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Biogenesis of Bacterial Membrane Vesicles

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Abstract

Membrane vesicle (MV) release remains undefined, despite its conservation among replicating Gram-negative bacteria both *in vitro* and *in vivo*. Proteins identified in *Salmonella* MVs, derived from the envelope, control MV production via specific defined domains that promote outer membrane protein-peptidoglycan (OM-PG) and OM protein-inner membrane protein (OM-PG-IM) interactions within the envelope structure. Modulation of OM-PG and OM-PG-IM interactions along the cell body and at division septa, respectively, maintains membrane integrity while coordinating localized release of MVs with distinct size distribution and protein content. These data support a model of MV biogenesis, wherein bacterial growth and division invoke temporary, localized reductions in the density of OM-PG and OM-PG-IM associations within the envelope structure, thus releasing outer membrane as MVs.

Keywords

surface organelle; membrane vesicle; outer membrane

INTRODUCTION

The release of membrane vesicles (MVs) is conserved among Gram-negative bacteria (Kuehn and Kesty, 2005), including many pathogens (Stephens *et al*., 1982; Brandtzaeg *et al*., 1992; Garcia-del Portillo *et al*., 1997; Fiocca *et al*., 1999; Hellman *et al*., 2000; Keenan *et al*., 2000; Namork and Brandtzaeg, 2002; Rosenberger *et al*., 2004; Marsollier *et al*., 2007; Necchi *et al*., 2007). MVs originate from the bacterial surface by an undefined process, and are composed of outer membrane (OM) and periplasmic constituents, including proteins, phospholipids, and lipopolysaccharides (McBroom and Kuehn, 12 May 2005, posting date; Kuehn and Kesty, 2005; Mashburn-Warren and Whiteley, 2006). MVs from bacteria have been observed in many environments, including *in vivo*, where the function of MVs is likely multifaceted: MVs act as primary delivery vehicles for bacterial toxins lacking typical signal sequences (Horstman and Kuehn, 2000; Wai *et al*., 2003), promote cell-cell communication via transit of signaling molecules (Mashburn and Whiteley, 2005), inhibit phagosome-lysosome fusion during macrophage infection (Fernandez-Moreira *et al*., 2006), and are rich in antigens that serve as initial targets for innate and adaptive immune recognition (Bergman *et al*., 2005), generating protective immunity against bacterial challenge when used as an immunogen (Sexton *et al*., 2004; Alaniz *et al*., 2007).

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The presence and importance of MVs released by bacteria growing on solid agar (Tetz *et al*., 1990), within biofilms (Beveridge *et al*., 1997; Schooling and Beveridge, 2006), *in vitro* (Garcia-del Portillo *et al*., 1997; Mashburn and Whiteley, 2005; Fernandez-Moreira *et al*., 2006), and *in vivo* (Stephens *et al*., 1982; Brandtzaeg *et al*., 1992; Fiocca *et al*., 1999; Marsollier *et al*., 2007; Necchi *et al*., 2007) have become increasingly evident. Although observed for decades (Knox *et al*., 1966; Work *et al*., 1966; Chatterjee and Das, 1967), the process by which Gram-negative organisms produce MVs is unknown. Several groups have investigated this question, proposing a range of models describing MV release. Early models suggested that MVs result from OM growth exceeding that of the peptidoglycan (Wensink and Witholt, 1981), or when fewer OM lipoprotein linkages to underlying layers are present (Hoekstra *et al*., 1976). However, direct supporting evidence of these proposed models, such as electron microscopic or quantitative methods to determine, for example, the rate of OM/peptidoglycan growth or the role or location of OM lipoprotein linkages, was not provided in these publications. Increased MV release has been observed in mutants lacking components of the *tol-pal* system, a group of envelope proteins exploited for entry of filamentous bacteriophages and group A colicins in *E. coli* (Webster, 1991; Bernadac *et al*., 1998). Drawing definitive conclusions about the role of these proteins in MV release, however, is confounded by comparing non-isogenic strains with multiple mutations, qualitative assessment of MV production, and electron microscopy of organisms during stationary phase (during which MV release is limited (Hoekstra *et al*., 1976; Bauman and Kuehn, 2006)). Furthermore, the suggestion that wild-type (WT) organisms do not produce MVs (Bernadac *et al*., 1998) is in direct conflict with observations of MV production by a variety of Gram-negative bacteria, including pathogens growing *in vivo* (Stephens *et al*., 1982; Brandtzaeg *et al*., 1992; Garcia-del Portillo *et al*., 1997; Fiocca *et al*., 1999; Hellman *et al*., 2000; Keenan *et al*., 2000; Namork and Brandtzaeg, 2002; Rosenberger *et al*., 2004; Marsollier *et al*., 2007; Necchi *et al*., 2007), and genetic evidence suggesting MV release cannot be abolished (McBroom and Kuehn, 12 May 2005, posting date; McBroom *et al*., 2006)). Subsequent investigations have proposed that MV release can act as an envelope stress response able to quickly rid the cell surface of misfolded proteins (McBroom and Kuehn, 2007), or that expression of modified forms of LPS (Kadurugamuwa and Beveridge, 1997; Nguyen *et al*., 2003) or interaction of hydrophobic molecules with the OM, such as the cell-cell communication molecule *pqs* (Mashburn and Whiteley, 2005; Mashburn-Warren *et al*., 2008), can stimulate MV production.

Multiple factors, some organism-specific, are apparently capable of inducing the release of MVs, yet the mechanism by which MVs are produced remains unknown (McBroom and Kuehn, 12 May 2005, posting date; Kuehn and Kesty, 2005; Mashburn-Warren and Whiteley, 2006), and quantitative approaches to this question have been underutilized to date. The conservation of this process among Gram-negative bacteria suggests the possibility of unifying factors or processes utilized by organisms demonstrating release of MVs. We used a multiphasic and quantitative approach to gain insight into the biogenesis of MVs, and demonstrate here that the release of MVs from actively dividing bacteria is specifically modulated by the density and distribution of highly conserved envelope protein interconnections.

RESULTS

Influence of protein constituents on MV release

The release of MVs by Gram-negative bacteria has been investigated for at least 40 years (Knox *et al*., 1966; Work *et al*., 1966; Chatterjee and Das, 1967), but only recently have more global approaches been applied to understanding the key components of this process (McBroom *et al*., 2006). In general, it is known that MVs contain OM and periplasmic (PP) proteins, phospholipids, and lipopolysaccharide (Kuehn and Kesty, 2005). Therefore, we

used proteomics as an unbiased approach to define the major protein constituents of MVs produced by WT bacteria, and then tested the hypothesis that these proteins were involved in MV release by developing quantitative methods to compare WT bacteria with isogenic mutants, each lacking one of the major MV proteins identified. Using 2D SDS-PAGE and MALDI-TOF mass spectrometry, we identified the major WT MV proteins as OmpC, OmpF, NmpC, OmpX, OmpA, LppAB, Pal, and TolB. We constructed targeted in-frame deletions of coding sequence of each corresponding gene and the mass of MVs produced by WT *Salmonella* and each mutant strain was quantified by a combination of tangential flow diafilatration and ultracentrifugation (see Experimental Procedures; Figure 1). MV release by mutants lacking OmpC, OmpF, NmpC, or OmpX was similar to WT ($p>0.05$), while mutants lacking OmpA, LppAB, Pal, TolB, or TolA (which closely associates with Pal and TolB) significantly increased MV production ($p<0.01$ for *ompA*, $p<0.001$ for *lppAB, pal, tolB*, and *tolA*). More in depth examination of MV size, reported to range from 10-200nm in diameter (Kuehn and Kesty, 2005), revealed that the size distribution of MVs released by WT *S. typhimurium* (Figure 2A), was unchanged in the absence of OmpC, OmpF, NmpC, or OmpX (Figure 2B, $p>0.05$). While the size distribution of MVs released by the *ompA* mutant was not significantly different than WT (Figure 2C, *ompA*, $p=0.059$), the population of MVs released by the *lppAB* mutant was shifted toward smaller MVs (Figure S1 *lppAB*, $p<0.001$). The *pal, tolB*, and *tolA* mutants, however, released MVs significantly larger than WT (Figure 2D, $p<0.001$).

Although MV release has been described in general terms as occurring at bacterial surfaces (McBroom and Kuehn, 12 May 2005, posting date), MV release by *Salmonella* had not been previously visualized. We sought to capture nascent MV formation by extensively surveying electron micrographs of actively dividing cells. Strikingly, WT bacteria released MVs at constricted division septa and at locations distributed along the cell body (Figures 2E, F, S2); similar release was seen in cells lacking OmpC, OmpF, NmpC, or OmpX (data not shown). However, the absence of OmpA or LppAB induced localization of MV release along the cell body at regularly spaced intervals (Figures 2G, 3A, S3A). Conversely, increased MV release was localized to constricted division septa in the absence of Pal, TolB, or TolA (Figures 2H, 3D, S3B).

Subsets of major MV proteins, therefore, similarly impact WT MV production. Integral outer membrane proteins (OMPs) OmpC, OmpF, NmpC, and OmpX did not alter WT MV release (Figures 1, 2B). Expression of LppAB and OmpA, OM proteins which bind PG via covalent (Braun and Sieglin, 1970) and non-covalent (De Mot and Vanderleyden, 1994;Koebnik, 1995) interactions, respectively (Figure 2I, middle panel, OM-PG linked), minimized cell body MV production (Figures 1,2C,G). Pal, TolB, and TolA bridge the IM and OM via protein-protein and protein-PG interactions (Clavel *et al*., 1998;Walburger *et al*., 2002;Cascales and Lloubes, 2004;Parsons *et al*., 2006) (Figure 2I, right panel, OM-PG-IM linked), restricting MV release and MV size at division septa (Figures 1,2D,H). Therefore, OM-PG linked proteins appear to control WT MV production at cell body locations (Figure 2G), supported by an even distribution of OM-PG linked proteins Lpp and OmpA along the cell body (Braun and Rehn, 1969;Lai *et al*., 2004), while OM-PG-IM complexes modulate MV production at division septa (Figure 2H), where the proteins Pal, TolA, and TolB, bridging the OM-PG-IM space, migrate during cell division (Gerding *et al*., 2007).

Interconnecting protein domains modulate MV release

One possible explanation for the impact of OM-PG and OM-PG-IM linked proteins on MV production is that their biophysical properties, resulting from their abundance in the envelope, contribute significantly to maintaining membrane stability. Their absence would result in envelope defects manifest as MV release. This seems unlikely, however, as MV

release is unchanged in integral OMP mutant strains (Figures 1, 2B), which lack proteins of similar abundance to OM-PG and OM-PG-IM linked proteins in the envelope (Chai and Foulds, 1977). Alternatively, OM-PG and OM-PG-IM linked proteins may act specifically via protein-PG and/or protein-protein interactions to modulate MV production. We investigated whether changes in MV production resulted from the absence of an abundant envelope protein, or the specific envelope interconnection, using Lpp and Pal as representative OM-PG and OM-PG-IM linked proteins (Figure 2I).

Lpp, the most abundant protein in the cell envelope, forms trimers *in vivo* (Braun *et al*., 1970; Inouye *et al*., 1972). One-third of each trimer covalently binds PG via the C-terminal lysine residue (Braun and Rehn, 1969), and removal of this single amino acid (aa) renders Lpp incapable of covalent PG interaction. Full length Lpp, as well as mutated Lpp protein lacking only the C-terminal lysine residue, were expressed in the *lppAB* mutant strain (Figure S4A) in the OM (Figure S4B). As in the *ompA* mutant (Figure 2G), a strain which also lacks OM-PG connections (Figure 2I), MV release was similarly increased along the cell body in the *lppAB* mutant (Figure 3A). Expression of full length Lpp ($Lpp_{1\text{-}58}$) restored covalent Lpp-PG interactions (Braun and Sieglin, 1970), WT MV production (Figures 3B, S4C, S5A), and WT MV size (Figure S5B; p=0.15 vs. WT (n.s.), $p=2.8\times10^{-5}$ vs. *lppAB*). While $pLpp_{1\text{-}58}$ expression did not fully regain WT MV production levels in these conditions (Figure S5A; p=0.006 vs. WT), the effect on MV mass was nonetheless a significant alteration from that of the *lppAB* mutant (p=0.006 vs. *lppAB*). However, expression of Lpp lacking the single PG-interacting lysine residue ($Lpp_{1\text{-}57}$) resulted in MV production that mimicked the *lppAB* strain (Figures 3C, S4C, S5A; p=0.0252 vs. WT, p=0.8012 vs. *lppAB* (n.s.)), and was unable to restore WT MV size distribution (Figure S5C; p=0.02 vs. WT, p=0.16 vs. *lppAB* (n.s.)), even when complemented to higher protein levels in the OM than $Lpp_{1\text{-}58}$ (Figure S4B).

Interaction between Pal and TolA is mediated by the C-terminal 30 aa of Pal, removal of which abolishes Pal-TolA bridges (Cascales and Lloubes, 2004). Full length Pal and mutated Pal protein incapable of interacting with TolA were expressed in the *pal* mutant strain (Figure S4A) in the OM (Figure S4B). Investigation of the *pal* mutant, lacking membrane-spanning OM-PG-IM interactions, revealed increased septal MV production as in the *tolA* mutant (Figures 2H, 3D). Expression of the full length Pal protein ($Pal_{1\text{-}153}$) restored OM-PG-IM complexes, returning septal MV release to WT levels (Figure 3E). Cells expressing the truncated form of Pal unable to interact with TolA ($Pal_{1\text{-}123}$) released large MVs at division septa as in the *pal* mutant (Figure 3F), despite abundant expression of $Pal_{1\text{-}123}$ in the OM (Figure S4B). Therefore, engagement of envelope tethers such as Lpp-PG and Pal-TolA (Figure 3B,E), and not simply the abundance of envelope proteins (Figure 3C,F), specifically impacts MV release along the cell body and at division septa.

Influence of envelope linkages on membrane integrity

Many pathogens release MVs *in vivo* (Stephens *et al*., 1982; Brandtzaeg *et al*., 1992; Garcia-del Portillo *et al*., 1997; Fiocca *et al*., 1999; Hellman *et al*., 2000; Keenan *et al*., 2000; Namork and Brandtzaeg, 2002; Rosenberger *et al*., 2004; Marsollier *et al*., 2007; Necchi *et al*., 2007), where growth and survival depend on surface modifications to maintain envelope integrity and enable them to resist a variety of host defense mechanisms (Gunn, 2000; Ernst *et al*., 2001). We therefore investigated whether major MV proteins influence envelope integrity by challenging WT and mutant strains to grow in the presence of the membrane chaotropic agent deoxycholate (DOC). DOC is a relevant environmental stimulus, as *Salmonella* must survive exposure to bile *in vivo* (Gunn, 2000). Both qualitative (Figure 4A,B) and quantitative (Figure 4C,D) methods of probing membrane integrity during growth in the presence of DOC demonstrated that WT *Salmonella* is resistant to DOC (Figure 4A,C, (Leifson, 1935)), which was not dependent on individual integral OMPs (Figure 4A,C,

p>0.05 vs. WT, Figure S6A). *pal*, *tolA*, and *tolB* mutants displayed DOC-sensitivity (Figure 4A,C, p<0.0001 vs. WT, Figure S6B). Resistance was restored only by expression of full length, but not truncated, forms of OM-PG-IM linked proteins (Figure 4B,D, Figure S6B; pTolA complementation was partial but significant compared to the *tolA* mutant, p<0.0001), indicating that OM-PG-IM tethers were essential for DOC resistance.

However, while cells lacking the OM-PG linked protein OmpA retained DOC-resistance (Figure 4A,C, p>0.05 vs. WT), the *lppAB* mutant strain exhibited a quantitatively intermediate level of DOC-resistance; *lppAB* mutant cells were less resistant to DOC than WT *Salmonella*, but more DOC-resistant than strains devoid of OM-PG-IM linked proteins (Figure 4A,C, *lppAB* vs. WT p=0 vs. WT p=0.0006, *lppAB* vs. *pal, tolB, and tolA* p<0.0001). This phenotype was dependent upon covalent Lpp-PG interactions, as expression of full length Lpp was required to completely restore resistance to DOC (Figure 4B,D; $Lpp_{1\text{-}57}$ p<0.001, $Lpp_{1\text{-}58}$ p>0.05 vs. WT). Covalent Lpp-PG connections (Braun and Sieglin, 1970), formed by 1/3 of Lpp molecules in the envelope (Inouye *et al*., 1972), are 2.5-times more abundant in the OM than non-covalent OmpA-PG linkages (Braun *et al*., 1970; Koebnik *et al*., 2000). Consequently, engagement of the more abundant, covalent Lpp-PG linkages (Braun and Rehn, 1969; Braun and Sieglin, 1970) is essential forcomplete DOC-resistance, while tethering of the less abundant, non-covalent OmpA-PG interactions (De Mot and Vanderleyden, 1994; Koebnik, 1995) is dispensable (Figure 4). Similarly, the contribution of covalent Lpp-PG linkages in minimizing MV release was greater than that of the non-covalent OmpA-PG linkages, as the *lppAB* mutant released more MVs than the *ompA* mutant (Figure 1, *ompA* vs. *lppAB* p=0.0121). Finally, expression of $Lpp_{1\text{-}58}$ in the *ompA* mutant strain was unable to restore WT MVproduction (Figure S7), demonstrating that OmpA and LppAB uniquely contribute to MV release, and reaffirming that both abundance of protein linkages and localized tethering modulate MV release (Figures 1, 2, 3, S7) and membrane stability (Figure 4). These data demonstrate bacteria can balance membrane loss and maintenance of envelope integrity: cells devoid of non-covalent OmpA-PG envelope interactions maintain membrane stability upon challenge (Figure 4A,C) and MV size is unaltered (Figure 2A,C), yet MV production is increased over WT (Figure 1, p<0.01).

MVs released from division septa and cell body

The ability of bacteria to replicate and for pathogens to cause disease is dependent upon growth and division, and constriction of division septa requires specifically coordinated protein redistribution and purposeful uncoupling of envelope linkages (Weiss, 2004; Gerding *et al*., 2007). As the Pal-TolB-TolA complex migrates to division septa during cell division (Gerding *et al*., 2007), and these OM-PG-IM linkages modulate MV production at septa (Figures 2H, 3D-F), we hypothesized that distribution of envelope connections, in addition to their abundance and nature of interaction, may influence MV release. Protein distribution was experimentally controlled by inducing filamentation to either promote (Figures S8A, S9A (Spratt, 1977; Botta and Park, 1981; Schmidt *et al*., 1981)) or prevent (Figure S8B (de Boer *et al*., 1989)) the generation of constricted division septa. MV populations were harvested from filamentous cells in each condition, which permits observation of MV formation in the absence of any envelope protein mutations or changes in total MV mass released (Figure S10). Sustained presence of division septa induced the release of an MV population enriched in large MVs (surface area ≥ $2100nm^2$) (Figure 5A, $p=1.1 \times 10^{-92}$ vs. WT; Figure S9B, $p=3.0 \times 10^{-64}$ vs. WT), and MVs released from non-septate filamentous *Salmonella* were enriched for small MVs (surface area ≤ $100nm^2$) (Figure 5B, $p = 1.3 \times 10^{-49}$ vs. WT). This biased size distribution of MVs released by septate and non-septate filamentous cells suggested that the size distribution of WT MVs (Figure 2A) may be the result of directed release of large and small MVs from specific

locations. Visualization of MV release by filamentous *Salmonella* revealed that large MVs were released at constricted septa (Figures 5C, S9A), and small MVs originated from the cell body (Figure 5D). Therefore, the distribution of proteins containing OM-PG-IM and OM-PG linkages directs WT MV release at septum (Figure 2E) and cell body (Figure 2F): large MVs are derived primarily from constricted septa during cell division as modulated by localized OM-PG-IM connections (Figures 2H, 5C, S9A), and small MVs originate most frequently along the cell body controlled by OM-PG associations (Figures 2G, 5D).

Localization of MV formation, modulated by the differential spatial distribution of envelope protein linkages, dictates MV size, and the integral involvement of protein movement in this process suggests the site of nascent MV release may also manipulate MV protein constituents. Proteins in septal-and cell body-derived MVs (harvested from filamentous cells; Figures 5, S8) were identified by LC-MS/MS and quantified (Fu *et al*., 2008). Significant differences in the abundance of specific proteins in each MV population were identified at a 90% confidence level. Representative results are reported from two independent experiments (Table 1), demonstrating that specific proteins are differentially represented in MVs released at septa or from cell body locations. For example, TolB, known to migrate to division septa during constriction (Gerding *et al*., 2007), was highly enriched in septal MVs. Cell body-derived MVs, however, were enriched in the flagellar cap and hook/filament junction proteins FliD and FlgK, as peritrichous flagella are manifest at locations other than the division site (Aizawa and Kubori, 1998). We also identified enrichment of metabolic (AceE, SucA, RNaseE) and transcriptional/translational (RpoD, RplS, RpsO, RpsP) proteins in MV populations. Although typically annotated as cytoplasmic constituents, these proteins have been identified previously in MVs (Vaughan *et al*., 2006;Vipond *et al*., 2006;Lee *et al*., 2007), and ribosomal proteins are thought to be at the cell surface to permit translation of envelope proteins simultaneously with their incorporation into the membrane (Herskovits *et al*., 2002;Lee *et al*., 2007;Chevance and Hughes, 2008). We have demonstrated specificity in size and protein content of MVs released at distinct cellular sites, dependent upon purposeful, not random, localization of envelope linkages. Therefore, quantity (Figure 4), quality (Figures 1, 3, 4), and distribution (Figure 5, Table 1) of envelope linkages all contribute to directing MV biogenesis.

DISCUSSION

Our data support a model of MV biogenesis (Figure 6) in which MV release occurs at cell envelope regions where the density of specific conserved protein associations has temporarily decreased. In wild-type *Salmonella* (middle panel), envelope proteins connect the OM to the PG and IM. During growth (left panel), localized envelope remodeling briefly induces regions with fewer OM-PG connections along the cell body, resulting in the release of small MVs. During the regulated process of cell division (right panel), IM and PG layers actively grow into the division septum (Weiss, 2004), temporarily disrupting OM-PG-IM linkages circumferentially at the septum. Prior to reattachment of OM-PG-IM connections and completion of cell division, the unassociated OM is able to be released as an MV. MV production, therefore, is not due to random membrane instability, but rather is the result of the essential processes of cell growth and division, supporting the observation that this process is conserved among Gram-negative bacteria.

The phenomenon of MV release has been recognized for decades (Knox *et al*., 1966; Work *et al*., 1966; Chatterjee and Das, 1967), prompting several groups of investigators to suggest models in which proteins and LPS were proposed to influence MV production. Many of these studies, however, qualitatively examined MV release in bacterial strains with multiply marked backgrounds and/or non-isogenic mutants, confounding interpretation of results. While our model shares elements of previously proposed mechanisms of MV production,

our studies quantitatively and directly address the mechanism of MV release. We present here the first demonstration of quantitative analysis of MV mass released by a truly WT Gram-negative organism and a panel of isogenic mutants harboring targeted deletions of the coding sequence of important envelope proteins (Figure 1). Quantitative alterations in MV mass, as well as size frequency distribution (Figures 2A-D, S1) and localization of MV release determined through electron microscopic analysis (Figures 2E-H, 3, 5, S2, S3, S7), implicated interconnected envelope proteins (Figure 2I) in modulation of MV production. We determined that the involvement of envelope proteins was specific to protein-PG and/or protein-protein interacting domains, as complementation with the full length, but not truncated, proteins regained WT MV production (Figures 3, S5) and resistance to the membrane chaotropic agent DOC (Figures 4, S6). In addition, attempts to complement the MV over-producing mutant *ompA* with expression of another OM-PG linked protein ($Lpp_{1\text{-}58}$) was unable to rescue the *ompA* mutant phenotypes (Figure S7), emphasizing the specificity of envelope interconnections modulating MV release. In bacteria without mutations in any envelope proteins, the redistribution of proteins during cell elongation and division resulted in localized MV release via two routes (Figures 5, 6, S8, S9), generating two MV populations with unique protein constituents (Table 1), determined by quantitative mass spectrometry. We have shown, therefore, that the influence of envelope proteins on MV release is specific and dependent upon not merely the presence or absence of an abundant protein in the membrane, but the quality, density, and spatial distribution of protein-PG and protein-protein interactions.

Analysis of the degree of envelope protein conservation in Gram-negative bacteria with a wide range of environmental niches, genera, and pathogenicity supports our conclusions that these proteins are likely a unifying factor in Gram-negative MV release. Thirty one Gram-negative genera encode at least one, if not all, of the interconnected proteins Lpp, OmpA, Pal, TolB, and TolA (Table S2, (BLAST;CMR)). While the overall identity of these protein sequences, as compared to that of *Salmonella*, varies from 10 to 100% (Tables S3-S7), there is extensive conservation of the domains known to be important for protein-PG and/or protein-protein interactions in the envelope. For example, *Vibrio* sp. express the OmpA protein, which has only 29% overall identity to *Salmonella* OmpA; however, the 4 amino acid residues known to interact non-covalently with PG are fully conserved (Table S4). Similarly, *Legionella pneumophila* expresses a Pal homologue with 100% conserved PG- and TolA-interacting domains, but only 33% overall identity to that of *Salmonella* Pal (Table S5). Thus, extensive conservation of protein-PG and protein-protein interacting domains exists among a wide range of Gram-negative organisms, including many well known to be prodigious MV-producers *in vitro* and *in vivo* (Kadurugamuwa and Beveridge, 1995,1997;Fiocca *et al*., 1999;Hellman *et al*., 2000;Schooling and Beveridge, 2006)

Further examination of the well studied MV-producing Gram-negative organism *P. aeruginosa* supports the involvement of Lpp, OmpA, Pal, TolB, and TolA as potentially unifying factors in MV release. *P. aeruginosa*, which is known to produce high levels of MVs from the cell surface (McBroom and Kuehn, 12 May 2005, posting date; Schooling and Beveridge, 2006) expresses the interconnected envelope proteins examined in our studies (Table S2). However, the Lpp homologue of *P. aeruginosa*, OprI, does not covalently interact with the PG via its C-terminal lysine residue (Hancock *et al*., 1981). Therefore, the observation that MV production occurs at high levels and is distributed over the cell body of *P. aeruginosa* (similarly to the *Salmonella lppAB* mutant in Figure 3A) further supports the idea that these envelope proteins modulate MV production among Gram-negative microbes.

While our data support the likelihood of interconnected envelope proteins as a unifying factor in the modulation of MV release, additional organism-specific factors may also

contribute to MV production, including bacterial cell size, shape, and LPS modifications. Cell size and shape have been integral in organism identification throughout the history of microbiology, yet the factors that determine and maintain these characteristics through many generations remain incompletely understood (Cabeen and Jacobs-Wagner, 2007; Osborn and Rothfield, 2007; Pichoff and Lutkenhaus, 2007). Although not yet explored, the biophysical properties of a curved (cocci) versus straight (bacilli) membranous surface should be considered as a potentially important organism-specific factor influencing nascent MV release from differently shaped Gram-negative organisms. This is especially intriguing given the knowledge that achieving the membrane curvature necessary to form an MV can be influenced by changes in envelope structures, such as LPS (Mashburn-Warren *et al*., 2008). For example, *N. meningitidis* is not only a Gram-negative diplococcus which produces abundant MVs *in vitro* and *in vivo* (Devoe and Gilchrist, 1973, 1974, 1975; Brandtzaeg *et al*., 1992; Nassif and So, 1995; Bjerre *et al*., 2000; Namork and Brandtzaeg, 2002), but does not encode homologues of Lpp, OmpA, Pal, TolA, or TolB (BLAST; CMR; Sturgis, 2001). We propose that the lack of OM-PG and OM-PG-IM interactions, combined with the yet to be defined effects of coccoid cell shape, may influence the abundance of MVs produced by *N. meningitidis*.

The influence of LPS-related factors on MV release has also been explored. The *Pseudomonas aeruginosa* quorum sensing molecule *pqs* stimulates MV release via alterations in LPS fluidity (Mashburn and Whiteley, 2005; Mashburn-Warren *et al*., 2008), and particular LPS species have been shown to be enriched in MVs (i.e. A band vs. B band LPS in *P. aeruginosa* MVs (Kadurugamuwa and Beveridge, 1995; Nguyen *et al*., 2003)). While modified LPS structures may influence MV release in organism-specific ways, the enrichment of particular LPS types in MVs does not exclude the possibility that particular LPS species are predominant in regions with fewer envelope interconnections and subsequently are released in MVs at these locations. In contrast to the conservation of interconnected envelope protein domains, analysis of LPS acyl chain number and length alone, in a wide range of Gram-negative bacteria, reveals little conservation (Table S8). Additional unexplored organism-specific factors likely to influence MV release are the extensive diversity of O-antigen structure and composition throughout Gram-negative bacteria (Smit *et al*., 1975; Lerouge and Vanderleyden, 2002), as well as other structural modifications, such as phosphorylation, to lipid A (Trent *et al*., 2006). Again, the widespread homology of envelope protein domains linking the OM-PG and OM-IM (Table S2-S7) among a wide variety of Gram-negative organisms supports the idea that interconnected envelope proteins play a conserved role in the release of MVs.

Many pathogenic bacteria handicap the immune response by actively inhibiting and/or killing host cells responsible for processing and presenting antigens (Brennan and Cookson, 2000; van der Velden *et al*., 2003; Alaniz *et al*., 2006; Tobar *et al*., 2006). However, MVs exist separately from live bacteria *in vivo* (Fiocca *et al*., 1999; Marsollier *et al*., 2007). Therefore, release and circulation of non-cytotoxic, antigen-rich MVs may represent important sources of bacterial antigens, betraying the presence of the bacterium to the host and making MVs a liability. *Salmonella* has evolved mechanisms to avoid immune recognition, which potentially includes restricting release of antigen-rich MVs (Figure 1). However, growth and division also rely on the directed redistribution of proteins (Weiss, 2004; Gerding *et al*., 2007), which not only results in MV release (Figures 5, 6), but leads to modulation of MV content (Figure 6, Table 1). The potential exists, therefore, for bacteria to hijack this inevitable physiologic process for its own means, such as toxin and/or cell signaling molecule secretion or inhibition of host cell processes (Horstman and Kuehn, 2000; Wai *et al*., 2003; Mashburn and Whiteley, 2005; Fernandez-Moreira *et al*., 2006).

Complete loss of OmpA-PG interactions increases MV release (Figures 1, 2G) without loss of membrane integrity (Figure 4A,C). Interestingly, MV release by an *ompA lppAB* mutant is significantly increased above either single mutant (data not shown; p=0.0002 vs. *ompA*, p=0.0011 vs. *lppAB*), and *Salmonella* down-regulates *lpp* and *ompA* transcripts during growth in macrophages (Eriksson *et al*., 2003). As we have demonstrated that *Salmonella* can balance MV release and maintenance of envelope stability (*ompA* mutant, Figures 1,4), these data suggest that modulation of envelope proteins, through gene regulation, could result in different ratios of interconnected proteins such as OmpA and Lpp. Such coordination would provide *Salmonella* with a tunable system to facilitate MV production in response to changing environments (pH, ionic strength, temperature, etc.), contributing to other well defined mechanisms of surface modifications (Ernst *et al*., 2001).

EXPERIMENTAL PROCEDURES

Bacterial strains and media

Bacterial strains and plasmids are listed in Table S1. Strains were grown in LB (Teknova) or Tryptic Soy Broth (VWR), with carbenicillin (100ug/ml) or kanamycin (50ug/ml) when necessary to retain plasmids, and IPTG (100uM) to induce gene expression.

Construction of mutants

Targeted chromosomal deletions of coding sequence were constructed via the Lambda Red recombination system as described previously (Datsenko and Wanner, 2000) (See Supplemental Experimental Procedures).

Plasmid construction

Chromosomal genes, either full-length or specific truncations, were PCR amplified and cloned into IPTG-inducible pTrc99a vector (Amann *et al*., 1988). Plasmids pDB173 (expressing *minCD*) and pTB6 (expressing periplasmic GFP) have been previously described (de Boer *et al*., 1989; Bernhardt and de Boer, 2004).

MV harvest and quantification

Bacteria were grown to late log phase (OD_{600}=0.6-0.8), cells were removed by centrifugation (45min @3800rpm), and culture supernatant filter-sterilized (0.22μm, Corning). Sterile supernatant was concentrated by molecular weight (100kDa MWCO, Pall), retentate ultracentrifuged (2hrs @35,000rpm), pellets resuspended in sterile water, and sterilized (0.22μm Spin-X column, Costar). Dry weight was measured by lyophilization and normalized to optical density of parent bacterial culture.

Mass spectrometry

WT MV proteins were separated by 2D SDS-PAGE (BioRad). Coomassie-stained protein spots were excised, trypsin digested, and identified by LC-MS/MS. Proteins in septal and cell-body MVs (50ug protein/sample) were separated by 15% Tris-glycine SDS-PAGE (BioRad) in triplicate, trypsin digested, and identified by LC-MS/MS (Proteomics Resource, Fred Hutchinson Cancer Research Center, Seattle, WA). Protein abundance was determined by a label-free method combining spectral index calculation with permutation analysis as previously described (Fu *et al*., 2008), with statistically significant cutoff values at the 90^{th} percentile confidence interval.

Electron microscopy

MVs were resuspended in 10 mM $MgCl_2$ and negatively stained with a 2% phosphotungstic acid solution (pH 7.3). Cells were fixed and embedded for TEM analysis of thin sections

(See Supplemental Experimental Procedures). Samples were observed with a JEM-1200EXII transmission electron microscope (JEOL-TEM) operated at 80KV.

MV size measurement

MV size was measured from at least 3 separate electron micrographs of 2 independent MV preparations per strain in Adobe Photoshop using the ruler tool. Long and short axes were measured for 400-750 individual MVs per strain (n noted on figures represents number of individual MVs measured), and surface area of each MV was calculated (long axis/2 * short axis/2 * pi). Data is presented in surface area ranges of $100nm^2$, where 100 represents $1\text{-}100nm^2$, and all MVs larger than $2100nm^2$ group in the final category.

Sensitivity assays

Sensitivity to deoxycholate (DOC) was qualitatively measured as previously described (Gerding *et al*., 2007). For quantitative measurement, backdiluted cultures were grown to late-log phase (OD_{600}=0.6-1.0), adjusted to equal OD_{600} values, and plated to LB +/- 0.1% DOC. CFU were quantified following overnight incubation at 37°C. Ability to grow in the presence of each agent was expressed as a percentage of each strain's growth on LB alone.

Filamentation

Treatment with a sub-lethal concentration of azlocillin (10ug/ml in water, Sigma) specifically inhibits FtsI (PBP3, (Botta and Park, 1981; Schmidt *et al*., 1981)) and induces septated filamenting bacteria. *ftsI*^{ts} (Spratt, 1977) pTB6 were grown initially at 30°C and then at 42°C to induce filamentation. Filamentation without septa was induced by deletion of *minCDE* and inducible expression of *minCD* from pDB173 (See above, Table S1, and Supplemental Experimental Procedures (de Boer *et al*., 1989)).

Cell fractionation

Cell fractions were harvested from cultures in late exponential phase as previously described (Bergman *et al*., 2005). Briefly, cells were washed in Tris/sucrose and EDTA/lysozyme solutions to harvest periplasm, lysed in a French pressure cell to separate cytoplasm, and treated with Sarkosyl to separate IM and OM (See Supplemental Experimental Procedures).

Confocal microscopy

Filamentous cultures were spotted onto glass slides and allowed to air dry, then treated with ProLong Gold antifade reagent (Molecular Probes) and covered for incubation overnight in the dark. Cover slips were sealed prior to viewing. GFP fluorescence was visualized with a Leica SL confocal microscope in the W. M. Keck Center for Advanced Studies in Neural Signaling (University of Washington, Seattle, WA).

Western blotting

Expression of Lpp and Pal in sonicated bacteria and OM fractions were compared by Western blot using standard techniques.

Statistical analysis

MV production and quantification of growth on DOC were analyzed using the Student's t test (unpaired samples, two-tailed), and the Chi-square test was used to analyze MV size distributions using GraphPad Prism version 4.0 for Macintosh (GraphPad Software, San Diego, California).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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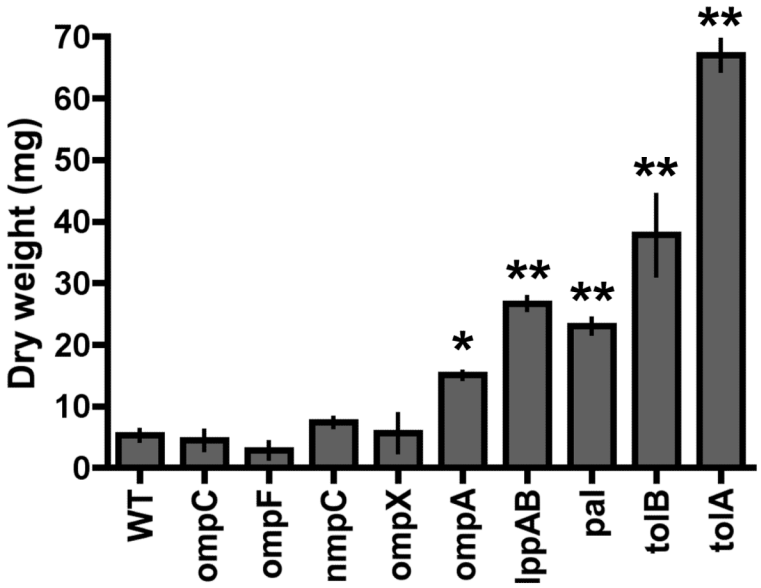

Figure 1. Major MV proteins influence MV production

MV production is quantitatively altered in bacteria lacking major MV proteins. Dry weight of MVs harvested from WT *Salmonella* and mutant strains was quantified (mean +/- standard error). *p<0.01 **p<0.001

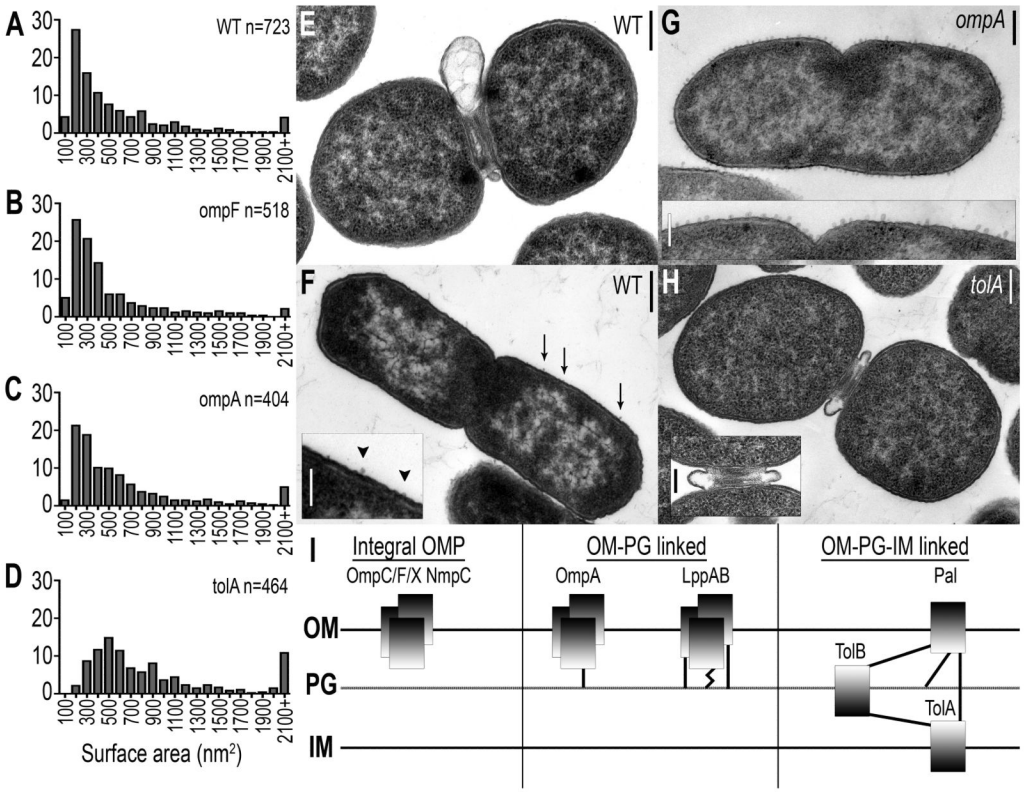

Figure 2. WT MV release localized to division septa and cell body; MV proteins control size and localization of MV release

(A) Size distribution of WT *Salmonella* MVs; x-axis represents MV size ranges (100 represents 1-100nm^2). Compared to WT MVs, size distribution of **(B)** *ompF* MVs and **(C)** *ompA* MVs is similar ($p>0.05$; also *ompC*, *nmpC*, and *ompX* MVs, data not shown), while **(D)** *tolA* MV size is significantly increased ($p<0.001$; also *pal* and *tolB* MVs, data not shown). **(E)** WT MVs are released at constricted division septa and **(F)** along the cell body. **(G)** MVs are released along the cell body in the absence of OmpA (shown) and LppAB (Figure 3A), and **(H)** MV release occurs at division septa in *tolA* (shown), *pal* (Figure 3D), and *tolB* (data not shown) mutant strains. **(I)** Major MV proteins classified by envelope interconnections: Integral OM proteins OmpC, OmpF, OmpX, and NmpC lack extensive connectivity to envelope components, Lpp and OmpA bind PG (OM-PG linked), and Pal, TolB, and TolA form membrane-spanning protein complexes (OM-PG-IM linked). Dark shading represents N-termini, straight lines represent non-covalent interactions, and zig-zag line denotes covalent interaction. Bars = 200nm (except F inset, bar = 100nm), n = number of individual vesicles examined.

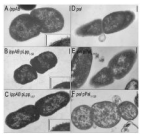

Figure 3. Envelope protein domains modulate MV production

(A) MV release along cell body in the *lppAB* strain is **(B)** complemented with full length $Lpp_{1\text{-}58}$, but **(C)** expression of abundant mutated $Lpp_{1\text{-}57}$ unable to bind PG retains *lppAB* mutant MV production. **(D)** MV release at division septa in *pal* strain is **(E)** restored to WT MV release upon expression of full length $Pal_{1\text{-}153}$, but **(F)** cannot be complemented by expression of truncated $Pal_{1\text{-}123}$ unable to bind TolA. Bar = 200nm

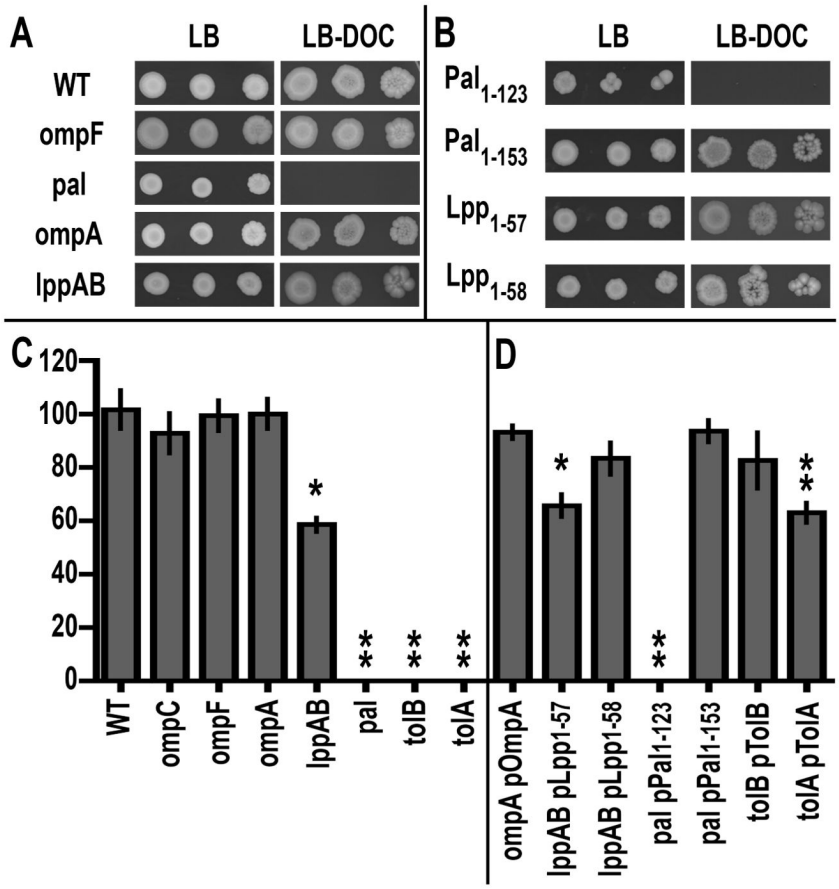

Figure 4. Envelope interconnections necessary for membrane integrity

Qualitative **(A,B)** and quantitative **(C,D)** analyses of membrane integrity. **(A,C)** WT *Salmonella* and mutants lacking integral OM proteins (OmpC, OmpF) are resistant to deoxycholate (DOC). OmpA-PG is dispensable for DOC-resistance, whereas loss of Lpp-PG and OM-PG-IM complexes (Pal, TolB, TolA) induces DOC-sensitivity. **(B,D)** DOC-sensitivity is dependent upon envelope linkages. Expression of full length, but not truncated, proteins complement deletions (complementation with pTolA is partial but significant compared to the *tolA* mutant; $p<0.0001$). For (A,B), three 10-fold dilutions shown from left to right. For (C,D), growth on DOC was quantified and adjusted to growth on LB alone; mean % growth +/- standard error is reported from at least three replicate experiments per strain. * $p<0.001$, ** $p<0.0001$

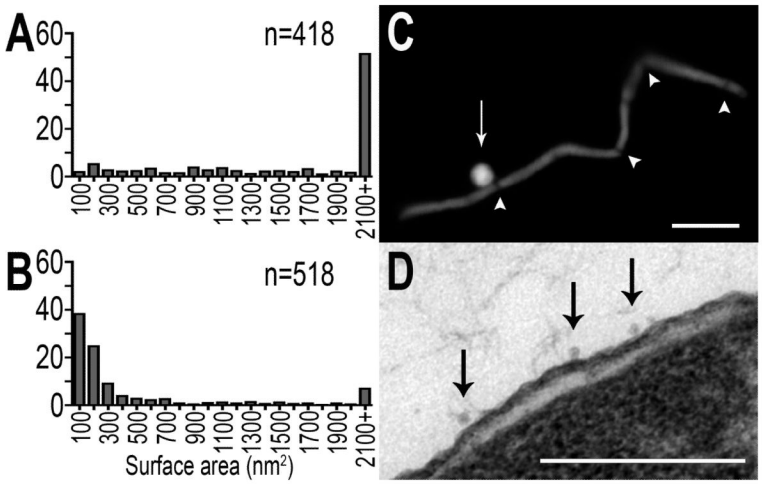

Figure 5. Quantitatively distinct septal- and cell body-derived MV populations

(A) Septal-derived MVs, harvested from septate filamentous cells, are significantly larger than WT MVs ($p=1.1\times10^{-92}$), and **(B)** non septal-derived MVs, harvested from non-septate filamentous cells, are significantly smaller than WT MVs ($p=1.3\times10^{-49}$). **(C)** Large MVs are released at constricted septa (Bar = 5um), and **(D)** small MVs are cell body derived (Bar=200nm). Arrowheads highlight constricted septa and arrows highlight MV release; n = number of individual vesicles measured.

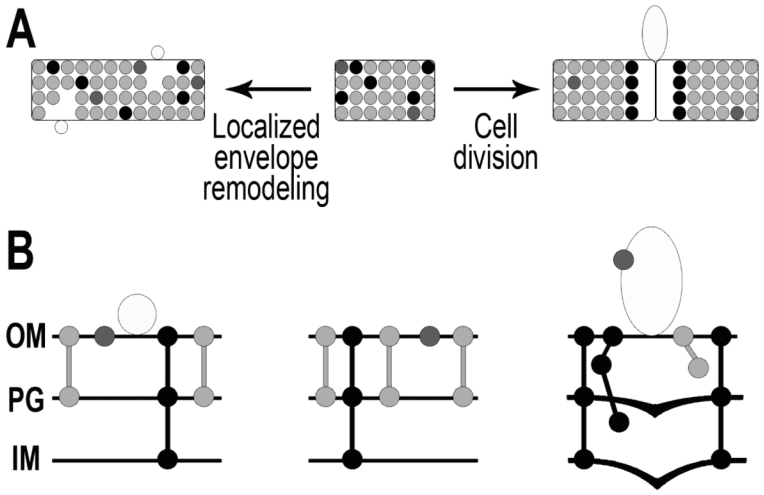

Figure 6. Model of MV biogenesis

(A) Whole-cell view, **(B)** close-up membrane view. *Middle panel*: Major MV proteins in the WT envelope: Integral OM proteins (dark gray), OM-PG linked proteins (light gray) and OM-PG-IM complex proteins (black). *Left panel*: Localized envelope remodeling induces release of small MVs at regions of lower density OM-PG connections along cell body. *Right panel*: Active invagination of IM and PG during cell division causes temporary disruption of septal OM-PG-IM complexes. OM release (MVs) occurs circumferentially at the septum due to lower density OM-PG-IM protein interconnections.

Table 1

Quantitative comparison of protein composition of septal- and cell body derived MVs

	Average # of peptides	
Septal MV proteins	Septal MVs	Body MVs
TolB	25.33	2.67
AceE	158.67	10.33
SucA	16.67	0.00
Rne	8.67	0.67
RpoD	7.33	0.00
Cell body MV proteins		
FliD	0.00	15.33
FlgK	4.33	47.67
RplS	3.00	28.33
RpsO	1.33	8.33
RpsP	0.00	12.33

Average number of peptides identified from each protein in MVs from septum and cell body.