ADDENDUM

Outer membrane vesicles in service as protein shuttles, biotic defenders, and immunological doppelgängers

Richard C. Laughlin and Robert C. Alaniz

Department of Biological and Health Sciences, Texas A&M University Kingsville, Kingsville, TX, USA; Department of Microbial Pathogenesis and Immunology, Texas A&M Health Science Center, College Station, TX, USA

ABSTRACT
Characterization of host microbial interactions typically occurs on the cellular or protein level. Recently, a more thorough and accurate appreciation of cellular interactions has come into better focus with improved understanding of membrane vesicles (OMV). While OMVs are documented primarily in Gram-negative bacteria, certain Gram-positive species generate these structures, despite the obvious physical limitations of the cell envelope. Here, we briefly review the current understanding of OMVs in content and function, their role in pathogenesis, and the consequences of somatic cell gene expression on these events.

The Gram–negative bacterial envelope is a heterogeneous mixture of proteins, lipids, and complex glycolipids consisting of outer and inner membranes surrounding a thin and flexible peptidoglycan cell wall. OMV formation in Gram-negative bacteria is highly dependent on the state of crosslinking and molecular interactions between several proteins of the outer membrane and the peptidoglycan layer. Interactions between the peptidoglycan cell wall and Braun’s lipoprotein (Lpp), outer-membrane protein A (OmpA), or the peptidoglycan-associated lipoprotein complex (Tol-Pal) are well characterized interactions central to membrane stability and rate of vesicle release. Functional impairment or deletion of Lpp, OmpA, or Tol-Pal resulted in increased membrane volatility and hyper-vesiculation. Alterations to membrane stabilizing crosslinking events can be coordinated locally or globally by specific cellular responses to environmental factors, signaling pathways, or other undefined perturbations. The importance of outer membrane lipid composition and asymmetry was highlighted by the hyper-vesiculation observed after disruption of the VacJ/Yrb ATP-binding cassette (ABC) transport system in Haemophilus influenzae, Vibrio cholerae, and Escherichia coli without compromising the outer membrane integrity. This transport system is purported to traffic phospholipids to the outer membrane, and its expression is down regulated during iron limiting environments. This is an important observation as it strongly links OMV production to environmental conditions likely encountered during the pathogenic lifecycle of H. influenzae, as well as other Gram–negative bacteria.

OMV release in Gram-negative bacteria is ubiquitous and associated with all facets of bacterial physiology including division, nutrient acquisition, toxin sequestration, antibiotic removal, formation and maintenance of bacterial communities/biofilms, cargo packaging and release – including delivery of virulence factors and pro-inflammatory molecules to host cells. One of the earliest OMV observations came from a study of Vibrio cholerae vegetative cultures in which “approximately spherical sacs” were observed blebbing from the bacterial surface by electron microscopy. Subsequent studies identified the Tol-Pal heteroprotein complex, which spans the Gram-negative cell envelope linking the outer membrane, peptidoglycan, and inner membrane layers, as a critical element for intra-envelope cross-linking during cell division. While a level of OMV release during cell division may be expected, interruption of the Tol-Pal complex by
component deletion or mutation results in failure of daughter cells to separate and an increase in OMV production, consistent with a loss of outer membrane integrity and crosslinking.8–10

Building from this biophysical phenomenon during cell division, OMVs also play important and diverse roles for within bacterial communities and biofilms in nutrient acquisition, communication, and competition. Numerous extracellular membrane structures, including outer membrane vesicles, were observed in a cultured environmental biofilm taken from the surface of sedimentary rock.11 This variety of membrane vesicles underscores the complexity of the extracellular environment, as well as the diversity of functions of OMVs. In Bacteroides spp., careful analysis of OMV protein content identified OMV-specific acidic glyco-sidases and proteases proposed to function in carbohydrate liberation and nutrient acquisition for the local microbial community.12 OMVs polymerize and form physical connections between cells in the soil bacterium Myxococcus xanthus, suggesting the intriguing scenario of novel cell-cell connections to shuttle cellular materials within a biofilm.13 M. xan-thus was previously shown to use OMVs to deliver alkaline phosphatase to lyse E. coli.14 The delivery of bio-active proteins for pathogenicity and environmental modification is of particular importance in the context of pathogenic bacteria - by far the most intensely studied group of bacteria, where several instances of OMV mediated delivery of virulence factors have been identified. Helicobacter pylori OMVs are enriched with the virulence factor HtrA, a protease secreted by numerous intestinal Gram-negative pathogens to interrupt the epithelial barrier.15 In the case of Salmonella enterica Typhimurium, several virulence factors have been linked to OMVs generated in conditions that replicate the intracellular portion of the life-cycle.16,17 Biophysical analysis of OMVs from Legionella pneumophila showed direct fusing of the bacterial vesicles with host liposomes and may facilitate cellular invasion and delivery of virulence factors to the host cell cytosol.18 Proteomic analysis of Vibrio cholera OMVs followed by selective gene deletion identified a decreased ability for biofilms formation upon deletion of the gene for the OMV protein degP.19

For any bacteria living in a mixed community and competing for resources – within an intestine, attached to a root system in the soil, or some other specified environmental niche – natural or pharmaceutical antimicrobial agents will provide selection pressure and challenge organismal fitness. OMVs serve an important role incountering these antimicrobials, acting as a decoy target away from the somatic cell, selectively removing the antibiotic from the surface of the cell, or shuttling antibiotic degrading enzymes into the milieu.1 For Pseudomonas syringae, a soil bacterium from shores of Lake Zub in Antarctica, OMVs provide protection against the antibiotics colistin and metillin, 2 antibiotics known to attack bacterial membranes.20 Similarly, OMVs isolated from E. coli MG1655 and then supplied to cultures of E. coli, Pseudomonas aeruginosa, and Acinetobacter radioresistens provided protection against the effects of membrane acting antibiotics.21 Further analysis of OMVs after incubation with the antibiotics revealed the membrane vesicles both sequester and degrade the antibiotics.21 In a similar fashion, an endogenous β-lactamase was found in OMVs from Bacteroides spp that provide protection to Salmonella enterica Typhimurium in susceptibility tests with the broad spectrum cephalosporin antibiotic, cefotaxime.22 This broadcast protection against antimicrobials by commensal microbiota provides interesting context when considering the true complexity of microbial population dynamics. While the protective effects of OMVs against antimicrobials are important, the role that these vesicles play in immune stimulation and host–pathogen interaction is significant. In addition to the protein cargo already discussed, OMVs contain proteins from the inner and outer membrane, periplasm, and cytosol, lipids, DNA, peptidoglycan, and RNA.23 Numerous reports have documented the immune modulatory effects of OMVs on host cells, eliciting the production of cytokines, activating pattern recognition receptor (PRR) signaling pathways, disruption of epithelial barrier, and activation of dendritic cells and macrophages.23,24 This immune and cellular activity can be robust in the case of OMVs isolated from known pathogens, or it can facilitate immune tolerance and prevent inflammatory responses. Bacteroides fragilis presents the capsular protein polysaccharide A to dendritic cells via OMVs to suppress inflammation and maintain homeostasis within the intestine.25 Notably, a recent report identified OMVs from Pseudomonas aeruginosa as a delivery mechanism for sRNA to reduce IL-8 secretion in vitro, and cytokine secretion in vivo.26 For pathogenic bacteria, OMVs are receiving increased attention for their contribution to the
immune modulatory properties and infectious life cycle. We have previously reported OMVs from *Salmonella* Typhimurium are immunologic facsimiles of the somatic cell and efficiently induce dendritic cell maturation and proinflammatory cytokine release. Importantly, inoculation of mice with *S. Typhimurium* OMVs generated a recall response and protection against future *Salmonella* infection. This effective immune stimulation has been seen with OMVs isolated from several other Gram-negative bacteria including *E. coli*, *Shigella flexneri*, and *Neisseria meningitidis*. Finally, OMVs can also serve as effective antigen delivery vehicles, stimulating dendritic maturation and both helper and cytotoxic T-cell responses against an otherwise non-immunogenic target such as ovalbumin.

Further analysis of *S. Typhimurium* OMVs demonstrated that the quality or magnitude of the host cell response was dependent on the somatic bacterial cell gene expression. The *S. Typhimurium* life cycle can be generally discussed as either invasive or intracellular. While invasive, the bacteria are extra-cellular, flagellated, and express an immunogenic Lipid A and gene products from the *Salmonella* Pathogenicity Island 1 (SPI-1) which facilitate cellular invasion. Once the pathogen gains access to the intracellular niche, it up regulates genes from SPI-2 which code for products with reduced immunogenicity and which facilitate the cessation of lysosomal maturation, recruitment of nutrients, and formation of intracellular structures. Expression of SPI-2 is controlled by the master regulator gene PhoP OMVs isolated from *S. Typhimurium* with a constitutively active PhoP were significantly less immunogenic than those isolated from WT *S. Typhimurium*, failing to induce dendritic cell maturation or stimulate T-cell activation as effectively as the OMVs from the WT. Complementary to this observation, protein constituents of OMVs from *S. Typhimurium* grown in SPI-1 or SPI-2 inducing cell culture correlated to the underlying genetic expression events of the somatic bacterial cell. The expression-dependent content of OMVs provides exciting possibilities for rationally designed and tunable protein expression and adjuvant properties. In an *in vivo* infection model, OMVs were observed during the initial stages of invasion in several intracellular *S. Typhimurium*, supporting the notion that OMV release plays a role during infection. With the already thorough understanding of the infectious cycle of *S. Typhimurium*, alterations in OMV immunogenicity provides an interesting avenue for further refinement and discovery of host-pathogen interactions and mechanisms of pathogenicity.

Given the multitude of functions documented for OMVs from other work using deletion mutants or *in vitro* systems, the significance and impact they have on infection and host-pathogen interactions will be an important area of future research focus. Already OMV research has demonstrated an intrinsic adjuvant and antigenic advantage over heterologous antigens that may be incorporated or combined in artificial formulations, further revealing the versatility to enable choice of administration via the mucosal or parenteral routes. Additional refinement and detail to the mechanisms controlling OMV biogenesis and recruitment of biologically active molecules will augment our understanding of these membrane vesicles, how their cargo is manipulated, and their interactions with host cells or other microbes.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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