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REVIEW

Bacterial outer membrane vesicles and the host–pathogen interaction

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Extracellular secretion of products is the major mechanism by which Gram-negative pathogens communicate with and intoxicate host cells. Vesicles released from the envelope of growing bacteria serve as secretory vehicles for proteins and lipids of Gram-negative bacteria. Vesicle production occurs in infected tissues and is influenced by environmental factors. Vesicles play roles in establishing a colonization niche, carrying and transmitting virulence factors into host cells, and modulating host defense and response. Vesicle-mediated toxin delivery is a potent virulence mechanism exhibited by diverse Gram-negative pathogens. The biochemical and functional properties of pathogen-derived vesicles reveal their potential to critically impact disease.

In nearly every case, virulence factors of Gram-negative pathogens are secreted products that enhance the survival of the bacteria and/or damage the host. Secretion of virulence factors by Gram-negative pathogens is complicated by the fact that the bacterial envelope consists of two lipid bilayers, the inner and outer membrane, and the periplasm in between. Gram-negative pathogens have developed many strategies, some specific to pathogens, to enable active virulence factors to gain access to the extracellular environment, typically the tissues or bloodstream of the host organism (Henderson et al. 2004). The Type II and Type V secretion systems are two-step processes in which proteins are transported first through the inner membrane (IM) and then through the outer membrane (OM). For secretion via the Type I, Type III, and Type IV secretion systems, the material is transferred directly into the extracellular milieu or into another cell. The Type III system is specific for the transport of factors by pathogenic bacteria. All of these secretion systems secrete individual proteins or small complexes. This review examines secretion via OM vesicles, a distinct "Type VI" mechanism that enables bacteria to secrete a large, complex group of proteins and lipids into the extracellular milieu.

Both pathogenic and nonpathogenic species of Gram-negative bacteria secrete vesicles (Mayrand and Grenier 1989; Kadurugamuwa and Beveridge 1997; Li et al. 1998; Beveridge 1999), including *Escherichia coli* (Hoekstra et al. 1976; Gankema et al. 1980), *Shigella* spp. (Kadurugamuwa and Beveridge 1999; Dutta et al. 2004), *Neisseria* spp. (Devoe and Gilchrist 1973; Dorward and Garon 1989), *Bacteroides* (including *Porphyromonas*) spp. (Grenier and Mayrand 1987; Mayrand and Holt 1988; Zhou et al. 1998), *Pseudomonas aeruginosa* (Kadurugamuwa and Beveridge 1995), *Helicobacter pylori* (Fiocca et al. 1999), *Vibrio* spp. (Chatterjee and Das 1967; Kondo et al. 1993), *Salmonella* spp. (Vesy et al. 2000; Wai et al. 2003), *Brucella melitensis* (Gamazo and Moriyon 1987), *Campylobacter jejuni* (Logan and Trust 1982; Blaser et al. 1983), *Actinobacillus actinomycetemcomitans* (Nowotny et al. 1982), *Xenorhabdus nematophilus* (Khandelwal and Banerjee-Bhatnagar 2003), and *Borrelia burgdorferi* (Shoberg and Thomas 1993). Studies of vesicles from diverse bacterial origins support a common function: Vesicles are a means by which bacteria interact with prokaryotic and eukaryotic cells in their environment.

Some of the best-characterized vesicles are those produced by pathogens. Biochemical analysis and functional characterization of pathogen-derived outer membrane vesicles demonstrate that this secretory pathway has been usurped by pathogens for the transport of active virulence factors to host cells (Table 1). Naturally produced OM vesicles from pathogenic bacteria contain adhesins, toxins, and immunomodulatory compounds, and they directly mediate bacterial binding and invasion, cause cytotoxicity, and modulate the host immune response. By participating in such diverse aspects of the host–pathogen interaction, OM vesicles are potent bacterial virulence factors.

Formation of bacterial OM vesicles

Naturally produced bacterial vesicles are discrete, closed OM blebs produced by growing cells, not products of cell lysis or cell death (Mug-Opstelten and Witholt 1978; Zhou et al. 1998; Yaganza et al. 2004; McBroom and Kuehn 2005). By electron microscopy, vesicles appear spherical with a bilayer membrane, electron-dense luminal content, and an average diameter of 50–250 nm, depending on the strain (Beveridge 1999). Vesicles are pelletable from a cell-free supernatant and, due to their lipid content, vesicles will fractionate into lighter density

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Table 1. *Virulence factors associated with bacterial OM vesicles*

Species	Virulence factor	Reference
Actinbacillus pleuropnemoniae	Proteases, ApxI	Negrete-Abascal et al. 2000
Actinobacillus actinomycetemcomitans	Leukotoxin	Nowotny et al. 1982; Kato et al. 2002
Enterotoxigenic *Escherichia coli* (ETEC)	LT	Wensink et al. 1978; Gankema et al. 1980; Wai et al. 1995; Horstman and Kuehn 2000
Shiga-toxin-producing *E. coli* (STEC) and Enterohemorrhagic *E. coli* (EHEC)	Shiga toxin	Kolling and Matthews 1999; Yokoyama et al. 2000
Enterohemorrhagic *E. coli* (EHEC)	ClyA	Wai et al. 2003
Helicobacter pylori	VacA	Fiocca et al. 1999; Keenan et al. 2000
Borrelia burgdorferi	OspA, OspD	Shoberg and Thomas 1993
Bacteroides fragilis	Haemagglutinin	Patrick et al. 1996
Pseudomonas aeruginosa	Peptidoglycan hydrolase, phospholipase C, hemolysin, alkaline phosphatase	Kadurugamuwa and Beveridge 1995; Li et al. 1998
Porphyromonas (Bacteroides) gingivalis	Arg-gingipain, Lys gingipain, proteases	Grenier and Mayrand 1987; Kamaguchi et al. 2003b; Duncan et al. 2004
Salmonella typhi	ClyA	Wai et al. 2003
Shigella flexneri	IpaB, IpaC, IpaD	Kadurugamuwa and Beveridge 1998
Shigella dysenteriae	Shiga toxin	Dutta et al. 2004
Treponema denticola	Proteases, dentilisin	Rosen et al. 1995; Chi et al. 2003
Xenorhabdus nematophilus	Chitinase, bacteriocin, adhesin, pore-forming toxin	Khandelwal and Banerjee-Bhatnagar 2003

fractions than solubly secreted proteins (Dorward et al. 1989; Horstman and Kuehn 2000; Allan et al. 2003). The OM of Gram-negative bacteria consists of proteins, such as porins, receptors, and pores, and an asymmetric distribution of lipids, with the outer leaflet composed primarily of lipopolysaccharide (LPS) and an inner leaflet containing phospholipids and lipoproteins. The periplasm contains the peptidoglycan (PG) layer, resident "housekeeping" proteins and enzymes, and resident and transient components of secretory pathways. Biochemical analysis of density gradient-purified OM vesicles demonstrated that native OM vesicles consist only of the protein and lipids of the OM and periplasm and do not contain IM and cytoplasmic components (McBroom and Kuehn 2005).

Enrichment and exclusion of vesicle membrane and soluble cargo as compared with their concentrations in whole bacteria point to vesicles as a specific secretory mechanism (Table 2). It is quite difficult to assess accurately the enrichment or exclusion of cargo. The localization of a protein in the whole cell influences its availability as vesicle cargo. For instance, a periplasmic protein that has affinity to the inner leaflet of the OM is more likely to appear "enriched" in vesicles when compared with the soluble periplasmic fraction of the whole cell, yet it may not be enriched when considering its concentration in the whole cell. Unfortunately, these affinities are often not known. Thus accurate enrichment/exclusion values are best determined by comparing the quantities of a component in vesicles and in whole cells relative to the quantities of other components (Kesty et al. 2004).

Vesiculation is a ubiquitous process for Gram-negative bacteria grown in a variety of environments including liquid culture, solid culture, and in biofilms (Beveridge 1999). In general, 0.2%–0.5% of OM and periplasmic proteins are packaged in vesicles from *E. coli* (Hoekstra et al. 1976; Mug-Opstelten and Witholt 1978; Kesty and Kuehn 2004), demonstrating that OM vesicle formation and release is an energy sink. In light of this, bacteria are unlikely to produce OM vesicles without function. In nonpathogenic bacteria, OM vesicles sometimes play a protective role: They contribute to bacterial survival by reducing levels of toxic compounds such as toluene and by aiding in the release of attacking phage (Loeb 1974; Loeb and Kilner 1978; Kobayashi et al. 2000).

Although OM vesicle production has been observed for more than 50 years, the machinery that allows vesicle secretion while maintaining bacterial viability remains elusive. Many theories exist on the mechanism of vesiculation based on biochemical and genetic data and are reviewed elsewhere (McBroom and Kuehn 2005). Based on budding events viewed by electron microscopy, outer membrane vesicles are thought to form when the OM bulges and pinches off, encapsulating soluble periplasmic cargo (Fig. 1; Chatterjee and Das 1967; Mayrand and Grenier 1989; Pettit and Judd 1992; Kadurugamuwa and Beveridge 1995; Li et al. 1998; Zhou et al. 1998). The maximum rate of vesicle production occurs during the end of log phase growth, as documented for *E. coli*, *Vibrio cholerae*, and *B. melitensis*, and vesicles are abundant at sites of cell division (Chatterjee and Das 1967; Hoekstra et al. 1976; Gamazo and Moriyon 1987).

As depicted in Figure 1, a reduction in the number of cross-links between the PG and the OM may govern sites of OM vesicle release (Hoekstra et al. 1976). The ties between the OM and PG are established during growth of the cell envelope, revealing how cell division could influence the quantity of vesicles produced (Chatterjee and Das 1967; Kadurugamuwa and Beveridge 1996;

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Table 2. *Examples of enriched and excluded factors in bacterial OM vesicles*

Species	Excluded factor	Enriched factor	Reference
Actinobacillus actinomycetemcomitans	Unidentified OM proteins	Leukotoxin, minor lipids	Kato et al. 2002
Bacteroides buccae	Unidentified OM proteins		Williams and Holt 1985
Borrelia burgdorferi	Oms28 porin		Cluss et al. 2004
Brucella melitensis	Group 2 OM proteins	Phosphatidyl-choline	Gamazo and Moriyon 1987
Campylobacter jejuni	Unidentified OM proteins	12 kDa surface protein	Logan and Trust 1982
E. coli K-12 modified to express ClyA	DsbA	ClyA	Wai et al. 2003
Enterotoxigenic *E. coli*		LT, OmpW, OmpX	Horstman and Kuehn 2000
Porphyromonas gingivalis		Unidentified OM proteins	Williams and Holt 1985
Pseudomonas aeruginosa	A-band LPS, unidentified OM proteins	Aminopeptidase (in cystic fibrosis sputum isolates), B-band LPS, unidentified OM proteins	Kadurugamuwa and Beveridge 1995; S.J. Bauman and M.J. Kuehn, unpubl.

Bernadac et al. 1998). The major lipoprotein Lpp contributes significantly to OM–PG linkages, and hypervesiculation results from a mutation in this gene (Bernadac et al. 1998; Cascales et al. 2002). However, the OM of the *lpp* mutant is also very fragile, and therefore the specific contribution to OM vesicle formation is unclear. Significant to the understanding of the mechanics of vesicle production, a deletion of *yfgL* causes a dramatic decrease in vesicle production in an adherent-invasive strain of *E. coli* (AIEC) as well as an *E. coli* K12 strain (Rolhion et al. 2005). *yfgL* encodes a lipoprotein involved in the syntheses and/or degradation of PG (Eggert et al. 2001). Its role in vesicle budding is proposed to be the result of an increase in PG production, or the down-regulation of lytic transglycosylases that leads to a loss of turgor pressure on the OM (Rolhion et al. 2005).

In general, pathogenic bacteria produce more vesicles than their nonpathogenic counterparts (Lai et al. 1981; Wai et al. 1995). Quantitative analysis of the cell-free supernatants of mid-log phase cultures revealed that enterotoxigenic *E. coli* (ETEC) produce ~10-fold more vesicles than nonpathogenic *E. coli* (Horstman and Kuehn 2002). Similar patterns in vesicle production occur for leukotoxic and nonleukotoxic *Actinobacillus actinomycetemcomitans*: The pathogenic strains produce >25-fold more vesicles (Lai et al. 1981). A mutation in *hns*, a global regulatory factor that regulates many virulence factors, causes a threefold increase in *E. coli* vesicle production (Horstman and Kuehn 2002). These results are consistent with the hypothesis that pathogenic bacteria have usurped the basic process of vesicle production to disseminate virulence factors and improve survival in the host.

LPS structure impacts vesicle biogenesis directly and indirectly. *P. aeruginosa* strain PAO1, which expresses two types of O-antigen side chain, produces vesicles enriched in the highly charged and longer "B-band" form (Kadurugamuwa and Beveridge 1995; Beveridge et al. 1997; Nguyen et al. 2003). B-band LPS enrichment in vesicles may occur due to budding from regions of the OM where adjacent B-band molecules are repulsed by charge, locally deforming the membrane (Kadurugamuwa and Beveridge 1996; Li et al. 1996). Consistent with this theory, PAO1 grown under oxygen stress conditions increases B-band LPS and demonstrates an increase in vesicle formation (Sabra et al. 2003). *Salmonella* and *P. aeruginosa* mutants missing the LPS O-antigen side chain also show increased vesicle formation (Smit et al. 1975; Meadow et al. 1978). The effects of core mutants on vesicle production are not yet resolved. Decreased OMP expression is associated with mutations in the core region of LPS (Ames et al. 1974; Smit et al. 1975; Schnaitman and Klena 1993); therefore, the vesiculation phenotypes seen in core LPS mutants (Meadow et al. 1978) could be indirect, the effect of altered outer membrane protein composition.

Vesicle production during infection

Vesicles from both intracellular and extracellular bacterial pathogens have been identified in diverse host tissues, revealing the ability of vesicles to access a variety of environments within the host. Because of their small size and the lack of known unique biological markers, OM vesicles are difficult to detect in biopsies and host specimens. Vesicle production and composition are influenced by environmental factors that pathogens experience inside the host.

Not surprisingly, vesicles are often observed near colonizing bacteria. Chronic *Helicobacter pylori* colonization of the stomach leads to peptic ulcers and, in some cases, cancer. Vesiculating *H. pylori* and vesicle binding to gastric cells have been detected in gastric biopsies and with tissue culture cells (Fiocca et al. 1999; Keenan et al. 2000). Vesicles containing vacuolating cytotoxin observed in *H. pylori*-colonized human gastric epithelium biopsies were similar in appearance and composition to *H. pylori* vesicles made in vitro (Fiocca et al. 1999; Keenan et al. 2000). Chlamydial OM vesicles were apparent in McCoy mouse fibroblast cells infected with either *Chlamydia trachomatis* or *Chlamydia psittaci* (Stirling and Richmond 1980). A *B. burgdorferi* isolate that causes Lyme disease was incubated with human skin for 24 h and was found to produce vesicles after invading in the dermis (Beermann et al. 2000).

Vesicles have also been detected in the fluids from

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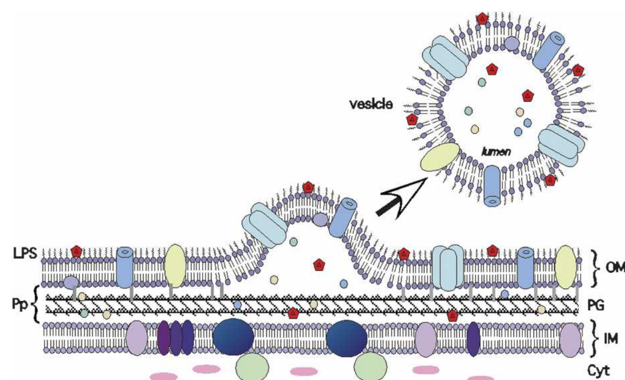

Figure 1. Model of vesicle biogenesis. OM vesicles are proteoliposomes consisting of OM phospholipids and LPS, a subset of OM proteins, and periplasmic (luminal) proteins. Proteins such as LT (red) that adhere to the external surface of the bacteria are associated with the external surface of vesicles. Proteins and lipids of the IM and cytosolic content are excluded from OM vesicles. Vesicles are likely to bud at sites where the links between the peptidoglycan and OM are infrequent, absent, or broken. (LPS) Lipopolysaccharide; (Pp) periplasm; (OM) outer membrane; (PG) peptidoglycan; (IM) inner membrane; (Cyt) cytosol.

infected hosts, demonstrating their ability to disseminate distant from the site of infection. Both vesiculating *Neisseria meningitidis* and freely diffused vesicles were detected in asymptomatic carriers and symptomatic patients, including in the cerebrospinal fluid from a patient with meningitis and blood from a patient who died from meningitis (Craven et al. 1980; Stephens et al. 1982; Brandtzaeg et al. 1992; Bjerre et al. 2000; Namork and Brandtzaeg 2002). Similarly, vesiculating *Borrelia* and free vesicles were detected in the urine and blood of *B. burgdorferi*-infected mice as well as in infected ticks (Dorward et al. 1991).

In some cases, structures similar to vesicles were detected but may not have been identified as vesicles. Bacterial vesicles are implicated in many aspects of periodontal disease including colonization, tooth destruction, inflammation, and disseminated sequelae (Grenier and Mayrand 1987; Mayrand and Grenier 1989; Fives-Taylor et al. 2000). Structures that appear to be vesicles were discovered in dental plaque (Williams and Holt 1985; Mayrand and Grenier 1989). LPS and outer membrane protein-containing bacterial fragments were present in the serum of septic human patients (Brandtzaeg et al. 1992) and septic rats (Hellman et al. 2000a,b; Hellman and Warren 2001). *E. coli* grown in culture with serum shed outer membrane material with the same composition as the material found in the human serum (Hellman et al. 1997). Several factors identify this outer membrane material as vesicles: They are composed of LPS, they contain outer membrane proteins including OmpA and lipoproteins, they are >0.1 µm in size, and they are stable.

The intracellular pathogen *Salmonella typhimurium* secretes OM vesicles when growing intracellularly as well as into the supernatant of bacterial cultures (Garcia-del Portillo et al. 1997; Vesy et al. 2000; Bergman et al. 2005). When actively growing *Salmonella* is in intracellular compartments that have lost lysosomal membrane glycoproteins, intracellular LPS accumulates in similar compartments (Garcia-del Portillo et al. 1997).

LPS serotype switching, oxygen stress, the availability of iron, and the presence of antibiotics are all pertinent to pathogenic bacteria living inside the host, and these conditions impact vesicle production, toxicity, and composition. Altering expression of LPS O-antigens is a well-characterized defense strategy for pathogens to evade the host response (Pier 2000; Lerouge and Vanderleyden 2002). As described in the previous section, the presence and type of LPS O-antigen as well as the indirect effect of oxygen stress on the LPS structure may influence the physical ability of the membrane to bulge and initiate the formation of vesicles. Iron regulation is also a critical aspect of virulence. Bacteria such as *H. pylori* colonize intact mucosal as well as ulcerative host tissue; thus, they encounter different levels of iron in the host. Compared with typical iron-containing media, growth in iron-limiting media reduces VacA associated with *H. pylori* vesicles and increases the concentration of vesicle-associated proteases, although OM vesicle production levels are not affected (Keenan and Allardyce 2000). Antibiotics also influence vesicle production. Treatment of *Shigella dysenteriae* with mitomycin C, a known inducer of Shiga toxin, causes an increase in OM vesicle production, size, and toxicity (Dutta et al. 2004). Other antibiotics (fosfomycin, ciprofloxacin, and norfloxacin) do not significantly alter vesicle production or increase toxicity.

Gentamicin treatment of *P. aeruginosa* induces a threefold increase in the formation of vesicular structures that have distinct characteristics (Kadurugamuwa and Beveridge 1998). These structures contain gentamicin, fuse to host cells infected by pathogenic bacteria, and kill the intracellular bacteria by the cytosolic delivery of the antibiotic. Gentamicin-induced vesicles are larger and contain IM and cytosolic components as well as OM and periplasm. Thus, the composition of gentamicin-induced vesicles differs significantly from native OM vesicles, and they are assumed to be generated via a vastly different mechanism. This is supported by the fact that gentamicin-induced *P. aeruginosa* vesicles are not enriched in the B-band LPS characteristic of native vesicles. Nevertheless, these studies reveal both the impact of the antibiotics on the number of released bacterial membrane structures, as well as the biochemical fusogenic capacity of the surface of vesicles that reflects on the ability of native OM vesicles to interact with neighboring cells.

Interbacterial activities

Some bacteria use vesicles as an offensive tactic to gain a growth advantage over other bacteria. Protease- and toxin-containing vesicles from *E. coli*, *Shigella*, *Actinobacillus*, *Pseudomonas*, and *Borrelia* can interact with bacteria possibly via a fusion or adherence mechanism

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(Gankema et al. 1980; Shoberg and Thomas 1993; Kadurugamuwa and Beveridge 1998, 1999; Saunders et al. 1999; Kato et al. 2002). Vesicle fusion delivers proteins, such as the autolysin murein hydrolase, that can lyse other ("non-self") Gram-positive and Gram-negative bacteria (Kadurugamuwa et al. 1998; Li et al. 1998). "Self"-fusion events between vesicles and the strain of bacteria from which they derive are not lethal due to the regulated activity of such proteins in the periplasm. Gentamicin-induced *P. aeruginosa* vesicles are also bacteriolytic (Kadurugamuwa and Beveridge 1996; Allan and Beveridge 2003). These studies demonstrate that vesicles could aid in securing a niche in a competitive bacterial environment, such as during the colonization of a host.

Vesicles also are a means to transfer beneficial material between bacteria and contribute to genetic diversity and bacterial survival. Native *P. aeruginosa* vesicles are able to transfer antibiotic-resistance enzymes to other bacteria (Ciofu et al. 2000), enhancing survival of neighboring bacteria. Vesicles carry luminal DNA as well as DNA on their surface. In several cases, vesicles were reported to package chromosomal, plasmid, and phage DNA, presumably from the periplasm (Dorward et al. 1989; Kolling and Matthews 1999; Yaron et al. 2000). The source and destination of this DNA may depend on the bacterial species or strain. Renelli et al. (2004) demonstrated that DNA from the environmental media is encapsulated by *P. aeruginosa* vesicles. In some instances, vesicle-associated, nuclease-resistant DNA is capable of transforming neighboring bacteria (Dorward et al. 1989; Kolling and Matthews 1999; Yaron et al. 2000), however, this is apparently not the case for all vesicle-associated DNA (Renelli et al. 2004). For *A. actinomycetemcomitans*, DNA is also associated with vesicles, but it is localized to the exterior surface, where it binds secreted leukotoxin (Ohta et al. 1991, 1993).

In addition to facilitating interbacterial material transfer, vesicles can mediate coaggregation of bacteria, enabling biofilm formation and colonization (Grenier and Mayrand 1987; Whitchurch et al. 2002). In particular, numerous oral bacteria produce vesicles that induce microbial aggregation, interact with host cells, and display toxic activities (Williams and Holt 1985; Mayrand and Grenier 1989). *Porphyromonas gingivalis*, one of the bacteria responsible for periodontal disease, produces vesicles with the potential to induce autoaggregation and coaggregation with other bacteria. In vitro, *P. gingivalis* vesicles coaggregated various oral microorganisms including *Eubacterium saburreum* with *Capnocytophaga ochracea*; *Staphylococcus aureus* with various *Streptococcus* spp.; *Actinomyces* spp. and mycelium-type *Candida albicans*; and *P. gingivalis* with *Prevotella intermedia* (Grenier and Mayrand 1987; Kamaguchi et al. 2003a,b). The proteolytic and adhesive activity of *P. gingivalis* vesicles is due to the presence of gingipains (Grenier and Mayrand 1987; Patrick et al. 1996; Kamaguchi et al. 2003b). Another oral bacterial species, *A. actinomycetemcomitans*, also produces vesicles with proteolytic and coaggregative abilities (Nowotny et al. 1982; Meyer and Fives-Taylor 1994; Negrete-Abascal et al. 2000). Hence, vesicles could have an impact on both the establishment and the longevity of a bacterial infection. In light of their bacteriocidal, aggregative, and/or transforming activities, vesicles produced by colonizing pathogens are likely to have a complex and as yet unexplored impact on the commensal or coinfecting bacterial population.

Adherence and entry into host cells and tissues

Membrane vesicles are natural vehicles, or bacterial "bombs," for directed intercellular transport of particular bacterial virulence factors into host cells and tissues. Toxins, protease, adhesins, and other virulence factors in addition to LPS (endotoxin) have been coisolated with native vesicles from the culture supernatants of many pathogens (Table 1). Vesicle surface factors can mediate adhesion to eukaryotic cells as well as subsequent internalization of vesicle material. The ability of native bacterial OM vesicles to fuse with host cells and deliver content directly into the cytosol has been theorized based on their ability to fuse with bacterial membranes; however, currently this mechanism is not substantiated. Based on their adhesive and proteolytic properties and their small size, OM vesicles can interact with specific host cells deep in tissues that are not readily accessible by the infecting bacteria.

Gram-negative pathogens often express surface adhesins, so it is not surprising that vesicles, which consist of a subset of OM proteins, also are adhesive. The method of vesicle binding and internalization generally reflects that of the bacterial cells. However, in many cases bacterial adhesins are fimbrial, and thus far no fimbriae (pili) have been detected to originate from purified vesicles. Unless there is a specific exclusion machinery, there is no reason why the OM protein-anchored Type I or P pili (Stathopoulos et al. 2000) would not be present in vesicles. In contrast, it is unlikely that Type IV pili are associated with vesicles, since these originate from an IM protein complex (Stathopoulos et al. 2000).

Surface factors of pathogens are genetically regulated during infection in order for the bacteria to adhere to host cells, enter host cells, and evade the immune response. The composition of vesicles from pathogens should reflect the state of the bacterial OM, and therefore depend on the site of culturing of the bacteria. Vesicles were purified from clinical isolates of *P. aeruginosa* from patients with cystic fibrosis (CF) and their composition was compared with vesicles from a laboratory *P. aeruginosa* strain (S.J. Bauman and M.J. Kuehn, unpubl.). In addition to the lack of LPS with O-antigen, typical of *P. aeruginosa* clinical isolates (Pier 2000), the vesicles are enriched for a surface-localized aminopeptidase and bind better to lung epithelial cells when compared with the laboratory strain-derived vesicles.

Toxins associated with the external vesicle surface act as adhesins for some vesicles, and the interaction between the toxin and the eukaryotic cell receptor governs subsequent uptake of the vesicle into the host cell. For instance, external, LPS-bound heat labile enterotoxin

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(LT) is the adhesin responsible for ETEC vesicle interactions with host cells (Horstman and Kuehn 2000; Kesty et al. 2004). The LT_B subunit responsible for the eukaryotic cell interactions is also responsible for binding LPS; however, a non-G_{M1}-binding LT_B mutant also bound LPS, indicating that the Kdo- and G_{M1}-binding areas of LT_B are distinct (Horstman and Kuehn 2002). These data are consistent with LT acting as a vesicle adhesin, a bridge between the LPS of the vesicle and G_{M1} of the host cell.

Association and internalization of ETEC vesicles into host cells depend specifically on the interactions between the LT and its receptor, G_{M1}; thus, LT acts as a host cell adhesin for vesicles. Because G_{M1} binding leads to lipid raft-mediated endocytosis, ETEC vesicles are internalized by this nondegradative, cholesterol-dependent pathway (Kesty et al. 2004). Multiple components of ETEC vesicles, including luminal components and structures that morphologically resemble bacterial vesicles, are found inside epithelial cells incubated with the vesicles, demonstrating that intact ETEC vesicles are taken up by intestinal cells (Kesty and Kuehn 2004).

Vacuolating toxin (VacA) is externally associated with *H. pylori* vesicles (Sommi et al. 1998; Fiocca et al. 1999; Heczko et al. 2000; Keenan et al. 2000), and, like soluble VacA, endocytosed vesicles accumulate in vacuoles. This suggests that vesicle-bound VacA directs the binding and internalization of *H. pylori* vesicles similarly to the LT-mediated binding and entry of ETEC vesicles.

In contrast, although leukotoxin is associated with the surface of *A. actinomycetemcomitans* OM vesicles, the toxin is not responsible for the adherence of the vesicles with HL60 human myeloid leukemia cells (Demuth et al. 2003). After a brief, 2-min incubation, *A. actinomycetemcomitans* OM vesicle antigens are found around the periphery of the cells. Based on the fast kinetics, the lack of intracellular label, and the insensitivity to cytochalasin D, the appearance of vesicle antigens in HL60 cell membranes is probably the result of adherent vesicles rather than their endocytosis.

Antibiotic-containing vesicles from *Shigella flexneri* adhere to and enter cultured Henle cells within 1 h, causing dramatic killing of intracellular *Shigella* by the delivery of their antibacterial soluble vesicle content (Kadurugamuwa and Beveridge 1998). Uptake of these vesicles by human intestinal cells is predicted to be catalyzed by the outer membrane invasins present in the vesicles, IpaB, C, and D.

Binding of vesicles to host cells can also influence binding of bacteria to those cells. *B. burgdorferi*, the spirochete responsible for the autoimmune Lymes disease, produces vesicles that bind human umbilical vein epithelial (HUVE), dendritic, and endothelial cells (Shoberg and Thomas 1993; Saunders et al. 1999). This binding competes for bacterial cell receptors and may be due to outer membrane proteins OspA and OspB (Shoberg and Thomas 1993). Rather than inhibiting bacterial binding, *Actinobacillus* vesicles enhance the adherence of *Actinobacillus* to oral KB epithelial cells (Meyer and Fives-Taylor 1994).

The ability of some bacteria to invade human intestinal epithelial cells is also linked to OM vesicles. A noninvasive AIEC bacterial mutant was discovered to have a defect in OM vesicle formation (Rolhion et al. 2005). It is proposed that the AIEC OM vesicles carry effectors into the host cell that lead to uptake of bacteria. The AIEC vesicles can work in *trans*, for example, OM vesicles from the wild-type AIEC strain, but not a K-12 strain, enable entry of the noninvasive AIEC bacterial mutant.

OM vesicles have been proposed to act as long-range virulence factors that can protect luminal cargo from extracellular host proteases and penetrate into tissues more readily than the larger bacteria. In support of this theory, purified *Treponema denticola* vesicles harbor dentilisin, a protease that allows *T. denticola* to penetrate the epithelial barrier, and the vesicles also disrupt the tight junctions of a Hep-2 epithelial cell monolayer (Chi et al. 2003). Proteins inside the vesicles are insensitive to protease treatment (Kolling and Matthews 1999; Horstman and Kuehn 2000).

Similar to bacterial–host cell interactions, vesicle–host cell interactions can be altered by manipulating the expression of outer membrane proteins in bacteria. The *Yersinia entercolitica* outer membrane protein Ail confers an invasive phenotype to a laboratory *E. coli* strain (Miller and Falkow 1988). The Ail adhesin/invasin expressed in laboratory *E. coli* is present in purified OM and OM vesicles produced by these strains. The Ail-containing vesicles display 10-fold higher association with host cells than vesicles from a laboratory strain not expressing Ail (Kesty and Kuehn 2004). The manipulation of vesicle adherence and entry is intriguing considering the prospective use of engineered vaccine strains for antigen delivery.

Toxin transporters

Active toxins are associated with several pathogen-derived vesicles (Table 1), and some of the toxins appear to be enriched in vesicles (Table 2). In some cases, vesicle-associated toxins are more active than the toxins alone, and some toxins are associated with the exterior surface of vesicles. The host cell response to the delivery of vesicle-associated toxin is likely to be quite different from that to the delivery of soluble toxin if the uptake of vesicle-associated toxin is coincident with the uptake with vesicles. This is anticipated for OM vesicles such as those produced by ETEC: Vesicle-associated LT is not only toxic, it also causes the internalization of other bacterial components, including membrane proteins, periplasmic proteins, and endotoxin, into the host cell (Fig. 2; Kesty et al. 2004).

Vesicles play a major role in the export and activity of some bacterial toxins. More than 95% of secreted LT activity is associated with vesicles (Horstman and Kuehn 2002). After secretion through the OM by the Type II general secretory pathway (Horstman and Kuehn 2002; Tauschek et al. 2002), LT becomes associated with LPS on the extracellular surface of the OM (Horstman and Kuehn 2002). Thus, when OM vesicles form, these

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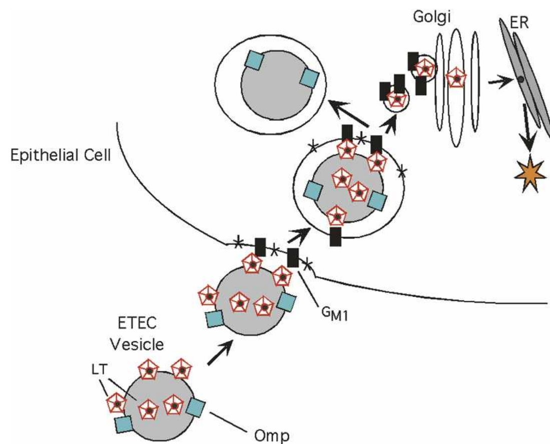

Figure 2. Model of toxin transmission to epithelial cells via ETEC vesicles. ETEC vesicles carry toxin (LT) on their surfaces and in their lumens. Surface-associated LT binds G_{M1} ganglioside (black rectangles), which is located in cholesterol-rich microdomains (caveolae) of host cell plasma membranes (asterisks). Vesicles are internalized into a nonacidified compartment, the toxin enters the Golgi, and the LT_A subunit of the toxin (black circle) is trafficked by retrograde transport via the endoplasmic reticulum (ER) to the cytosol, where it is active. Accumulation of the bacterial vesicles inside the cells accompanies LT-mediated vesicle entry.

contain luminal LT originating from the periplasm, as well as external, LPS-associated LT (Fig. 2). The minimal structure recognized by LT is the Kdo (3-deoxy-D-*manno*-octulosonic acid) portion of LPS, and intriguingly, phosphorylation of Kdo inhibits binding by LT as well as its close homolog, cholera toxin (CT) (Horstman et al. 2004). The difference in soluble secretion of CT by *V. cholerae*, compared with the vesicle-associated secretion of LT by ETEC, is therefore explained by the expression of phosphorylated Kdo on the LPS of *V. cholerae*.

The secreted form of cytolysin A (ClyA) is found in vesicles released from *E. coli*, and an in-depth study of ClyA secretion via vesicles revealed a unique property of OM vesicles (Wai et al. 2003). ClyA in the periplasm is oxidized, with cysteines participating in disulfide bonds that render it unable to oligomerize and become inactive. The reduction of the cysteines is critical for the conversion of ClyA into active oligomers, and interestingly, the vesicle lumen has a different redox state than the periplasm due to uncoupling of periplasmic and IM factors of the Dsb pathway. As a result of the reducing environment of the vesicle, possibly in addition to an increase in local concentration, ClyA forms active oligomeric pore structures in the vesicles. Large polymeric ClyA structures are clearly visible by electron microscopy on the surface of *E. coli* vesicles. Vesicle-associated ClyA was more toxic than a similar concentration of purified periplasmic toxin, presumably due to its proper folding into an active structure.

Leukotoxin, which kills human polymorphonuclear leukocytes and monocytes, is enriched in *A. actinomycetemcomitans* vesicles; the vesicles fuse to eukaryotic cell membranes and can cause cell lysis; and vesicle-associated leukotoxin is more potent than leukotoxin associated with OM (Kato et al. 2002). Unlike LT for ETEC vesicles, leukotoxin binds nucleic acid on the OM surface (Ohta et al. 1991, 1993; Demuth et al. 2003). *A. actinomycetemcomitans* vesicles also have calcium-releasing and bone-resorption activities associated with tooth loss (Nowotny et al. 1982; Meyer and Fives-Taylor 1994; Negrete-Abascal et al. 2000).

Complexes that include endotoxin and bacterial lipoproteins, consistent with the characteristics of vesicles, are released by *S. flexneri* and are cytotoxic to macrophages, demonstrating a role for vesicles in key apoptotic events during dysentery (Aliprantis et al. 2001). Enterohemorrhagic *E. coli* O157:H7 and *S. dysenteriae* produce vesicles that contain active Shiga toxin (Kolling and Matthews 1999; Yokoyama et al. 2000; Dutta et al. 2004). Vesicle-associated Shiga toxin appears partially protease sensitive and partially protease protected, suggesting both vesicle surface and luminal localization.

OM vesicles produced by enteric insect pathogens have also been analyzed. *X. nematophilus* OM vesicles are insecticidal for neonatal larvae and are more potent than purified OM proteins (Khandelwal and Banerjee-Bhatnagar 2003). These vesicles harbor chitinase activity.

Vacuolating toxin, VacA, has been detected closely associated with the membrane of *H. pylori* vesicles (Keenan et al. 2000). Vesicular VacA is stable in the eukaryotic cell, and its toxicity, which is urease dependent, can be induced days following internalization (Sommi et al. 1998). Since urease is secreted by *H. pylori* into the gastric mucosa but vesicles do not carry urease (Keenan et al. 2000), this may be part of an elaborately regulated virulence mechanism that is not yet fully understood.

Modulation of host response

In addition to specific virulence factors, vesicles contain compounds that are recognized by eukaryotic cells in the innate and acquired immune response pathways. Considering that properties of the OM change during a bacterial infection (such as the O antigen of LPS), the impact of vesicles on the modulation of the immune system will also vary during the course of infection. B-cells activate upon coincubation with vesicles from *B. burgdorferi* (Whitmire and Garon 1993). *N. meningitidis* vesicles were highly immunogenic when administered intranasally into mice, eliciting a mucosal and systemic bactericidal antibody response (Saunders et al. 1999). This is not surprising since OM vesicles contain a natural adjuvant (endotoxin) and surface-localized bacterial antigens. Mice infected with *S. typhimurium* generate $CD4^+$ T cells that recognize antigens in *Salmonella* OM vesicles (Ernst et al. 2001). Intriguingly, *Salmonella* OM vesicles carry antigens in addition to LPS that are also regulated by PhoP/Q, suggesting that the *Salmonella* coordinately reduces recognition for both innate and adaptive immune systems (Bergman et al. 2005).

An overstimulated inflammatory response to a pathogen is destructive for the host. LPS, lipoprotein, and

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OMPs present in vesicles are all biologically active molecules that can activate immune cells via Toll-like receptors (TLRs) and induce leucocyte migration (Galdiero et al. 1999; Akira et al. 2001). Oral bacteria often cause an inflammatory response, and vesicles from oral bacteria are active in modulating host responses (Mayrand and Holt 1988; Duncan et al. 2004). IL-8 production from gastric epithelial cells is stimulated by *H. pylori* vesicles, and stimulation is independent of the VacA toxin (Ismail et al. 2003). Therefore, bacterial vesicles that do not carry exotoxins can nevertheless cause damage due to the host inflammatory response.

Vesicles may enable bacteria to escape immune detection during colonization. *Neisseria* vesicles have been proposed to act as decoys to the immune system, binding and removing cell-targeted bactericidal factors in serum (Pettit and Judd 1992). The gingipain proteases on *P. gingivalis* vesicles cause CD14 degradation on O937 human macrophages that could lead to disarming the responsiveness of the immune system in periodontal disease (Duncan et al. 2004) *P. gingivalis* vesicles also induce membrane expression of E-selectin and ICAM-1 on vascular endothelial cells, whereas they inhibit synthesis of interferon γ MHC class II synthesis (Srisatjaluk et al. 1999, 2002).

As a result of severe periodontitis, dental inflammation, or dental treatments, oral bacteria may disseminate through the bloodstream. Here, vesicles also are predicted to play important roles. *P. gingivalis* vesicles induce murine macrophage foam cell formation, which is thought to be causal to coronary thrombosis by loosening plaques in the arterial wall (Qi et al. 2003). *P. gingivalis* vesicles also are potent activators and aggregative factors for murine platelets, whereas vesicles from other oral bacteria do not show these activities (Sharma et al. 2000).

Summary and future directions

As a bacterial secretion system, vesicles are unique. Vesicles enable the extracellular dispersal of specific proteins as part of complexes of proteins and lipids that can act synergistically to activate toxic and immune pathways in the host. By virtue of their small size, adhesive properties, endotoxic component, and ability to carry and deliver toxic components into host cells, outer membrane vesicles are likely to play a significant role in disseminating virulence factors for Gram-negative pathogens. Vesicles can also help carve out a niche in the environment by modulating interactions between neighboring bacteria and between bacteria and the immune system.

The activation of the ClyA toxin within secreted *E. coli* vesicles reveals an unprecedented level of sophistication for OM vesicles to participate in the generation of a toxic virulence factor. In the case of ETEC vesicles, the use of the toxin as a vesicle adhesin complicates the scenario of toxin delivery into host cells by the concomitant introduction of stimulatory bacterial compounds inside host cells that are not normally phagocytic. Consequently, whereas it will not be surprising to find other examples of vesicle involved in mediating virulence factor activity, the molecular details by which this occurs remain unpredictable.

In the future, studies of vesicles will likely turn toward the gene products involved in their formation and the basis by which virulence factors, membrane, and periplasmic cargo are included or excluded. In addition, it will be important to understand which host factors influence vesicle release, and how the environmental conditions are translated into the mechanics of OM vesicle production. The data thus far guarantee many more interesting and surprising discoveries regarding the impact and significance of bacterial OM vesicles in the host–pathogen interaction.

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