Environmentally-Controlled Bacterial Vesicle-Mediated Export

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Summary

Over the past two decades, researchers studying both microbial and host cell communities have gained an appreciation for the ability of bacteria to produce, regulate, and functionally utilize outer membrane vesicles (OMVs) as a means to survive and interact with their cellular and acellular environments. Common ground has emerged, as it appears that vesicle production is an environmentally-controlled and specific secretion process, however it has been challenging to discover the principles that govern fundamentals of vesicle-mediated transport. Namely, there does not appear to be a single mechanism modulating OMV export, nor universal "markers" for OMV cargo incorporation, nor particular host cell responses common to treatment with all OMVs. Given the diversity of species studied, their differences in envelope architecture and composition, the diversity of environmentally regulated bacterial processes, and the variety of interactions between bacteria and their abiotic and biotic environments, this is hardly surprising. Nevertheless, the ability of bacteria to control exported material in the context of a packaged insoluble particle, a vesicle, is emerging as a significant contribution to bacterial viability, biofilm communities, and bacterial-host interactions. In this review, we focus on detailing important, recent findings regarding the content and functional differences in bacterially secreted vesicles that are influenced by growth conditions.

Introduction

The process of OMV-mediated secretion

Recent reviews have focused on mechanistic models of OMV production (Kulp *et al.*, 2010, Bonnington *et al.*, 2013, Klein *et al.*, 2014, Kulkarni *et al.*, 2014a, Haurat *et al.*, 2015, Schwechheimer *et al.*, 2015a), and therefore this topic is not the focus here. Overall, the process appears simple: OMVs form from where the outer membrane (OM) buds outward and these portions of the bacterial envelope are released in the form of membrane vesicles enclosing soluble components. However, many studies now support the concept that the process of OMV generation is controlled and regulated. In a variety of cases where OMVs have been carefully characterized, OMVs have been found to exhibit a) selective protein and lipid content compared to envelope, b) variance in the amount and content of OMV released depending on growth conditions, and c) gene-dependent variation in production levels and content (Bernadac *et al.*, 1998, Berlanda Scorza *et al.*, 2008, Bager *et al.*, 2013, Bai *et al.*, 2014, Elhenawy *et al.*, 2014, Cahill *et al.*, 2015, Kulp *et al.*, 2015, Mantri *et al.*, 2015,

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Elhenawy *et al.*, 2016, Roier *et al.*, 2016). This is in contrast to the generation of vesicles which result from the encapsulation of soluble material by lysed bacterial membranes whose function in biofilms has been described recently (Turnbull *et al.*, 2016).

Many mechanistic questions remain regarding the OMV-mediated secretion process. For instance, what type of energy is used to generate scission? Is a specific, conserved (set of) protein(s) needed for this process? Is cargo incorporation an active process, or does cargo selectivity result from particular sites of the envelope that are more prone to budding as a result of heterogeneity in the membrane and/or envelope composition? Other types of vesicles and extensions of the envelope are noted to exist from Gram-negative bacteria, e.g. inner-outer membrane vesicles (I-OMVs), nanopods, nanotubes, and nanowires (Schwechheimer *et al.*, 2015a) but these are not discussed further here. How these structures mechanistically and functionally relate to OMVs also remains unknown.

Current challenges and goals

Progress to establish commonalities in the OMV field is hampered due to the variety of analytical methods used to study OMV generation and composition. For instance, there are inconsistencies between studies in the quantification methods used to measure OMV production in mutants or under specific growth conditions. The quantitative methods include counting particles by particle tracking, FACS, confocal microscopy, or transmission electron microscopy techniques (Van Der Pol *et al.*, 2010, Devos *et al.*, 2015); measurement by absorbance of phospholipids in the sample after chloroform extraction (Stewart, 1980, Schertzer *et al.*, 2010, Wessel *et al.*, 2013); quantification of lipid content using lipid probes such as FM4–64 (McBroom *et al.*, 2006); and quantification of protein content by SDS-PAGE staining and densitometry of outer membrane protein, or assays of total protein by Bradford reagent or bicinchoninic-acid assay (BCA) (Sharpe *et al.*, 2011, Chen *et al.*, 2016). Each of these methods has strengths and weaknesses. OMV counting is limited by particle size and the ability to distinguish between protein complexes and vesicles. Growth conditions and genetic mutations can change lipid to protein ratios and composition in the bacterial membrane, skewing lipid- or protein-only based quantitative comparisons of OMV production. Furthermore, samples may not consist of pure OMVs so the use of total protein quantification in samples is reliable only if OMV purity has been established. Depending on the preparative method or culture conditions, cell-free supernatants may be contaminated with protein from lysed cells, flagellin, cytoplasm, or cytoplasmic membrane that co-pellet with OMVs upon ultracentrifugation. Density gradient purification of OMVs reduces contaminants but also impacts quantitative yield, therefore it is most useful for quantitation as a means to identify which protein bands should be used in SDS-PAGE densitometry measurements of a less pure sample. Finally, OMV production data also must be normalized to the number of bacteria in the culture, either by utilizing optical density (OD) or colony forming units (CFU). A combination of quantitative techniques is often useful to establish OMV production. For instance, OMV lipid quantification can be coupled to measurements of outer membrane protein (OMP) by SDS-PAGE and densitometry, and normalized to bacterial counts (Chutkan *et al.*, 2013, Schwechheimer *et al.*, 2015b, Roier *et al.*, 2016).

The lack of a universal methodology extends to studies of OMV composition. There is a growing need to establish a "baseline" OMV protein and lipid composition for a particular bacterial species grown in a specific growth phase and media conditions (Table 1). This would allow invaluable comparisons of OMV composition, function, and production from the same species in different media conditions, or of OMVs produced by different species in the same growth phase. Also, in order to identify OMV cargo enrichment and subsequently study the mechanics and functional consequences of cargo selectivity, OMV content must be compared with purified outer membrane (OM) and periplasmic fractions using quantitative proteomic and lipidomic methods, which are typically cost-prohibitive. However, with the current rate of technical progress, these goals are becoming increasingly realistic.

A major goal in the field is to elucidate how differing bacterial growth conditions impact OMV functional properties such as enabling viability, host cell adhesion, cytotoxicity, and inter-species communication. This review will exclusively focus on studies in which functional analyses of vesicles grown in specific conditions (e.g. a change in media or antibiotic exposure) revealed important roles for vesicles or vesicle secretion in that particular environment (Fig. 1, Table 2).

Environment controls OMV content and production

Achieving a simple definition of OMV-related activities, even for those derived from a single species, will be impossible without including a caveat for growth conditions. Growth phase, solid or liquid media, or the composition of the media, are well-known to affect virulence-associated bacterial traits such as adherence and toxin secretion, and impact membrane composition. Similarly, OMVs are complex entities composed of environmentally and temporally-controlled factors. Therefore, we can best focus on elucidating the functional importance of OMVs produced in a particular environment to bacteria growing in that setting. Here we highlight some recent examples demonstrating the environmental control of OMVs.

Iron availability

Iron is an essential element for bacterial survival, however, its bioavailability is strictly controlled within the host. Several reviews have highlighted the importance of iron acquisition during infection (Caza *et al.*, 2013, Ferreira *et al.*, 2016) and in light of recent evidence, OMVs might potentially serve as vehicles for iron uptake during host invasion.

Evidence for environmental influence over OMV production for *M. tuberculosis* comes from a study by Rosales et al. in which vesiculation was assayed in conditions of high and low iron concentration using minimal media (Prados-Rosales *et al.*, 2014). While OMV size did not change significantly between both conditions, vesicle production was enhanced in iron-limiting conditions. Furthermore, OMVs from low iron conditions were enriched for the lipid siderophore mycobactin whereas its precursor, with lower iron binding affinity, was the main siderophore found in OMVs from high iron conditions. Therefore, iron deprivation stimulated the production of OMVs enriched in the siderophore mycobactin which was also able to deliver iron to iron deficient mutants of *M. tuberculosis*. The selective packaging of mycobactin in vesicles grown in iron limiting conditions and the ability of these vesicles to

donate iron and support the growth of mutants deficient in iron uptake, suggest a crucial role for OMVs during stringent conditions.

In addition, many proteins associated with iron acquisition were found in biofilm OMVs of *P. aeruginosa* (Toyofuku *et al.*, 2012). Given that iron limiting conditions are also known to inhibit biofilm formation (Singh *et al.*, 2002, Cai *et al.*, 2010), iron uptake mechanisms in the OMVs might also be important for the transition from a planktonic to a biofilm lifestyle, allowing the bacteria to thrive in this type of community.

In the case of *H. pylori*, it was observed that iron-limiting conditions also caused a marked increase in vesicle production and influenced the expression of the OMV-associated virulence factor VacA. Reduced levels of VacA were found in vesicles from iron limiting conditions and iron salt was able to restore this decrease (Keenan *et al.*, 2000). Similarly, studies of OMV production by *Haemophilus influenzae*, *E. coli*, and *Vibrio cholerae*, found iron-limiting conditions corresponded to increased OMV production, and that a deletion in the iron-sensitive *fur* transcription factor also lead to increased OMV levels (Roier *et al.*, 2016). In this case, it was found that the absence of the transcriptional activating activity of *fur* caused a decrease in iron-activated genes such as *vacJ* and *yrbE* which control phospholipid transport. Phospholipid and fatty acid analysis of the OMVs and OM from wildtype and mutants of these genes in *H. influenzae* suggested that *vacJ*-and *yrbE*-dependent changes in envelope lipid composition could be the cause of the observed iron-regulated OMV formation. Together, these findings point to a link between iron availability and modulation of both vesicle production and cargo.

Biofilm vs planktonic lifestyle

Many studies highlight differences in both planktonic and biofilm OMV proteomes of *P. aeruginosa* (Toyofuku *et al.*, 2012, Park *et al.*, 2015). In particular, a recent proteomic analysis revealed differences in the amount of the antibiotic secondary metabolite and pigment, pyocyanin, in OMVs over time (Park *et al.*, 2015). Vesicles from late biofilm cultures (96 hrs) were found to contain higher levels of pyocyanin, in comparison to OMVs from early planktonic cultures (24 hrs). Given that in early planktonic cultures, supernatants had low levels of pyocyanin as did later biofilm supernatants, the authors argue OMVs are serving as a reservoir for pyocyanin in the context of biofilms. Consistent with this finding is the enrichment for phenazine biosynthetic proteins in early biofilms in comparison to planktonic cultures (Park *et al.*, 2015). Although the quantification of pyocyanin in OMVs might point to a possible mechanism for the delivery of this toxin, it is yet to be determined whether pyocyanin within the vesicles can interact or inhibit growth of competing bacterial species.

In the case of *H. pylori* biofilms, OMVs seem to play more of a structural role. Extracellular DNA (eDNA) is a component of *H. pylori* biofilms and using PicoGreen staining, Grande et al. observed increased levels of double stranded DNA (dsDNA) associated with biofilm OMVs in comparison to planktonic OMVs (Grande *et al.*, 2015). It is argued that the association of eDNA with vesicle membranes explain the protection observed for *H. pylori* biofilms treated with DNase I (Grande *et al.*, 2011). Thus, as bacterial cells transition from a planktonic to a biofilm lifestyle, OMVs could not only have a role in toxin storage but also

in protection of eDNA from degradation, maintaining the integrity of the extracellular matrix of the biofilm. It is important to mention that although many studies have detected DNA as well as RNA as a component of OMVs (Table 3), there is still a great deal of debate regarding how nucleic acids enter into OMVs, their association with OMVs, and their role in host-microbe interactions.

Oxygen availability

Oxygen availability impacts bacterial physiology in biofilms as well as in liquid anaerobic conditions, and the ability of oxygen to modulate OMV production has been studied most extensively in *P. aeruginosa*. Most studies of OMV production in *P. aeruginosa* use aerobically grown cultures in which the Pseudomonas quinolone signal (PQS) which is identified to be involved in *P. aeruginosa* OMV production (Mashburn-Warren *et al.*, 2008) is active. However, Toyofuku et al. focused on monitoring OMV production under denitrifying conditions (Toyofuku *et al.*, 2013). *P. aeruginosa* undergo anaerobic respiration via denitrification, utilizing nitrates or oxidized forms of nitrogen (Arai, 2011, Arat *et al.*, 2015). This is particularly relevant during biofilm growth, due to the generation of anaerobic microenvironments because of inherent oxygen gradients within the biofilm (Xu *et al.*, 1998, Werner *et al.*, 2004).

Firstly, the authors determined that *P. aeruginosa* is able to produce OMVs in the absence of oxygen, conditions lacking PQS. They further observed marked differences in OMV production under anoxic conditions in different media. While anoxic cultures of *P. aeruginosa* produced very few vesicles in brain heart infusion (BHI) medium, anoxic Luria Bertani (LB) broth-grown cultures produced up to six-fold more vesicles in comparison to the aerobic cultures (Toyofuku *et al.*, 2013), as measured by FM4–64 dye incorporation. This reduction in vesicle production is consistent with a previous study that revealed reduced vesicle production under anaerobic conditions in BHI (Schertzer *et al.*, 2010) despite the fact that the vesicle isolation and purification methods for these two studies were slightly different. When the same group examined the anaerobic OMV proteome, various pyocin-related proteins were found in the vesicles while pyocin structures were not observed. Pyocins are bacteriocins produced by *P. aeruginosa* which are toxic to other strains of this species. Additional experiments with mutants also uncovered a connection between OMVs and pyocin production under denitrifying conditions (Toyofuku *et al.*, 2013). In the anaerobic conditions tested, nitric oxide (NO) was identified as an inducer of vesicle production given that a mutant for nitrite reductase (NIR) displayed a decrease in pyocin production as well as vesicle production. In accordance with these results, a microarray analysis by Chang et al. revealed oxidative stress induced pyocin genes in *P. aeruginosa* (Chang *et al.*, 2005). Furthermore, treatment of *P. aeruginosa* with hydrogen peroxide has been shown to cause an increase in OMV production (Macdonald *et al.*, 2013). These results are all consistent with the idea that oxygen availability modulates OMV production, however, it has yet to be determined whether the enrichment of pyocin synthesis proteins in OMVs provides *P. aeruginosa* vesicles with particular functions such as modulating stress resistance or pyocin production.

SOS response and antibiotic exposure

Bacteria encounter antibiotics in their native as well as human host environments, and antibiotics often result in activation of stress responses which can be tied to OMV production and function. A recent study by Maredia et al, revealed that the SOS response in *P. aeruginosa* increases OMV production (Maredia *et al.*, 2012). A wildtype strain treated with ciprofloxacin to induce the SOS response, experienced a 100-fold increase in OMVs, as measured by Bradford assay, in comparison to untreated cells. Tests with a LexA (SOS repressor) noncleavable strain indicated that both the SOS response and the antibiotic itself contributed to the increase in OMV levels. To exclude the possibility of co-precipitated proteins in the OMV samples, Maredia et al. also performed a lipid analysis which revealed that ciprofloxacin treated wildtype OMVs were heavier than those of the *lexAN* mutant strain (Maredia *et al.*, 2012). Moreover, a proteomic analysis identified SOS-regulated proteins in OMVs of antibiotic-treated cells. The authors suggest then that in the presence of antibiotics, OMV production is enhanced and SOS plays a role in such a mechanism.

Whether ciprofloxacin-induced OMVs improve bacterial viability or resistance in the presence of the antibiotics was not studied by Mareida et al, however another study demonstrated that OMVs can improve survival by absorbing antibiotics in the environment (Manning *et al.*, 2011). A hypervesiculating mutant strain grew better than wildtype when treated with cyclic cationic antimicrobial peptides (AMPs) polymyxin B and colistin, but not when treated with antibiotics that target peptidoglycan synthesis and protein synthesis. Therefore, hypervesiculation was found to be protective against antibiotics that more specifically target the outer membrane. Furthermore, treatment of wildtype cultures with polymyxin B or colistin in the presence of purified OMVs resulted in increased survival in comparison to cultures without OMVs. Once again, OMVs were not protective against ampicillin, ceftriaxone, or tetracycline. In the same study, purified OMVs from an enterotoxigenic strain of *E. coli* (ETEC) or from K12 increased growth of ETEC in the presence of polymyxin B (Manning *et al.*, 2011). This is consistent with a model in which antibiotic exposure induces the production of vesicles that interact with the stressor molecules and confer protection, increasing survival.

In a consequent set of studies, treatment of *S. maltophilia* with the β-lactam antibiotic imipenem was found to not only increase in OMV production, but also, analysis of the OMV proteome demonstrated that the vesicles contained β-lactamase (Devos *et al.*, 2015, Devos *et al.*, 2016). The presence of this molecule within the OMVs points to a protective role for vesicles when cells are faced with antibiotic stress. Whether the enzyme is selectively enriched in these OMVs or whether its increased abundance results from the bulk-flow incorporation of an increased amount of enzyme in the periplasm due to stress, is unknown.

The β-lactamase-containing OMVs observed by Devos *et al.* were further shown to be capable of improving bacterial survival during antibiotic exposure (Devos *et al.*, 2016). Ampicillin-related vesicle functions have been studied for several other bacteria and are also consistent with their ability to confer protection to self or bystander bacteria. In the case of *S. aureus*, addition of β-lactamase-containing vesicles from this Gram-positive bacterium mediated survival of ampicillin-susceptible *E. coli* DH5α and *S. aureus* strains in the presence of ampicillin. The same effect was observed for *S. enterica* serovar *enteridis*, *E.*

coli O157:H7 and *S. epidermis* ATCC 12228 on LB supplemented with ampicillin (Lee *et al.*, 2013). Notably, vesicle-mediated resistance was antibiotic-specific. Only when OMV "donor" *E. coli* DH5α cells were transformed with a plasmid encoding β-lactamase, were the OMVs able to mediate the survival of an *E. coli* DH5α ampicillin-susceptible strain (Lee *et al.*, 2013).

Recently, the Devos group also treated bacterial cultures with penicillin G to induce the packaging of β-lactamase into *S. maltophilia* vesicles (Devos *et al.*, 2016). OMVs from these stimulated cultures where then added to cultures of *P. aeruginosa*, and *B. cenocepacia*, bacterial species known to cohabitate with *S. maltophilia*. For both species, OMVs caused a 100-fold increase in the minimum inhibitory concentrations for other β-lactam antibiotics (imipenem, amoxicillin, and ticarcilin) (Devos *et al.*, 2016). These results demonstrate that OMVs of resistant bacteria exposed to antibiotics can package active antibiotic-degrading enzymes, and reduce the antibiotic susceptibility of bystander pathogens.

Host factors

During invasion and colonization, bacteria encounter a variety of host factors that can potentially affect OMV production as well as content and cytotoxicity. For instance, CFTR inhibitory factor (Cif) is commonly found in the *P. aeruginosa* OMV proteome (Bomberger *et al.*, 2009). Cif is an epoxide hydrolase enzyme, capable of altering the recycling of CFTR, a mammalian ABC transporter relevant to the development of cystic fibrosis (Bomberger *et al.*, 2011). It has been previously established that this virulence factor is regulated by direct binding and repression by CifR, an epoxide-responsive repressor (Ballok *et al.*, 2012). Epoxides are toxic compounds secreted as by-products of eukaryotic metabolism with roles in cellular signaling. Notably, human-derived epoxides are produced by leukocytes in the lung, a niche for *P. aeruginosa* during infection (Hayakawa *et al.*, 1986, Gómez *et al.*, 2007). Given that Cif is known to be packaged into OMVs of *P. aeruginosa*, Ballock et al. set to examine whether epoxides mediate the packaging of Cif into vesicles. Treatment with a model epoxide, epibromohydrin (EBH) induced changes in OMV shape and density, however, more interestingly, it altered the packaging of proteins into the vesicles, supporting the idea of selective sorting of cargo into OMVs. Furthermore, additional experiments revealed that vesicles formed in the presence of EBH had reduced epithelial cell cytotoxicity. Therefore, during host invasion, exposure to human-derived epoxides could cause significant changes to bacterial vesicles that can influence host-pathogen interactions.

Besides the lungs, *P. aeruginosa* can colonize humans in other sites and is known to be the most common cause of corneal infection in contact-lens wearers (Stapleton *et al.*, 1995). However, the mechanism of infection has not yet been fully elucidated (Tam *et al.*, 2010, Evans *et al.*, 2013). Given differences in models of bacterial infection in the presence and absence of contact lenses, several studies have indicated that bacteria can release additional virulence factors in the eye-lens environment (Tam *et al.*, 2010). In experiments with *P. aeruginosa* exposed to human tear fluid, Metruccio et al. revealed that OMV production is induced in the presence of both solutions in comparison to buffer only and epithelial cell lysates controls (Metruccio *et al.*, 2016). As abundant components of human tear fluid, lysozyme and lactoferrin were tested, and, lysozyme specifically, was found to trigger OMV

production to levels comparable to tear fluid. OMVs from the lysozyme-induced conditions also had a similar protein-banding pattern as human tear-induced vesicles (Metruccio *et al.*, 2016). In lactate dehydrogenase (LDH) release assays to measure host cell lysis, these lysozyme-induced OMVs induced cytotoxicity of corneal epithelial cells to a greater degree than vesicles from uninduced conditions. These cytotoxicity results were consistent with in an *in vivo* model of mouse corneas. In the same *in vivo* study, recruitment of Ly6G/C myeloid cells was also observed as a result of the vesicle treatment. Furthermore, intact lysozyme-induced vesicles promoted association of *P. aeruginosa* to mouse corneas in an *ex vivo* model (Metruccio *et al.*, 2016). These results suggest that the ocular environment can modulate the release of OMVs by *P. aeruginosa* during a corneal infection and affect cell adhesion as well as cytotoxicity towards host cells.

Considering that Gram-negative bacteria can alter the structural composition of LPS present in their OM in response to various environmental stimuli, a system was developed to track the native dynamics of lipid A change in *Salmonella enterica* serovar Typhimurium following environmental shift to PhoP/Q- and PmrA/B-inducing conditions which mimic effects of host cell infection and colonization (Bonnington *et al.*, 2016). Growth conditions altering pH and Mg concentrations influenced OMV production, size, and lipid A content. Interestingly, lipid A content of OMVs did not fit with a stochastic model of content selection, revealing the significant OM retention as well as OMV enrichment of lipid A species in the different host-like conditions. The concept that OMV composition and production reflects changes occurring in the OM during host-induced OM remodeling was also studied by an investigation of the effect of overexpression of PagL, a PhoP/Q controlled OM-localized lipid A deacylase in *S.* Typhimurium (Elhenawy *et al.*, 2016). They found that deacylated lipid A was found exclusively in the OMVs, not the OM, of cells overexpressing PagL. This result suggests that PagL activity is extremely localized to small patches of the OM, or that PagL is active only after OMVs are formed. The former possibility is supported by the fact that PagL overexpression also corresponded to increased levels of OMVs, and these data lead the authors to suggest that the deacylated LPS alters the OM geometry to promote budding.

Growth phase and media composition

Several studies have examined changes in OMV production and cargo under various growth conditions in addition to altering iron concentrations which were discussed in an earlier section. Whereas data from OMVs produced by the *P. putida* DOT-T1E strain in particular, revealed few changes to the OMV proteome under varying conditions (Baumgarten *et al.*, 2012a, Baumgarten *et al.*, 2012b), studies of other strains and bacterial species have demonstrated media- and growth phase-dependent changes in OMV proteomes and production. For example, a study by Choi et al. on the proteome of *P. putida* KT2440 (See Table 1), assessed OMV production of cells grown in different media: LB broth, Minimal medium with 10 mM succinate, or minimal medium with 5 mM benzoate. OMV production in LB media was found to be more than three-fold increased than in the other two conditions. Interestingly, OMVs from cells grown in benzoate-containing media contained proteins involved in benzoate degradation. However, it was not determined here whether the vesicles from the different conditions conferred some sort of protection against stress-

inducing conditions. On the other hand, they did observe that OMVs derived from LB-rich media decreased cell viability of A549 carcinomic human alveolar basal epithelial cells, but it was not determined whether vesicles from the other two conditions are also able to confer cytotoxicity (Choi *et al.*, 2014). In a study of *G. anatis*, changes in medium composition also affected vesicle production. Addition of 1 mM EDTA to BHI medium or growth in RPMI 1640 HEPES medium caused a reduction in OMV production in comparison to growth in BHI (Bager *et al.*, 2013). Again, it remains unknown whether the reduction in OMV production is a functional advantage to cells grown in those specific conditions.

The impact of growth conditions on OMV production and function should also be thought of in a temporal context, as bacteria alter their composition throughout their cell cycle, and OMVs are likely to be similarly affected. While OMVs are produced at all stages of bacterial growth, the maximum rate of vesicle production has been known to occur at the end of log phase for various species of bacteria (Chatterjee *et al.*, 1967, Hoekstra *et al.*, 1976, Gamazo *et al.*, 1987). Interestingly, *P. aeruginosa* vesicles from different growth phases also exhibit altered physical properties. For instance, vesicles from stationary phase cultures contained higher levels of PQS and were found to be more negatively charged in comparison to OMVs from the exponential phase (Tashiro *et al.*, 2010). Also, based on the idea that vesicles may potentially play a role in cell to cell communication (Kadurugamuwa *et al.*, 1995), the same study employed FTIC labeled vesicles to test whether growth phase impacted association of vesicles with *P. aeruginosa* cells and observed that OMVs from stationary cells more readily associated with the bacterial cells. This could be explained by the fact that more negatively-charged vesicles have an increased potential to form salt-bridges to the Ca^{2+} or Mg^{2+} surface of bacteria (Tashiro *et al.*, 2010). Therefore, as bacteria transition between growth phase, vesicles can acquire inherently different properties that may influence their interactions with neighboring cells and this might be crucial for cell-cell communication.

Concluding remarks

The process of OMV biogenesis has gained much recent attention given the potential of vesicles for vaccine development and their role in bacterial pathogenesis (Baker *et al.*, 2014, Toyofuku *et al.*, 2015, van der Pol *et al.*, 2015). Consequently, many studies have focused on vesicle engineering to design OMVs suited for drug and antigen delivery while others have characterized vesicle protein, lipid, and nucleic acid composition (See Tables 1 and 3) in order to better understand bacterial physiology and improve on current antimicrobial therapeutic approaches. While there has been a substantial and increasing number of proteomic studies of bacterial OMVs, there is a concomitant need to characterize the function of vesicles produced by bacteria grown under various physiologically-relevant conditions. It is well established in biology that environmental changes affect bacterial gene expression, metabolism, and composition, allowing adaptation to new conditions. The studies highlighted in this review indicate that such environmental conditions similarly affect not only the process of vesiculation, both the amount of vesicles and their composition, but also the specific functional roles for the vesicles (Fig 1). Uncovering functions for vesicles produced under specific conditions will not only help us understand how bacteria thrive in

different environments, but will also allow us to define specific conditions in which vesicles play a crucial role in bacterial survival, further defining their role in bacterial pathogenesis.

Acknowledgments

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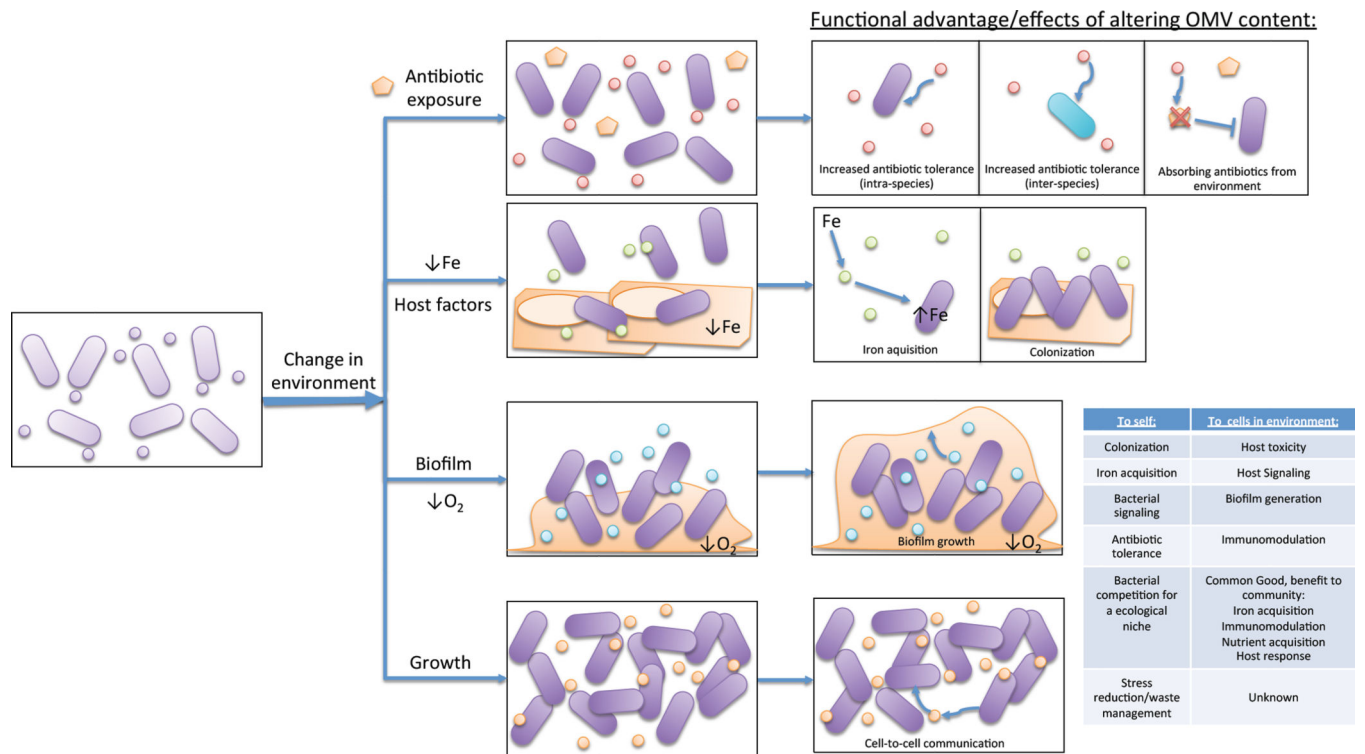
Figure 1. Environment shifts elicit changes in functional roles for OMVs

Environment changes such as antibiotic exposure, release of host factors, low iron, transition to biofilm lifestyle, anaerobic conditions, and changes in growth phases can cause modulation of OMV production. In many cases, vesicles produced upon the shift in environmental conditions exhibit functional roles that can help the mother cell or other bystander cells adapt to the new environment, survive future stressors, and/or interact with host cells or co-inhabitant cells differently.

Table 1

Summary of recent OMV proteomic analyses[1]

Species	Size (nm)[2]	Growth Phase[3]	Medium	Proteome[4]	Functional Analysis[5]	Immunological Activity Analysis[6]	Reference
Acinetobacter baumanii Clinical isolate A38	30 – 140	early stationary	LB broth	148	✓	✓	(Li *et al.*, 2015)
A. baumanii Clinical isolate 5806				138	✓	✓	
A. baumanii ATCC 19606	40 – 70	log	LB broth	ND		✓	(Jun *et al.*, 2013)
A. baumanii ATCC 17978	ND	in host	Mouse pneumonia model	112		✓	(Jin *et al.*, 2011)
A.baumanii Clinical isolate DU202	20 – 160	early stationary	LB broth	132	✓		(Kwon So, 2009)
A. radioresistens Clinical isolate MMC5	10 – 150	early stationary	LB broth	71	✓		(Fulsundar *et al.*, 2015)
Aggregatibacter Actinomycetemcom itans serotype e strain, 173	ND	NS	Blood agar plates	151			(Kieselbach *et al.*, 2015)
Bacteroides fragilis NCTC 9343	30 – 80	stationary	Supplement ed BHI, or	115	✓		(Elhenawy *et al.*, 2014)
B thetaiotaomicron VPI-5482	ND	stationary	supplement ed basal medium	21			
Campylobacter jejuni ATCC 700819	50	mid log	BHI	134			(Jang *et al.*, 2013)
Escherichia coli MG1655	80 – 100	stationary	LB broth	316	✓		(Kulkarni *et al.*, 2015)
E. coli Nissle 1917	20 – 60	early log	LB broth	192			(Aguilera *et al.*, 2014)
E. coli DH5α	20 – 40	stationary	LB broth	141			(Lee *et al.*, 2007)

Species	Size (nm)[2]	Growth Phase[3]	Medium	Proteome[4]	Functional Analysis[5]	Immunological Activity Analysis[6]	Reference
Delftia sp. Cs1–4*	ND	biofilm	Defined mineral salts medium	19			(Shetty *et al.*, 2011)
Francisella novicida ATCC 15482	43 – 125	stationary	0.1%l-cysteine HCl BHI broth	416	✓		(Pierson *et al.*, 2011)
F. philomiragia ATCC 25015		stationary	Chamberlain's medium	238	✓		(McCaig *et al.*, 2013)
F. novicida ATCC 15482	50 – 300, OM tubes: 0.3–1.5 μm	log, early stationary	BHI	99, 286	✓	✓	(Sharpe *et al.*, 2011)
Haemophilus influenzae Clinical isolate 86–028NP	20 – 200	stationary	BHI	142		✓	(Roier *et al.*, 2016)
H. influenza (several mutants)	100	late exponential	BHI broth	163	✓		(McCaig *et al.*, 2016)
H. parasuis Nagasaki	50 – 200	early stationary	Supplemented BHI (liquid and plate grown)	86 (liquid), 250 (plate grown)		✓	(Olofsson *et al.*, 2010)
H. parasuis D74		early stationary		93 (liquid), 251 (plate grown)			(Lee *et al.*, 2012)
Helicobacter pylori	50 – 110	early stationary	Blood agar plates of Brucella agar, Brucella broth	306	✓		(Ayalew *et al.*, 2013)
Klebsiella pneumoniae ATCC 13883	20 – 200	early stationary	LB broth	159	✓	✓	(Schaar *et al.*, 2011)
Mannheimia haemolytica serotype S1 strain 89010807N	10 – 20	early stationary	BHI	226		✓	
Moraxella catarrhalis	50 – 150	stationary	BHI	57	✓		

Species	Size (nm)[2]	Growth Phase[3]	Medium	Proteome[4]	Functional Analysis[5]	Immunological Activity Analysis[6]	Reference
Mycobacterium tuberculosis H37Rv	20 – 250	early stationary	Minimal media	287			(Lee *et al.*, 2015)
M. bovis BCG	40 – 250	10 days	Defined minimal media?	66	✓	✓	(Prados-Rosales *et al.*, 2011)
M. smegmatis				64	✓	✓	
Myxococcus xanthus DK1622 WT	30 – 100	18 hrs in plates Vegetative / Developmental cells	CTT medium	107/124			(Kahnt *et al.*, 2010)
M. xanthus	ND	late log	DCY-rich medium	75			(Whitworth *et al.*, 2015)
Neisseria gonorrhoeae§	ND	late log	GCBL Medium+	168			(Zielke *et al.*, 2014)
N. meningitidis (MC58csb− and 2120css− strains)	ND	10 h	gonococci agar	172			(Lappann *et al.*, 2013)
Porphyromonas gingivalis W50	60 – 120	log	Supplemented Tryptic soy-enriched BHI broth	151			(Veith *et al.*, 2014)
P. gingivalis ATCC 53978							
P. gingivalis 33277	ND	log	Tryptic soy broth	67			(Mantri *et al.*, 2015)
P. gingivalis W83				70			
Prochlorococcus MIT9313	70 – 100	mid/late log	Constant light flux conditions, Pro99 media	27			(Biller *et al.*, 2014)
Prochlorococcus MED4	ND			40	✓		
Pseudomonas aeruginosa	~<200 nm	in biofilm	LB broth	76			(Toyofuku *et al.*, 2012)
		stationary		194			
P. aeruginosa PAO1	ND	biofilm (24, 48, and 96 hrs)	Tryptic soy broth	18, 78, 98	✓		(Park *et al.*, 2015)
P. aeruginosa PAO1	50 – 250	early log	Lysogeny broth	338			(Choi *et al.*, 2011)
P. putida KT2440	25 – 75	log	LB broth	243	✓		(Choi *et al.*, 2014)

Species	Size (nm)[2]	Growth Phase[3]	Medium	Proteome[4]	Functional Analysis[5]	Immunological Activity Analysis[6]	Reference
P. putida KT2440			Minimal medium + 10 mM succinate	359			
P. putida KT2440			Minimal medium + 5 mM benzoate	456			(Kulkarni *et al.*, 2014b)
P. syringae Lz4W	60 – 100	stationary	Antarctic bacterial medium	429	✓		(Chowdhury *et al.*, 2013)
P. syringae pv. Tomato T1	120 – 125	early stationary	King's broth medium	139			(Perez-Cruz *et al.*, 2013)
Shewanella vesiculosa	25 – 250	late log	Tryptic soy agar	46			(Chen *et al.*, 2014)
Shigella flexneri 2a str	50 – 200	log	Tryptic soy broth	148			(Ferrer-Navarro *et al.*, 2016)
Strenotrophomonas maltophilia ATCC13637	ND	early stationary	LB broth	274			
S. maltophilia M30	ND	early stationary	LB broth	133			(Devos *et al.*, 2015)
S. maltophilia 44/98 (LMG 26824)	NS	early stationary	LB broth	234			
Vibrio cholerae biotype strain C6706	ND	OD_{600} = ~0.9	Hepes-buffered LB	90			(Altindis *et al.*, 2014)

[1] From publications in 2010–2016.

[2] Diameter range or average.

[3] Growth phase when OMVs were isolated for analysis.

[4] Total proteins identified in sample, studies where only most abundant were identified are not included in this table.

[5] One or more of the following: OMVs were found to have some activity via supplementation of OMVs to another culture; OMVs conferred some sort of protection (e.g. antibiotic tolerance); OMVs were tested for cytotoxicity.

[6] OMVs elicited immune response (e.g. cytokine secretion) when incubated with mammalian cells.

*
Strain is flagella production mutant

+
See reference for details

§
Many OMV studies on this bacterium are focused on detergent-derived vesicles and not included in this table

ND, not determined; NS, not shown; LB, Luria–Bertani; BHI, Brain-heart infusion; DCY, Double casitone yeast

Table 2

Environmental changes demonstrated to affect vesiculation (e.g. levels, activity or contents)

	Reference
Media	(Bager *et al.*, 2013, Choi *et al.*, 2014)
Growth phase	(Tashiro *et al.*, 2010)
Biofilm vs planktonic mode	(Grande *et al.*, 2015, Park *et al.*, 2015)
Temperature	(Macdonald *et al.*, 2013, Fulsundar *et al.*, 2014)
Host cell factor	(Ballok *et al.*, 2014, Metruccio *et al.*, 2016)
Antibiotic exposure	(Maredia *et al.*, 2012, Macdonald *et al.*, 2013, Devos *et al.*, 2015, Devos *et al.*, 2016)
Anoxic conditions	(Toyofuku *et al.*, 2013)
Iron depletion	(Keenan *et al.*, 2000)

Table 3

Recent studies where lipid analysis was performed on OMVs and/or DNA was detected in vesicle samples.

Species	Lipid Analysis	DNA Presence	Reference
Acinetobacter radioresistens MMC5 strain	✓*		(Fulsundar *et al.*, 2015)
A. baylyi		✓	(Fulsundar *et al.*, 2014)
Ahrensia kielensis		✓	(Hagemann *et al.*, 2013)
Bacteroides fragilis	✓		(Elhenawy *et al.*, 2014)
B. thetaiotaomicron	✓		
Delftia sp. Cs1–4	✓		(Shetty *et al.*, 2011, Shetty *et al.*, 2014)
Francisella novicida ATCC 15482		✓	(Pierson *et al.*, 2011)
F. philomiragia ATCC 25015		✓	
Haemophilus influenzae		✓	(Sharpe *et al.*, 2011)
Haemophilus influenzae (several mutants)	✓✓		(Roier *et al.*, 2016)
Helicobacter pylori	✓✓		(Olofsson *et al.*, 2010)
Kingella knigae		✓✓	(Maldonado *et al.*, 2011)
Moraxella catarrhalis		✓✓	(Schaar *et al.*, 2011)
Mycobacterium bovis BCG	✓✓	✓✓	(Prados-Rosales *et al.*, 2011)
M. smegmatis	✓✓	✓✓	
Prochlorococcus sp.	✓✓	✓✓	(Biller *et al.*, 2014)
Pseudoalteromonas marina		✓✓	(Hagemann *et al.*, 2013)
Pseudomonas aeruginosa	✓✓		(Tashiro *et al.*, 2011)
P. syringae Lz4W	✓✓		(Kulkarni *et al.*, 2014b)
P. syringae pv. Tomato T1	✓✓		(Chowdhury *et al.*, 2013)
Porphyromonias gingivalis		✓✓	(Ho *et al.*, 2015)
Shewanella vesiculosa		2713✓	(Frias *et al.*, 2010, Perez-Cruz *et al.*, 2013)
Shigella flexneri 2a str			(Chen *et al.*, 2014)

*Only fatty acid analysis