Roles of membrane vesicles in bacterial pathogenesis

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To my dear mother/
Моей дорогой маме посвящается

“The role of the infinitely small in nature is infinitely great”.

-Louis Pasteur
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Abstract

The production of membranous vesicles is observed to occur among organisms from all domains of the tree of life spanning prokaryotes (bacteria, archaea) and eukaryotes (plants, animals and fungi). Bacterial release of membrane-derived vesicles (MVs) has been studied most extensively in cases of Gram-negative species and implicating their outer membrane in formation of extracellular MVs. However, recent studies focusing on Gram-positive bacteria have established that they also undergo MV formation. Membrane vesicles are released during normal bacterial growth, they are derived from the bacterial membrane(s) and may function as transporters of different proteins, DNA and RNA to the neighbouring bacteria or to the cells of a mammalian host. The transport of virulence factors in a condensed manner via MVs to the host cells presumably protects these proteins from degradation and, thereby, targets the host cells in a specific manner.

The aim of my thesis is to investigate secretion of MV-associated virulence factors and to study interactions of MVs produced by two selected Gram-negative and Gram-positive bacteria, i.e. *Vibrio cholerae* and *Listeria monocytogenes*, with eukaryotic host cells. Depending on whether the bacterium acts as an extracellular or intracellular pathogen, MVs may be considered to have specific functions, which may lead to the different outcomes of MV-host interactions.

*V. cholerae* transport systems for virulence factors include the Type VI secretion system and MVs (also referred to as the “Type 0” secretion system). We have identified that the biologically active form of PrtV protease in different *V. cholerae* serogroups is transported via MVs. PrtV protease is essential for *V. cholerae* environmental survival and protection from natural predator grazing. We demonstrated that PrtV is primarily translocated via the inner membrane to the periplasmic space, where it undergoes autoproteolysis, and the truncated version of PrtV protein is packaged inside the MVs and released from the surface of bacteria. MV-associated PrtV protease showed a contribution to bacterial resistance towards the
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Antimicrobial peptide LL-37, thereby, enhancing bacterial survival by avoiding this innate immune defense of the host.

We also studied another virulence factor of *V. cholerae*, the pore-forming toxin VCC, which was found to be transported by MVs. MV-associated VCC is biologically active and triggers an autophagic response in the target cells. We suggested that autophagy serves as a cellular defense mechanism against the MV-associated bacterial virulence factor of *V. cholerae*.

*Listeria monocytogenes* is a Gram-positive intracellular and facultative anaerobic food-borne pathogen causing listeriosis. It causes only sporadic outbreaks in healthy individuals, however, it is dangerous for a fetus or newborn child, and for pregnant and immunocompromised people, leading to a deadly infection in one third of the cases. We have analyzed MVs produced by *L. monocytogenes* and their interaction with eukaryotic cells. Confocal microscopy analysis showed that MVs are internalized into HeLa and HEK293 cells and are accumulated in lysosomes. Moreover, *L. monocytogenes* produces MVs inside the host cells and even inside the phagosomes. We found that the major virulence factor of *L. monocytogenes*, the cholesterol-dependent pore-forming protein listeriolysin O (LLO), is entrapped inside the MVs and resides there in an oxidized inactive state. LLO is known to induce autophagy by making pores in the phagosomal membrane of targeted eukaryotic cells. In our studies, we have shown that MVs effectively abrogated autophagy induced by Torin1, by purified LLO or by another pore-forming toxin from *V. cholerae*. We also found that MVs promote bacterial intracellular survival inside mouse embryonic fibroblasts. In addition, MVs have been shown to have a strong protective activity against host cell necrosis initiated by pore-forming toxin. Taken together, these findings suggested that in vivo MVs production from *L. monocytogenes* might be a relevant strategy of bacteria to manipulate host responses and to promote bacterial survival inside the host cells.
### List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMPs</td>
<td>antimicrobial peptides</td>
</tr>
<tr>
<td>CDC</td>
<td>cholesterol-dependent cytolysin</td>
</tr>
<tr>
<td>CTX</td>
<td>cholera toxin</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>IM</td>
<td>inner membrane</td>
</tr>
<tr>
<td>InlA</td>
<td>internalin A</td>
</tr>
<tr>
<td>InlB</td>
<td>internalin B</td>
</tr>
<tr>
<td>LLO</td>
<td>listeriolysin O</td>
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<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
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<tr>
<td>MVs</td>
<td>membrane vesicles</td>
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<tr>
<td>NF-κB</td>
<td>nuclear factor kappa-light-chain-enhancer of activated B cells</td>
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<tr>
<td>NOVC</td>
<td>non-O1, non-O139 <em>V. cholerae</em></td>
</tr>
<tr>
<td>OM</td>
<td>outer membrane</td>
</tr>
<tr>
<td>OMV(s)</td>
<td>outer membrane vesicle(s)</td>
</tr>
<tr>
<td>p.i.</td>
<td>post-infection</td>
</tr>
<tr>
<td>PAMP</td>
<td>pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PFT</td>
<td>pore-forming toxin</td>
</tr>
<tr>
<td>PRR</td>
<td>pattern-recognition receptor</td>
</tr>
<tr>
<td>QS</td>
<td>quorum sensing</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SEM</td>
<td>scanning electron microscopy</td>
</tr>
<tr>
<td>SLAP(s)</td>
<td>spacious <em>Listeria</em>-containing phagosome(s)</td>
</tr>
<tr>
<td>SLO</td>
<td>streptolysin O</td>
</tr>
<tr>
<td>T1SS</td>
<td>Type I secretion system</td>
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<tr>
<td>T2SS</td>
<td>Type II secretion system</td>
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<tr>
<td>T3SS</td>
<td>Type III secretion system</td>
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<tr>
<td>T4SS</td>
<td>Type IV secretion system</td>
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<td>T5SS</td>
<td>Type V secretion system</td>
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<tr>
<td>T6SS</td>
<td>Type VI secretion system</td>
</tr>
<tr>
<td>T7SS</td>
<td>Type VII secretion system</td>
</tr>
</tbody>
</table>

Syftet med detta avhandlingsarbete är att undersöka MV-associerad virulensfaktorsekretion och att studera interaktioner mellan vesiklar som utsöndras av Gram-negativa (Vibrio cholerae) eller Gram-positiva (Listeria monocytogenes) bakterier och eukaryota värdceller. Beroende på vilken patogen det är (extracellulär eller intracellulär), kan MV ha olika funktioner och det kan i sin tur leda till olika konsekvenser av MV-värdcellsinteraktionen.


I den andra artikeln (II) analyserar vi en annan virulensfaktor i V. cholerae, det porbildande toxinet VCC, som också transporteras av MV i biologiskt aktiv form och orsakar autofagi i värdceller. Vi föreslår att autofagi
fungerar som en försvarsmekanism i cellen mot en MV-associerad bakteriell virulensfaktor hos *V. cholerae*.

Papers in this thesis

Papers included in the thesis:


* These authors contributed equally


Manuscript not included in the thesis:


* These authors contributed equally
1. Introduction

Earth, ocean, soil, human, animals, plants, life and evolution: everything brings us back to bacteria. Life started with bacteria. No niche on earth that supports life can exist without bacteria. Considering their biodiversity and importance for the environment one can definitely say that bacteria shape the planet. Their ability to survive for billions of years and quickly evolve arose from adaptation in response to the changing environment or host immune response. This constant evolution and adaptation is possible due to the short generation time of bacteria, their large population size and their genome plasticity. Point mutations, DNA rearrangements and horizontal gene transfer contribute to the genome plasticity of bacteria. These mechanisms allow a bacterium to acquire new pathogenic properties and resistance genes against antimicrobials to become a “superbug” [1].

Different bacteria occupy different niches, which determine their mutation and adaptation rates. Amazingly, the human body contains ten times more bacterial cells than human cells. These bacteria called commensal are usually harmless and can have a mutualistic relationship with the host. Certain bacteria, which are normally benign for a healthy host, can cause disease once the environment changes (during pregnancy, compromised immune system etc.) and are called opportunistic pathogens, for example, *Listeria monocytogenes, Staphylococcus aureus, Pseudomonas aeruginosa*. The ability of bacteria to cause disease determines their virulence. It is the measure of organism pathogenicity, in other words. The development of bacterial infection and disease in the host is called bacterial pathogenesis.

1.1. Concept of bacterial pathogenesis

The word “pathogenesis” originates from the Greek word “pathos” (disease) and “genesis” (creation). To be able to treat infectious disease, first
it is important to understand the molecular mechanisms behind the bacterial pathogenesis. Despite the fact that different bacterial pathogens occupy different niches and can cause different infections, they share in principle common mechanisms: adherence, invasion, damage to host cells and tissues, surviving host defense systems and establishing acute or persistent infection. Thus, virulence factors produced by bacteria play critical roles [2].

Virulence factors that help bacteria to establish infection can be divided into the following categories:
- adherence and colonization factors
- invasion factors
- capsules and other anti-phagocytic factors
- siderophores
- endotoxins and exotoxins.

The initial step of infection is adherence of pathogens to host surfaces (skin, mucous layers of oral cavity, nasopharynx, urogenital tract, and deeper layers of lymphoid tissue, gastric and intestinal epithelia), which occurs with the help of adhesins. Most of Gram-negative bacteria (Vibrio cholerae, E. coli, P. aeruginosa etc.) use fimbriae (pili) for attachment, while some Gram-positive bacteria (Staphylococcus spp. and Streptococcus spp. etc.) can also form pili but predominantly use teichoic acids.

The easiest way to enter phagocytic cells is through phagocytosis, but pathogens have also evolved other molecular strategies to enter host cells, including non-phagocytic cells. Mainly, there are two strategies for pathogen invasion: the “zipper” mechanism and the “trigger” mechanism. The first mechanism involves tight binding of a bacterial surface molecule to the host receptor, thereby, promoting “zippering” of the bacterium into the host cell. For instance, the Gram-positive food-borne pathogen L. monocytogenes expresses invasins called internalins InlA and InlB, where InlA binds E-cadherin on the host cell surface and InlB binds the hepatocyte growth factor receptor Met, mediating the bacterial internalization. The “trigger” mechanism includes a large-scale activation and rearrangement of the actin cytoskeleton, causing significant membrane ruffling. This is triggered in response to injection of bacterial effectors by the Type III secretion system
(T3SS) into the host cell, which alters host cell Rho GTPases, whose activity controls actin polymerization levels. After the pathogen internalization, other effectors stimulate actin cytoskeleton recovery and normalization of its architecture. *Salmonella* and *Shigella* spp. use this type of mechanism for host invasion [2, 3].

In order to avoid recognition by the host immune response and becoming phagocytosed, some Gram-positive and Gram-negative bacteria synthesize a **capsule** that covers the bacterial surface (*Streptococcus pneumoniae, Neisseria meningitidis, P. aeruginosa* etc.). Capsular polysaccharides also contribute to biofilm formation, leading to the development of chronic infection and resistance to antibiotics [4]. Other virulence factors can be secreted in order to block phagocytosis, i.e., the *Yersinia* outer membrane proteins Yops or the M-related protein (Mrp) of *S. pyogenes* [5, 6].

A range of **siderophores** varies among different microbial species (enterobactin, salmochelin, yersiniabactin) and are mainly aimed to scavenge iron from the host during infection. Additionally, other functions of siderophores have been discovered, such as protection from oxidative stress and chelating other metals such as copper and zinc. Since iron is necessary during bacterial colonization, siderophores play an important role in inter-bacterial competition, modulating host cellular pathways and defining a “replicative niche” during bacterial infection [7, 8].

Bacterial toxins are the real power of bacteria during infection and at the same time they are extensively used as targets for vaccines development. Toxins can be classified as **exotoxins**, which are commonly secreted by bacteria into the milieu, and **endotoxins**, which are released upon bacterial cell death and lysis, and cause septic shock. Mainly endotoxin refers to lipopolysaccharide (LPS), a large amphiphilic carbohydrate molecule embedded in the outer membrane of Gram-negative bacteria, which activates the host cell response through release of proinflammatory cytokines, complement and coagulation cascade activation. This occurs due to the toxic part of LPS, lipid A. An alternative for LPS in Gram-positive
bacteria is peptidoglycan fragments and teichoic acids (polymers of sugar alcohol phosphate), which can also elicit septic shock in the host [9, 10].

Exotoxins according to their structure and function can be classified as: (1) A-B toxins, (2) membrane disrupting toxins and (3) superantigens.

(1) A-B toxins consist of the A subunit, which has the enzymatic activity, and the B subunit, which binds a host cell receptor and deliver toxin into the host cell. Examples of A-B toxins are cholera toxin (CT) of V. cholerae, diphtheria toxin of Corynebacterium diphtheriae and exotoxin A of P. aeruginosa [2].

(2) Membrane-disrupting toxins oligomerize and become inserted into the host cell membrane forming pores and disrupting cellular homeostasis. Examples of pore-forming toxins (PFTs) are listeriolysin O (LLO) of L. monocytogenes, streptolysin O (SLO) of S. pyogenes, V. cholerae cytolsin (VCC), repeats-in-toxin Rtx toxin HlyA of uropathogenic E. coli etc. [11]. More about pore formation and pore-forming toxins is discussed in 1.3 section of this thesis.

(3) Superantigens elicit non-specific, antigen-independent cell response and T-cell stimulation through binding to their receptor and to the major histocompatibility (MHC) class II molecule on the surface of antigen-presenting cells (APCs) [12].

In order to transport exotoxins from the cytoplasm outside the cell or even inject them directly into the host cell, bacteria use different secretion systems.

1.2. Bacterial membrane-derived vesicles (MVs)

1.2.1. Protein secretion systems in bacteria

The difference between Gram-negative and Gram-positive bacteria lies in the difference of their cell wall structure, defined by Gram staining using crystal violet and safranin. The cell envelope of Gram-negative bacteria consists of an outer membrane, inner membrane and periplasmic space in between, while the envelope of Gram-positive bacteria has only a
cytoplasmic membrane and a thick peptidoglycan layer (Fig. 1). In order to overcome these barriers, bacteria use secretion systems to facilitate the protein export. So far, eight types of secretion systems have been discovered named Type 0 to VII.

**Figure 1. Differences in Gram-positive and Gram-negative cell envelope.**

Protein secretion in Gram-negative bacteria can occur either in one or two steps. One-step protein secretion occurs via Type I, III, IV and VI secretion systems, where proteins are transported directly from the bacterial cytoplasm into the extracellular space or into a target cell. Two-steps secretion starts first with translocating proteins into the periplasmic space via the Sec pathway or the twin-arginine translocation Tat pathway, and then farther from the periplasm these proteins are secreted into the extracellular space by one of the secretion systems [13]. The Sec and Tat pathways are also commonly used by a number of Gram-positive bacteria for protein transport across the membrane. The Sec system, however, transports unfolded proteins, while the Tat system transports already folded proteins [14, 15].

The Type I secretion system (T1SS) spans the inner and outer bacterial membranes together with the periplasmic space and represents a three-component translocon. T1SS substrates are mainly polypeptides, such as
RTX toxins and lipases of various bacteria. For instance, uropathogenic *E. coli* uses T1SS to secrete hemolysin HlyA, *V. cholerae* – to secrete MARTX toxin, and *Serratia marcescens* - to secrete the hemophore HasA [13, 16-18].

The Type II secretion system (T2SS) is more complex than the T1SS, consisting of at least 12 proteins. Even though, it also spans both inner and outer membranes, T2SS-dependent protein secretion occurs in two steps: first, unfolded proteins are translocated through the inner membrane via the Sec or Tat pathway, then folded in the periplasm and finally transported through the outer membrane with T2SS. Virulence factors secreted with the T2SS are, for example, the ADP-ribosylating toxins of enterotoxigenic *E. coli* (heat labile toxin), cholera toxin of *V. cholerae*, exotoxin A of *P. aeruginosa* and enzymes of some plant pathogens [19-21].

The Type III secretion system (T3SS), also called an injectisome or needle-like apparatus due to its structure, is used by a variety of pathogens to transport substrates across bacterial membranes and inject them into a target eukaryotic host cell, in order to subvert host cell defense and establish its own niche. T3SS machinery has nine highly conserved core proteins and structurally resembles flagellum [22, 23]. *Yersinia, Shigella* and *Salmonella* species secrete a number of virulence factors (Yops, Ipas and Sips, respectively) via the T3SS [24].

The unique property of the Type IV secretion system (T4SS), which spans both membranes, is its ability to transport not only proteins, but also nucleic acids and DNA/protein complexes, directly into mammalian or bacterial cells. The T4SS has mainly been studied in Gram-negative bacteria, such as *Neisseria gonorrhoeae, Helicobacter pylori, Legionella pneumophila*, but has also been discovered in Gram-positive bacteria, as streptococci and enterococci, *Clostridium difficile, L. monocytogenes* etc. [25, 26].

A large group of secreted proteins in Gram-negative bacteria are secreted by the Type V secretion system (T5SS), which, like the T2SS, also
operates in a two-step manner. Protein secretion across the inner membrane of both Gram-positive and Gram-negative bacteria occurs mainly via the Sec-dependent pathway, and proteins are either further translocated into the periplasmic space in the case of Gram-negative bacteria, or can be released into the extracellular environment or incorporated into the cell wall in the case of Gram-positive bacteria. Proteins secreted via the T5SS include toxins such as VacA of Helicobacter pylori, IgA proteases of N. gonorrhoeae and N. meningitidis, SepA of Shigella flexneri and PrtS of S. marcescens etc. [20, 27].

The Type VI secretion system (T6SS) shares structural homology with the tailed bacteriophage, it is a large secretion system that upon contact translocates toxic effector proteins into eukaryotic or other bacterial cells, playing a significant role in pathogenesis of bacteria and interbacterial competition. The T6SS has a minimal set of 13 core component genes conserved among many Gram-negative bacteria and a subset of multiple accessory proteins that are directed to either facilitate assembly of the secretion machinery or play roles in regulation or host adaptation. The T6SS is required for virulence of many Gram-negative pathogens, such as V. cholerae, P. aeruginosa, S. marcescens, Proteus mirabilis, Francisella tularensis, Edwardsiella tarda, Burkholderia mallei etc. [13, 28, 29].

The Type VII secretion system (T7SS) was first identified in the pathogen Mycobacterium tuberculosis, but genes encoding T7SS were later discovered in other bacteria, albeit Gram-positive pathogens: Bacillus subtilis, L. monocytogenes, S. aureus, C. diphtheriae etc. Studies have shown that a M. tuberculosis T7SS mutant was not able to replicate in macrophages and had growth defects in the early stage of mouse infection. However, the mechanisms and structure of the T7SS are not well understood and remain under investigation [30, 31].

An extensively studied secretion system in both Gram-negative and Gram-positive bacteria is membrane vesicle (MV)-associated secretion, which is also named as the “Type 0 secretion system” [32, 33].
1.2.2. Membrane vesicle characterization

Extracellular vesicle secretion is conserved through all domains of life: Bacteria, Eukarya and Archaea - and are often referred to as membrane vesicles, exosomes, microvesicles and virus-like particles. So far, all types of bacteria that have been studied produce MVs. Vesicle secretion occurs in various environments during bacterial growth: fresh and salty water, biofilms, inside eukaryotic cells and at the site of infection, and in planktonic cultures [34].

1.1.2.1. MV biogenesis

Since MV production has been intensively studied in Gram-negative species, while Gram-positive bacteria were thought for a long time not to produce MVs, it is not surprising that the biogenesis of MVs has been mainly investigated in Gram-negative bacteria. Considering differences in the cell envelope structures of Gram-positive and Gram-negative bacteria (Fig. 1), the manner in which MVs are formed and released from the surface of Gram-positive bacteria currently remains unclear. MVs are spherical particles, commonly 20-300nm in diameter, that contain periplasmic components, LPS, nucleic acids and a variety of proteins, enzymes and virulence factors.

Several models have been proposed for MVs biogenesis in Gram-negative species. In general, cell envelope stability depends on the crosslinks between the peptidoglycan and the outer membrane (OM). One model suggests that reduced cross-linking between the OM and peptidoglycan promotes MV biogenesis, as was shown for *Acinetobacter baumannii* and *E. coli* [35, 36]. Another model suggests that accumulation of periplasmic material, misfolded proteins or LPS can increase vesiculation [37]. Moreover, deletion or repression in response to iron starvation of the VacJ/Yrb ABC (ATP-binding cassette) transport system, a proposed phospholipid transporter, results in increased MV biogenesis in *V. cholerae* and *Haemophilus influenzae*. Phospholipid accumulation in the outer leaflet of the OM, thereby, initiates the bulging of the OM and pinching off of the
MVs [38]. Environmental changes also affect levels of vesiculation, as does temperature in the case of *S. marcescens* and *E. coli*, and antibiotics in the case of *Pseudomonas* [37, 39, 40].

An interesting model for MV formation was proposed for *Shewanella vesiculosa*, Gram-negative bacterium, where double-bilayered MVs were observed by transmission electron microscopy. They were formed out of both the inner and outer membrane, incorporating cytoplasmic components and DNA [41]. This model suggests that formation of MVs in Gram-positive bacteria can also involve cytoplasmic membrane. However, it is unclear how a thick layer of peptidoglycan is cleaved during MV biogenesis. Some hypotheses exist, such as the idea that MVs might be forced through the cell wall by a turgor pressure, or cell wall-modifying enzymes might facilitate MV release, or MV release might occur at natural “break points” of the cell wall that undergo thinning during daughter cell budding [42].

Every study suggests its own general MV biogenesis mechanism, however, it is more probable that vesiculation can occur by different mechanisms, depending on the type of bacteria and the environment. A simple generalized model of MV biogenesis is introduced in Figure 2.
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Figure 2. Simplified depiction of MV biogenesis model in Gram-negative bacteria. IM – inner membrane, OM - outer membrane, PG – peptidoglycan, MV - membrane vesicle. This model is based on reduced cross-linking between the OM and peptidoglycan, which leads to the curvature of the OM and pinching off the MV. Formed MVs contain outer membrane proteins and periplasmic proteins entrapped inside the MV lumen and/or associated on the MV surface.

1.1.2. MV entry into host cells

MV, as one of the bacterial secretion systems, are used by bacteria to deliver diverse cargo (small molecules, nucleic acids, proteins, lipids) to other bacterial or eukaryotic cells. MVs can enter host epithelial cells via different mechanisms involving fusion with the host cell membrane and/or endocytosis in a lipid raft-dependent or independent manner (Table 1). During endocytosis the intact MV is internalized into the host cell and further trafficked intracellularly. Meanwhile, MVs from some of the bacteria can fuse with the eukaryotic plasma membrane and deliver the vesicle contents inside the cells. For some bacteria the entry of MVs inside the cells was shown to occur by different pathways, as in the case of P. aeruginosa.
Different studies showed that *P. aeruginosa* uses both membrane fusion and endocytosis to deliver virulence factors inside the host cells [43-45].

**Table 1. Types of MV internalisation into the host cells.**

<table>
<thead>
<tr>
<th>Endocytosis</th>
<th>Clathrin-dependent</th>
<th>Caveolin-mediated</th>
<th>Membrane fusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipid raft-dependent</td>
<td><em>Vibrio cholerae</em>, <em>Pseudomonas aeruginosa</em>, <em>Helicobacter pylori</em>, <em>Acinetobacter baumanni</em>, <em>Campylobacter jejuni</em></td>
<td><em>Helicobacter pylori</em>, <em>Enterohemorrhagic Escherichia coli</em>, <em>Enteroaggregative Escherichia coli</em>, <em>Aggregatibacter actinomycetemcomitans</em></td>
<td><em>Enterotoxigenic Escherichia coli</em>, <em>Vibrio cholerae</em>, <em>Haemophilus influenza</em>, <em>Moraxella catarrhalis</em>, <em>Pseudomonas aeruginosa</em>, <em>Legionella pneumophila</em>, <em>Aggregatibacter actinomycetemcomitans</em></td>
</tr>
</tbody>
</table>

The most studied type of endocytosis is clathrin-mediated endocytosis. It requires a set of adaptor and accessory proteins, as well as GTPase dynamin, which promotes scission of the clathrin-coated vesicle from the plasma membrane [46]. MVs can be internalized as well via caveolin-mediated endocytosis, through formation of flask-shaped invaginations, which are cell-type specific. The size of the MVs might also dictate how they will be taken up: up to 200 nm in diameter - in clathrin-coated particles, while up to 50-80 nm – in caveolae. Other types of clathrin- and caveolin-independent endocytosis include macropinocytosis, phagocytosis and membrane fusion [43, 47]. In addition, several studies have shown that entry of some of the MVs require cholesterol-rich lipid rafts for endocytosis, as in the case of MVs produced by enterotoxigenic *E. coli*, *H. pylori* and *Porphyromonas gingivalis* [48, 49]. In the case of MVs from *P. aeruginosa* and *Aggregatibacter actinomycetemcomitans*, MVs also require lipid rafts, however, they deliver their cargo via fusion with lipid rafts on the eukaryotic cell membrane [50]. Additionally, the Gram-positive pathogen *S.*
Introduction

*aureus* delivers its key virulence factor α-hemolysin via cholesterol-dependent fusion with the host cell plasma membrane [51].

Upon entry, MVs depending on their size and heterogeneity can be trafficked to different cellular compartments. Thus, enterotoxigenic *E. coli* MVs accumulate in non-acidified compartments, *P. gingivalis* MVs are transported to early endosome antigen 1 (EEA1)-associated compartments and further sorted to lysosomal compartments, while *P. aeruginosa* MVs colocalize with the ER [45, 49, 52].

1.1.2.3. MV-mediated cargo transport

Named the Type 0 secretion system, MVs transport a large number of cargo to the host cell or neighboring bacterial cell [32]. The priority of this secretion system over other secretion systems is the long-distance delivery of virulence factors in a concentrated, protected manner. The MV structure resembles that of the bacterial OM and contains LPS and lipoproteins, peptidoglycan, phospholipids, periplasmic and OM proteins, DNA, RNA, adhesins, enzymes and toxins, while being depleted in cytoplasmic and inner membrane content, indicating that MVs are not a product of bacterial cell lysis [53-55]. The biological mechanism of MV formation as a phenomenon, that is not a random pinching off, is also confirmed by enrichment and exclusion of certain proteins and lipids in the MV content. Some of the proteins use it as a main secretion system, as in the case of leukotoxin, which is enriched in *A. actinomycetemcomitans* MVs [56]. Moreover, certain toxins are more biologically active in association with MVs, than as freely secreted toxins, as reported for the leukotoxin of *A. actinomycetemcomitans*; ClyA, pore-forming toxin of *E. coli*; or Cif, hemolytic phospholipase C, of *P. aeruginosa* [44, 57, 58]. In contrast, other situations have also been observed - free-soluble vacuolating toxin VacA of *H. pylori* shows higher activity than MV-associated VacA [59].

To date, no single mutant completely deficient in MV production has been created, although certain gene deletions can increase or reduce MVs production [60]. The vesiculation level can also be increased under bacterial
stress, which happens during colonization of host tissues. For instance, the
general stress transcription factor $\sigma^B$ was shown to regulate MV production
in *L. monocytogenes*, since the isogenic sigB mutant produced nine times
less MVs than wild-type [61]. A similar pattern was observed for *E. coli*,
where the envelope stress response $\sigma^E$ pathway was disrupted resulting in
MV overproduction [60]. In *V. cholerae*, small regulatory RNAs VrrA, the
expression of which is controlled by membrane stress sigma factor $\sigma^E$,
regulate MV secretion [62]. In addition, the probiotic *E. coli* Nissle 1917
tolR mutant (cell envelope protein) exhibited a significant increase in
vesiculation compared to the wild-type strain, as well as decreased
internalization in Caco-2 cells due to lower cell-binding capacity [63].

**1.2.3. Gram-negative MVs**

The majority of studies have been focused on MVs production in
Gram-negative species, in which MVs are also termed outer membrane
vesicles (OMVs). OMVs transport a large number of virulence factors that
contribute to bacterial pathogenesis and benefit the microbe. Examples of
virulence factors associated with Gram-negative OMVs are represented in
Table 2.

**Table 2. Examples of OMV-associated virulence factors in Gram-negative
bacteria**

<table>
<thead>
<tr>
<th>Bacterial species</th>
<th>Virulence factors</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Acinetobacter baumannii</em></td>
<td>Outer membrane protein A</td>
<td>[64]</td>
</tr>
<tr>
<td><em>Aggregatibacter actinomycetemcomitans</em></td>
<td>Leukotoxin</td>
<td>[56], [50]</td>
</tr>
<tr>
<td></td>
<td>Cytotoxic distending toxin Cdt</td>
<td></td>
</tr>
<tr>
<td><em>Bacteroides fragilis</em></td>
<td>capsular polysaccharide PSA</td>
<td>[65]</td>
</tr>
<tr>
<td><em>Borrelia burgdorferi</em></td>
<td>OspA, OspD</td>
<td>[66]</td>
</tr>
<tr>
<td><em>Campylobacter jejuni</em></td>
<td>Cdt</td>
<td>[67]</td>
</tr>
<tr>
<td><em>Enterohemorrhagic</em> <em>Escherichia coli</em></td>
<td>ClyA</td>
<td>[57]</td>
</tr>
<tr>
<td><em>Enterotoxigenic</em> <em>Escherichia coli</em></td>
<td>Heat-labile enterotoxin</td>
<td>[68]</td>
</tr>
</tbody>
</table>
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<table>
<thead>
<tr>
<th>Extraintestinal <em>Escherichia coli</em></th>
<th>α-hemolysin</th>
<th>[69]</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Helicobacter pylori</em></td>
<td>Vacuolating cytotoxin VacA</td>
<td>[70]</td>
</tr>
<tr>
<td><em>Porphyromonas gingivalis</em></td>
<td>Gingipain cysteine proteinases</td>
<td>[71]</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>β-lactamase, alkaline phosphatase, hemolytic phospholipase C, and Cif</td>
<td>[44]</td>
</tr>
<tr>
<td><em>Shigella flexneri</em></td>
<td>IpaB, IpaC, IpaD</td>
<td>[72]</td>
</tr>
<tr>
<td><em>Vibrio cholerae</em></td>
<td>RTX toxin, Bap1, PrtV protease</td>
<td>[73, 74, 75]</td>
</tr>
</tbody>
</table>

#### 1.2.4. Gram-positive MVs

Gram-positive MVs are distinct in composition from Gram-negative MVs, since they do not include OM components. They contain many cytosolic proteins instead, which implicates the involvement of the cytosolic membrane in MV formation in Gram-positive bacteria. Nevertheless, they also carry proteins involved in virulence, stress response, metabolism and transport proteins. Examples of virulence factors associated with Gram-positive MVs are represented in Table 3.

### Table 3. Examples of MV-associated virulence factors in Gram-positive bacteria

<table>
<thead>
<tr>
<th>Bacterial species</th>
<th>Virulence factors</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus anthracis</em></td>
<td>Lethal factor, edema toxin, anthrolysin</td>
<td>[76]</td>
</tr>
<tr>
<td><em>Listeria monocytogenes</em></td>
<td>Internalin B, listeriolysin O</td>
<td>[61]</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>α-hemolysin, β-lactamase</td>
<td>[51, 77]</td>
</tr>
<tr>
<td><em>Streptococcus pneumoniae</em></td>
<td>Lipoproteins (ABC transporters, foldase PrsA), pneumolysin</td>
<td>[78]</td>
</tr>
<tr>
<td><em>Streptomyces coelicolor</em></td>
<td>Phospholipase C, antibiotic actinorhodin</td>
<td>[79]</td>
</tr>
</tbody>
</table>

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1.2.5. Role of MVs during infection

Secretion of MVs occurs also during the infectious process. As such, MVs have been detected in cerebrospinal fluid of a child diagnosed with *N. meningitidis* and from a case of fatal meningococcal septicaemia caused by *N. meningitidis* serogroup B [80, 81]. Blebbing of the vesicles was also observed in clinical isolates of *H. pylori* [82]. In addition, *S. aureus* was shown to produce MVs *in vivo* in the extracellular milieu of mouse lung tissue [83]. MV content, such as toxins, cell wall components, and nucleic acids, is protected from degradation within a lipid bilayer of MVs. The content is released upon MV rupture inside the host cells, which, in turn, elicits a range of host responses. MVs contain pathogen-associated molecular patterns (PAMPs), which are recognized by pattern-recognition receptors (PRRs) in the host. Peptidoglycan moieties D-glutamyl-meso-diaminopimelic acid (DAP) and muramyl dipeptide (MDP) act as PAMPs and are present in Gram-positive and Gram-negative MVs. They activate, consequently, NOD1- and NOD2-responses in the cell. Further, NOD1 and NOD2 activation leads to RIP2 kinase recruitment and NF-κB pathway activation, which mediates a pro-inflammatory response [84, 85]. Additionally, LPS and flagellin, carried by MVs, also activate NF-κB, MAPK and the interferon signaling cascades, through MyD88/TRIF, and, consequently, affect expression of interferons, pro-inflammatory cytokines, chemokines and antimicrobial peptides. In this way, MVs are a powerful trigger of innate immunity and further the adaptive immune response. Interestingly, *V. cholerae* MVs mediate innate immune responses through quorum sensing. At low cell density the negative master regulator HapR is degraded, which activates virulence gene expression; MVs contain little peptidoglycan, leading to a decrease in the NOD-induced inflammatory response [86]. However, MVs can also limit inflammation, as do gingipains in MVs of *P. gingivalis* by degrading IL-8 (CXCL8 chemokine) [71]. Not only are immune cells affected, *N. meningitidis* and *P. gingivalis* MVs induce platelet aggregation contributing to bacteria-induced thrombosis [87, 88].
Apart from immune responses, PRR signalling stimulated by MVs also mediates antimicrobial peptide (AMP) production by host epithelial cells. For instance, OMVs from *N. gonorrhoeae*, *P. aeruginosa* and *H. pylori* stimulate human β-defensins *HBD2* and *HBD3* expression [89]. AMPs (defensins, cathelicidins) are the first line of host defense that are mainly produced by intestinal epithelium and help to clear bacterial infection. On the other hand, MVs can sequester AMP and, in that manner, decrease its antimicrobial activity on the bacterial cell, serving as a "decoy". Duperthuy *et al.* showed *in vitro* that, when subjected to a sub-lethal concentration of polymyxin B, MVs secreted by *V. cholerae* contain Bap1 protein bound to OmpT porin on the MV surface. Bap1 protein sequestered LL-37 peptide (cathelicidin), and as a result bacteria were exposed to a lower concentration of LL-37 [74].

MVs can also mediate biofilm formation through co-aggregation of bacteria, thereby, promoting bacterial resistance to antimicrobial agents [90]. Antimicrobial resistance is also mediated by MVs through horizontal gene transfer of antibiotic resistant genes [91].

**Figure 3.** Examples of Gram-positive and Gram-negative MV functions.
Introduction

Depending on the MV-associated virulence factors, MVs can elicit a wide range of genotoxic or cytotoxic effects on the eukaryotic cell, which might lead further to carcinogenesis. Thus, *E. coli* MVs induce eukaryotic cell proliferation, DNA damage, reactive oxygen species production, aneuploidy and chromosomal instability [92]. *H. pylori* OMVs transporting vacuolating cytotoxin (VacA) promote genomic instability in gastric epithelial cells and contribute to *H. pylori*-induced gastric carcinogenesis [93]. Moreover, *H. pylori* MVs also induce apoptosis in gastric epithelial and Jurkat T cells [93, 94]. Apoptosis is also induced by *S. aureus* MVs (carrying protein A) and enterohemorrhagic *E. coli* MVs (carrying hemolysin) [82, 95]. Another host cell response, autophagy, is induced by MV-associated *V. cholerae* cytolysin VCC [96]. Additionally, MVs facilitate the establishment of infection and inflammation in the host cells. MVs produced by adherent-invasive *E. coli*, which colonizes the ileal lesions of patients with Crohn's disease, are able to facilitate bacterial colonization, potentially contributing to further complications in patients with Crohn's disease [97].

Not only do MVs interact with the host and cause many toxic effects, but they also can interact with commensal bacteria and other pathogenic bacteria in order for bacteria that release them to establish and occupy a niche in the host. Interbacterial killing mediated by MVs, which are able to kill other Gram-negative and Gram-positive bacterial species, occurs through the delivery of peptidoglycan-degrading enzymes or fusion with the competing bacteria [94–96].

Cellular protective and immunoregulatory roles have also been identified for bacterial MVs. For instance, the gut commensal *B. fragilis* releases a capsular polysaccharide (PSA) within MVs, sensed by dendritic cells, resulting in enhanced regulatory T cell responses and increased anti-inflammatory cytokine production, which provides protection against experimental colitis [65].
1.2.6. The importance of MVs in medicine

Due to their immunogenicity, uptake by immune cells and cargo delivery in a protected manner, MVs serve as promising candidates for vaccine development and are used as tools in nanotechnology and drug delivery. It is possible to modify MVs, such as for LPS reactogenicity, to produce a safe and effective product. A MV-based vaccine against meningitis serogroup B (Bexasero®, Novartis) was licensed by the European Medical Association (EMA) and approved in Europe and Australia in 2013, and in the U.S. in 2015 [97, 98]. Many other studies are focused on the development of new MV-based vaccines against bacterial infections, such as, for example, *H. influenzae, Pasteurella multocida, V. cholerae, enterotoxigenic E. coli, N. meningitidis, Bordetella pertussis* and *S. typhimurium* [99-104]. Additionally, bacterial MVs are currently studied as cell-specific drug-delivery vehicles for treating various cancers. This idea is based on the fact that systemic injection of siRNA-packaged MVs can silence specific genes and induce tumor growth regression [105]. The next exciting approach will be to use MV-associated miRNAs for delivery and targeting, for example, intestinal cancer using orally administered MVs from native enterobacteria [106]. Therefore, studying MVs opens new opportunities in medicine and biotechnology.
1.3. Bacterial pore-forming toxins

Pore-forming toxins (PFTs) secreted by bacteria are the most common cytotoxic proteins, which are required for bacterial virulence. Pore-forming proteins are an ancient protein family found in all kingdoms of life. Their main function is to create pores in a membrane of other bacteria (colicins) or mammalian cells. Through the alteration of plasma membrane permeability of the target cell, PFTs usually cause cell death. However, pore formation may also lead to a range of intracellular effects, due to induction of response pathways against the pore formation [11, 107].

1.3.1. Classification

Based on whether the secondary structure of the membrane-spanning elements consists of α-helices or β-barrels, PFTs are classified as α-PFTs and β-PFTs. Three families of α-PFTs (colicins, actinoporins and ClyA-like) and three families of β-PFTs (haemolysins, aerolysins and cholesterol-dependent cytolysins) were identified [11]. The small-pore-forming repeat-in-toxin (RTX) toxins are a large group of PFTs but their mechanism of pore formation remains unclear. Cholesterol-dependent cytolysins (CDCs), produced mainly by Gram-positive bacteria, form large pores and belong to a β-PFT subclass. Sizes of small pores range between 0.5 to 5 nm, whereas sizes of big pores range between 20 to 100 nm [108].

1.3.2. Mechanism of pore formation

β-PFTs are commonly secreted as monomeric soluble proteins. Upon binding to the mammalian cell surface through specific receptors, β-PFTs monomers oligomerize and undergo conformational changes. The majority of these receptors are lipids or lipid-anchored proteins. Therefore, cholesterol on lipid rafts is the receptor for CDCs (streptolysin O, perfringolysin O, listeriolysin O), glycosylphosphatidylinositol (GPI)-anchored proteins are the receptors for Aeromonas aerolysin and C. septicum α-toxin, while the components of tight junctions, claudins, are the
receptors for enterotoxin of *C. perfringens* [107]. Oligomerization of the β-toxin happens at the membrane surface, which leads to the formation of a pre-pore ring-like structure. Further, pre-pore is rearranged into a β-barrel, which is hydrophobic on the outside, and becomes spontaneously inserted into the lipid membrane bilayer [11].

All PFTs rearrange their structures upon pore formation in order to donate β-strands to the formation of a transmembrane β-barrel, however, only CDCs are known to switch their secondary structure [109]. CDCs can also form intermediate structures shaped as and called “arcs”, as was shown on the example of listeriolyisin O [110].

Pore formation by α-PFTs occurs through binding to a receptor, unfolding and insertion of the pore-forming domain. The insertion occurs simultaneously with oligomerization, leading to formation of either a complete pore or a partially formed but active pore [11]. However, the insertion pathways and pore structures of α-PFTs are poorly understood compared to those of the β-PFTs [109].

The first consequence of pore formation by PFTs is host membrane permeabilization and consequent changes in the concentration of ions and small molecules within the cytosol of the target cell, which further lead to the downstream effects [111].

### 1.3.3. Host cell response to pore formation

PFT-induced pore formation in the plasma membrane leads to the primary effects, consisting of rapid efflux of potassium and massive calcium influx into the cell. Calcium, potassium, and in some cases ATP have been considered as the major players in the cellular response to plasma membrane damage [111]. Cytoplasmic potassium decrease serves as a central master regulator for the downstream effects [111]. Upon plasma membrane damage cells either trigger signaling pathways aimed for recovery of plasma membrane integrity and ion homeostasis or they enter into a low-energy-consumption mode. However, this usually happens at low concentration of PFTs. High PFT doses lead to cell death, which, depending on the cell type and toxin, can be apoptotic, necroptotic, necrotic, or pyroptotic [115-118].
Sublytic concentrations of PFTs can activate different sets of signaling pathways, such as autophagy, protein translation arrest and storage of energy in lipid droplets (for energy conservation); the MAPK pathways and inhibition of host protein SUMOylation (for membrane recovery), NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) pathway, the inflammasome, the Unfolded Protein Response, histone dephosphorylation, among others [119]. The cross-talk between all the activated pathways remains unclear. Cells also try to eliminate the toxin and the formed pores by endocytosing them or through bleb formation in a calcium-dependent manner. However, pore elimination occurs only in the case of large pores, caused mainly by CDCs, but not small pores caused by aerolysin or S. aureus α-hemolysin. The pore-containing membrane domain vesiculates outwards, forming a bleb, and is released from the surface [111]. Due to the ability of PFTs to cause cell death, they are attractive targets in oncology, as a new class of therapeutic reagents promoting suicide in malignant cancer cells [112, 113].

1.4. *Vibrio cholerae*

*V. cholerae* is a Gram-negative, facultative anaerobic, curved rod-shaped bacterium, which is the causative agent for cholera disease and gastroenteritis. It belongs to the genus *Vibrio*, family *Vibrionaceae*, class Gammaproteobacteria. It is motile due to a polar flagellum. *V. cholerae* has two chromosomes: large (3.0 to 3.3 Mb), which is more conserved among *Vibrio* spp., and small (0.8 to 2.4 Mb), which has higher genetic plasticity [114].

1.4.1. Epidemiology, etiology and pathogenesis

Cholera is a pandemic disease, and between 1817 and 1961 six pandemics of cholera were recorded. It still remains a dangerous threat in many developing countries. Cholera outbreaks cause estimated 120,000 deaths per year worldwide, and the main affected group is children [115]. *V. cholerae* was first discovered as the cause of cholera in 1854 by Filippo
Pacini, but became widely recognised due to Robert Koch 30 years later. John Snow discovered in 1854 that cholera is transmitted through contaminated water or food, not air.

These pandemics were caused by the O1 lipopolysaccharide serotype, classified into 2 biotypes: classical (O395, Ogawa serotype) and El Tor (C6706, Inaba serotype). However, not all *V. cholerae* strains cause cholera. Non-O1 strains were considered to cause a mild diarrhea, extraintestinal infection or to be avirulent. However, the etiological agent of a new outbreak of cholera in India and Thailand in 1992 was a newly discovered O139 serotype. This serotype is derived from a strain closely related to O1 El Tor as a result of horizontal gene transfer between a non-O1 and an O1 strain [116]. All the other known serogroups of *V. cholerae*, which are not O1 and O139, are recognized as non-O1, non-O139 [117].

During the infection, *V. cholerae* colonizes the small intestine with the help of intestinal colonization factors (including the toxin coregulated pili (TCP), haemagglutinins, accessory colonization factor etc.). It adheres and secretes cholera toxin across the bacterial outer membrane into the extracellular environment, disrupting ion transport by intestinal epithelial cells. Following that, the main manifestation of cholera appears to be watery diarrhea and dehydration. Consequently, the main treatment of cholera is the replacement of fluids and electrolytes [115].

### 1.4.2. Virulence factors

Earlier, cholera was considered a simple and well-understood disease, which symptoms were mainly due to three virulence factors: flagella, which helps the bacteria to swim to mucosal surfaces; pili, which mediate bacterial adherence to the mucosal surfaces; and cholera toxin (CTX), which causes severe diarrhea. However, recent studies showed that the molecular mechanisms of *V. cholerae* colonization and virulence, as well as interaction with the host, are much more complex.
1.4.2.1. Intestinal colonization factors – Tcp and HapA

Tcp pili are virulence factors produced by *V. cholerae*, essential for intestinal colonization and pathogenicity. Tcp mutants are non-virulent in human. Tcp belong to Type IV pili family and mediate attachment to the host cell, protection against bile, and microcolony formation by mediating bacterium-bacterium interactions [118]. Additionally, Tcp serve as receptors for bacteriophage CTXΦ, which carries the gene for CTX [119]. In addition, Tcp play a role in bacterial serum resistance, protecting *V. cholerae* from complement-mediated cytolysis [120].

*V. cholerae* secretes the zinc- and calcium dependent metalloprotease haemagglutinin/protease (HapA). It is important for *V. cholerae* to detach from shedding mucosal cells and reattach to new mucosal cells. In this process, HapA is responsible for the detachment of bacteria from the cells by digesting several putative receptors for *V. cholerae* adhesins. Moreover, HapA causes hemagglutination of erythrocytes, and degrades a number of host proteins, i.e. mucin, fibronectin, lactoferrin, and even the A subunit of CTX itself [121].

1.4.2.2. Cholera toxin CTX

Cholera toxin is the major virulence factor, an A-B type ADP-ribosylating toxin, consisting of one A (enzymatic) subunit and a pentameric ring of B (binding) subunits. The A subunit consists of A1 and A2 domains linked by a disulfide bond. A and B subunits are assembled in the periplasm and CTX is secreted via T2SS [122]. B subunits bind to G_{M1} ganglioside receptors on the lipid rafts of enterocytes and the CTX complex is delivered into the endoplasmic reticulum (ER) of the host cell via retrograde transport. The catalytic A1 subunit is then released from the toxin, exits the ER and ADP-ribosylates GTPase G protein, which stimulates the host cell adenylate cyclase activity and, therefore, raises the cAMP level within the host cells. As a result, a rapid flow of Cl⁻ ions through cAMP-dependent Cl⁻ channels followed by Na⁺ ions and water passive flow from the extracellular fluid into
the bowel lumen leads to a severe watery diarrhea, which can reach up to 20 litres per 24-hour period [123].

1.4.2.3. *V. cholerae* cytolysin VCC

*V. cholerae* cytolysin (VCC), also known as hemolysin A (HlyA), because it causes sheep erythrocyte hemolysis, is encoded by the *hlyA* gene. It plays a major role in *V. cholerae* pathogenesis, especially in non-CTX-producing strains. VCC forms anion channels on the apical membrane of enterocytes and induces efflux of chloride from human intestinal mucosa, thus contributing to diarrhea [124]. It belongs to the family of β-barrel pore-forming toxins (β-PFTs). The water-soluble VCC monomer is secreted as an inactive 79-kDa pro-VCC, and upon proteolytic cleavage by different proteases (i.e. HapA, host proteases), which remove an N-terminal segment, forms a mature active 63-kDa toxin. VCC hemolysin is closely related to Gram-positive staphylococcal cytolysins (α-toxin and leukocidin) and also aerolysin, since they have a common cytolytic core and putative membrane-spanning region. Interestingly, VCC has a lectin domain, which is not present in other cytolysins [125].

Upon binding as a monomer to the target cells, in the presence of cholesterol, VCC oligomerizes, forming heptameric oligomers, inserting into the membrane and creating transmembrane pores ranging in size between 1.2 to 1.6 nm [126]. Pore formation leads to cytotoxic activity, causing cell death in a range of cell lines, either by colloid osmosis due to ion disbalance or through apoptosis induction in a caspase-dependent manner [127, 128]. Sublytic concentrations of VCC, however, activate a range of responses in host cells and trigger pathways directed to promote cell survival [129]. One survival strategy of cells is to activate autophagy against VCC, directed to degrade VCC within the autophagolysosomes. Gutierrez *et al*. demonstrated that secreted VCC induced an extensive vacuolation in epithelial cells, which is likely derived from double-membrane LC3-labeled autophagic vesicles. Vacuolation was not induced in *Atg5−/−* cells, indicating autophagy involvement. Autophagy blocking showed decreased survival of Caco-2 cells
upon VCC treatment, suggesting that autophagy acts as a cell defence response against pore-forming toxin VCC [130].

VCC is present in almost all *V. cholerae* strains, including those lacking Tcp and CTX. Besides acute gastroenteritis, non-O1, non-O139 strains can cause extraintestinal infections, wound infections, meningitis, bacteremia, skin and soft tissue infections, pneumonia, acute cholecystitis, endophthalmitis, peritonitis, urinary tract infection, splenic abscess, liver abscess, intracerebral abscess, among other infections [131, 132]. This makes the role for VCC, especially in non-cholera toxigenic strains, particularly interesting and important for pathogenesis.

1.4.2.4. PrtV protease

*V. cholerae* is a natural inhabitant of estuarine and coastal waters and can survive in different conditions, even in the presence of predators, such as flagellates and ciliates. PrtV protease, secreted by *V. cholerae*, plays a major role for the survival of bacteria in this environment.

The PrtV protease is an extracellular Zn$^{2+}$-binding metalloprotease that belongs to the M6 metalloprotease family. The PrtV-encoding gene is located on chromosome II. PrtV is synthesized as a 102-kDa pre-pro-protein, but undergoes N- and C-terminal processing steps during *V. cholerae* envelope translocation and prolonged incubation. The full-length PrtV contains an M6 peptidase domain, a zinc-binding domain and two C-terminal Polycystic Kidney Disease (PKD) domains, as represented in Figure 4. Purified processed PrtV pro-protein has a size of 81 kDa, which upon calcium ion removal results in rapid autoproteolysis. Calcium-dependent autoproteolysis leads to formation of the catalytically active 37-kDa and 18-kDa M6 domains. Crystallisation of the PKD1 domain revealed a Ca$^{2+}$-binding site near the linker regions between domains, where Ca$^{2+}$ binds and controls domain linker flexibility. Therefore, the presence of Ca$^{2+}$ is important for PrtV stabilization [133-135]. The role of PKD domains in *V. cholerae* is not clear, since not many studies have been done on PrtV. Originally, the PKD domain was found in polycystin-1, a large eukaryotic cell-surface glycoprotein potentially involved in protein-protein and protein-
carbohydrate interactions [136]. PKD domains are also found in other bacterial species as structural parts of collagenases, cellulases and chitinases [137, 138].

Figure 4. Schematic representation of domain organisation for *V. cholerae* PrtV protease. The full-length 102-kDa (1-918 amino acids) pre-protein, processed 81-kDa pro-protein and autoproteolytic 37 and 18-kDa forms are shown.

The PrtV protease was shown to be important for environmental survival and during infection. PrtV is essential for *V. cholerae* survival from grazing by the flagellate *Cafeteria roenbergensis* and the ciliate *Tetrahymena pyriformis*. Moreover, PrtV is essential for killing the nematode *Caenorhabditis elegans* upon intestinal colonization [139]. During infection of human intestinal HCT8 cells, PrtV showed a cytotoxic effect leading to cell death, probably due to degradation of substrate proteins in host tissues leading to cell rounding and detachment. Among some of the identified host substrate proteins degraded by PrtV are the extracellular matrix and fibrin system components fibrinogen, fibronectin and plasminogen [133]. Interestingly, another substrate for PrtV is VCC secreted by *V. cholerae*. Controlling VCC activity allows bacteria potentially to modulate hemolysis of erythrocytes and inflammatory responses, maintaining low reactogenicity inside the host [140]. Interestingly, the PrtV protease contributes to biofilm reinforcement by processing the biofilm matrix RbmA protein. Processed RbmA binds bacterial cells lacking exopolysaccharide, which results in recruitment of new cells to the surface where biofilm is formed [141]. In our study, we focused more on analysis of
PrtV secretion in order to gain a deeper understanding of the role(s) of PrtV during infection.

1.4.3. Regulation of virulence

Virulence and biofilm formation in *V. cholerae* are tightly controlled through a regulatory hierarchical network of transcriptional activators (ToxT, AphA, VpsR and VpsT), transcriptional repressors (HapR and H-NS), RNA polymerase sigma factors (RpoN, RpoS and RpoE), small RNAs and autoinducers. Being an aquatic bacterium and intestinal pathogen, *V. cholerae* needs to survive in environments differing in: temperature, osmolarity, bile salts, pH, nutrient availability etc. [142]. Therefore, the first step in virulence regulation is the ability to sense environmental cues. In response to environmental stimuli, hierarchical chain of events leads to an activation of ToxT. ToxT activates the transcription of the major virulence factors CTX and Tcp through DNA binding. Expression of ToxT is dependent on transcriptional activators ToxR and TcpP. Transcriptional activators ToxR, in conjunction with ToxS, and TcpP, in conjunction with TcpH, work synergistically to regulate expression of ToxT. The expression of tcpP, in its turn, is activated by the cytoplasmic regulatory proteins AphA and AphB, which bind to different regions of the tcpP promoter (Fig. 5)[143].

![Diagram](image)

**Figure 5. Overview of the connection between virulence regulation, biofilm and quorum sensing in *V. cholerae*. Modified from [144].**

AphA serves as a link between quorum sensing and virulence gene expression, since its expression is negatively controlled by HapR [143]. HapR, in turn, is the main negative regulator not only of virulence but also of biofilm formation. HapR activates a few genes, such as those encoding
HapA, the PrtV protease and haemolysin co-regulated pili (Hcp), but downregulates VCC and biofilm formation (Fig. 5). Quorum sensing (QS) plays a role in biofilm formation by *V. cholerae*. At low bacterial cell densities, with low autoinducer concentrations, the membrane-bound sensor histidine kinases LuxQ and CqsS initiate a phosphorelay event that results in phosphorylation and activation of the response regulator LuxO. Phosphorylated LuxO and RpoN activate transcription of small QS regulatory RNAs, which together with the RNA chaperone Hfq, destabilize mRNA of *hapR*, resulting in low HapR levels at low cell densities. Virulence gene expression is then increased. In contrast, at high cell densities, HapR is active; therefore, biofilm and virulence genes are repressed [142]. In addition, *V. cholerae* MVs then contain more peptidoglycan, which induces an inflammatory response in the host cell during infection [86]. Therefore, *V. cholerae* tightly regulates gene expression and uses bacterial cross-talk to regulate genes involved in virulence versus biofilm formation.
1.4.4. MV production by *Vibrio cholerae*

*V. cholerae* produces an abundant amount of outer membrane vesicles (OMVs) during growth (Fig. 6). Besides contributing to biofilm formation, *V. cholerae* OMVs have many other functions.

![Electron micrograph representing MV release from *V. cholerae*.](image)

The picture was taken using *V. cholerae* O1 El Tor strain A1552 by Akemi Takade, Kyushu University, Fukuoka, Japan. Scale bar 100nm.

MVs carry biologically active proteases HapA and VesC, which are transported to epithelial cells and cause a range of cytotoxic and inflammatory responses (apoptosis, necrosis, enterotoxicity of mouse ileal loop) depending on the cell type [145]. OMVs induce pro-inflammatory responses via NOD1 activation, causing MAPK and NF-κB pathways activation, as well as dendritic cell activation, which primes CD4⁺ T cells and induces the Th2/Th17 pathways [146]. OMV-associated DegP protease, a periplasmic chaperone involved in the processing and maturation of proteins, selectively influences the composition of OMVs and bacterial virulence [147]. Apart from proteins, *V. cholerae* OMVs also carry RNAs.
originating from intergenic regions, whose targets remain unknown so far [55]. Since V. cholerae OMVs induce an immunogenic response, they have been tested as vaccine antigen candidates against cholera [101, 148].

1.5. *Listeria monocytogenes*

*L. monocytogenes* is a Gram-positive, facultative anaerobic, non-spore-forming, motile, rod-shaped bacterium discovered in 1926. The bacterium belongs to the genus *Listeria*, family *Listeriaceae*, class *Bacilli*, and phylum *Firmicutes*. *L. monocytogenes* is motile at 30°C and below, due to its flagella; but at 37°C it moves within the eukaryotic cells through polymerization of actin filaments.

1.5.1. Epidemiology, etiology and pathogenesis

*L. monocytogenes* is an opportunistic food-borne pathogen, an etiologic agent of listeriosis. It is particularly dangerous for immunocompromised patients, the elderly, and pregnant women; in these high-risk groups its fatality rate is up to 30%. In the European Union, human listeriosis cases increased by 74% from 2001 to 2006, and have remained stable since then [149].

As a food-borne pathogen, *L. monocytogenes* is acquired via ingestion of contaminated food: raw and smoked meat and fish products, unpasteurized dairy products, soft cheeses, and ready-to-eat food. *Listeria* can grow at refrigerator temperatures and can even survive at -20°C. The number of increased listeriosis outbreaks is probably due to a change in food consumption and the availability of more products with a longer shelf life: “ready-to-eat” and “ready-to-cook” products.

In healthy individuals, *L. monocytogenes* can cause febrile gastroenteritis with short incubation times, flu-like symptoms and muscle ache. If infection spreads to the nervous system, headache, stiff neck, loss of balance or convulsions can occur. However, in a risk group of patients it can
be a cause of perinatal infections, septicemia, encephalitis, meningitis, meningoencephalitis, brain abscess, miscarriage and stillbirth [150].

1.5.2. **Intracellular life cycle of* L. monocytogenes**

Before reaching the intestine, *Listeria* is able to partially withstand the harsh acidic environment of the stomach. Next, *L. monocytogenes* invades the intestinal epithelium and penetrates the intestinal barrier. Bacteria that crossed this barrier are carried through the blood/lymphatic system and can infect the mesenteric lymph nodes, liver and spleen. The bacterium is able to cross the placental and blood-brain barrier as well, causing the above-mentioned manifestations [151].

1.5.2.1. **Internalization**

*L. monocytogenes*, being a facultative intracellular bacterium, is able to survive inside macrophages and non-phagocytic cells, such as epithelial cells, fibroblasts, hepatocytes, nerve cells and endothelial cells. Internalization starts by adhering to the eukaryotic cell surface and further cell invasion using the "zipper" mechanism. *L. monocytogenes* can recognize a wide range of host receptors, including the transmembrane glycoprotein E-cadherin, the hepatocyte growth factor receptor Met, the C1q complement component receptor and components of the extracellular matrix, such as heparan sulfate proteoglycans and fibronectin [152-155].

1.5.2.2. **Intracellular replication and cell-to-cell spread**

Once internalized, *L. monocytogenes* becomes surrounded by a tight phagocytic vacuole, which it can lyse with the help of listeriolysin O (LLO), and subsequently escape into the host cell cytoplasm facilitated by phospholipases. In the cytoplasm, the bacteria have a higher replication rate. Further, to be able to spread from one cell to another, *L. monocytogenes* uses the protein ActA. ActA rearranges the actin cytoskeleton, forming an actin "comet tail" at one of the bacterium poles, which helps to propel the bacterium to the cell periphery. Upon internalization into neighboring
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The Listeria bacteria become entrapped in a double-membrane phagosome, from which it escapes to restart its life cycle [151]. The Listeria-containing phagosome fuses with the lysosome and becomes acidified, due to hydrogen ions from the cytoplasm, which leads to degradation of the bacteria. LLO can block phagosome–lysosome fusion by generating membrane pores that uncouple pH and calcium gradients across the phagosome membrane [156].

Different fates of bacteria can occur during the intracellular life cycle: degradation inside the autophagolysosomes; escape from the phagosome; or survival and slow replication inside non-acidic, non-degradative, spacious Listeria-containing phagosomes (SLAPs) [157].

1.5.3. Virulence factors

1.5.3.1. Internalins

Internalin A (InlA) and B (InlB) are surface-exposed virulence factors belonging to the family that comprises 23 additional internalins [152]. InlA and InlB are essential for L. monocytogenes adhesion and invasion within non-phagocytic cells. InlA interacts with the receptor E-cadherin on the host cell surface, whereas InlB interacts with the Met receptor. InlA mediates stringent cell tropism, since E-cadherin is expressed by limited cell types [158]. Meanwhile, InlB mediates bacterial internalization into a wide range of cell types, since the Met receptor is ubiquitously expressed in many cell types [152]. Another internalin InlC, secreted during the infection, dampens the host innate immune response by interacting with the NF-κB pathway and preventing translocation of NF-κB into the nucleus [159].

1.5.3.2. Listeriolysin O

LLO, encoded by hly gene, is a pore-forming toxin of the cholesterol-dependent cytolysin family and a key virulence factor of L. monocytogenes. LLO plays multifaceted roles during intracellular infection. Pore formation causes an ion disbalance (potassium efflux, calcium influx) and triggers a cascade of reactions in the eukaryotic cell. LLO potentiates bacterial entry inside the host cells, induces autophagy, delays phagosome maturation,
inhibits phagosome-lysosome fusion, mediates primary and secondary phagosome lysis, inhibits reactive oxygen species (ROS) production through preventing localization of NADPH oxidase to phagosomes, causes histone modifications, inflammasome and NF-κB activation, mitochondrial fragmentation and spacious Listeria-containing phagosomes (SLAPs) formation [157, 160-164].

Within the host cell, the activity of LLO is tightly regulated; LLO activity is increased inside the phagosome due to the acidic pH and decreased inside the cytosol in order to prevent lysis of the host cell membrane and loss of its intracellular niche, where bacteria can survive and replicate [165]. LLO activity is also regulated by the bacterium through an N-terminal PEST-like sequence (P, Proline; E, Glutamic acid; S, Serine; T, Threonine) of LLO, which is essential for bacterial virulence and intracellular compartmentalization, possibly through phosphorylation of its PEST residues. The PEST-like sequence targets LLO for degradation, and thus inactivation, within the host cell cytosol, as a mechanism for controlling LLO levels in the cytosol, and restricts LLO activity to the host-cell vacuole [166]. Nonetheless, intracellular LLO is ubiquitinated and rapidly degraded via host proteasomes independently from the PEST-like sequence [167].

Activity of LLO is cell-type dependent and is regulated additionally by host cell factors. Thus, two factors highly expressed in macrophages can mediate LLO activity: a lysosomal thiol reductase GILT (γ-interferon-inducible lysosomal thiol reductase), which activates LLO by maintaining it in a reduced state, and a chloride channel CFTR (cystic fibrosis transmembrane conductance regulator), which transiently increases phagosomal chloride concentration, potentiating LLO pore formation and vacuolar escape of bacteria [168, 169].

Being a hemolysin and cytolysin, LLO mediates hemolysis of erythrocytes and cell fate. Depending on the cell type and concentration of LLO, it can cause different types of cell death. At non-lytic concentrations, LLO can activate caspase-dependent (rapid) and independent (slow) apoptotic pathways in T cells, dendritic cells and lymph node lymphocytes [170-173].
Additionally, the LLO molecule is immunogenic and a major source of CD4 and CD8 T cell epitopes during *Listeria* infection. Together with its cytotoxic properties, LLO has become a promising anti-tumor vaccine candidate, used as an effective adjuvant for tumor immunotherapy [174, 175].

### 1.5.3.3. Phospholipases

In addition to LLO, *L. monocytogenes* secretes two phospholipases C (PLCs) that contribute to bacterial virulence and the ability to escape into the cytosol. PlcA or phosphatidylinositol-specific PLC (PI-PLC) is encoded by the *plcA* gene, whereas PlcB or a broad-spectrum PLC (PC-PLC) is encoded by *plcB* gene. The latter hydrolyzes phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and sphingomyelin, and is synthesized as a pro-enzyme, activated by a secreted zinc metalloprotease [151]. Recent studies showed that PlcA and PlcB contribute to *L. monocytogenes* escape from host autophagy by inhibiting autophagic flux and pre-autophagosomal structure maturation [176].

### 1.5.3.4. ActA

After escape from the phagosome, *L. monocytogenes* recruits the ActA protein, which hijacks the host cell actin cytoskeleton. ActA, encoded by the *actA* gene, binds and activates the Arp2/3 complex, catalyzing actin nucleation. The protein promotes actin assembly at one pole of the bacterium, forming actin “comet tails”, which mediate bacterial motility and spreading from one cell to another *in vivo* [177]. Binding to the Arp2/3 complex occurs via the amino-terminal ActA domain, while the central ActA domain recruits members of the Ena/VASP family, which control the efficiency of bacterial motility [178].

ActA-mediated recruitment of both the Arp2/3 complex and Ena/VASP to the bacterial surface disguises *Listeria* from autophagic recognition, protecting the bacterium from ubiquitination and further
autophagic degradation. This mechanism represents one of *L. monocytogenes* anti-autophagic strategies for survival inside host cells [179].

**1.5.4. Regulation of virulence**

To be able to survive under different conditions, ranging from the environment (in the saprophytic state) to inside the host (in the pathogenic state), expression of virulence genes in *L. monocytogenes* must be tightly regulated. Alternative sigma factor (σ^B^) and Positive regulatory factor A (PrfA) are the main transcription regulatory factors controlling a subset of virulence and stress response genes. Stress-related factor σ^B^ is restricted to the gastrointestinal phase before entry into the host cell, whereas PrfA is activated mainly in the blood and during the intracellular phase of the bacterial life cycle [180]. Sigma B, encoded by the *sigB* gene, within RNA polymerase, is recruited to the target promoter region leading to gene expression. Modulation of σ^B^ activity happens through anti-sigma and anti-anti-sigma factors [181].

PrfA belongs to the cAMP receptor protein (Crp)/fumarate nitrate reductase regulator (Fnr) family of bacterial transcription factors and influences directly or indirectly expression of 145 genes, including transporters, metabolic enzymes, regulators, and proteins of unknown function. Among these genes are key virulence determinants of *Listeria* located along with prfA on the *Listeria* pathogenicity island-1 (LIPI-1): *hly*, *inLA*, *inLB*, *inLC*, *actA*, *plcA*, *plcB* and *mpl* [180].

Interestingly, expression of the *inLA* and *inLB* genes is controlled by both PrfA and σ^B^ [151, 182]. Since PrfA is a master regulator and essential for virulence, its regulation occurs at different levels. Three promoters control PrfA expression at the transcriptional level: PplcA, P1 and P2, where the P2 promoter is regulated by transcription factor σ^B^ [180]. At the translational level, a transcript from the P1 promoter has a thermoswitch that at 37°C allows the ribosome to bind to the Shine-Dalgarno sequence and initiate translation, which is inhibited otherwise at 30°C [180]. Post-translationally, PrfA expression changes during starvation conditions and in the presence of non-phosphorylated sugars [183].
1.5.5. MVs production by *Listeria monocytogenes*

Not much is known about MV production by *L. monocytogenes*. Lee and colleagues found that the wild-type strain produced nine times more vesicles than a ΔsigB strain, highlighting the importance of transcription factor σB in MV production. Proteomic analysis identified 130 proteins in wild-type MVs and 89 in sigB mutant MVs. Among the σB-dependent proteins, there were transporters (OpuCA and OpuCC), proteins involved in stress response (Kat), metabolism (LacD), translation (InfC), and cell division (FtsZ). Importantly, InlB and LLO were found to be secreted from the bacterium in association with MVs [61].

With the help of scanning electron microscopy (SEM) we have shown MVs released from the surface of *L. monocytogenes*, predominantly at the sites of bacterial division (Fig. 7).

![Figure 7. Scanning electron micrograph representing MVs release from *L. monocytogenes*. The picture was taken by Cheng Choo Lee, UCEM, Umeå University, Sweden. Scale bar 500 nm.](image-url)
1.6. Autophagy

1.6.1. Cell fates during infection

As a result of infection, eukaryotic cells can undergo different types of cell death. The most studied types of cell death are necrotic, apoptotic, autophagic and pyroptotic types. Cell death is usually determined through cell morphology, enzyme activity, functional or immunological aspects of cell processes [184]. Necrosis is a traumatic uncontrolled type of cell death, where cells demonstrate swelling of organelles, plasma membrane rupture and cell lysis [184].

Apoptotic cell death is a type of programmed cell death, which involves rounding up of the cell, nuclear fragmentation, chromatin condensation and formation of apoptotic bodies. Apoptosis can be initiated either through the intrinsic (intracellular mechanism during cell stress, mitochondria-mediated; caspase-8-dependent) or extrinsic (extracellular signalling, cell-surface death receptor-mediated; caspase-9 dependent) pathways [185]. Using the caspase-inhibitor zVAD-fmk is a common way to differentiate apoptosis from the other types of cell death.

Besides caspase-dependent apoptosis, programmed cell death can include caspase-independent apoptosis, necroptosis and autophagic cell death [186]. To identify necroptosis, it is common to use the necroptosis inhibitor Necrostatin-1 (Nec-1) [184]. Similar to apoptosis, necroptosis involves death-domain-receptor activation, however, necroptosis is not regulated by caspases and requires RIP1 and RIP3 kinases [187]. Pyroptotic cell death involves activation of caspase-1 or caspase-5 and IL-1β-induced inflammation, which exhibits morphological features of both apoptosis and necrosis [188]. Autophagy is mainly a pro-survival strategy of cells, directed to maintain cell homeostasis through degradation of unnecessary molecules or organelles during starvation, stress or infection. However, autophagy can also promote cell death. Autophagic cell death is characterized by a massive autophagic vacuolization of the cytoplasm in the absence of chromatin condensation. It can be unclear, however, whether the cell death is
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autophagy-associated or autophagy-induced. In order to identify autophagy-induced cell death, one can use either pharmacological or genetic inhibition of autophagy [189].

1.6.2. The process of autophagy

Autophagy (in Greek: “self-devouring”) is an evolutionarily conserved catabolic pathway inside cells, which through the formation of autophagosomes mediates the degradation and recycling of cytoplasmic components, protein aggregates and organelles. Starvation, nutrient deprivation, growth factor depletion and hypoxia, as well as infection, are known to activate autophagy. However, a basal level of autophagy plays an important role in maintaining healthy cells [190].

1.6.2.1. Autophagosome biogenesis and degradation

The main feature of autophagy is the formation of an autophagosome. The process starts with isolation of the autophagic membrane (phagophore), its elongation and closure, and engulfment of intracellular cargo while forming a double-membraned autophagosome. Further, the autophagosome fuses with the endosome, forming an amphisome, prior to fusion with the lysosome to form autolysosome, where lysosomal hydrolases degrade the autophagosomal content. The degradation products are transported further into the cytoplasm, where they are re-used for further metabolism. Therefore, autophagy is a self-recycling process supplying energy to the cell; it plays a central role during stress conditions, bacterial and virus infection, aging, inflammation, immunity, tumour suppression and genome stability (Fig. 7) [190-193]. Autophagy involves more than 30 autophagy-related proteins (Atg), which are associated with different steps of the autophagy pathway.

1.6.2.2. Autophagy regulation

Mammalian target of rapamycin (mTOR), a serine/threonine kinase, is the negative master regulator of autophagy. mTOR forms two complexes:
the mTORC1 complex, which is important for cell growth and proliferation, biosynthesis of proteins, lipids and organelles; and the mTORC2 complex, which participates in cell survival, metabolism, proliferation and cytoskeleton organization. Downstream targets of mTORC1, which are phosphorylated by active mTOR, are the eukaryotic initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) and the p70 ribosomal S6 kinase (p70S6K). These targets are important key players in protein synthesis. Phosphorylation of 4E-BP1 prevents its binding to eIF4E, allowing eIF4E to stimulate cap-dependent translation, whereas phosphorylation of p70S6K results in increases in mRNA biogenesis, cap-dependent translation and elongation, and the translation of ribosomal proteins [194].

During cell starvation or treatment with mTOR inhibitor (rapamycin, Torin1), mTOR inhibition leads to the activation of the ULK1-Atg13-FIP200 complex, which induces autophagosome formation [195]. In response to lowered cellular ATP levels and ATP/AMP ratio, activation of adenosine 5′-monophosphate (AMP)-activated protein kinase (AMPK), which is upstream of mTOR, leads to activation of the ULK1 complex as well [196].

Class III PI3 kinases, such as Vps34 (vesicular protein sorting 34) and binding partner Beclin-1, are important for phagophore elongation and a rise in PI3P level, using phosphatidylinositol (PI) as a substrate. Subsequently, PI3P generation leads to more Atg proteins recruited. The Atg12-Atg5-Atg16L ubiquitin-like conjugation complex when formed, promotes closure of the autophagosome. Later, when the phagosome is formed, Atg5–Atg12–Atg16L is dissociated from the membrane. Another ubiquitin-like system involved in phagosome formation includes microtubule-associated protein light chain 3 (LC3B), a mammalian homologue of Atg8 in yeast. Upon autophagy stimulation, LC3B is proteolytically cleaved by cysteine protease Atg4, generating LC3-I. LC3B is lipidated by binding to phosphatidylethanolamine (PE) in the autophagosomal membrane, being, thereby, converted from LC3-I (non-lipidated; cytosolic form) to LC3-II (lipidated; membrane-bound form). For this reason, LC3 is commonly used as autophagy marker (Fig. 8) [190].
Independent from the mTOR pathway, during starvation, phosphorylation of the eukaryotic initiation factor 2α (eIF2α) by a conserved family of protein kinases leads to a decreased protein translation and induced autophagy [192].

![Image of autophagy process]

**Figure 8. Schematic overview of autophagy process.** Several steps of autophagy are shown including key proteins associated with each of these steps.

1.6.2.3. Different types of autophagy

Depending on how the material for degradation is delivered to lysosomes, autophagy is classified in three forms: macroautophagy (xenophagy), microautophagy and chaperone-mediated autophagy. Macroautophagy, or autophagy, where the autophagosome fuses with the lysosome, is described in 1.6.2.1. In microautophagy, cellular components are directly sequestered by the lysosome through invagination of the lysosomal membrane. In chaperone-mediated autophagy, heat-shock cognate proteins deliver substrates to lysosomes for degradation [189].

Additionally, sometimes formation of autophagosomes does not involve the whole subset of Atg proteins. This type of autophagy is called
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non-canonical. Unlike canonical autophagy described in 1.6.2.2., non-canonical autophagy can occur without some proteins involved in elongation and closure (ATG7, ATG5 and LC3) or proteins important for initiation (ULK1) and nucleation (Beclin-1). In certain cases of non-canonical autophagy, autophagosome formation can bypass initial steps including AMPK–mTORC1–ULK1 [196, 197]. LC3-associated phagocytosis (LAP) involves the direct recruitment of LC3 to single-membrane phagosomes, which has been shown for E. coli, S. typhimurium, B. pseudomallei and L. monocytogenes infections [198].

1.6.2.4. Methods for monitoring autophagy

In order to monitor autophagy, guidelines have been developed for the use and interpretation of autophagy assays. Several methods are well-established and frequently used for identifying and visualizing different steps of autophagy. Among them the most commonly used are:

- visualizing autophagic vacuoles by transmission electron microscopy (TEM);
- Atg8/LC3 detection via fluorescence microscopy (through GFP-LC3 puncta) or immunoblotting (as LC3-I to LC3-II conversion);
- Atg8/GABARAP detection;
- measuring autophagic flux (LC3 turnover in the presence of lysosomal inhibition or interference with autophagosome-lysosome fusion);
- mTOR and its downstream targets 70S6K and 4E-BP1 phosphorylation, AMPK and Atg1/ULK1 phosphorylation;
- identification of additional autophagy-related protein markers [199, 200].

Additionally, a recently developed and efficient method for monitoring autophagy is a macroautophagic cargo sequestration assay. The assay measures macroautophagic activity through transfer of the autophagic cargo marker enzyme lactate dehydrogenase (LDH) from the cytosol to autophagic vacuoles [201, 202].
1.6.3. Autophagy in infectious diseases

The role of autophagy in infectious diseases remains a subject of intense interest. Cells use autophagy as a host cell defence to eliminate pathogens. However, many bacterial pathogens have developed strategies to evade or subvert autophagy.

One of the common ways for bacteria to avoid being killed by autophagy is to escape into the cytoplasm by disrupting the surrounding vacuole, a strategy used, for example, by *B. pseudomallei, L. monocytogenes, Mycobacterium marinum, Rickettsia conorii* and *Shigella flexneri*. Unlike *Shigella*, which directly inhibits autophagic proteins, *Listeria* recruits host cell proteins in the cytosol that mask the bacterium from autophagic recognition [179, 203, 204].

Some invasive bacterial pathogens can benefit from autophagy by establishing an intracellular niche, a strategy employed by *L. pneumophila, Coxiella burnetii* and *Brucella abortus*. Blocking or altering phagosome maturation allows bacteria to reside in vacuoles. Despite *S. aureus* being an extracellular pathogen, it can also prevent the maturation of the autophagosomes, avoiding both acidification and fusion with the lysosomes, and allowing it to establish persistent and chronic infection [205]. Similarly, *L. pneumophila* resides in ER-derived vacuoles and uses the RavZ effector to irreversibly deconjugate PE from LC3 [203]. *Anaplasma phagocytophilum* inhibits phagosome-lysosome fusion, exhibiting another strategy to avoid destruction by autophagy. While, *C. burnetii* delays fusion of bacteria-containing phagosomes and lysosomes, favouring its own replication [205].

1.6.4. Autophagy induced by pore-forming toxins

Activation of autophagy is a common defense mechanism of the host cell against bacterial pore-forming toxins. Pore formation in the plasma membrane leads to a loss of potassium, which acts as a master activator of host responses, including autophagy. In fact, amino acid starvation and energy loss lead to AMPK phosphorylation, as well as eIF2α phosphorylation
via GCN2 and PKR kinases, which are hallmarks of PFT-activated autophagy for *S. aureus* α-toxin, *V. cholerae* VCC, LLO, SLO, and *E. coli* hemolysin HlyA [162, 176, 206, 207].

1.6.5. Importance of autophagy in health and disease

Autophagy plays an important role not only in infection, but also in inflammation, innate and adaptive immunity, autoimmunity, and pathogenesis of many diseases. Induced by PRRs, autophagy regulates interferon production, inflammasome activation, contributes to antigen presentation, B-cell survival, lymphocyte development and T-cell homeostasis. Dysfunctions in Atg genes are linked to human diseases, such as Parkinson’s disease, Crohn’s disease, systemic lupus erythematosus and cancer [208-211]. Interestingly, autophagy plays different roles at different stages of cancer: tumour prevention or suppression at the early stage, but once the tumour is developed, autophagy can be used by cancer cells for their cytoprotection and tumour progression [212].
2. Aims of the thesis

The main objective of this thesis was to study the transport and functions of virulence factors associated with membrane vesicles of Gram-negative and Gram-positive bacteria, using *Vibrio cholerae* and *Listeria monocytogenes* as models.

The more specific aims were:

- To study the transport and biological activity of PrtV metalloprotease associated with OMVs produced by *Vibrio cholerae*;

- To investigate secretion and biological activity of *Vibrio cholerae* pore-forming cytolysin VCC associated with OMVs;

- To elucidate roles of *Listeria monocytogenes* MVs in autophagy and cell death associated with pore-forming toxins.
3. Results and discussion

3.1. Paper I. Outer membrane vesicle-mediated export of processed PrtV protease from *Vibrio cholerae*

Bacteria produce OMVs, long-distance vehicles that deliver virulence factors in a protected manner to neighbouring bacterial cells or host eukaryotic cells, where they cause a range of biological effects [67, 76, 85, 145, 213, 214]. In this study, we investigated the mechanism of PrtV translocation and secretion in *V. cholerae*, its biological activity in association with OMVs and its role in *V. cholerae* resistance against host antimicrobial response.

PrtV, a Zn$^{2+}$-binding metalloprotease from the M6 family, was earlier discovered in our laboratory as a factor needed for *V. cholerae* survival against the flagellates and ciliates in natural environment, as well as the factor necessary for *C. elegans* killing [139]. However, the function of PrtV is not limited only to the environmental setting; it is also important during infection. Previous studies demonstrated that PrtV is biologically active, although the mechanism of its secretion remained unknown.

In this study, we isolated OMVs from different *V. cholerae* strains, including non-O1, non-O139, O1 El Tor clinical isolates the classical O1 strain and environmental O1 isolates. Interestingly, PrtV secretion in association with OMVs was detected in all of these strains, suggesting that it is not strain specific, but rather an evolutionarily conserved process. Moreover, most of the secreted PrtV fraction is associated with OMVs upon secretion. Using the O1 El Tor C6706 clinical isolate for OMV isolation and gradient-density purification, we determined that PrtV is tightly associated with OMVs. The association with OMVs was visualised by electron microscopy with immunogold labeling using wild-type and a ΔprtV strain. This result was additionally confirmed by proteinase K susceptibility assay, which revealed that the 81-kDa full-length PrtV protein is localised on the external OMV
surface, whereas proteolytically cleaved 37-kDa PrtV form is entrapped inside the OMV lumen, probably upon discharging from the bacterial surface. As reported earlier, the inactive PrtV pro-protein is autoproteolytically cleaved upon removal of calcium ions, generating an active 37-kDa form and an unstable 18-kDa form. This mature 37-kDa protein is sufficient for cleaving PrtV substrates, such as fibrinogen [134]. In line with this report, our results suggest that OMVs deliver to the target site an active PrtV form that is protected from proteases.

During bacterial fractionation, we detected the 37-kDa PrtV in the periplasmic fraction by immunoblotting, which suggested that full-length PrtV might be cleaved in the periplasmic space. Cholera toxin, proteases and chitinases of *V. cholerae* are translocated via T2SS [215]. Similar to the above-mentioned proteins, we observed that PrtV secretion is abolished or decreased in T2ss mutants and restored upon complementation. Therefore, based on our data, we suggest that PrtV undergoes autoproteolytic cleavage in the periplasm, resulting in the truncated 37-kDa version. This truncated PrtV is entrapped within the OMV lumen during its formation. Meanwhile, uncleaved PrtV is translocated from the periplasm through the outer membrane into the extracellular milieu via T2SS, and further might become re-associated on the surface of OMVs.

Structurally, PrtV consists of the M6 metalloprotease domain, as well as PKD1 and PKD2 domains. Crystallography revealed a Ca$^{2+}$-binding site in the PKD1 domain, which controls domain linker flexibility and PrtV stability. The role of the PKD2 domain remains unknown [135]. Therefore, in our study we aimed to investigate the role of PKD domains in PrtV reassociation on the OMV surface, considering especially that PKD domains might be involved in protein-protein and protein-carbohydrate interactions based on the predicted domain structure. Interestingly, the processed 37-kDa form was associated with OMVs independently from PKD domains. However, the full-length 81-kDa form was detected as a free, secreted but not OMV-associated form in the absence of PKD domains. Together, our results
suggest that PKD domains are essential for PrtV reassociation on the OMV surface.

Pure PrtV has biological activity as demonstrated by the degradation of plasma components and HCT8 cell cytotoxicity [133]. Considering that the active PrtV form is mainly transported by OMVs, we asked the question of whether OMV-associated PrtV is biologically active, i.e., whether it has any biological significance. OMVs entered the HCT8 cells after 1 hour, demonstrating cytotoxicity only after 12 hours, as indicated by cell rounding and detachment. Morphological cell changes occurred in response to the 37-kDa form, which was delivered by OMVs to the cells. It would be of interest to detect whether this biological effect happens due to proteolysis or due to initiation of programmed cell death. In either case, it would be important to detect novel substrates for PrtV or initiation cascade(s) of cell death.

Interestingly, *Vibrio spp.* use OMVs to trap antimicrobial peptides or modify bacterial membranes in order to prevent the action of AMPs on these membranes. Consistently, we found that OMVs isolated from a PrtV-overexpressing strain protected *V. cholerae* growth from the human AMP cathelicidin LL-37 (hCAP18), which is commonly expressed in the epithelial cells of human gastrointestinal tract. LL-37 stimulates free and OMV-associated PrtV release, as we have shown by immunoblotting (Fig. 9B), which presumably occurs due to transient pore-forming activity of LL-37 at sub-lethal concentrations. OMV-associated PrtV, therefore, contributes to a bacterial protective response and resistance against host AMPs. However, the exact mechanism of PrtV contribution remains to be further investigated.

Together, in this study, we have investigated the transport of PrtV metalloprotease in *V. cholerae* strains and its biological activity in association with secreted OMVs, which plays a role during infection.
3.2. Paper II. Outer membrane vesicles mediate transport of biologically active *Vibrio cholerae* cytolysin (VCC) from *V. cholerae* strains

In this paper, we used a non-O1, non-O139 (NOVC) V:5/04 *V. cholerae* strain, which lacks cholera toxin, the cause of extraintestinal life-threatening infections (wound, septicemia, bacteremia, meningitis). Here, we have demonstrated biological activity of OMV-transported VCC, as shown by hemolytic activity, cell toxicity and autophagy induction.

VCC, a pore-forming cytolytic toxin, was earlier studied as a free secreted soluble virulence factor. We isolated and purified OMVs following optiprep density gradient ultracentrifugation, analyzing the association of VCC with OMVs. Using SDS-PAGE and immunoblotting, as well as TEM analysis, we demonstrated that approximately 45% of VCC is secreted in association with OMVs released from the *V. cholerae* surface. Moreover, we also detected that VCC is enclosed within the OMV lumen, rather than just associated on the external OMV surface, based on the results of dissociation assay and proteinase K protection assay. This result was additionally confirmed using intact and sonicated OMVs of wild-type and an isogenic *vcc* mutant, which were analyzed by VCC-immunogold labeling and TEM. Ruptured by sonication wild-type vesicles were studded with gold particles, unlike OMVs from the *vcc* mutant strain or unsonicated OMVs from the wild-type strain. As in the case of PrtV, a sub-cellular fractionation assay also detected VCC in the periplasmic fraction, suggesting a possible role for T2SS prior to transport through OMVs. Based on our results, we could conclude that VCC is entrapped within the OMV lumen during formation and blebbing, and not due to subsequent re-association on the vesicle surface.

Having membrane-damaging cytolytic activity, VCC can cause cell death due to colloid-osmotic dysbalance in the target cells or due to caspase-dependent apoptosis induction. Sublytic VCC concentrations initiate cell survival pathways, including autophagy [129]. We tested cytotoxicity and
hemolytic activity of OMV-associated VCC on HeLa cells and erythrocytes, respectively. Interestingly, OMV-associated VCC demonstrates higher dose-dependent hemolytic activity compared to physiologically relevant amount of purified VCC. Moreover, using the HEK293-GFP-LC3 cell line we detected that OMV-associated VCC is more active in autophagy induction based on GFP-LC3 conversion and confocal microscopy analysis, compared to purified VCC. These results led us to the idea that VCC is protected from external proteolytic degradation by the OMV structure and, thereby, it has higher biological activity than free secreted VCC. Purified pore-forming toxins are known for their instability. Therefore, vesicle transport of VCC plays an important role in V. cholerae pathogenesis, particularly in NOVC strains, where VCC was also suggested to be a virulence factor causing diarrhea [124]. Additionally, OMV-mediated VCC release is common for the other V. cholerae serogroups, as well as being detected in an E. coli K-12 strain MC1061 heterologously expressing the vcc gene.

VCC has an affinity for cholesterol- and ceramides-enriched membranes, where it forms heptameric channels. In this paper, we showed for the first time that host cells employ autophagy as a defense mechanism against an OMV-associated virulence factor of extracellular pathogens. Studying mechanisms by which free and OMV-bound VCC stimulates autophagy and pore formation can provide better insight into V. cholerae pathogenesis, a knowledge that may also be valuable in therapeutics development.
3.3. Paper III. A novel role of *Listeria monocytogenes* membrane vesicles in inhibition of autophagy and cell death

Until recently, the greater research focus on Gram-negative MVs has kept the existence of Gram-positive MVs relatively unknown. Even though the number of studies on Gram-positive MVs has significantly increased in the past few years, knowledge about the formation, release and functions of Gram-positive MVs remains limited. The only study about *L. monocytogenes* MVs showed that the general stress transcription factor σB contributes to MV production. In addition to transporters, stress response, metabolic, translational and cellular processing proteins, the authors identified LLO and InlB as being secreted in association with MVs [61].

In order to understand the role(s) of *L. monocytogenes* MVs and MV-associated LLO during the infection, we investigated in this paper MV production both *in vitro* and *in vivo*. Using atomic force microscopy and scanning electron microscopy we showed MV release by bacteria *in vitro*. Using thin section electron microscopy we demonstrated the release of MVs by bacteria *in vivo*, intracellularly, particularly within phagosomes. This result highlights the importance of MV production by *L. monocytogenes* during the intracellular phase of its life cycle.

In line with the previous report, we detected that a major virulence factor, the pore-forming toxin, LLO, is released in tight association with MVs [61]. Using both Optiprep density-gradient purification and dissociation assay, we showed that LLO is mainly localized inside the MV structure or embedded in the MV membrane, while some portion of LLO is localized on the outer leaflet of the MV membrane. Due to its location inside the MV lumen, LLO is maintained there in an oxidized, inactive state, which was shown using a hemolytic assay. Only upon adding a reducing agent, LLO could cause hemolysis of erythrocytes and even then to a much lower extent than purified LLO toxin. Temporal and spatial regulation of LLO activity by
L. monocytogenes, known from previous reports, is necessary to protect cells from extreme damage. Thus, LLO translation is tightly controlled within the cytosol of the host cell, while optimal LLO activity is detected in the acidic pH of the vacuole (pH 5.5) and LLO is activated within the vacuole by the host factor GILT [168, 216-218]. Thereby, our results suggest another level of LLO activity control: by inactivating toxin via entrapment within MVs.

In paper II, we have identified that MV-associated PFT of V. cholerae, VCC, can induce autophagy. In paper III, we aimed to investigate the relationship between MVs/MV-associated LLO and autophagy. Interestingly, we discovered that MV-associated LLO was unable to induce autophagy. However, MVs inhibited autophagy stimulated by purified or MV-associated VCC or by purified active LLO. This was observed via confocal microscopy of HEK293-GFP-LC3 cells as a decrease in GFP-LC3 puncta or via immunoblotting as a reduction in LC3 conversion.

It was not clear for us whether MVs can enter host cells or deliver their cargo via fusion, or if MVs can directly interact with autophagosomes, inhibiting, for example, LC3 lipidation. Therefore, we tested MV internalization inside HEK293 cells and HeLa cells. Co-localization with endosomes at early time points and with lysosomes at early and especially later time points, together with the absence of co-localization with phagosomes, demonstrated that MVs entered host cells, were quickly recycled within endosomes and accumulated within lysosomes. Considering the fact that mTORC1 also can be localized to lysosomes, we investigated the impact of MVs on mTORC1 upon LLO-stimulated autophagy [219]. Consequently, L. monocytogenes MVs reversed LLO-induced inhibition of mTORC1 activity, i.e. they reversed dephosphorylation of mTORC1 and its downstream targets. Additionally, MVs reversed phosphorylation of eIF2α and AMPK. This finding suggests that upon internalization and accumulation within lysosomes MVs might interfere with mTORC1.

In order to determine whether MVs inhibit only PFT-stimulated autophagy, we used Torin1 as an inducer of canonical autophagy, in combination with Bafilomycin A1, a V-ATPase inhibitor, which blocks
phagosome maturation and autophagosome-lysosome fusion. Interestingly, MVs also inhibit autophagy induced by Torin1, a chemical substance analogous to rapamycin. Immunoblotting, confocal microscopy analysis and macroautophagic cargo sequestration assay showed a significant decrease in Torin1-stimulated autophagy, but not basal autophagy. Thus, the MV inhibitory effect on autophagy is not only limited to PFTs and pore formation, but might have a more complex interaction with mTOR and autophagy pathways.

We further performed an extraction of proteins and lipids from *L. monocytogenes* MVs and identified that MV-associated proteins, but not lipids, are responsible for PFT-induced autophagy inhibition. Interestingly, MVs isolated from the constitutively active mutant PrfA (G145S) strain, which exhibits virulence gene overexpression independent of environmental conditions, showed a significantly stronger inhibitory effect on LLO-induced autophagy inhibition [220]. Thus, confocal microscopy analysis and SEM revealed that an 8-fold lower concentration of MVs from the PrfA mutant (G145S) showed the same inhibition effect as MVs from wild-type strain (data not shown). This indicates that MV-associated protein(s) important for inhibition of autophagy are at least under PrfA regulation. Further studies will be important in order to identify the protein(s) involved.

In lower concentrations LLO can induce autophagy, while at higher concentrations the toxin can cause cell death. After only 15 minutes of exposure to LLO, HEK293 cells exhibited cell necrosis, which was completely prevented by addition of *L. monocytogenes* MVs. Therefore, MVs prevent not only autophagy induced by pure active LLO, but also cell necrosis caused by a high concentration of LLO. Considering that inhibition of LLO-induced cell necrosis by MV treatment occurs within a short period of time, we hypothesized that MVs act on the level of pore formation. This hypothesis is supported by the fact that AMPK phosphorylation occurs as an immediate response to pore formation and is an upstream part of the signaling cascade. Indeed, measuring propidium iodide influx through the pores using flow cytometry demonstrated that inhibition of PFT-stimulated autophagy and
cell death occurs through the inhibition of pore or pre-pore formation on the eukaryotic cell surface. These findings were additionally confirmed by SEM, where the surface of HEK293 cells was visualized after pure LLO treatment with and without the addition of LLO-deficient MVs (Fig. 9). It was previously shown for SLO, secreted by S. pyogenes, that as a response to pore formation caused by SLO and a part of membrane repair, eukaryotic cells can shed toxin and pores through blebbing of plasma membrane, so called ectosytosis [221]. Considering similarities in structures between SLO and LLO, it is not surprising that we have observed similar blebs on the surface of HEK293 cells after LLO treatment (Fig. 9) [222]. However, in the case of MV pre-treatment, we have observed neither pores, nor blebs. Together, this data supports our idea that MVs might either participate in pore sealing or prevent insertion of PFT-induced pre-pore complexes/pore formation, probably due to the modification of cholesterol regions where PFTs bind. This phenomenon can be used by bacteria during infection. Indeed, we detected that MVs promote intracellular survival of L. monocytogenes after 2 and 8 hours p.i. In conclusion, we suggested that MV release by intracellular pathogen L. monocytogenes is another level of LLO activity regulation: by entrapping and maintaining it in an inactive (oxidized) state within MVs; and by preventing phagosomal and plasma membrane damage by active LLO. Furthermore, MVs might be important for inhibition of LLO-induced autophagy and cell necrosis in order to modulate intracellular survival and protection of eukaryotic cells against extreme pore-induced damage.
Figure 9. Scanning electron micrographs of HEK293 cell surfaces after MV with and without LLO treatment. Low magnification (1,000x) is represented in panels A-D. High magnification (50,000x) is represented in panels E-H. A and E – mock-treated cells; B and F – LLO-treated cells; C and G – Δhly MV treated cells; D and H – Δhly MV treated cells prior to LLO. Blebs are visualized on panel B (indicated with arrows), pores on the blebs are visualized on panel F (indicated with arrows). The pictures were taken by Cheng Choo Lee, UCEM, Umeå University, Sweden. Scale bars: 10 µm (A-D), 200 nm (E-H).
Main findings in this thesis

**Paper I**

- OMVs released from the surface of *V. cholerae* transport a biologically active processed form of PrtV protease to host cells
- PrtV is translocated to the periplasmic space via the Type II secretion system prior to secretion externally in association with OMVs
- OMV-associated PrtV contributes to cell cytotoxicity
- PKD domains are essential for PrtV association with OMVs
- OMV-associated PrtV contributes to the resistance of *V. cholerae* against the host antimicrobial peptide LL-37.

**Paper II**

- VCC is tightly associated and transported inside *V. cholerae* OMVs in a protected manner
- VCC release by OMVs is a conserved common mechanism among *V. cholerae* strains
- OMV-associated VCC demonstrates higher biological and autophagy-inducing activity compared to free, soluble VCC.

**Paper III**

- *L. monocytogenes* produces MVs during its growth both *in vitro* and *in vivo*
- *L. monocytogenes* MVs enter HeLa and HEK293 cells and accumulate in lysosomes
- *L. monocytogenes* MVs can inhibit PFT-induced autophagy and partially Torin1-induced macroautophagy
- MVs inhibit cell necrosis caused by LLO
- MVs reverse LLO-induced inhibition of mTORC1 activity
- Inhibition of LLO-induced autophagy and cell death by MVs is due to the inhibition of pore formation
- MVs contribute to *L. monocytogenes* survival inside eukaryotic cells, possibly through the inhibition of LLO activity.
Future perspectives

The interplay between autophagy and bacteria is very complex and depends on many factors. In this thesis, I have shown that depending on whether it is an extracellular or intracellular bacterium, the interaction between MV-associated PFT and autophagy can differ. Still many questions remain to be answered: which protein(s) within *L. monocytogenes* MVs are responsible for autophagy inhibition, what is the exact mechanism of pore inhibition by MVs, at which level MVs interact with canonical autophagy, does autophagy inhibition occur throughout all stages of the infectious process or is it modulated at a certain stage? Nevertheless, our findings open a new page in autophagy manipulation by bacteria. Since autophagy can play either protective or destructive roles in different diseases, even at different stages of the same diseases, pharmacological manipulation of autophagy has received much attention. The ability of MVs to be internalized within eukaryotic cells, and their antigenicity, as well as their cost-effective production, temperature stability and ease in genetic manipulation are factors that make MVs an interesting drug delivery vehicle for cancer therapy.
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To my dear mother...
References


S1-S2. PubMed PMID: 19278550; PubMed Central PMCID: PMCPMC2654662.


45. Bauman SJ, Kuehn MJ. Pseudomonas aeruginosa vesicles associate with and are internalized by human lung epithelial cells. BMC Microbiol.


54. Wessel AK, Liew J, Kwon T, Marcotte EM, Whiteley M. Role of Pseudomonas aeruginosa peptidoglycan-associated outer membrane


References


87. Mirlashari MR, Hagberg IA, Lyberg T. Platelet-platelet and platelet-leukocyte interactions induced by outer membrane vesicles from N.
References


96. Kadurugamuwa JL, Beveridge TJ. Membrane vesicles derived from Pseudomonas aeruginosa and Shigella flexneri can be integrated into the


104. Alaniz RC, Deatherage BL, Lara JC, Cookson BT. Membrane vesicles are immunogenic facsimiles of Salmonella typhimurium that potently activate dendritic cells, prime B and T cell responses, and stimulate protective
References


References


References

10.1371/journal.ppat.1003126. PubMed PMID: 23436993; PubMed Central PMCID: PMCPMC3578741.


