter cells, bind DNA, and consequently fluoresce was used as another assay to assess membrane integrity (Cowles et al., 2011; Schwechheimer and Kuehn, 2013).

Cultures were grown overnight (16-18 h). All cultures were 25 mL and grown at 37 °C or 40°C depending on OMV purification conditions. Log-phase cultures were inoculated to an OD$_{600}$ of ~0.02 (t=0) and grown to an OD$_{600}$ of ~0.4. All cultures were 25 mL and grown at 40°C. Untreated 5 mL cultures were grown overnight (37°C, 16-18 h). For D-AA treated cultures, 5 mL cultures were inoculated (1:100 dilution), incubated (2 h,
40°C) until they reached OD₆₀₀ ~0.4, treated with the indicated concentration of D-AA or the same volume of LB (for the untreated controls), and incubated for an additional hour at 40°C.

One mL of treated or untreated culture was centrifuged (Microcentrifuge, 16 000 g), resuspended in 600 µL 10 mM Tris-HCl, pH 8.0, and 3 µM Sytox Green (Invitrogen) dissolved in DMSO was added. The mixture was incubated in the dark for 10 min at room temperature and fluorescence was measured (excitation: 500 nm, emission: 550 nm). The fluorescence values were divided by the OD₆₀₀ of the original culture and this value was divided by the fluorescence of the WT or untreated control strain to determine relative fold fluorescence change.

This data is summarized in Table 1-3.

**8.6.4. Electron Microscopy**

Cells were grown ~ 16-18 h in LB at 40°C, fixed in the cold for 1 h in 2.0% paraformaldehyde, 2.5% gluteraldehyde, 1% tannic acid, 0.1M sodium cacodylate (EM Sciences) buffer, pH 7.3 then washed 3x 5 min in 0.1M sodium cacodylate, pH 7.3 and rinsed in 50% ethanol for 5 min. For dehydration the cells were incubated 2 x 5 min in 70%, 80% and 90% ethanol followed by 3x 5 min 100% ethanol (using a fresh solution each time). Infiltration was done by 2 x 5 min incubation in propylene oxide followed by overnight incubation in 1:1 Embed 812 Mixture (EM Science) and propylene oxide with tubes capped. Next, the cells were incubated in 3:1 Epon and propylene oxide for 2 h, followed by 2 x 2 h in fresh Epon. To embed the cells, they were incubated in fresh Epon for 2 d at 60°C then 1 d at 70°C. For sectioning, the blocks were trimmed, and then cut on a Leica Ultracut Microtome at 60 nm. Sections are post stained with 2% uranyl acetate in 50% ethanol followed by Sato Lead Stain. The microscope used for imaging is a Tecnai
12, made by FEI. Digital images were recorded on a Gatan Multi-Scan Camera Model 794.

8.7 Periplasm assays

Periplasm was isolated using an adapted method (Kesty and Kuehn, 2004). Overnight broth cultures or log phase cultures were grown to an OD$_{600}$ of ~ 0.4 (100 ml) and cells pelleted with the Beckman Avanti J-25 centrifuge (JLA-16.500 rotor, 10 min, 10 000 g, 4°C). The pellets were weighed and resuspended in 20 mM Tris-Cl (pH 8), 20% sucrose buffer (2 ml/ 1 g of cell pellet), 0.1 M EDTA (100 µL/ 1 g of cell pellet) and 0.5 mM PMSF (phenylmethylsulfonyl fluoride) protease inhibitor (Sigma) in ethanol. The cell suspension was incubated on ice for 1 h, and then 0.5 M MgCl$_2$ (160 µL/ 1 g of cell pellet) was added and mixed well. Lastly, the mixture was centrifuged (Microcentrifuge, 8100 g, 20 min, room temperature) to pellet cell debris, and the supernatant containing the periplasm was collected. To quantitate periplasmic protein, a Bradford assay (Coomassie stain, Thermo Scientific) was used with a standard curve generated with known concentrations of bovine serum albumin diluted in water.

8.8 OMP assays

8.8.1 Differential Labeling Assay

To detect surface-exposed OMPs, purified OMVs from stationary phase cultures were incubated with a membrane impermeable dye, Texas Red-X succinimidyl ester (Invitrogen) (dissolved in DMSO at a concentration of 2.5 mg/ml), at a 1:50 ratio (dye to OMVs) for one hour at room temperature in the dark due to the dye being light sensitive. To quench the unbound dye 100 µL of 50 mg/ml Lysine was added and thoroughly mixed. The OMVs were pelleted with the Beckman Optima TLX
Ultracentrifuge (TLA 100.3 rotor, 41 000 g, 1 h, 4°C) and washed twice with DPBSS and stored in DPBSS. To account for the potential loss of OMVs during the wash steps, the absorbance at 280 nm ($A_{280}$) was measured in the original OMV sample and at the end of the surface staining procedure. The surface stained samples were separated using 15% SDS-PAGE and labeled protein detected with UV light. OMVs from ΔompR and ΔompA strains were used to identify the bands corresponding to OMPs C/F and A that were used for quantification by densitometry. The total amount of OMPs C/F and A (folded and misfolded) was determined by SYPRO Ruby Red staining as mentioned above under OMV quantitation. For each strain, the same amount of OMVs based on $A_{280}$ was loaded for surface labeled and SYPRO Ruby Red detection. Finally, the ratio between surface stained and total OMPs was normalized to the wild-type strain.

### 8.8.2 Gel Shift Assay

Purified OMVs from WT and hypervesiculating mutant cultures (stationary phase, 37°C) were either prepared with native or denaturing sample buffer and equal protein amounts based on $A_{280}$ were separated by SDS-PAGE Ruby-stained bands corresponding to misfolded OMPs present in the native preparations were quantified by densitometry and normalized to the amount in native preparations of the WT. The protocol of Krojer et al. (Krojer et al., 2008) was used.

### 8.9 PG purification, digestion and quantitation of covalently crosslinked Lpp

Unless otherwise indicated, media (500 mL) was inoculated (1:250 dilution) from overnight 37°C bacterial cultures and cultures grown at 37°C until they reached $OD_{600} \approx 0.4$. For D-AA treated cultures, 500 mL cultures were inoculated (1:125 dilution), incubated (2 h, 40°C) until they reached $OD_{600} \approx 0.4$, treated with the indicated...
concentration of D-AA or the same volume of LB (untreated controls), and incubated for an additional hour at 40°C until they reached OD$_{600}$ ~ 1. PG was isolated from broth cultures based on the protocol by Lam et al. (Lam et al., 2009). Briefly, cells were pelleted and resuspended in PBS after which the ice-cold suspensions were dropped in an equal volume of vigorously stirring, boiling 10% SDS. Samples were boiled for 4 h and then incubated at 37°C, continuously shaking, overnight. The following day, the PG was pelleted with the Beckman Optima TLX Ultracentrifuge (TLA 100.3 rotor, 80 000 g, 15 min, 304°C), resuspended in 1% SDS followed by another 2 h of boiling. PG was washed four times with deionized water and finally resuspended in equal volumes of deionized water.

Equal fractions of the purified sacculi were digested with 15 mg/mL chicken egg lysozyme (Sigma-Aldrich) in 10 mM Tris-HCL, pH 8, at room temperature for 2 days. Lysozyme digested PG was separated by 15% SDS-PAGE and Lpp was detected by immunoblotting and quantified by densitometry (NIH Image J software). The Lpp density values were divided by the OD$_{600}$ of the original culture to calculate the amount of Lpp that was covalently crosslinked to PG, and this value was divided by the PG-crosslinked Lpp of the WT or untreated control strain to determine relative fold of bound Lpp.

8.10 FM4-64 Lipid quantification of OMVs

To determine the lipid to OMPs ratio one portion of the purified WT, ΔrfaC, ΔrfaG, and ΔrfaP OMVs were incubated with FM4-64 (Invitrogen), 3.3 g/ml in phosphate-buffered saline (PBS) for 10 min at 37°C. FM4-64 incubated in PBS was used as a negative control. The fluorescence signal was measured with a Molecular Devices SpectraMAX GeminiXS fluorometer (excitation: 506 nm, emission: 750 nm). To
determine the OMPs concentration, a second portion of OMVs was treated as explained above under OMV purification and quantitation. Lastly the lipid value was divided by the OMP value and normalized to the WT strain.

8.11 Statistics

Parameters used for the T-test are equal variance due to the comparison of identical experimental repetitions or unequal variance due to different experimental repetitions and a two-tail distribution. For direct sample size comparison the paired T-test was used and for fold comparison the unpaired. The T-test’s value < 0.051 was considered statistically significant, if the value was lower then that it was indicated in the significance value under the corresponding data. The number each experiment was repeated (n) is stated in the figure legend.
References


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Biography

Carmen Schwechheimer was born March 11, 1981 in Heidelberg Germany. She grew up in Edingen-Neckarhausen and attended Elizabeth von Thadden Highschool in Heidelberg from which she graduated in 2000. She attended Diablo Valley College in Pleasant Hill, CA from 2002 to 2005, where she met her husband Robert Carter. In 2005 Carmen transferred to the University of California, Berkeley from which she graduated with a B. A. in Molecular and Cell Biology with an emphasis in Biochemistry in 2007. Fall of the same year, she started her graduate studies at Duke University in the Biochemistry Department and joined the Kuehn laboratory in the spring of 2008. Carmen received the American Society of Microbiology Conference Travel Fellowship, the Kamin Travel Fellowship, the Duke Pre-Dissertation Travel Award, and the Duke Conference Travel Fellowship. After defending her thesis on July 24, 2014, she will move to the San Francisco area and pursue postdoctoral research with Dr. Fitnat Yldiz at UC Santa Cruz.

Publications


