Published in final edited form as:
Geobiology. 2008 June ; 6(3): 214–219. doi:10.1111/j.1472-4669.2008.00157.x.

Gram-negative outer membrane vesicles: beyond the cell surface

L. MASHBURN-WARREN[1], **R. J. C. MCLEAN**[2], and **M. WHITELEY**[1]

[1]Department of Molecular Genetics and Microbiology, The University of Texas at Austin, Austin, TX 78712, USA

[2]Department of Biology, Texas State University-San Marcos, San Marcos, TX 78666, USA

Abstract

Considerable interest has recently mounted regarding the biological roles of Gram-negative outer membrane vesicles (MVs). The first discovery of MVs was made over four decades ago, and it is now clear that most Gram-negative bacteria produce MVs, with *Pseudomonas aeruginosa* and *Escherichia coli* as the most extensively studied. Much of our knowledge of the biological roles of MVs and mechanism of MV formation is due to T.J. Beveridge and colleagues. Beveridge pioneered the field of MV research not only by enhancing our understanding of MV function, but also through the application of a wide variety of physical, chemical, and genetic techniques to complement his elegant electron microscopy investigations. Here we review the contributions of Beveridge's group to our understanding of MV biology.

INTRODUCTION

'The unity of bacterial cell design must play an important role in the ecological success of prokaryotes. It is one that has lasted at least 3.5 billion years and therefore has survived the test of time. It is an economy of design, and one that I, and others, find pleasing and graceful.' (Quotation from T.J. Beveridge, presented at many of his talks). The outer membrane plays an important role for Gram-negative bacteria. It represents the cell-surface component by which these bacteria contact their environment, and is the outermost structure that controls access into and out of the cell. Damage to this structure by compounds such as lactoferrin and EDTA has been associated with increased sensitivity to antimicrobials (Hancock, 1984; Ellison *et al*., 1988). In this context, it is surprising that many Gram-negative bacteria shed their outer membrane via membrane vesicles (MVs). Yet, this phenomenon has been described for at least 40 years (Beveridge, 1999). Indeed, the relatively small size of MVs may have led them to be incorrectly referred to as independent life forms (i.e. nanobacteria; Folk, 1993).

MVs are released into the surrounding environment by most Gram-negative bacteria through bulging and 'pinching off' of the outer membrane (Fig. 1). MVs range in size from 50 to 250 nm in diameter and are primarily composed of outer membrane components such as phospholipids, proteins, and lipopolysaccharide (LPS), but also contain periplasmic components. MVs are found in planktonic cultures, biofilm communities in solid and liquid media, as well as natural environments (Beveridge, 1999). It has long been argued by Beveridge and others that based on their metabolic 'cost' and ubiquity, it is likely that MVs have important biological functions, which may differ depending on the organisms from

Corresponding author: M. Whiteley. Tel.: 512-471-5493; fax: 512-471-7088; mwhiteley@mail.utexas.edu. .

which they originate. Through their investigations, Beveridge's group identified an array of biological roles for MVs (Fig. 2).

MVs CARRY VIRULENCE FACTORS AND ARE 'PREDATORY'

Kadurugamuwa and Beveridge showed convincingly that MVs liberated from *Pseudonomas aeruginosa* contain many virulence factors and are likely important in disease pathogenesis. Factors packaged into *P. aeruginosa* MVs include phospholipase C, proteases, alkaline phosphatases, and hemolysins. Some of these virulence factors are enriched in MVs, suggesting a sorting mechanism for packaging specific factors into MVs (Kadurugamuwa & Beveridge, 1995). Although the benefit of packaging toxins into MVs is unknown, Beveridge proposed two potential advantages: (i) MVs may serve to concentrate virulence factors and allow focused delivery to target cells; and (ii) virulence factors within the lumen of MVs may protect them from degradation and/or recognition by either host factors or other microorganisms.

Li and Beveridge later showed that *P. aeruginosa* MVs not only contain factors critical for killing host cells, but also several antibacterial factors, including murein hydrolases (autolysins; Li *et al*., 1996). During normal bacterial growth, these autolysins function in cell wall turnover and cell division. Li *et al*. (1996) showed *P. aeruginosa* MVs are enriched with a 26-kDa autolysin, further supporting the hypothesis that some MV components are preferentially packaged into MVs (Li *et al*., 1996). Interestingly, autolysin-containing MVs were able to degrade the peptidoglycan walls of Gram-negative and Gram-positive bacteria. This degradation ultimately leads to cell lysis and death; thus, Beveridge coined the term 'predatory' MVs (Kadurugamuwa & Beveridge, 1996; Beveridge *et al*., 1997; Li *et al*., 1998). In addition to *P. aeruginosa*, MVs released by 15 other Gram negatives were able to lyse a variety of Gram-negative and Gram-positive bacteria, with *P. aeruginosa* having the most extensive killing spectrum (Li *et al*., 1998). The lytic ability of these 'predatory' MVs was dependent upon the peptidoglycan chemotype of the target cell, where chemotypes closely resembling the MV-producing cell were more susceptible to hydrolysis (Li *et al*., 1998). Li and Beveridge proposed that autolysins display higher activity against their own peptidoglycan chemotypes, since their normal function is cell wall turnover during cell division (Li *et al*., 1998).

These studies by Beveridge and colleagues provided the first evidence that MVs not only contain virulence factors but also antimicrobial agents. It is tempting to speculate that inclusion of virulence factors within MVs is important in establishing and maintaining infections by protecting and concentrating these components for host cell delivery. Predatory MVs may also provide Gram-negative bacteria with a competitive advantage in polymicrobial environments through lysis of 'nonself' bacteria. Lysis of neighboring cells might also liberate available nutrients to enhance growth of predatory bacteria.

MVs CONTAIN DNA

In an elegant study, Beveridge showed that *P. aeruginosa* wild-type MVs also contain DNA, and that MVs protect DNA from degradation when treated with extracellular DNases (Kadurugamuwa & Beveridge, 1995; Renelli *et al*., 2004). Not only was chromosomal DNA packaged into MVs, Beveridge also demonstrated that *P. aeruginosa* was able to package plasmid DNA into MVs. These plasmid-containing MVs were unable to successfully transform the DNA into nonplasmid containing cells (Kolling & Matthews, 1999; Yaron *et al*., 2000). This may be a result of the transformation conditions utilized since studies using MVs from other species have successfully transformed DNA into recipient cells (Renelli *et al*., 2004). Packaging DNA into MVs is counterintuitive since DNA is normally found in the cytoplasm. Beveridge proposed two models to explain DNA packaging into MVs. The first

model involves DNA release by autolysing bacterial cells and uptake of this extracellular DNA by MVs. Beveridge's group provided support for this model by demonstrating MVs could take up 'naked' plasmid DNA, and subsequently protect the DNA from DNase treatment (Renelli *et al*., 2004). The second model involves DNA packaging into MVs before they 'bud' from the bacterial cell. Beveridge proposed that these models are likely not mutually exclusive and that both naturally occur.

MVs ASSOCIATE WITH GRAM-NEGATIVE AND GRAM-POSITIVE BACTERIA

To deliver its cargo, MVs must associate with the target cell membrane, otherwise the contents would not be able to penetrate the cell membrane barrier (Kadurugamuwa & Beveridge, 1996). Kadurugamuwa and Beveridge demonstrated that MVs are able to fuse to the outer membrane of Gram-negative cells. Since they are composed of the outer membrane, MVs are able to fuse to Gram-negative bacteria without completely reorganizing the outer membrane (Kadurugamuwa & Beveridge, 1999). Using transmission electron microscopy (TEM) and immuno-gold labeling, they illustrated that MVs liberated from *P. aeruginosa* and *Shigella flexneri* were able to incorporate into the outer membranes of *Salmonella typhi*, *S. enterica*, and *Escherichia coli*. The integration of the MVs was immediate and stable, with *P. aeruginosa* MVs being more efficient in membrane integration than *S. flexneri* (Kadurugamuwa & Beveridge, 1999). Attachment and fusion of MVs into the outer membrane allowed them to open and deliver their components into the periplasm of target cells. This delivery was critical for the predatory nature of MVs as it provided the MV autolysin with direct access to the underlying peptidoglycan layer (Kadurugamuwa & Beveridge, 1999).

Due to their structural makeup, MVs readily fuse with Gram-negative membranes but not Gram-positive membranes. Kadurugamuwa and Beveridge showed that *P. aeruginosa* MVs did not fuse, but did attach to the surface of the Gram-positive pathogen *Staphylococcus aureus*. Using TEM, they demonstrated that MVs release their components upon interaction with the Gram-positive cell wall (Kadurugamuwa & Beveridge, 1996). The mechanism of MV attachment to the Gram-positive exterior is unknown. Kadurugamuwa and Beveridge proposed that charge–charge interactions play a role in adherence. The surface of Gram-positive bacteria has an overall negative charge and like Gram-negative bacteria, divalent cations form salt bridges to stabilize these charges. Because MVs are composed of LPS, it was proposed that salt bridges may stabilize interactions between MVs and the Gram-positive cell wall. This salt bridging would change the curvature of the circular MV causing it to burst and release its contents to a localized area on the cell (Kadurugamuwa & Beveridge, 1996).

MacDonald and Beveridge extended these initial observations by examining the ability of *P. aeruginosa* MVs to attach and lyse several Gram-positive species including Bacillus subtilis, S. aureus, Listeria monocytogenes, and *Enterococcus hirae* (MacDonald & Beveridge, 2002). These species have different cell surface hydrophobicities and peptidoglycan chemotypes which might affect the lytic ability of *P. aeruginosa* MVs. *P. aeruginosa* MVs were able to attach immediately to all cells, with a higher number of MVs attaching to the hydrophilic surface of *B. subtilis*. MV autolysin susceptibility was more prominent on the same peptidoglycan chemotypes, with the exception of *S. aureus*. This study showed the importance of MV attachment and 'predatory' activities (MacDonald & Beveridge, 2002), and it was proposed that MVs likely play a key role in providing *P. aeruginosa* with a competitive advantage during growth in polymicrobial populations.

MVs ARE TRANSPORT VEHICLES

Gentamicin is an antibiotic that targets bacterial protein synthesis and is often used to treat *P. aeruginosa* infections. Beveridge showed that gentamicin induced a threefold increase in MV production by *P. aeruginosa*, and remarkably, some of the gentamicin was packaged into MVs (Kadurugamuwa & Beveridge, 1995). These results demonstrated that gentamicin not only acted by disrupting protein synthesis, but also caused outer membrane perturbation. Because gentamicin was packaged within MVs, this enhanced the 'predatory' activity of the MVs produced by *P. aeruginosa* due to the presence of both the autolysin and the antibiotic (Kadurugamuwa & Beveridge, 1995).

These findings led to an array of studies by Beveridge's group aimed at utilizing MVs as therapeutic delivery vehicles. The delivery of antibiotics via MVs is enticing since it would allow direct and concentrated delivery to bacterial cells. This delivery mechanism would be particularly useful for treating intracellular bacterial infections since many antimicrobials are unable to reach the interior of host mammalian cells. Kadurugamuwa and Beveridge illustrated that soluble gentamicin was unable to target the intracellular pathogen *S. flexneri* when growing in mammalian cells; however, gentamicin-loaded MVs were able to deliver the antibiotic to the cytoplasm of host cells and reduce the number of viable *S. flexneri* (Kadurugamuwa & Beveridge, 1998). This proof of principle experiment suggested that MVs may have practical uses in a clinical setting as trafficking vehicles for delivery of antimicrobials to target cells (Kadurugamuwa & Beveridge, 1998).

MVs ARE A MAJOR COMPONENT IN BIOFILMS

The majority of bacteria in nature do not live as free-swimming planktonic organisms, but instead in bacterial biofilms. Biofilms are communities of cells attached to a surface surrounded by a self-produced extracellular polymeric matrix (Hall-Stoodley *et al*., 2004). This matrix contains DNA, polysaccharides, cell debris such as flagella and pili, proteins, and phage and serves as a protective 'coating' for biofilm bacteria. Based partly on this matrix, biofilm bacteria are more resistant to killing by antimicrobials and phagocytosis than planktonic bacteria. Schooling & Beveridge (2006) demonstrated that the extracellular matrix of biofilms is also composed of MVs. TEM analysis of laboratory-grown biofilms revealed that MVs were present when biofilm were grown with several different *in vitro* biofilm systems, with areas of high and low MV density present within the biofilm matrix (Hunter & Beveridge, 2005; Schooling & Beveridge, 2006).

In a significant technological advancement, MVs were subsequently purified from biofilm matrices (Schooling & Beveridge, 2006). Biofilms were shown to produce significantly more MVs than planktonic bacteria, and biofilm MVs were lighter in color and more gelatinous than those from planktonically grown cells. The protein profiles of biofilm MVs were also distinct from planktonic MVs, suggesting that different proteins were packaged into MVs based on the mode of growth (biofilm or planktonic). Schooling & Beveridge (2006) subsequently expanded on these initial studies of laboratory-grown biofilms demonstrating that MVs were found to be a major element in the matrix of naturally occurring biofilms (Fig. 3). These latter studies hypothesized that in naturally occurring biofilms, MVs could act as 'decoys or sponges' that would decrease the levels of harmful agents such as antibiotics and immune factors that can penetrate the biofilm (Schooling & Beveridge, 2006). These initial studies have shown convincingly that one of the next major steps in MV biology is to understand their roles in naturally occurring biofilms.

PROPOSED MECHANISMS OF MV FORMATION

The molecular mechanism of MV formation is currently unknown. Beveridge proposed that *P. aeruginosa* MV formation is likely affected by the anionic nature of the outer membrane. *P. aeruginosa* LPS contains two types of O-antigen: a smaller more neutrally charged A-band, and a longer more negatively charged B-band. Beveridge has shown that naturally occurring MVs are primarily composed of B-band O-antigen and proposed that MVs are less likely to form in areas on the outer membrane where A-band LPS is mostly present (Kadurugamuwa & Beveridge, 1995, 1996; Li *et al*., 1996). Instead, areas rich in B-band LPS have a higher propensity to bleb due to charge-to-charge repulsion of the longer and negatively charged side chains, where not all negative charges are masked due the complex and rigid structure of B-band chains. He also hypothesized that areas rich in B-band LPS would cause the membrane to bulge out and form 'high curvature structures.' These structures would eventually pinch off to form MVs (Kadurugamuwa & Beveridge, 1995, 1996; Li *et al*., 1996). This 'anionic repulsion' hypothesis provided the first molecular model for MV formation. Revised molecular models have been subsequently proposed by us and others (Kuehn & Kesty, 2005; Mashburn-Warren & Whiteley, 2006), incorporating aspects of this original model.

CONCLUSIONS

From Beveridge's work, it is clear that most Gram-negative bacteria produce MVs and that these once-perceived innocuous entities have very important biological roles. Some of the features mentioned above will undoubtedly be investigated further. From a geological aspect, one issue that may arise may be a role for MVs in mineral nucleation and dissolution. Certainly, this concept has been described by Gorby *et al*. (2006). During the 1990s, R.L. Folk described small bacteria-shaped spherical particles that were found in association with travertine. As these particles were considerably smaller than bacteria, the term nannobacteria (also spelled nanobacteria) was coined (Folk, 1993; Sillitoe *et al*., 1996), and the prospect was raised that they may in fact be a new class of independent life. While subsequent work may question the origin of nanobacteria as independent life forms (Kirkland *et al*., 1999; Gorby *et al*., 2006), they may in fact represent MVs.

Thanks to Beveridge and colleagues, we have an enhanced understanding of the importance of MVs. His studies inspired an array of interest in MV biology including our own. Our interest in MV formation was initiated by examination of MVs as trafficking vehicles for bacterial cell–cell signals (Mashburn & Whiteley, 2005). Shortly after this discovery, I contacted Dr Beveridge to consult his opinion on our results. I received an immediate reply which began with, 'This is a fascinating story you tell about your MVs and, to be quite honest, I am not surprised.' Although at the time I had not met Dr Beveridge, we had several interactions through e-mail, which encouraged us to continue our investigations. The final correspondence from Dr Beveridge was, 'I encourage you to keep up this particularly interesting line of research and keep in touch'. T.J. Beveridge was an excellent scientist and will be greatly missed.

Acknowledgments

The authors gratefully acknowledge the financial support of the National Science Foundation (M.W.) and the National Institutes of Health (M.W.) to study membrane vesicle biology.

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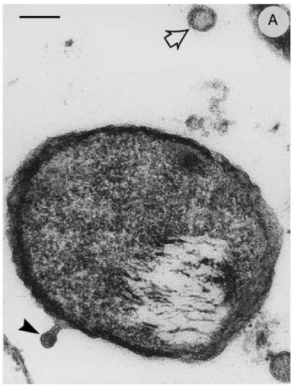

Fig. 1.

Transmission electron micrograph showing MVs budding from the outer membrane of a *Pseudonomas aeruginosa* cell (from Kadurugamuwa & Beveridge, 1995). The dark arrow shows an MV budding from the outer membrane, and the open arrow indicates an MV that has been released into the surrounding medium. The scale bar (upper left) is 100 nm.

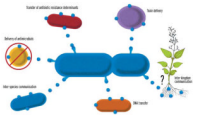

Fig. 2.
Proposed biological roles for MVs (from Mashburn-Warren & Whiteley, 2006).

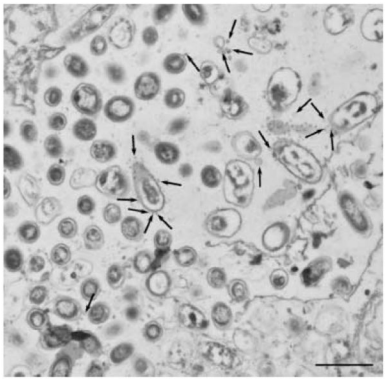

Fig. 3.

Transmission electron micrograph of a naturally occurring biofilm (from Schooling & Beveridge, 2006). Arrows indicate MVs present within the biofilm matrix. The scale bar (bottom right) is 1 μm.