Chapter 7
Delivery of Virulence Factors by Bacterial Membrane Vesicles to Mammalian Host Cells

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Abstract Bacterial membrane vesicles represent a universal secretion mechanism enabling both Gram-negative and Gram-positive organisms to transfer cargo to eukaryotic cells, as well as to other bacterial cells. Bacterial vesicles can deliver to target cells an extremely wide range of virulence factors, including exotoxins, lipids, nucleic acids, and small molecules. Although there has been extensive research to decipher the mechanisms regulating cellular uptake of Gram-negative bacterial outer membrane vesicles (OMVs), much less is known about the cellular uptake of Gram-positive bacterial membrane vesicles (MVs). This chapter focuses on a selection of major bacterial pathogens and summarizes the present knowledge of OMV and MV-mediated virulence factor delivery, as well as mechanisms of bacterial vesicle–host cell interaction and uptake by mammalian cells.

7.1 Bacterial Membrane Vesicle-Mediated Protein Delivery

Bacterial pathogenicity is enhanced by secretion systems that export virulence factors, either by secretion or injection, into the environment or adjacent host cells. Once delivered, these virulence factors then interfere with or stimulate host cellular processes. Eight bacterial secretion systems designated types I–VIII have been characterized to date (Green and Mecsas 2016). Both Gram-negative and Gram-positive bacteria of several different bacterial species release membrane vesicles to augment their pathogenic potential. Release of bacterial membrane vesicles, a very basic and relevant mode of protein transport, presumably also occurs during infection. Compared to other secretion mechanisms, membrane vesicle release has special implications since vesicles can deliver cargo, e.g., virulence factors, over much

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longer distances than secretory systems dependent upon direct bacterial contact. Effectively, these bacterial membrane vesicles become vehicles of multifunctional cargo, delivering a multitude of virulence factors, including metabolites, several protein toxins, nucleic acids, and immune modulators such as peptidoglycan (Berleman and Auer 2013).

7.1.1 Escherichia coli

Bacterial membrane vesicles were initially discovered as a product of Gram-negative bacterial outer membrane blebbing, and are therefore often referred to as outer membrane vesicles (OMVs). However, in 1976, Hoekstra et al. reported that membrane fragments, consisting of essentially unmodified outer membranes, were present in the culture supernatant of *E. coli* during normal growth (Hoekstra et al. 1976). Since then, a number of studies have described *E. coli* OMV biogenesis as well as the physiological cargo of these OMVs. When newly synthesized OMVs are released from *E. coli,* they contain active heat-labile enterotoxin (ELT) (Gankema et al. 1980; Wai et al. 2003). Because ELT is associated with lipopolysaccharide (LPS) on the OMV surface, host cell uptake of enterotoxigenic *E. coli* (ETEC) OMVs is allowed by binding to LT-receptor (GM1) (Horstman and Kuehn 2000, 2002; Kesty et al. 2004). The LeoA protein, a homolog of eukaryotic GTPase, secretes ELT from the periplasm of ETEC bacteria and it has been suggested that LeoA contributes to OMV formation and protein content (Brown and Hardwidge 2007).

Earlier studies demonstrating that a cytotoxic protein, cytolysin A (ClyA), in *E. coli* was exported through OMVs pointed to the potential physiological relevance of *E. coli* OMVs (Wai et al. 2003). In a process that involves redox-dependent oligomerization, ClyA is incorporated into OMVs, thus appearing to possess an intrinsic ability to translocate to the bacterial periplasm. ClyA incorporated into OMVs has considerably higher cytotoxicity toward mammalian cells compared to ClyA purified from the bacterial periplasm. Thus, protein localization in OMVs may play a direct role in activating and delivering virulence effector proteins (Wai et al. 2003).

Additional studies in *E. coli* examining vesicle-mediated export of bacterial virulence factors revealed the capacity of OMVs to deliver toxigenic cargo. For example, during infection, *E. coli* OMVs may represent an alternative pathway to deliver type I-secreted alpha-hemolysin from bacteria to host cells (Balsalobre et al. 2006). Another study reported that OMVs are a vehicle for bacteria to transfer cytotoxic necrotizing factor-1 (CNF1) to the environment and to infected tissue (Kouokam et al. 2006). Similarly, Enterohemorrhagic *E. coli* (EHEC) use OMVs to release hemolysin toxin (EHEC-Hly), a typical repeats-in-toxin protein (RTX) that lyses host cells through a mechanism of pore formation (Aldick et al. 2009). The toxin can exist as free EHEC-Hly and as EHEC-Hly associated with OMVs, and both forms are released during EHEC growth. Free EHEC-Hly is lytic toward human endothelial cells, whereas OMV-associated EHEC-Hly is not lytic toward microvascular endothelial cells (HBMEC) and the colon epithelial cell line Caco-2, although it can trigger apoptosis

(Bielaszewska et al. 2013). Research into whether an MV-associated genotoxin from intestinal *E. coli* can promote cancer development revealed that *E. coli*-derived OMVs are readily internalized into target cells. Within these target cells, OMVs have the potential to induce oxidative stress, which would lead to DNA damage, replication, and aneuploidy in susceptible cells (Tyrer et al. 2014).

During hyper-biofilm formation, the *kil* gene, located in a three-gene cluster on the *E. coli* ColE1 plasmid, induces release of proteinous materials and aberrant OMVs into the extracellular environment (Nakao et al. 2018). A variety of pathogen-associated molecular pattern molecules are enriched in OMVs isolated from *E. coli.* These include LPS, lipoproteins, CpG DNA, flagellin, and peptidoglycan, most of which are Toll-like receptor (TLR) and nucleotide-binding and oligomerization domain (NOD) ligands (Ellis et al. 2010). Therefore, bacterial OMVs are capable of activating epithelial cells, endothelial cells, macrophages, and dendritic cells to release TNF-α, IL-1β, IL-6, and IL-8 (Bauman and Kuehn 2006; Bielaszewska et al. 2018; Canas et al. 2018; Lee et al. 2018). OMVs from pathogenic *E. coli* have also been known to cause sepsis-induced cardiac dysfunction, demonstrated both in vitro and in vivo (Svennerholm et al. 2017).

OMVs secreted by clinical isolates of EHEC O157 cause cell death by delivering into host cells a cocktail of virulence factors, such as Shiga toxin 2a (Stx2a), cytolethal distending toxin V (CdtV), EHEC hemolysin, and flagellin (Bielaszewska et al. 2017). Interestingly, OMVs from the nonpathogenic *E. coli* strain Nissle 1917 (EcN) can cause anti-inflammatory responses by reinforcing epithelial barrier integrity, thus affecting intestinal homeostasis (Alvarez et al. 2016; Behrouzi et al. 2018; Fabrega et al. 2017). In contrast, OMVs from the Nissle 1917 strain can also cause eukaryotic DNA double-stranded breaks (Canas et al. 2016). This strain harbors a cluster of genes that encode for proteins involved in the biosynthesis of hybrid non-ribosomal peptide-polyketide(s). It has been suggested that polyketides may be involved in inducing these eukaryotic DNA double-stranded breaks (Olier et al. 2012). In addition to host inflammatory responses, in colon cancer cells, OMVs from nonpathogenic commensal *E. coli* can induce epigenetic modifications (Vdovikova et al. 2018). Furthermore, OMVs from nonpathogenic *E. coli* can suppress the growth of established tumors as well as prevent tumor metastasis. These activities occur via an interferon-γ-mediated antitumor response, whereby OMVs deliver trypsin-sensitive surface proteins to the target cancer cells (Kim et al. 2017). Taken together, these studies demonstrate the enormous potential of bacterial OMVs from nonpathogenic *E. coli* as novel therapeutic agents against various cancers.

7.1.2 Vibrio cholerae

The formation of OMVs by *Vibrio cholerae* and *Vibrio parahaemolyticus* was first observed by researchers analyzing the cell structure of *V. cholerae* and *V. parahaemolyticus* using electron microscopy with freeze-substitution (Kondo et al. 1993). *V. cholerae*, the causal agent of the diarrheal disease cholera, possesses

cholera toxin (CT) as its major virulence factor. In addition to CT, many other secreted protein toxins and enzymes that are important to *V. cholerae* pathogenesis have been reported to be associated with OMVs. These include *Vibrio* cytolysin (VCC) (Olivier et al. 2007), metalloprotease of *Vibrio* (PrtV) (Vaitkevicius et al. 2006), Zn-dependent hemagglutinin protease (HAP) (Ghosh et al. 2006; Hase and Finkelstein 1991), accessory cholera enterotoxin (Ace) (Kaper et al. 1995), and trypsin-like serine protease (VesC) (Syngkon et al. 2010).

OMVs from the *V. cholerae* strain O395 secrete biologically active CT (Chatterjee and Chaudhuri 2011). Using a GM1-independent mechanism, CT-containing OMVs are trafficked to host cells. This GMI-independent mechanism represents a secondary mechanism for CT secretion, in addition to the well-studied type II secretion system (Chatterjee and Chaudhuri 2011). Future studies to elucidate the functional details of this secondary mechanism of CT delivery are important to fully understand *V. cholerae* pathogenesis (Rasti et al. 2018).

Non-O1 and non-O139 *V. cholerae* (NOVC) serogroups are the causal agents of gastroenteritis and extraintestinal infections in humans; however, the virulence of NOVC strains is not well understood. OMVs from NOVC strains elicit NOD1- and NOD2-mediated immune responses in mammalian hosts. Quorum-sensing machinery attenuates OMVs' inflammatory potential and thereby influences the immune responses (Bielig et al. 2011a, b).

Biologically active VCC, a pore-forming toxin, is released from the *V. cholerae* NOVC strain V:5/04 together with OMVs. OMV-associated VCC induces target cell autophagy, demonstrating that autophagy may play a role in cellular defense against an OMV-associated virulence factor (Elluri et al. 2014). The metalloprotease PrtV, a type II secretion system substrate protein, is also secreted from the *V. cholerae* strain C6706 together with OMVs (Rompikuntal et al. 2015). The biological activity of OMV-associated PrtV has been demonstrated in human colon carcinoma cells. Furthermore, the OMV-associated PrtV protease facilitates bacterial resistance toward the antimicrobial peptide LL-37 (Rompikuntal et al. 2015). HAP and VesC proteases are also released along with *V. cholerae* OMVs and the biologically active form of these proteases are delivered into human intestinal epithelial cells, causing cytotoxic and inflammatory responses (Mondal et al. 2016). Taken together, these studies demonstrate that the ability of *V. cholerae* to deliver virulence factors into host cells via OMV-mediated secretion is, therefore, a seemingly widespread feature among different *Vibrio* strains.

7.1.3 Pseudomonas aeruginosa

During normal growth, the Gram-negative bacterium *Pseudomonas aeruginosa* also releases OMVs. *Pseudomonas* OMVs carry and release several toxins and enzymes, including hemolysin, phospholipase C, alkaline phosphatase, protease, and elastase, which contribute to the organism's pathogenicity (Kadurugamuwa and Beveridge 1995). Peptidoglycan hydrolases associated with OMVs that are naturally released

by several Gram-negative bacterial strains, including *Enterobacter*, *Citrobacter*, *Salmonella*, *Shigella, Escherichia*, *Klebsiella*, *Morganella*, *Pseudomonas*, and *Proteus*, enable the lysis of both Gram-negative and Gram-positive bacteria (Kadurugamuwa and Beveridge 1998; Li et al. 1998). This predatory interaction indicates that within biofilms, where bacteria compete for growth with other bacteria in the surrounding microflora, OMVs might play a fitness role, providing an increased survival benefit (Kadurugamuwa and Beveridge 1997). This hypothesis is supported by the finding that *P. aeruginosa* OMVs play an important role in the formation of biofilms (Beveridge et al. 1997; Murphy et al. 2014). Cif, the cystic fibrosis (CF) transmembrane conductance regulator (CFTR) inhibitory factor, is associated with *P. aeruginosa* PA14 OMVs (MacEachran et al. 2007). The zinc-dependent leucine aminopeptidase PaAP, an enzyme involved in bacterial association with host cells, is also found in OMVs from two *P. aeruginosa* clinical strains, PAO1 and CF2 (Bauman and Kuehn 2006). The major outer membrane proteins (OMPs) that are associated with OMVs have been identified by mass spectrometry to be OprE, OprF, OprG, OprH, OprI, PcoB, and PagL (Bauman and Kuehn 2006; Choi et al. 2017a; Tashiro et al. 2010). Previously, it was thought that OMVs contain only outer membrane and periplasmic proteins; however, proteomic analysis of *P. aeruginosa* revealed the possible presence of cytoplasmic proteins in naturally released OMVs (Choi et al. 2017a). *P. aeruginosa* primarily occupies the mucus layer of the lung epithelium in cystic fibrosis patients. Released OMVs can deliver virulence factors into the cytoplasm of host cells, resulting in modified innate immune responses. A recent study investigated whether the antibiotic tobramycin, which is commonly used to treat CF patient lung infections caused by *P. aeruginosa*, affects the abundance of virulence factors in OMVs. The study demonstrated that in CF patients, tobramycin may improve lung function by decreasing the abundance of several key virulence factors in OMVs, which restores chloride ion secretion necessary for bacterial clearance from the lungs (Koeppen et al. 2019). It is thus likely that OMVs carrying virulence factors are important contributors to the in vivo survival and adaptability of *P. aeruginosa* in CF lung infection.

7.1.4 Acinetobacter baumannii

Acinetobacter baumannii is an opportunistic pathogen responsible for a wide range of nosocomial infections. *A. baumannii* secretes OMVs that contain phospholipases and exhibit both hemolytic and leukocytic activities against target host cells (Jha et al. 2017). In a lung infection mouse model, surface proteins of *A. baumannii* OMVs can induce pro-inflammatory immune responses (Jun et al. 2013). Release of the elongation factor Tu (EF-Tu) from bacterial cells associated with OMVs from *A. baumannii* may contribute to fibronectin-mediated binding on the host cell (Dallo et al. 2012). Vaccinating a sepsis mouse model with OMVs purified from *A. baumannii* triggers high levels of IgM, IgG1, and IgG2c immunoglobulins, while levels of the pro-inflammatory cytokines IL-1β and IL-6 remain low (McConnell et al. 2011).

OMV vaccination protects mice against challenge with the *A. baumannii* ATCC 19606 strain (McConnell et al. 2011). Variation in OMV production is associated with a unique feature of *A. baumanii*, the reversible switching between formation of opaque and translucent colonies. In experiments assessing immune response in macrophages, OMVs from the *A. baumannii* opaque colony form appear to be more immunogenic than those from the translucent colony form (Ahmad et al. 2019). The rise in extensive antibiotic resistance to *A. baumannii* highlights the potential need for a vaccine against this organism (Li et al. 2006; Lei et al. 2019). Therefore, OMVs from *A. baumannii* represent a promising vaccine candidate due to its immunogenic properties.

7.1.5 Porphyromonas gingivalis

Porphyromonas gingivalis is a major pathogenic cause of adult periodontitis. The Gram-negative anaerobic bacterium *P. gingivalis* releases OMVs that contribute to pathogenesis due to their high proteolytic and hemagglutinating activities, as well as their ability to promote inter- and intra-bacterial species adherence (Olsen and Amano 2015). Multiple studies have shown that OMV-associated toxins and proteolytic enzymes have a major contribution to periodontal diseases (Bourgeau and Mayrand 1990; Duchesne et al. 1995; Ellen and Grove 1989; Kamaguchi et al. 2003; Patrick et al. 1996; Singh et al. 1989; Smalley et al. 1991). The specific OMV-associated virulence factor(s) involved in OMV-mediated pathogenesis of *P. gingivalis* are not well known despite the clinical importance of *P. gingivalis*. It is known that *P. gingivalis* produces gingipain proteinases that are preferentially packed into OMVs (Haurat et al. 2011; Veith et al. 2014). OMV-associated FimA, hemagglutinin A, and heat-stress protein (HtrA) are involved in the attachment of *P. gingivalis* to host cells and subsequent invasion (Belanger et al. 2012; Zhang et al. 2011). Also, major *P. gingivalis* outer membrane proteins are associated with OMVs, which are used to efficiently invade host cells (Ho et al. 2015; Mantri et al. 2015; Veith et al. 2014). Thus, it has been suggested that OMVs may be involved in the development of atherosclerosis and represent a "Trojan horse" strategy to cause an effect without employing intact bacterial cells (Xie 2015).

7.2 OMV-Mediated Virulence Factor Delivery by Other Gram-Negative Bacteria

OMVs from several different Gram-negative bacterial species effectively transport multifunctional cargo over long distances. For example, OMVs from *Aggregatibacter actinomycetemcomitans* carry proteins that function in antibiotic targeting, nutrient acquisition, and immune evasion, representing both offensive and defensive activities

(Kieselbach et al. 2015). This phenomenon was described for the first time in *Bordetella pertussis*, whose OMVs carry a virulence complex that includes an adhesin (Imagawa et al. 1979). A causal organism of acute respiratory tract infection, *B. pertussis* harbors a wide range of virulence factors, including pertussis toxin, filamentous hemagglutinin, adenylate cyclase hemolysin, and tracheal cytotoxin, which are secreted in association with OMVs (Hozbor et al. 1999). OMVs carrying adenylate cyclase toxin can induce murine macrophage and CHO-K1 cell death independent of the toxin's receptors (Donato et al. 2012). Immunization with *B. pertussis* OMVs may represent an effective next-generation pertussis vaccine strategy as evidenced by its ability to protect against bacterial colonization by eliciting antibody and Th1/Th17 type immune responses (Raeven et al. 2016).

One of the major virulence factors of *Campylobacter jejuni* and *A. actinomycetemcommitans*, cytolethal distending toxin (CDT), is secreted primarily from bacterial cells in association with OMVs, suggesting evolutionary conservation of this mode of CDT delivery (Berlanda Scorza et al. 2008; Lindmark et al. 2009; Rompikuntal et al. 2012). OMVs from *C. jejuni* carry three proteases, HtrA, Cj0511, and Cj1365c, and these OMV-associated proteases can cleave occludin and E-cadherin of T84 colon carcinoma cells (Elmi et al. 2016). In humans, *C. jejuni* can cause gastroenteritis, while in avian hosts, colonization is asymptomatic. The body temperature difference between human (37 °C) and avian (42 °C) hosts suggests that growth of *C. jejuni* at 37 °C potentially cues expression of bacterial virulence factors. Proteome analyses comparing OMVs from *C. jejuni* grown at 37 °C and at 42 °C revealed more virulence-related proteins associated with OMVs isolated from the bacteria grown at 37 °C (Taheri et al. 2018). The presence of bile in the growth medium also influences the selective packing of virulence factors in *C. jejuni* OMVs (Taheri et al. 2018), suggesting that the protein cargo of OMVs may also be regulated by the host environment.

The release and intracellular uptake of *Bacteroides fragilis* OMVs can activate caspase-11-dependent cell death and IL-1 responses to LPS (Vanaja et al. 2016). OMVs from *B. fragilis* carry polysaccharide A capsular antigen (PSA). PSA induces TLR2-mediated signaling in dendritic cells, which results in regulatory T cell maturation by production of the immunoregulatory cytokine IL-10 (Shen et al. 2012), thus implicating OMVs as an important mediator in establishing mutualism.

Bacteria employ various secretion systems to deliver virulence factors to target cells. The field of bacterial OMVs has become an exciting research area that is poised to improve our understanding of bacterial pathogenesis and provide alternative strategies to control infectious disease. Further investigation into the mechanisms and roles of OMV secretion systems may uncover novel targets and strategies for developing new antimicrobial therapies.

7.3 Delivery of Bacterial Nucleic Acids by OMVs

7.3.1 DNA

In 1989, *Neisseria gonorrhoeae* was shown to release RNA and DNA in association with OMVs (Dorward and Garon 1989). It was suggested that the RNA and linear DNA are associated with the exterior of the vesicles because nuclease treatment eliminated them from OMV preparations. However, circular DNA inside the OMVs, and thus resistant to nuclease treatment, was capable of transforming recipient cells. Hence, it was suggested that OMVs can act as a mechanism by which cells can exchange genetic information (Dorward et al. 1989). Since the first description in 1989, an increasing number of reports have described the OMV-associated release of plasmid DNA and/or chromosomal DNA (Biller et al. 2014; Lee et al. 2007; Perez-Cruz et al. 2015; Renelli et al. 2004; Yaron et al. 2000). DNA purified from *E. coli* O157:H7 OMVs contain the virulence genes *stx1, stx2*, *eae*, and *uidA* (Kolling and Matthews 1999). Among bacteria, OMVs can contribute to antibiotic resistance spread by two different mechanisms (Ciofu et al. 2000; Mashburn-Warren and Whiteley 2006). Spread of antibiotic resistance may occur by direct transfer of a resistance protein (e.g., β-lactamase) to neighboring cells or by lateral transfer of resistance genes following fusion of the OMV with the recipient cell membrane (Fulsundar et al. 2014; Renelli et al. 2004; Rumbo et al. 2011; Yaron et al. 2000). In the case of *P. aeruginosa*, it has been suggested that OMVs can also be formed after cell lysis, when membrane fragments and cytosolic contents including DNA are released from spontaneously lysed bacteria (Turnbull et al. 2016). DNA associated with OMVs contributes to establishing bacterial biofilms to facilitate bacterial host colonization (Liao et al. 2014). OMVs can deliver DNA into eukaryotic cells, suggesting a role for bacteria–host cell interactions and demonstrating potential for OMV-based DNA vaccines (Bitto et al. 2017). Interestingly, integration of bacterial DNA has been detected in the host genome, suggesting transfer of bacterial genetic material into human somatic cells (Riley et al. 2013). It remains to be determined, however, if OMV-associated DNA integrates into the host genome. Further studies are needed to investigate whether OMVs are capable of delivering DNA into the host nucleus as well as whether OMV-delivered DNA can integrate into the host genome or modulate the innate immune response via DNA sensors (Hornung 2014). Earlier studies indicated that among similar bacterial species, i.e., *N. gonorrheae*, it may be possible for genetic material to be transferred by OMVs (Dorward and Garon 1989). Moreover, OMVs can transfer carbapenem-resistance genes to surrounding *A. baumannii* bacterial isolates (Rumbo et al. 2011). Research on Gram-positive bacterial membrane vesicles (MVs) has shown that *Clostridium perfringens* releases MV-containing DNA and protein components that can be internalized by macrophages and induce dramatic pro-inflammatory cytokines both in vitro and in vivo (Jiang et al. 2014). In addition to providing a potential mechanism for exchange of genetic material between prokaryotes and eukaryotes, these studies reveal a new

perspective on the immunogenic properties of bacterial OMV and MV-based DNA vaccines.

7.3.2 RNA

Bacterial membrane vesicles share similarities with exomes, cell vesicles secreted by most mammalian cell types. Bacterial membrane vesicles and exosomes both carry payloads of proteins, lipids, and genetic material enclosed in membrane-bound spherical structures of similar size ranges. Both bacterial membrane vesicles and exosomes can deliver functional molecules to distant extracellular compartments and tissues. Exosomes are involved in the horizontal transfer of genetic material, such as mRNAs and miRNAs, from the donor cells to recipient cells (Valadi et al. 2007; Zhong et al. 2011; Zomer et al. 2010). Investigation of whether bacterial RNA associates with OMVs by us and other researchers revealed that RNA is indeed encapsulated inside the OMVs in the form of RNase-resistant secondary structures and/or is associated with proteins in RNase-stable complexes (Blenkiron et al. 2016; Choi et al. 2017a, b; Resch et al. 2016; Sjostrom et al. 2015). Emerging evidence indicates that OMVs contain short RNAs (sRNAs) that are differentially packaged and have the potential to target the function and/or stability of host mRNA. Interestingly, via a regulatory OMV-associated sRNA, a new mechanism of pathogen–host interaction attenuates the innate immune response in human airway epithelial cells as well as in mouse lung. A specific bacterial sRNA (sRNA52320) is transferred from *P. aeruginosa* OMVs to host cells, where in human airway epithelial cells it attenuates OMV-stimulated IL-8 secretion, and in the lungs of a mouse model it attenuates keratinocyte-derived cytokine secretion and neutrophil recruitment (Koeppen et al. 2016).

Different classes of RNA are present in OMV-associated fractions of *Salmonella enterica* serovar Typhimurium and are exported. These include rRNAs, mRNAs, tRNAs, and other ncRNAs (Malabirade et al. 2018). However, RNA associated with OMVs is clearly different when the bacteria are grown under host-mimic cultural conditions in comparison with ordinary laboratory culture media. At least a fraction of the extracellular RNA associated with OMVs is present as full-length transcripts, indicating that OMVs can protect RNA and that this RNA might be functionally active (Malabirade et al. 2018). Export of full-length transcripts via OMVs opens the possibility of numerous functional implications for bacteria–bacteria and bacteria–host communication.

7.3.2.1 How Does RNA Associate with OMVs?

Several possibilities for RNA association with OMVs have been suggested (Blenkiron et al. 2016). First, extracellular RNA released by general bacterial cell lysis may be tightly reassociated to the OMV surface after secretion from bacterial

cells. Second, RNA incorporation into OMVs could occur through an active and selective mechanism. Third, RNA association with OMVs may merely represent nonspecific envelopment of RNA in the cytoplasm within vesicle blebs. Finally, the phenomenon may be due to RNA riding as passengers on OMV-bound proteins, as bacterial mRNAs are frequently found at the sites of their future protein products (Nevo-Dinur et al. 2012). There are indeed many mRNAs that encode many membrane proteins present in OMVs. These include mRNAs for *ompA*, *lpp*, and *tonB* in OMVs from uropathogenic *E. coli* and mRNAs for *ompU*, *ompA*, and *tolC* in OMVs from *V. cholerae* (Blenkiron et al. 2016; Sjostrom et al. 2015). The ability of OMVs to deliver their associated RNA cargo into host cells poses the interesting question of whether these RNAs can function as novel signaling molecules in bacteria–host interactions.

7.4 Bacterial Lipid Release in Association with OMVs

OMVs contain bacterial phospholipids such as phosphatidylglycerol, phosphatidylethanolamine, and cardiolipin. Moreover, the phospholipid composition of OMVs generally resembles that of the outer membrane (OM) from which they are derived (Horstman and Kuehn 2000). Phospholipid and fatty acid compositions between OMVs and the cellular OM of *E. coli* do not differ significantly (Hoekstra et al. 1976). However, the phospholipid head groups and acyl chains compositions between the bacterial OM and OMVs from *P. aeruginosa* are quite different. Therefore, the OMV membrane is considered rigid compared to the cellular OM of *P. aeruginosa*. Thus, it has been suggested that the OMV blebbing mechanism may not be conserved among Gram-negative bacteria (Tashiro et al. 2011). OMVs contain, in addition to phospholipids, abundant LPS, which normally comprise the majority of the OM outer leaflet. *P. aeruginosa* can express both a common antigen (A-band) and serotype-specific antigen (B-band) in the O-antigen portion of LPS. Thus, these OMVs are highly enriched in B-band LPS, in contrast to the lipid composition of the OM (Kadurugamuwa and Beveridge 1995). Based on these differences, B-band LPS has been proposed to sort into OMVs, similar to the sorting of LPS and proteins seen in *Porphorymonas gingivalis* (Haurat et al. 2011).

7.5 Small Molecule Delivery Via OMVs

In addition to lipids, proteins, and nucleic acids, small molecules associate with OMVs as well. *Pseudomonas putida* strains that are resistant to toluene produce more OMVs upon exposure to toluene and release toluene-enriched OMVs as a detoxification system (Kobayashi et al. 2000). In *P. aeruginosa*, PQS (*Pseudomonas* quinolone signal), a quorum-sensing molecule, associates with OMVs (Mashburn and Whiteley 2005). Because PQS is more hydrophobic than the quorum-sensing

signal acylhomoserine-lactone of *P. aeruginosa*, it is concentrated in the OMV membrane where it can interact specifically with LPS. It appears that such interactions contribute to physically stimulate the formation of vesicles into which the PQS is subsequently packaged (Mashburn and Whiteley 2005).

Gram-negative bacteria can employ OMVs to deliver peptidoglycan to cytosolic nucleotide-binding oligomerization domain-containing protein 1 (NOD1) in host cells. OMVs (containing peptidoglycan) purified from *P. aeruginosa*, *H. pylori*, and *Neisseria gonorrheae* can upregulate NF-κB and NOD1-dependent responses in vitro (Irving et al. 2014; Kaparakis et al. 2010). Moreover, when administered to mice intragastrically, *H. pylori* OMVs trigger NOD1-dependent but TLR-independent innate and adaptive immune responses (Kaparakis et al. 2010). In mammalian cells, *V. cholerae* OMVs induce NOD1- and NOD2-mediated immune responses. Quorum-sensing machinery attenuates the inflammatory potential of OMVs, playing an important role in regulating this process during infection (Bielig et al. 2011a, b). In human embryonic kidney cells, *A. actinomycetemcomitans* OMVs strongly induce NOD1- and NOD2-dependent NF-κB activation. Moreover, in myeloid THP1 cells, NOD1, the primary sensor of peptidoglycan delivered by MVs, contributes to the overall inflammatory responses induced by the vesicles (Thay et al. 2014).

OMVs from *P. aeruginosa*, *Shigella flexneri*, and *Myxococcus xanthus* contain molecules with bacteriolytic properties (Evans et al. 2012; Kadurugamuwa and Beveridge 1995, 1997, 1999). Thus, OMVs are critical to intra- and inter-species communication, although in bacterial cell–cell interactions occurring via OMVs, the selectivity of the interaction between MVs and bacterial cells is not fully understood. Recently, employing OMVs isolated from the Enterobacterium *Buttiauxella agrestis*, OMVs selectively interacted with target bacterial cells (Tashiro et al. 2017). These results offer a new avenue by which particular bacterial species can be controlled using bacterial OMVs in microbial communities.

7.6 Gram-Positive Bacteria Membrane Vesicles (MVs)

MVs are also released by Gram-positive bacteria such as *Staphylococcus aureus* (Lee et al. 2009), *Enterococcus faecium* (Wagner et al. 2018), *Streptococcus pneumoniae* (Codemo et al. 2018), *Streptococcus pyogenes* (Resch et al. 2016), *Mycobacterium ulcerans* (Marsollier et al. 2007), *Bacillus anthracis* (Rivera et al. 2010), *Listeria monocytogenes* (Vdovikova et al. 2017), and *Lactobacillus* (Dean et al. 2019) (see Chap. 3). These MVs are released both in vivo and in vitro as spherical, bilayered structures with a diameter of approximately 20–150 nm (Gurung et al. 2011; Rivera et al. 2010; Vdovikova et al. 2017).

7.6.1 Staphylococcal Species

7.6.1.1 *Staphylococcus aureus*

S. aureus is responsible for a wide spectrum of human infections that range from superficial cutaneous infections to life-threatening bacteremia (Lowy 1998). MVs from *S. aureus* have been isolated and analyzed by mass spectrometry. Proteins identified include the IgG-binding protein, ferritin, ferrichrome-binding lipoprotein precursor, ABC transporter extracellular binding protein, β-lactamase, and membrane protein OxaA (Gurung et al. 2011). *S. aureus* MVs deliver protein A to host cells by interacting with host cell plasma membranes through a cholesterol-rich microdomain in the membrane (Gurung et al. 2011; Rivera et al. 2010). *S. aureus* α-toxin (α-hemolysin), a 33-kDa pore-forming protein, is also associated with MVs. *S. aureus* α-toxin can lyse a wide range of human cells and induce apoptosis in T cells (Berube and Bubeck Wardenburg 2013). *S. aureus* MVs containing α-toxin are cytotoxic to HeLa cells and induce erythrocyte lysis (Thay et al. 2013). *S. aureus* MVs have also been reported to contain δ-hemolysin (Hld), γ-hemolysin, leukocidin D, exfoliative toxin C, and exfoliative toxin A, identified by proteomic analysis (Jeon et al. 2016). Comparative proteomics identified a total of 131 and 617 proteins in MVs from *S. aureus* grown in Luria-Bertani and brain-heart infusion broths, respectively, suggesting that culture media components can influence MV protein composition (Askarian et al. 2018). A study of the roles of MVs in bacteria–host interactions led to the suggestion that during systemic infection, *S. aureus* MVs can influence bacteria–host interactions and that they provide protective immunity in murine infection models (Askarian et al. 2018).

7.6.1.2 *Staphylococcus haemolyticus*

S. haemolyticus is a skin commensal microorganism. *S. haemolyticus* nosocomial isolates are the most antibiotic-resistant members of the coagulase-negative Staphylococci. However, little is known about *S. haemolyticus* virulence factors. Potential virulence proteins associated with MVs have been compared to the *S. haemolyticus* total secretome. This comparison revealed that the cargo carried by MVs is enriched in proteins involved in adhesion, acquisition of iron, and antimicrobial resistance (Cavanagh et al. 2018).

7.6.2 Streptococcal Species

7.6.2.1 *Streptococcus pyogenes*

Comprehensive studies have been performed on MVs produced by the Gram-positive human pathogen *S. pyogenes*, the etiological agent of necrotizing fasciitis

and streptococcal toxic shock syndrome. These studies have provided an explanation for the MV-associated secretion of *S. pyogenes* macromolecules, including RNAs, lipids, and proteins, as well as described a two-component system that modulates *S. pyogenes* MV production (Resch et al. 2016; Biagini et al. 2015).

7.6.2.2 *Streptococcus pneumoniae*

S. pneumoniae, a major Gram-positive respiratory pathogen, produces MVs that may serve as a vehicle for many bacterial proteins. Pneumolysin, a cytosolic pore-forming toxin, is significantly enriched in MVs (Codemo et al. 2018). Pneumococcal MVs are internalized into A549 lung epithelial cells and human monocyte-derived dendritic cells, where they trigger pro-inflammatory cytokine responses independent of pneumolysin content. It has been suggested that *S. pneumoniae* MVs act in an immunomodulatory manner by enabling transfer of vesicle-associated proteins and other macromolecules into host cells. In addition, MVs bind tightly to serum complement system components, sequestering complement factor C3 in human serum and decreasing pneumococcal opsonophagocytosis (Codemo et al. 2018).

7.6.3 Mycobacterial Species

The etiologic agent of Buruli ulcers, the mycobacterium *Mycobacterium ulcerans* is slow-growing and infects the skin and subcutaneous tissues (George et al. 1999). Mycolactone, a poliketide-derived macrolide, is the only virulence factor known to be responsible for Buruli ulcers (George et al. 1999). MVs from *M. ulcerans* are cytotoxic to mouse macrophages because the vesicles contain mycolactone (Marsollier et al. 2007). MVs from *M. bovis* BCG and *M. tuberculosis* H37Rv are enriched in proteins associated with bacterial virulence, revealed by proteomic analysis. These proteins include a remarkable abundance of putative Toll-like receptor 2 (TLR2) ligands, such as 19 kDa *Mycobacterium* lipoproteins LpqH, LprA, and LprG. Interaction of MVs from either *M. bovis* BCG or *M. tuberculosis* H37Rv with murine macrophages induces TLR2-dependent cytokine and chemokine release. This evidence demonstrated that mycobacterial vesicles serve as a delivery mechanism for immunologically active molecules that contribute to the virulence of mycobacteria (Prados-Rosales et al. 2011). It was recently reported that the protein VirR (encoded by the gene rv0431) in *M. tuberculosis* (Mtb) regulates the amount of Mtb-derived MVs containing TLR2 ligands such as the lipoproteins LpqH and SodC, suggesting that VirR plays a role in immunomodulating properties of Mtb via MVs (Lee et al. 2013; Rath et al. 2013).

7.6.4 Enterococcus faecium

E. faecium is a commensal organism that is inherently resistant to several antimicrobial agents and can become a bacteremia-causing pathogen. Like other Gram-positive bacteria, *E. faecium* strains produce MVs (Gao et al. 2018). *E. faecium* MV-associated proteins include virulence factors, such as biofilm-promoting proteins, extracellular matrix-binding proteins, and antimicrobial resistance-related proteins, suggesting that *E. faecium* may utilize MVs to release proteins promoting virulence, pathogenicity, and antimicrobial resistance (Wagner et al. 2018).

7.6.5 Bacillus anthracis

B. anthracis, a spore-producing bacillus, causes anthrax in a range of vertebrates. *B. anthracis* releases vesicles that contain components of the anthrax toxins, the protective antigen (PA), lethal factor (LF), and edema toxin (ET), as well as anthrolysin (Rivera et al. 2010). Immunizing mice with *B. anthracis* MVs protects them against subsequent challenge with *B. anthracis* (Marsollier et al. 2007).

7.6.6 Listeria monocytogenes

L. monocytogenes, a Gram-positive pathogen, causes listeriosis, an illness transmitted through the consumption of contaminated food. Similar to other Gram-positive bacteria, MVs are released by *L. monocytogenes* in a process that is regulated by the general stress transcription factor σB (Lee et al. 2013). Internalin B (InIB), which is responsible for *L. monocytogenes* entry into target cells, and listeriolysin O (LLO), a pore-forming toxin, were identified in MVs from *L. monocytogenes* (Lee et al. 2013). In a detailed study, Vdovikova et al. demonstrated that *L. monocytogenes* produces MVs both in vitro and in vivo (Vdovikova et al. 2017). The pore-forming hemolysin LLO is a major virulence factor that is tightly associated with MVs in an oxidized, inactive form. Autophagy induced by pure LLO, by other bacterial pore-forming toxins or by Torin1-stimulated macroautophagy is effectively abrogated by MVs. Thus, it has been suggested that *L. monocytogenes* may survive inside host cells by controlling LLO activity and avoiding destruction from the autophagy system via intracellular release of MVs (Vdovikova et al. 2017).

7.6.7 Lactobacillus

MVs from three different *Lactobacillus* species have been characterized for their physiochemical properties and protein compositions. A recent study identified more than 80 protein components from *Lactobacillus*-derived MVs, including bacteriocin, which is enriched in MVs, suggesting that the vesicles serve as vehicles for delivery of the antimicrobial molecule (Dean et al. 2019). Collectively, these studies highlight the role of MVs in the pathogenesis of Gram-positive bacterial infections.

7.7 Entry and Trafficking of OMVs and MVs into Host Cells

Bacterial OMVs and MVs can enter host cells using various pathways, including clathrin- or caveolin-mediated pathways, or through fusion with plasma membranes (Bielaszewska et al. 2017; Mulcahy et al. 2014; Olofsson et al. 2014). Despite extensive research to understand the mechanisms that regulate cellular uptake of OMVs, little is known about the cellular uptake of MVs. Interestingly, Gram-negative (*V. cholerae*) and Gram-positive (*L. monocytogenes*) bacterial vesicles were efficiently internalized into the intracellular compartments of epithelial cells, which accumulated primarily in the lysosomal compartment of host epithelial cells (Fig. 7.1a and b). Importantly, a recent study showed that *E. coli* O157 (EHEC) OMVs are quickly internalized into intracellular compartments, where they deliver a cocktail of bacterial factors to different host cell compartments (Bielaszewska et al. 2017).

Endocytosis allows small molecules to traverse a cells membrane bilayer (Doherty and McMahon 2009). Host cells internalize OMVs and MVs from several bacteria mainly via various endocytic pathways. As described in recent reviews, endocytosis involves cell membrane invagination, and occurs through several different pathways that depend on the composition and cargo of the OMVs to be internalized (Bitto and Kaparakis-Liaskos 2017; Kaparakis-Liaskos and Ferrero 2015; Pathirana and Kaparakis-Liaskos 2016). Three primary cellular mechanisms regulating the cellular uptake of OMVs and MVs are: (i) clathrin-mediated endocytosis, (ii) cholesterol-enriched microdomains, also known as caveolae or lipid rafts, and (iii) F-actin-coated vacuoles, also known as macropinocytosis and phagocytosis (Table 7.1). These pathways produce endosomal compartments that allow cargo transfer to various subcellular sites in the host cell cytoplasm (Doherty and McMahon 2009). Furthermore, the size of OMVs has been recently shown to play an important role in the preferred mode of entry into host cells (Turner et al. 2018).

OMVs from several microorganisms, *H. pylori*, *A. actinomycetemcomitans*, *enterohemorrhagic E. coli* (EHEC), *enteroaggregative E. coli* (EAEC), and *Brucella abortus*, use clathrin-mediated endocytosis as their major mode of entry into host cells (Bielaszewska et al. 2013; Canas et al. 2016; O'Donoghue and Krachler 2016; Olofsson et al. 2014; Pollak et al. 2012; Thay et al. 2014). In addition to clathrin-

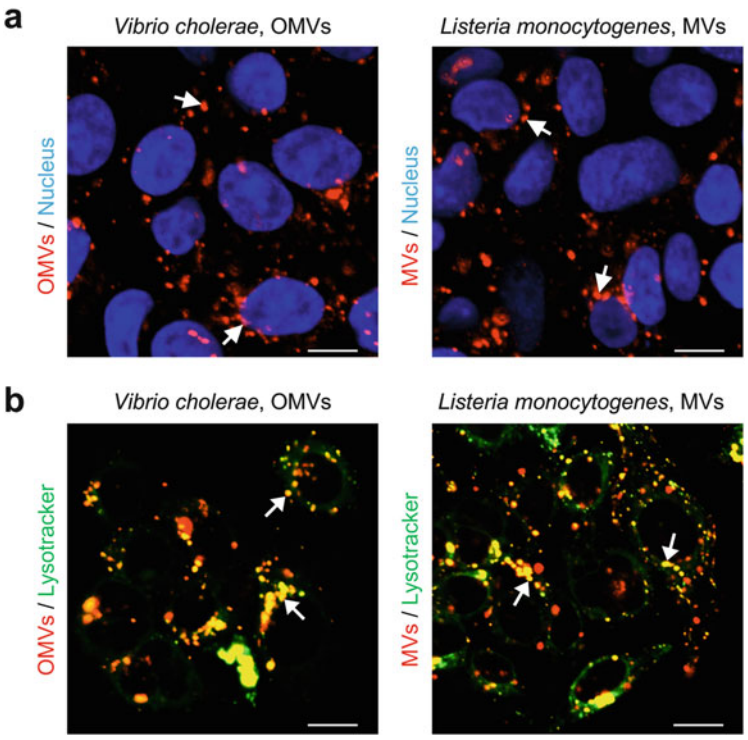

Fig. 7.1 Lysosomal accumulation of bacterial OMVs and MVs. (**a**) Cellular uptake of PKH2-labeled OMVs (Red) isolated from *V. cholerae* or MVs from *L. monocytogenes*. Arrow head indicates their vesicular uptake into HCT8 cells. Nucleus is counter-stained with Hoechst 33342. Scale bars = 10 μm. (**b**) Co-localization of OMVs or MVs (red) with lysosomal marker, Lysotracker (green). Arrow head indicates co-localized spots, seen as yellow in the cytoplasm of HCT8 cells. Scale bars = 10 μm

mediated endocytosis, it has been proposed that dynamin also plays an important role in host cell-mediated uptake of OMVs (Bielaszewska et al. 2017; Kunsmann et al. 2015).

Membrane microdomains, called lipid rafts, are dynamic and abundant in several types of lipids, including cholesterol and sphingolipids, and proteins such as caveolin. The importance of membrane cholesterol for delivery of OMV cargo to the intracellular compartment of host cells has been reported by several investigators. OMVs from *V. cholerae* (Mondal et al. 2016), *V. vulnificus* (Kim et al. 2010), *A. actinomycetemcomitans* (Rompikuntal et al. 2012), *ETEC* (Johnson et al. 2009), *H. influenzae* (Sharpe et al. 2011), *P. gingivalis* (Furuta et al. 2009), *Moraxella catharralis* (Schaar et al. 2011), *H. pylori* (Kaparakis et al. 2010; Olofsson et al. 2014), and *C. jejuni* (Elmi et al. 2012) rely on lipid rafts to mediate internalization by host cells via endocytosis. Bacteria shed OMVs and MVs of different sizes, with the larger OMVs being engulfed by the host cell through ruffled cell membrane

Table 7.1 Cellular mechanisms regulating host cell association and uptake of bacterial vesicles

Mechanisms involved in vesicle uptake	Bacterial strain	References
Clathrin		
	H. pylori	Olofsson et al. (2014), Turner et al. (2018)
	A. actinomycetemcomitans	Thay et al. (2014)
	EHEC	Bielaszewska et al. (2013)
	EAEC	Canas et al. (2016)
	B. abortus	Pollak et al. (2012)
Lipid raft		
	V. cholerae	Mondal et al. (2016)
	V. vulnificus	Kim et al. (2010)
	A. actinomycetemcomitans	Rompikuntal et al. (2012)
	P. aeruginosa	Bauman and Kuehn (2009)
	H. influenzae	Sharpe et al. (2011)
	M. catarrhalls	Vidakovics et al. (2010), Schaar et al. (2011)
	ETEC	Johnson et al. (2009)
	C. jejuni	Elmi et al. (2012)
	H. pylori	Olofsson et al. (2014), Kaparakis et al. (2010)
	P. gingivals	Furuta et al. (2009)
	S. aureus	Gurung et al. (2011)
Membrane fusion		
	P. aeruginosa	Bomberger et al. (2009)
	A. actinomycetemcomitans	Rompikuntal et al. (2012)
	S. aureus	Thay et al. (2013)
	L. pneumophila	Jager et al. (2015), Galka et al. (2008)
Macropinocytosis		
	H. pylori	Turner et al. (2018)
	P. aeruginosa	Bomberger et al. (2009)

protrusions driven by actin polymerization (Kaparakis-Liaskos and Ferrero 2015). Inhibiting actin polymerization using cytochalasin D or wiskostatin decreases entry of *P. aeruginosa* OMVs into the epithelial cells lining the airway tract (Bomberger et al. 2009).

The different pathways involved in entry of vesicles into host cells have been elucidated through experiments employing a large repertoire of inhibitors/binders specific for different components of each pathway, e.g., dynasore for dynamin, chlorpromazine for clathrin, filipin III, wortmannin, nystatin for lipid rafts, and cytochalasin D for pinocytosis (Amano et al. 2010; Canas et al. 2016; O'Donoghue and Krachler 2016; Rompikuntal et al. 2012). Vesicles make ideal delivery vehicles due to their ability to enter eukaryotic host cells and transfer their cargo to

intracellular compartments. In addition, both Gram-positive and Gram-negative bacteria benefit from using vesicles for pathogenesis, intracellular communication and regulating host immunity (Bitto and Kaparakis-Liaskos 2017). Thus, discovering strategies to block vesicle entry into host cells may inhibit membrane vesicle-mediated pathogenesis of bacterial infections.

Membrane fusion is a mechanism by which Gram-negative and Gram-positive bacterial vesicles are internalized into host cells. Membrane fusion enables vesicles to deliver multiple virulence factors directly and simultaneously into the host cell cytoplasm in a coordinated fashion. This phenomenon was first reported by Bomberger et al. (2009) who demonstrated delivery of β-lactamase, alkaline phosphatase, hemolytic phospholipase C, and Cif by *P. aeruginosa* OMVs into human airway epithelial cells. Membrane fusion between *P. aeruginosa* vesicles and epithelial cells appear to occur preferentially at lipid raft domains on target host cells. Concomitantly, using filipin III, which sequesters cholesterol and disrupts lipid rafts, the membrane fusion events can be eliminated (Bomberger et al. 2009). *A. actinomycetemcomitans* OMVs deliver cytolethal distending toxin (CDT) in its biologically active form, and other proteins, including OmpA, into HeLa cells and human gingival fibroblasts, respectively. The OMV-mediated delivery of these proteins occurs in a cholesterol-dependent manner (Rompikuntal et al. 2012). Membrane fusion as a mechanism to deliver virulence factors into host cells has also been observed with MVs from Gram-positive bacteria, i.e., delivery of α-toxin (Hla) by *S. aureus* MVs into HeLa cells that occurs in a cholesterol-dependent manner and triggers death of the host cell (Thay et al. 2013). Despite the utility of filipin III in studying the dependence on lipid rafts for fusion of bacterial membrane vesicles with host cell vesicles, there is a limitation in its use. Filipin III affects a major component of eukaryotic cell plasma membranes, thus its inhibition of membrane fusion may also extend to processes beyond lipid rafts. In a study of the interaction of *L. pneumophila* OMVs with model membranes, the membrane material of the MV became incorporated into liposomes composed of different eukaryotic phospholipids, revealing that MVs have an inherent tendency to fuse with eukaryotic membranes (Jager et al. 2015).

7.8 Conclusions

Taken together, the present literature provides ample evidence that OMVs and MVs are capable of employing multiple routes to enter mammalian host cells. Due to their small size, adhesive properties, immunomodulating activity, and ability to carry and deliver specific effectors into mammalian cells, membrane vesicles of bacterial pathogens are well-suited to contribute significantly in the host interaction. Bacterial membrane vesicles allow the extracellular dispersal of particular proteins, as part of complexes of proteins, as well as lipids that can function synergistically to activate different pathways, either toxic or protective, in the host. Further investigations to dissect mechanisms of vesicle adhesion and entry, vesicle trafficking, and vesicle-

associated contents will provide a critical foundation for future exploitation of OMVs and MVs for medical use. To date, innovative approaches based on engineered bacterial membrane vesicles have shown great clinical potential, and progress is being made to gain further insight and "know how" in using bacterial membrane vesicle-based technologies to enhance global human health.

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