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Chapter

The Secretome of *Vibrio cholerae*

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Abstract

Vibrio cholerae is a facultative human pathogen responsible for the cholera disease which infects millions of people worldwide each year. *V. cholerae* is a natural inhabitant of aquatic environments and the infection usually occurs after ingestion of contaminated water or food. The virulence factors of *V. cholerae* have been extensively studied in the last decades and include the cholera toxin and the coregulated pilus. Most of the virulence factors of *V. cholerae* belong to the secretome, which corresponds to all the molecules secreted in the extracellular environment such as proteins, exopolysaccharides, extracellular DNA or membrane vesicles. In this chapter, we review the current knowledge of the secretome of *V. cholerae* and its role in virulence, colonization and resistance. In the first section, we focus on the proteins secreted through conventional secretion systems. The second and third sections emphasize on the membrane vesicles and on the secretome associated with biofilms.

Keywords: *Vibrio cholerae*, secretome, secretion system, membrane vesicles, biofilm

1. Introduction

Vibrio cholerae is a Gram-negative bacterium responsible for the cholera disease, which infects millions of people per year worldwide. In the environment, *V. cholerae* is a common inhabitant of aquatic ecosystems. Over more than 200 serotypes of *V. cholerae* have been described, but only two are responsible of the pandemics, *i.e.* O1 and O139 serotypes. The O1 serotype is divided in two biotypes, classical and El Tor. The *V. cholerae* O1 El Tor is responsible for the ongoing 7th pandemic [1]. The infection usually begins with the ingestion of contaminated water or food. Once inside the human host, *V. cholerae* colonizes the small intestine where biofilm-like structures have been observed [2]. The colonization and virulence inside the host are highly correlated with the secretion of a panel of proteins, including the cholera toxin (CT). The toxin is responsible for the misfunction of the calcium channel of the host epithelial cells, leading to the cholera characteristic massive loss of water and the diarrhea [3]. This review aims to focus on the secretome of *V. cholerae* and the secretion systems used by this bacterium to colonize the human host, compete with other bacteria, and survive in the environment.

2. Secretion systems

V. cholerae possesses as many as five multicomponent secretion systems, allowing secretion or translocation of a broad range of molecules into the extracellular milieu

or directly into the neighbouring cells. These molecules are essential for niche competition in the environment and for persistence in the host.

2.1 Type II secretion system for virulence and environmental fitness

The type II secretion system (T2SS) shares many structural characteristics with the type IV pilus (T4P) and is conserved among Gram-negative bacteria for delivery of colonization and virulence factors in the extracellular milieu [4, 5]. In *V. cholerae*, it is used in the aquatic environment and in the human host to secrete exoproteins from the periplasm to the extracellular milieu or to anchor the bacteria to the host cells [4, 6]. The loss of T2SS altered growth, biofilm formation, antimicrobial resistance, and cell envelope integrity, suggesting that the T2SS has an essential role in this bacterium, which makes it a suitable target for therapeutic development [7–9]. The T2SS genes are referred to as extracellular protein secretion (*eps*) [7]. Hydrolyzation of ATP is required to provide energy for secretion [5]. The T2SS is anchored in both bacterial membranes and is distributed all over the bacterial surface [10, 11].

The growth defect of mutants lacking essential components or regulators of the T2SS shows that it is a vital component for *V. cholerae*, mostly since all the proteins secreted by the T2SS seem to act together to facilitate *V. cholerae* colonization and survival in ecological niches or in the human host. Majority of experiments occur in controlled laboratory conditions which do not represent the complexity of the intestinal nor the marine niches. These conditions might influence the type of proteins that are secreted by the bacteria, as seen in the Sikora et al. study where the CT has not been detected in the supernatant while it is a known T2SS secreted protein [6].

2.1.1 Structure and secretion through the T2SS

The structural components [5] and secreted proteins [4] of the T2SS have recently been the object for reviews. Briefly, the T2SS assembles in 4 complexes; (i) the secretin, a pore located in the outer membrane, (ii) the inner membrane anchoring platform, (iii) the intracytoplasmic ATPase complex and (iv) the pseudopilus. Even though the exact sequence of biogenesis is still unknown, a general pathway of assembly has been suggested.

The targeted proteins with signal peptides are firstly translocated to the periplasm by Sec or Tat, where they are assembled to acquire a secretion competent conformation [12, 13]. Then, it has been proposed that they bind to the pseudopilin trimeric tip and to the inner membrane platform. This interaction activates the ATPase hydrolysis activity, thus the pseudopilus elongation by addition of pseudopilin subunits and leads to the thrust of the secreted protein through the secretin channel as a piston [5]. It has been proposed that the signals for T2SS transportation are dependent on the protein conformation on the N-terminal signal peptides, but they have not been clearly identified yet [14].

2.1.2 Genes and regulation

The T2SS apparatus is composed of a dozen types of proteins, which are encoded on the *eps* operon (*epsC* to *epsN*), plus *epsAB* and the *vcpD* (*pilD*) genes in *V. cholerae* [7, 10]. Few studies have concentrated on the regulation of the T2SS in *V. cholerae*. Under laboratory conditions, the T2SS is constitutively expressed in *V. cholerae* following the growth rate of the bacteria with a higher expression at 25°C than at 37°C [15]. In addition, studies on the T2SS regulation suggest that several major regulatory pathways, including the quorum sensing, the c-di-GMP, the σE envelope stress response, might be involved [15, 16]. Finally,

as more than 20 extracellular proteins with important activities throughout the infection are secreted through the T2SS [6], this system must be tightly controlled over time to allow their synchronized secretion. Therefore, the expression of the cargo proteins is regulated by a panoply of regulators.

2.1.3 Secreted proteins

In *V. cholerae*, the T2SS ensures the transportation of more than 20 proteins with extracellular activities such as enzymes, toxins, virulence and colonization effectors [6]. The T2SS is essential for survival in the aquatic environment and to infect the human gut. An essential process to colonize both environments is the ability to adhere to abiotic and biotic surfaces such as copepods and zooplankton exoskeletons and epithelial cells, respectively [17–19]. The surface-exposed GlcNAc binding protein (GbpA - VCA0811) is secreted by the T2SS and is an adhesion factor used by *V. cholerae* to bind chitinous surfaces, intestinal epithelial cells and mucin [18–21]. Chitin is the second most abundant polymer in nature and consists on N-acetyl-D-glucosamine (GlcNAc) monomers linked in β-1,4 and the main component of copepods and zooplankton exoskeleton [22]. Attachment to marine crustacea and zooplankton is an advantage for nutrients acquisition, survival and dispersion in aquatic environment. GlcNAc-containing glycoconjugates are often beared by some glycoproteins at the surface of the intestinal epithelial cells and might insure adhesion to the epithelial cells [17, 23, 24]. GbpA possesses 4 domains, the 1 and 4 being the most important for chitin binding, while only the 1 is needed for mucin binding [18]. As a secreted protein, GbpA must be able to interact with *V. cholerae* to allow its adhesion to the substrate. This function is assumed by the domains 3 and 4 of GbpA, that bind to the bacterial surface [18]. GbpA is regulated by the quorum sensing and produced at low cell density [25]. At high cell density, HA/P and PrtV digest GbpA to allow cell detachment and propagation [25, 26]. Also, higher temperatures increase the production of GbpA, promoting cell adhesion [27]. GbpA induces mucin secretion by intestinal epithelium, and mucin increases expression of GbpA [21]. Studies also determined that GbpA can induce necrosis of intestinal cells by increasing their membrane permeability [28]. Recently, a chitin cleavage activity under copper saturation has been described for GbpA and would therefore make it a lytic polysaccharide monooxygenase, a metalloenzyme copper-dependant capable of polysaccharide cleavage by oxidation [29]. Taken together, these findings suggest that GbpA is not only an early adhesion factor but might also have a more important role in pathogenesis.

In the aquatic environment, after binding to zooplankton, copepods and insect egg masses, *V. cholerae* can use chitin as source of carbon and nitrogen [14, 30]. To do so, *V. cholerae* secretes at least 2 chitinases: ChiA-1 and ChiA-2. The chitinase-1 (ChiA-1 - VC1952) and 2 (ChiA-2 – VC0027) are secreted by the T2SS and synergistically hydrolyzes the β1,4 bond between the GlcNAc monomers in the extracellular milieu [6, 14, 31]. The expression and activity of *V. cholerae* chitinases are influenced by environmental factors such as pH, salinity or temperature [32]. In the extracellular milieu, ChiA-1 expression is induced by chitin via the sensor kinase of the orphan two-component system ChiS [31]. In the intestine, the expression of the chitinases is constitutive and a role for ChiA-2 in mucin degradation and in virulence has been reported [33]. Besides ChiA-1 and ChiA-2, other proteins might have a role in chitin utilization including the VCA0140 gene that encodes for the spindolin-related protein, the VC0769 gene product and the chitin oligosaccharide deacetylase (COD - VC1280) [6, 31, 34]. All of them are secreted by the T2SS [6]. Regarding COD, Xibing Li *and coll*. demonstrated that it removes the GlcNAc from chitin oligosaccharides [34].

Besides chitin, collagen can also be used as carbon source by *V. cholerae*. Collagen is one of the most abundant components of host tissues and aquatic animals, and can therefore be found in aquatic environments in association with marine life and sedimentation of decomposing animals [35]. Its degradation provides a nitrogen source, giving a growth advantage to collagenases producing bacteria [35]. The collagenase (VchC - VC1650) is a metalloprotease that degrades type I collagen, providing another carbon source for *V. cholerae* [36]. VchC has been recognized as a T2SS dependant extracellular protein [36]. In other *Vibrio* species, collagenases are recognized as virulence factors as it facilitates their dispersion by degradation of the cellular basal lamina, but this role has not been attributed to VchC yet [36].

After the ingestion, *V. cholerae* navigates through the digestive tract, where it survives many physical and chemical barriers such as gastric acid, peristaltic movement, bile, mucin and microbiota. In the small intestine, it crosses mucin using its flagellum and the mucinase complex, that includes the vibriolysin, a zinc dependant metalloprotease hemagglutinin/protease (HA/P - VCA0865) secreted *via* the T2SS as a free protease or in a cell associated form [26, 37]. The structure, regulation, secretion mechanism and functions of HA/P have been reviewed recently [37]. Briefly, HA/P is expressed when cell density is high or when there is nutrient limitation through the HapR and RpoS regulators [38, 39]. It is translated as an inactive protein and chaperones ensure its inactive state inside the cytoplasm, then the secretion occurs in 2 steps; (i) HA/P is translocated *via* Sec into the periplasm, (ii) the T2SS exports the protease in the extracellular space where an autocatalytic event activates HA/P [37]. HA/P has multiple targets to facilitate spreading of *Vibrio* and increases its virulence [37]. *V. cholerae* gains access to the intestinal epithelial cells by degradation of the mucus layer, lactoferrin and fibronectin by HA/P in order to release toxic effectors into the epithelial cells [26]. In addition, HA/P can cleave toxins such as CT and lactoferrin to activate or increase their activity [26, 40, 41] and disrupts tight junctions between intestinal epithelial cells by occludin cleavage [42]. HA/P participates in *V. cholerae*'s release into the stool by degradation of mucin to detach bacteria from epithelial cells [43].

A second important component of the mucinase complex is the neuramidase (VCNA - VC1784). The sialidase, or neuraminidase, is encoded on the pathogenicity island of every toxigenic *V. cholerae* strains and is secreted by the T2SS [44]. VCNA removes the sialic acid that hides the ganglioside GM1, which is the receptor of the CT, on the surface of epithelial cells [45]. It binds to sialic acid to modify it by its N-terminal lectin domain [46]. Multiple enzymes (VesA, HA/P, and VCNA) appear to work synergistically and with redundancy, ensuring access to the receptor and the activation of the toxin immediately after its secretion [6].

The CT (VC1456-57) is an AB5 toxin secreted by the T2SS in the intestinal lumen, which represents the main virulence factor of *V. cholerae* found in O1 and O139 strains [3, 47]. The subunits are individually translocated by Sec into the periplasm, and the assembled toxin is translocated to the extracellular milieu by the T2SS [7]. The toxin is secreted in an inactive form and must be cleaved by human or bacterial protease to be activated [40]. CT is composed of five B subunits linked in a ring shape that bind to the ganglioside membrane receptor GM1 on the apical surface of intestinal epithelial cells [3]. CT is internalised and transits to the endoplasmic reticulum. The A subunit is heterodimeric with the A2 as the linker between B and A1 subunits, and A1 as a mono-ADP-rybosyltransferase [3]. A1 is released in the endoplasmic reticulum by disulfide isomerase and translocated to the cytosol where it activates the adenylate cyclase G protein by addition of an ADP. Thereby, the activated adenylate cyclase increases the intracellular levels of cyclic AMP, which in turn, activates the protein kinase A (PKA). Finally, PKA activates the chloride anion (Cl^-) excretion by phosphorylation of the chloride channel, that leads to major water secretion by osmose [3]. *ctxA* and *ctxB* genes are organized as

an operon on the integrated CTXφ lysogenic phage [48]. The secretin complex of the T2SS is required by CTXφ to exit the bacteria, which makes the T2SS essential for virulence and horizontal transfer of CT [49]. CT is expressed when the cell density is low, inversely to HA/P, which is why it has been suggested that HA/P could cleave the remaining non-activated CT when the cell density rises [50].

Prior to GM1 binding, the CT must be processed by extracellular proteases to be activated. These proteases are therefore important for virulence and colonization. Besides their capacity to activate the toxin, they have a role in finding a substrate (modification of integrin) and nutrients, and in deactivating host defense mechanisms. Among the T2SS secreted proteins, 3 serine proteases with 30% homology between them have been identified, the *Vibrio* extracellular serine proteases (VesA - VCA0803; VesB - VC1200; VesC - VC1649) [6]. All three proteins have a N-terminal protease domain [6, 51]. VesB has a similar structure and specificity to trypsin [51]. Mutation of *vesABC* allowed to identify that VesB is the main responsible of the proteolytic activity, while VesA and VesC are responsible of 20% of the total proteolytic activity [6]. VesABC do not require bivalent ions for their enzymatic activity [6]. Under laboratory growth conditions, VesA, and in a lesser extend VesB and HA/P, activate the CT in the extracellular milieu [6, 40]. VesC induces a hemorrhagic response in rabbit ileal loop model, which might also reflect a role in virulence [52]. VesB and VesC are expressed at low cell density while VesA is expressed at high cell density [4].

Other virulence factors secreted by the T2SS have been identified in *V. cholerae*. The extracellular metalloprotease (PrtV) is a Zn-dependant metalloprotease [53, 54]. Its activity depends on several autocatalytic events occurring inside and outside the cell for activation [55]. PrtV uses two mechanisms of secretion, in association with membrane vesicles (MV) and *via* the T2SS [6, 56]. PrtV cleaves host proteins such as extracellular matrix and substrate proteins, inducing a change in host cell conformation leading to cell death [53]. In addition, PrtV is necessary for killing of *Caenorhabditis elegans* and protection against predators [57]. To do so, PrtV has many substrates such as, but not limited to, fibronectin, fibrinogen and plasminogen [53]. PrtV is composed of two domains usually known to allow protein-protein or protein-carbohydrates interactions [55, 58]. Thus, PrtV is important for the colonization of ecological niches and in pathogenesis.

The cytolytic toxin cytolysin/hemolysin A (VCC or HlyA - VCA0219), is secreted by the T2SS [6]. All *V. cholerae* strains produce VCC, an iron-dependant secondary toxin activated by cleavage [59]. VCC leads to cell death by vacuolization of the target cell, after production of anions channels in the membrane [60]. Since VCC leads to chloride efflux in intestinal cells, and subsequently to sodium and water by osmosis, it has been suggested that VCC is the major factor responsible of diarrhea in non-producing CT strains.

Leucine aminopeptidase (Lap - VCA0812) and aminopeptidase (LapX - VCA0813) are other secreted proteases using T2SS [6]. Lap is a zinc dependant metallo-exopeptidase that cleaves leucin in N-terminal position, while the role of LapX remains unknown [61]. Both Lap and LapX have no role in virulence in a *C. elegan*s model [57]. While the TagA-related protein (Tarp - VCA0148), the unidentified VCA0583 and VCA0738 proteins, as well as the putative lipoprotein VC2298, have been recognized as T2SS secreted proteins, their role in *V. cholerae* is still unknown [6].

Finally, several proteins involved in biofilm formation and dissemination are also secreted by the T2SS in *V. cholerae*. Biofilm protects bacteria from antibiotics, immune system and poor environmental conditions, thus allows their survival in diversified range of ecological niches. Many components are secreted into the extracellular milieu to form the matrix. Among them, Biofilm associated protein 1 (Bap1 - VC1888) and rugosity and biofilm structure modulator A (RbmA - VC0928) and C (RbmC - VC0930) are the matrix proteins and are secreted by the T2SS [9]. In addition, the DNase Xds, an exonuclease would also be secreted by the T2SS [62].

Xds is expressed at the late stage of infection, can contribute to survival against neutrophiles NET traps, to acquisition of new DNA and dispersion of the biofilm [62, 63]. More details about the roles of the matrix proteins and nucleases in biofilm formation are presented in the Biofilms and Flagella section.

2.2 Type VI secretion system for competition and DNA acquisition

The type VI secretion system (T6SS) is a versatile syringe-like apparatus with homology to the phage T4 and produced by more than 25% Gram-negative bacteria that, upon contact with a target cell, punctures its cell wall, allowing translocation of toxic effectors directly into the neighboring cells [64, 65]. The cellular targets of these effectors are multiple; peptidoglycan, actin, cellular membrane, nucleic acids and immune system components, for instance [66]. As the target cells release their DNA into the extracellular milieu upon lysis, another function of the T6SS is to capture the extracellular DNA (eDNA) in order to acquire new features such as antibiotic resistance factors and new effectors or immunity proteins [67]. Bacteria use this device as a competition effector to take over the environmental niche and a single bacterium can possess as much as 6 different types of T6SS [65]. In *V. cholerae*, the T6SS is as efficient at killing bacterial competitors as it is at delivering toxic effectors to eukaryotic host cells, making it an important colonization and virulence factor [68].

2.2.1 Structure and secretion through the T6SS

The T6SS is anchored in the cell membrane and contains 4 distinct domains; (i) the membrane complex, (ii) the baseplate, (iii) the contractile sheath and (iv) the syringe. The current knowledge on the structure of the T6SS have been reviewed elsewhere [64].

Valine glycine rich proteins G 1, 2 and 3 (VgrG1-3) and a single proline-alanine-alanine-arginine repeated motif protein (PAAR) form the tip of the syringe [69]. There are multiple PAAR proteins in *V. cholerae*'s genome but only one binds and folds in order to form a sharpened tip and it has been shown to be essential for an efficient secretion by the T6SS [69]. The PAAR proteins also have toxic effector functions. The syringe is a tube composed of multiple hexameric rings of hemolysin-coregulated protein (Hcp) [70]. Almost simultaneously, the syringe is wrapped by the helical contractile sheath made of VipA and VipB [71] which polymerizes in an extended conformation. This high-energy conformation provides enough energy, upon contraction signal, to propel the Hcp syringe, the VgrG-PAAR tip and the associated effectors into the extracellular milieu or directly into a near target cell by contraction and rotation of VipB [71]. VipA would function as a chaperone for the VipB subunits [71]. The contracted arrangement of VipB would expose the ClpV binding sites on VipB, which are hidden in the extended conformation. ClpV is the ATPase responsible for recycling the sheath components that can be reused for further effectors translocation [72, 73]. Adaptor proteins are required to load the effectors on the tip of the syringe; however, they are not secreted by the T6SS [74].

2.2.2 Genes and regulation

In *V. cholerae*, the core genes are organized in a main cluster operon that includes *vipAB*, *hsiF*, *vasA* to *vasM* and *clpV*, on the small chromosome [68]. It contains most of the structural components of the T6SS, except for Hcp, in addition to the regulator VasH and recycling protease ClpV. At least 2 auxiliary clusters (Hcp –1 and –2), harbouring Hcp, VgrGs, adaptor and effector/immunity proteins, are distributed in the genome [75]. Some strains, including pandemic strains, have an additional

auxiliary cluster (Aux –3) coding for a second PAAR protein and extra effector/immunity module set [75]. Recently, two other auxiliary clusters, Aux –4, coding for the predicted Tse4, and Aux –5, coding for Hcp, a VgrG protein, an adaptor protein and effector/immunity module set, have been identified [75, 76]. While the genes from the main cluster are highly conserved, the auxiliary clusters, even from the pandemic strains harbouring the same effectors/immunity module sets, only share about 30 % homology between them [77].

The complexity of the apparatus and its organization require a fine regulation to insure its efficiency and recycling. The transcriptional regulation of the T6SS in *V. cholerae* is strain dependant [78, 79], complex and not entirely understood yet. As the environmental strains constitutively express the T6SS to control the surrounding bacterial populations and survive predators of the ecological niche, the pathogenic strains tightly regulate their T6SS [79, 80]. Quorum sensing, the chitin and bacterial competence pathways, osmolarity and other environmental conditions are involved in the regulation of the T6SS (for a more detailed review of the T6SS see: [64]).

2.2.3 Secreted proteins

As mentioned before, the T6SS apparatus carries toxic effectors directly into the target bacterial or eukaryotic cells. A single contraction event allows the translocation of many of these effectors at the same time into the target cell [69]. The cellular targets for these effectors are multiple; they go from peptidoglycan to cellular membrane, actin and nucleic acids [64]. To protect themselves against the toxic effectors they produce, bacterial cells express immunity proteins, which brings the notion of strains compatibility (for more information see: [81]). The secreted effectors and structure components can be reused by recipient cells to form a new T6SS [82].

Hcp is one of the proteins transported by the T6SS into the target cell, in addition to be part of its structure by forming the inner tube and serving as a chaperone to the effector molecules [83]. Hcp is encoded by two different yet functional genes (VC1415; VCA0017) producing the same protein [68, 84]. Both genes must be knocked out to suppress the T6SS activity [68]. Hcp is co-expressed with HlyA, and its secretion was observed before the discovery of the T6SS [84].

Similarly to Hcp, the VgrG proteins (VC1416; VCA0018; VCA0123) are part of the T6SS structure and are secreted into the target cell upon contraction of the T6SS [68, 85]. VgrG-1 has an actin cross-linking activity in eukaryotic cells, thus preventing cytoskeleton reorganization and phagocytosis [86]. VgrG-2 is homologous to VgrG-1, but without a functional C-terminal effector domain [85]. Both appear to be essential for secretion of other T6SS components as a mutational inactivation of one of these gene makes the mutant unable to secrete any T6SS-dependant effectors [85]. Since its toxicity is exclusive to eukaryotic cells, no immunity coupled protein is required against VgrG-1. The VgrG-3 protein is known to be active against other bacteria by hydrolyzing peptidoglycan with its lysozyme-like domain, after a translocation to the periplasm [85, 87]. It might also have a muramidase activity, which could be useful in its aquatic niche to gain access to chitin or in infection to cross mucin [88]. TsiV3 (VCA0124) acts as the antitoxin for VgrG-3 by biding to it and prevents the degradation of the cell wall in the predator bacteria [87]. Thus, VgrG-3 might be important for infection by killing gut microbiota and by hydrolysing mucin.

The PAAR proteins (VCA0284; VCA0105), along with VgrGs, form the tip of the syringe of T6SS, bind the effectors and are therefore essential for T6SS effectors' secretion. There are two proteins with a PAAR domain in *V. cholerae*'s genome with enzymatic activities that could be toxic for eukaryotic or prokaryotic cells, thus acting as effectors [69]. PAAR proteins are secreted by the T6SS by caping the tip of the syringe, the PAAR domain allowing the bond with the VgrG trimeric tip. As Hcp, VgrG

and PAAR proteins can bind and load effectors, the multiple effector translocation VgrGs (MERV) model has been proposed, suggesting that the T6SS spike (Hcp-VgrG-PAAR) can deliver different cargo effectors at the same time into the targeted cell [69].

The cargo effector VasX (VCA0020) acts as a colicin and targets the inner bacterial membrane or eukaryotic membrane in which it is believed to form pores, increase permeability and lead to its disruption [89]. It is encoded downstream of Hcp-2 and VgrG-2 and is regulated by VasH [89]. Its immunity coupled protein is TsiV2 (VCA0021) [88]. The VasW (VCA0019) protein encoded right upstream VasX is an adaptor protein that plays a role in secretion of VasX and an accessory role to VasX bactericidal activity [90].

The type six effector Lipase (TseL - VC1418) is another cargo effector and its secretion depends on the presence of VgrG-3. It carries a phospholipase domain that is believed to cause damage to cell membranes in both eukaryotic and prokaryotic cells [88, 91]. Its immunity coupled protein is TsiV1 (VC1419).

The type six effector Hydrolase (TseH - VCA0285) is encoded next to the PAAR protein and its secretion is dependant of the T6SS [92]. It has been shown that TseH is able to degrade peptidoglycan, a main component of the bacterial cell wall, by hydrolysis and would therefore make it an important effector as for interbacterial competition. Its immunity coupled protein is the type six immunity hydrolase (TsiH - VCA0286), which prevents cell wall degradation.

Recently, another lipase, the Type VI lipase effector *Vibrio* (TleV1) has been discovered in environmental *V. cholerae*'s genome [75]. TleV1 has a toxic activity in bacteria, mainly in periplasm, by targeting phospholipids and destabilizing the cellular membrane. Two immunity coupled proteins are associated with TleV1, TliV1a and TliV1b (type VI lipase immunity Vibrio 1a and 1b), but only TliV1a has an effective neutralizing effect against TleV1.

It is most likely that, as genome analysis of more *V. cholerae* strains will occur, new effector/immunity modules will be identified as they can be transferred between strains by genetic transfer or acquisition of eDNA upon target cell lysis [67, 75, 76, 93]. All the pandemic strains encode the same effector/immunity module sets (TseL/TsiV1, VasX/TsiV2 and VgrG3/TsiV3, called AAA), as a result of intraspecific competition [93]. Some strains harbour immunity genes without the coupled effector that they acquired from gene transfer, named orphan immunity proteins, allowing their survival from multiple toxic effectors [80]. The modules found within strains may differ from each other, however, their diversity and their omnipresence testify of their value for virulence and competition of the niche.

2.3 Type I secretion system, a tool for auxiliary toxins secretion

The type I secretion system (T1SS) is used by Gram-negative bacteria to secrete, in a one-step process using ATP, proteins directly into the extracellular milieu.

2.3.1 Structure and secretion through the T1SS

The most studied T1SS is the hemolysin A associated T1SS (HlyA-T1SS) from *E. coli* and its general structure has been reviewed elsewhere [94]. Briefly, the T1SS are composed of 3 proteins encoded on the same operon, next to their associated secreted protein and activator; (i) an outer membrane protein (TolC), (ii) an ATP binding cassette (ABC) transporter in the inner membrane (HlyB), and (iii) a linker protein (HlyD) anchored in the inner membrane, linking the two other components.

The secreted proteins carry a C-terminal secretion signal sensed by the inner membrane proteins upon binding [95]. The porin TolC is then recruited to the complex, and the proteins pass through the HlyB and HlyD channel. The binding

of TolC to the inner membrane complex allows its opening and the secretion of the protein to the extracellular milieu, whereafter TolC leaves the complex [94]. As the inner membrane proteins bind to specific substrates, the TolC can be used by multiple T1SS within a cell [96]. The secreted proteins have a functional domain in N-terminal and are secreted shortly after their translation in their unfolded state. In *V. cholerae*, the T1SS structure is atypical and is composed of 4 components: the periplasmic linker RtxD, the outer membrane protein TolC, and 2 ATPases RtxB and RtxE, which most probably form heterodimers in the inner membrane instead of the conventional homodimer ATPase [97].

2.3.2 Genes and regulation

The *rtx* gene cluster is encoded near the CTXφ phage, but their regulation is not linked [98]. RtxA is secreted by its own unorthodox T1SS that requires two ABC transporters for the secretion [97]. The *rtx* locus is composed of 2 operons, the first one is left oriented and contains a conserved hypothetical gene VC1449, the activator (*rtxC*) and the toxin (*rtxA*). The second, right oriented, contains the ABC transporter (*rtxB*) and the fusion protein (*rtxD*) genes, plus the extra ABC transporter *rtxE*. The *tolC* is encoded further. The RtxA toxin secretion is optimal during the exponential phase of growth but is inhibited in stationary phase [98].

2.3.3 Secreted proteins

The repeat in toxin (RTX) proteins are a class of proteins exclusively secreted by the T1SS [99]. They include the HlyA of *E. coli* (but not of *V. cholerae* – see T2SS) and RtxA and FrhA of *V. cholerae*. Briefly, these proteins contain glycine and aspartate-rich sequence in C-terminal, before their T1SS secretion signal, and a functional domain originally associated with toxin activities. They require activation by the acetyl transferase activator encoded within the operon. The RTX region offers many Ca^{2+} binding sites. Once bound to the sites, the Ca^{2+} generates a sudden conformation change and formation of the secondary structure of the RTX protein. As the Ca^{2+} concentrations are low inside the cells, the RTX proteins keep their unfolded state until they reach the extracellular milieu, where the Ca^{2+} concentrations are higher.

One T1SS has been described in *V. cholerae.* It is associated to the RTX toxin (RtxA), a large toxin found in many *V. cholerae* strains, including O1 El tor, O139 and non-O1/O139 strains, but not in the O1 classical strains that contain a deletion into the gene cluster [98, 100]. The omnipresence of RtxA toxin among currently circulating strains lets us think that it is an important virulence factor that could be responsible for the non-O1/O139 strains' emergence [100]. RtxA leads to the depolarization of actin, by cross-linking the actin monomers into dimers, trimers or multimers, which causes rapid rounding of host cells [101–104].

Three other T1SS could be found in *V. cholerae*'s genome. The first one is associated to two putative RTX toxins with hemolytic activity (RtxL1 and RtxL2) that have been discovered in the genome of many *V. cholerae* strains [105]. They induce cell rounding and cytotoxicity and, unlike RtxA, also have a hemolytic activity. The locus has been identified but the secretion pathway has not, although all RTX proteins are secreted through a T1SS, the RtxL associated T1SS is yet to be described. Another of the putative T1SS of *V. cholerae* is associated to another RTX-like toxin, the Flagellum-regulated hemagglutinin A (FrhA - VC1620) [106]. The decreased hemagglutination in non-motile *V. cholerae* mutant has led to the discovery of FrhA. FrhA contains a RTX-like domain and a T1SS signal and has a role in hemagglutination, adhesion to human host cells and chitin, thus in colonization and biofilm formation [107]. Its regulation is comprised in the four-step hierarchy regulation of

motility, which includes the regulation of several virulence factors. FrhA is encoded in a gene cluster harbouring components with homology to TolC (FrhC - VC1621) and HlyB (VC1628). However, no HlyD homolog has been found in the surrounding genes and the homology of VC1618 to HlyB is poor, as the ATP binding site, essential to the translocation process through the T1SS, is missing [106]. This secretion system is yet to be described. The retention module-containing protein (CraA - VCA0849) also contains a glycine rich module used as T1SS secretion signal, but its secretion system remains to be described [108]. CraA is an adhesin that has a role in early stage of biofilm formation by binding chitin. It has some homology to RtxA. In other Gram-negative bacteria, it serves as an adhesin on the bacterial surface.

2.4 Type III secretion system for colonization and injection of effectors into eukaryotic host cells

The Type III secretion system (T3SS) is a multicomponent device translocating various effectors directly into the neighbouring eukaryotic host cells and is found in many *Vibrio* species, including *V. cholerae* [109]. Many non O1/O139 strains, which can lead to severe diarrhea even though they do not produce the CT and toxin coregulated pilus (TCP), possess a T3SS [110, 111]. Unlike the pandemic strains, the diarrhea induced by non O1/O139 strains shows damages to the intestinal epithelium [112]. The T3SS would in fact be essential for intestinal colonization and invasive diarrhea in those strains. The T3SS is composed of a basal structure that shares similarities with the flagella, and a needle, connecting the bacterial cytoplasm to the eukaryotic cell using a pore at its end [113]. While the structure of the T3SS is conserved among Gram-negative bacteria, the effectors encoded differ from one another, subsequently to the intended host. In *V. cholerae*, the translocated effectors are multiple, and their accumulation disrupts host cellular processes with a key role in the early stages of infection, such as cytoskeletal rearrangement and cytotoxicity, resulting in intestinal epithelial damages and colonization of the gut [109, 114].

2.4.1 Structure and secretion through the T3SS

The T3SS is a multicomponent apparatus spawning both bacterial membranes. While the effectors' secretion through T3SS is Sec independent, the translocation of the membrane components of the injectosome requires it [113]. The T3SS uses ATP for the active translocation of the effectors through both bacterial membranes directly into host cell cytoplasm. The T3SS consists of an injectosome with structural and genetic homology to the flagellum and a molecular syringe, the structure has been reviewed elsewhere [113]. In brief, the syringe connects the membrane complex to the host cell cytoplasm. It is composed of (i) a basal needle, (ii) a tip and, at its end, (iii) a translocation pore. The membrane complex is composed of an assembly of concentric rings creating a channel through both bacterial membranes. It includes an outer membrane ring connected, in the periplasm, to the inner membrane ring, in addition to a cytoplasmic portion, made of a cytoplasmic ring and an ATPase complex. The exact T3SS assembly in *V. cholerae* has not been studied yet, and some components remain to be identified [109].

2.4.2 Genes and regulation

While some *Vibrio* species (*V. parahaemolyticus*) possess two T3SS (T3SS1 and T3SS2), only one, with similarities to the T3SS2 of *V. parahaemolyticus*, has been found in *V. cholerae*'s genome [111]. The T3SS genes are located on a genomic island of approximatively 49kb, which includes an integrase, structural components,

effectors and regulators ($vttR_A$, $vttR_B$) [114]. The T3SS genomic island is acquired by horizontal transfer [115]. The core region contains most of the structural components and some effectors, while the upstream and downstream regions, more affected by the gene transfer, harbor a variety of effectors [109]. The *Vibrio* type three regulators $VttR_A$ and $VttR_B$ share similarities with ToxR, an important virulence regulator in *V. cholerae* [116]. The regulators $VttR_A$ and ToxR control the expression of $VttR_B$, which, afterwards, controls the expression of the T3SS structural genes in presence of bile [117]. The deletion of either of these regulators leads to a decreased T3SS-dependant cytotoxicity. $VttR_A$ and $VttR_B$ might also regulate genes outside the T3SS island [110].

2.4.3 Secreted proteins

The presence of T3SS in non O1/O139 strains leads to intestinal epithelium damages, such as alteration of the brush border and disruption, as seen in the infant rabbit model of infection [112]. It is the result of the translocation of many effectors into the eukaryotic host cytoplasm by the T3SS. In *V. cholerae*, there are 7 effectors encoded within the T3SS core genomic island and at least 5 others have been identified in the up and downstream regions [109]. The first effector to be identify is *Vibrio* outer protein F (VopF - NT01VC2350) [118, 119]. VopF possesses 2 actin binding domains, the formin homology-1 like and WASP homology 2 domains, that intervene in actin polymerization of the host intestinal epithelial cells. It has been shown to be essential for virulence in infant mouse model of infection [119]. VopF has a homolog in other non O1/O139 strains, VopN, that shares 55% similarity [119]. Just like VopF, VopN disturbs actin polymerization by nucleation, but unlike VopF, locates in the stress fibers by binding filamin. Both would also have an anti-apoptotic effect.

A total of 11 proteins that use the T3SS for their secretion have been identified by using a FRET technique to visualize the translocation of proteins in HeLa cells, including an effector specific to *V. cholerae,* VopX (A33_1663) [114]. VopX has been found to be essential for colonization in infant mouse model of infection and to induce an important growth defect in *S. cerevisiae* by destabilization of the cell wall through Cell Wall Integrity MAP kinase pathway activation [114, 120].

Another of the secreted effectors is VopE (A33_1662) [121]. VopE is translocated to the mitochondria after its secretion by the T3SS, where it acts as a GTPase-activating protein. Its presence in the mitochondria intervenes with the normal process of Rho GTPases Miro1 and 2, thus with the immune response using mitochondrial signalisation pathways [121, 122]. Along with VopF, VopE would lead to the loosening of the tight junctions, a primordial structure of the intestinal epithelium [119]. VopM (A33_1684) is another effector secreted by the T3SS that leads to actin stress fibers formation and brush border effacement [110].

Other effectors have been identified, but their function remains unclear, such as VopZ (A33_1704), VopW (A33_1690), VopA (A33_1680), VopG (A33_1697), VopI (A33_1687), VopY (A33_1700), VopH (A33_1678) and VopK (A33_1699) [110, 114]. VopW is known as a hydrophilic translocator that would both have structural and effector roles [114]. Despite the lack of information, a study on multiple effectors brought some light on their potential role in infection [110]. It stated that VopA, VopM, VopW and VopH seemed to be required for intestinal colonization in infant mouse model of infection, as mutants of these effectors where not recovered from infected animals. VopA could also have a role in adhesion to the intestinal cells in the early stages of infection. Along with VopH, VopI and VopW, VopA could be part of the structural apparatus as it is essential for other effectors secretion.

2.5 Type IV secretion system, a crucial virulence factor

Three T4Ps can be found at the surface of *V. cholerae*, TCP, the chitin regulated pilus (ChiRP) and the mannose sensitive hemagglutinin pilus (MSHA). T4P have structural similarities with the T2SS, and their structure has been reviewed elsewhere [123]. An inner membrane complex, docking an ATPase cytoplasmic complex, recruits a secretion pore in the outer membrane. The pilin subunits are then assembled and secreted to form a strong but malleable filament. They have a role in many biological processes leading to virulence such as, in *V. cholerae*, acquisition of mobile genetic elements (MGE), micro-colonies formation in the intestinal lumen, adhesion to abiotic surfaces or chitin and biofilm formation [123]. The bacterial aggregation by pilus-pilus interaction with TCP, in form of micro-colonies, allows concentration of the toxin at the site of colonization and protection of the immune system (as would a biofilm) [124]. Most T4Ps have cytoplasmic ATPases that allow their elongation and retraction, which can lead to eDNA capture and motility. The main secreted components of the T4P are the pilin subunits.

2.5.1 The toxin coregulated pilus

The pandemic virulence potential of *V. cholerae* resides in its MGE, harbouring both the CT and TCP apparel on the integrated CTXφ phage and *Vibrio* Pathogenic Island 1 (VPI-1), respectively [125]. TCP is essential for effective colonization of the intestinal epithelium in pandemic O1/O139 strains [126]. The VPI-1 harbours the receptor for the CTXφ phage, the major pilin of TCP (TcpA), allowing its entry into *V. cholerae*. It also regulates the CT production with ToxT, which also regulates TCP expression [48, 125]. It is believed that acquisition of both these MGE is enough to convert environmental strains into pathogenic strains [125]. Considering this information, it is clear that the gene acquisition by horizontal transfer is important for the toxigenic potential of *V. cholerae*. Obviously, TcpA, being the major component of the filament, is responsible for the pilus:pilus interaction that leads to the formation of the micro-colonies [124]. TCP also has a minor pilin, TcpB, which is also secreted and initiates pilus polymerization and retraction, despite the lack of a retraction ATPase in TCP [127]. TcpB would also bind to CTXφ minor coat protein and then leads to its internalization into *V. cholerae* by initializing the retraction of the T4P [127].

2.5.2 The mannose sensitive hemagglutinin pilus

MSHA is produced by O1 El Tor and O139 strains, but not by the O1 classical strains, and is important for adhesion to chitinous surface and biofilm formation, although it does not seem to play a role in virulence nor colonization in humans [27, 31, 126, 128]. Its filament is composed of the single major pilin MshA [129]. The dynamic of retraction/polymerization of the MSHA is controlled by c-di-GMP [129].

2.5.3 The chitin regulated pilus

The third T4P identified in *V. cholerae* is ChiRP [31]. Because, in its marine life, *V. cholerae* can use chitin as a carbon source, the capacity to colonize shellfish is then primordial to acquire this element. The expression of PilA, the major pilin of ChiRP, is induced when the bacteria are grown in presence of chitin [31]. The absence of PilA, thus of ChiRP, decreases, but does not suppress, the ability of *V. cholerae* to colonize crab shell, even though it has no effect in infant mouse model nor on adhesion to human cells [130]. It suggests that ChiRP has a role in adhesion to chitin in collaboration with other chitin binding structures and proteins (MSHA, GbpA). The

colocalization of ChiRP at the pole of *V. cholerae* along with the T2SS, secreting chitinases required for chitin acquisition, would increase the effectiveness of chitin uptake by limiting the secretion to an adhesion site [31]. In other *Vibrio*, ChiRP could also have a role in biofilm formation by mediating bacterium:bacterium interactions, a phenomenon that has also been observed in *V. cholerae* and that could further increase chitin uptake [131, 132]. It is important to note that the chitin utilization pathway is linked with natural competence pathway and that ChiRP is implied in eDNA uptake [131]. eDNA uptake is used by bacteria to gain new functions, such as virulence and resistance factors, and to increase their fitness and survival in environment.

2.6 Other secreted molecules

The cholix toxin (ChxA) is a eukaryotic elongation factor-2 specific ADP-rybosyl transferase that induces cell death [133]. ChxA is produced by many *V. cholerae* strains [134, 135].

Accessory cholera enterotoxin (Ace - VC1459) and zonula occludens toxin (Zot - VC1458) are accessory toxins that are both encoded near the CT genes on the CTXφ phage [136, 137]. Zot leads to the disruption of the tight junctions between intestinal epithelial cells, an important structure in the intestinal permeability [138, 139]. It is translocated and anchored in the outer membrane and has two functional domains. The N-terminal domain is important for CTXφ phage's morphogenesis and the C-terminal domain is cleaved by proteases. Once released into the intestinal lumen, the C-terminal domain acts as a toxin [139, 140]. Thus, Zot does not employ a conventional secretion system for its release into the extracellular milieu. Regarding Ace, it leads to fluid secretion in rabbit ileal loop model by unbalancing calcium secretion and the secretion mechanism has not been determined yet [138].

3. Membrane vesicles, the type 0 secretion system

Most bacteria, including Gram-negative and Gram-positive bacteria, release MV, also known as the type 0 secretion system [141]. Different types of MV can be produced including the outer membrane vesicles (OMV), the outer-inner membrane vesicles (OIMV), the cytoplasmic membrane vesicles (CMV) and the tube-shaped membranous structures (TSMS). The different types of MV differ in their composition and their biogenesis mechanisms, which will not be presented here since they have already been reviewed elsewhere [142]. *V. cholerae* can secrete OMV and OIMV, which contain lipopolysaccharides, phospholipids, proteins [143], DNA and RNA [144, 145]. An hypervesiculation has been reported in *V. cholerae* at the early stages of intestine colonization by silencing the phospholipid transporter VacJ/Yrb involved in the maintenance of the outer membrane lipid asymmetry. This hypervesiculation is characterized by a drastic modification of the membrane composition and a better colonization of the host intestine [146].

In vitro, the protein cargo of MV is highly dependent on the bacterial growth conditions [147]. The protein cargo of the MV secreted by *V. cholerae* El Tor O1 has been determined under virulence activating conditions. A total of 90 proteins associated to MV have been identified, 50 % being outer membrane or periplasmic proteins [143]. Among these proteins, some are secreted in association with the vesicles, such as the membrane and periplasmic proteins, while others are secreted independently and associated with the vesicles in the extracellular compartment, such as Bap1 [148]. The proteins associated with the vesicles can have a role in resistance (antimicrobials, plasma and bacteriophages), in biofilm formation or in virulence.

3.1 Membrane vesicles and resistance

A role for the MV in antimicrobial peptides (AMP) resistance has been reported in several Gram-negative bacteria including *V. cholerae*. Our previous studies demonstrated that PrtV-associated MV can protect *V. cholerae* from the lysis by LL-37, a cathelicidin secreted by the epithelial cells in response to *V. cholerae* infection [56]. In addition, the matrix protein Bap1 can bind to OmpT, a porin located in the outer membrane, on the surface of the MV of *V. cholerae* in presence of polymyxin B and confer cross-resistance to LL-37 [148]. Interestingly, the hypervesiculation observed during the early stages of infection leads to a decrease of OmpT in the outer membrane correlated with an increase in OmpT abundance in the MV [146]. The authors suggest that the hypervesiculation is a process used by *V. cholerae* to quickly modify the outer membrane protein content in order to increase the intestinal colonization fitness. Therefore, the hypervesiculation *in vivo* may contribute to the Bap1 mediated AMP resistance in the intestine where analogues of polymyxin B are secreted by the microbiota. In *V. cholerae*, the expression of *ompT* is negatively correlated with the expression of *ompU* through the ToxR switch [149, 150]. During the hypervesiculation process, *ompU* expression increases, leading to an accumulation of OmpU in the membrane [148]. A role of OmpU in AMP and bile resistance has been reported in *V. cholerae* [151]. Therefore, the hypervesiculation in *V. cholerae* might represent a double advantage in terms of AMP resistance through vesicles associated OmpT-Bap1 and membrane bounded OmpU.

Besides AMP, MV are also involved in serum resistance in *V. cholerae*. Septicemia caused by *V. cholerae* has been reported, especially in patients suffering liver disorders, which can lead to a 50% mortality rates [152]. In an elegant study, Aung *et al*. demonstrated that IgG present in the serum of healthy people can recognize OmpU of *V. cholerae*, which leads to the recruitment of the complement through C1q binding and to the clearance of *V. cholerae* [153]. However, the presence of OmpU in the MV can sequester the anti-OmpU IgG before they reach the bacterial cells, leading to an increased resistance of the bacteria to serum [153].

A role of the MV in resistance to bacteriophages has also been demonstrated in *V. cholerae* [154]. The authors proposed that, similarly to Bap1 and AMP and to OmpU and IgG, the presence of MV is used as bacterial decoy to lure the phages before they can reach the bacterial cell. In this case, it is the presence of phage receptors on the surface of the MV that is responsible for the sequestration of the phages [154].

3.2 Membrane vesicles and biofilm

A significant part of antimicrobial resistance is associated with the biofilm lifestyle of bacteria. The bacteria growing in a biofilm are up to 1000 times more resistant to antimicrobials and disinfectants than their planktonic counterparts [155]. It has been demonstrated that MV are involved in the formation of biofilms in several Gram-negative bacteria [156]. In *V. cholerae*, the association of Bap1, PrtV and eDNA with the MV might have a role in biofilm formation by strengthening the structure and by recruiting planktonic bacteria. More information on the role of MV in biofilm is provided in the Biofilms and Flagella section.

3.3 Membrane vesicles and virulence

The MV can also carry virulence factors including the CT, the major virulence factor of *V. cholerae* [157]. After secretion, The B subunits interact with the GM1 receptor at the surface of the epithelial cells and the toxin is endocytosed. The A

subunit dissociates from the rest of the toxin in the endoplasmic reticulum and spontaneously unfold. The unfolded form of the A subunit is responsible for the toxic activity of the CT. The vesicle-associated toxin is biologically active although only A subunits are encapsulated [158]. It has been demonstrated that the MV can enter inside the host cells using different mechanisms involving clathrin coated pits, formation of caveolae, use of lipid rafts and direct fusion with host cell membrane [159]. Therefore, it is likely that the lack of B subunits is not an issue for the delivery of active A subunits of the CT while encapsulated inside the MV.

Besides the CT, other biologically active virulence factors can also be transported to the host cells through MV. It is the case for HA/P and VesC [160], PrtV metalloprotease [56] and the VCC [161]. Therefore, the MV of *V. cholerae* can carry a concentrated arsenal of virulence factors that can be efficiently delivered to the host cells and have a role in *V. cholerae* pathogenesis.

4. Biofilms and flagella

Most of the bacteria, including pathogens, form biofilm to survive and persist in different environments. Biofilms are organized bacterial communities attached to a surface and producing a matrix. *V. cholerae* form biofilms at different stages of its life cycle. An increasing number of evidences suggest that *V. cholerae* forms biofilm during the gut infection and biofilm-like aggregates display a hyper-infective phenotype [162]. To persist in the environment, *V. cholerae* forms biofilm on different biotic and abiotic substrates such as floating aggregates, ship hulls, microalgae and copepods [163]. The transition between planktonic motile and non-motile biofilm states is highly regulated [162, 164]. The composition and abundance of the secreted factors involved in biofilm formation, maturation and dispersion largely depend on environmental conditions and on surface composition. In this section, we will review the current knowledge about the biofilm secretome in *V. cholerae* at different stages of the biofilm formation and the interplay between biofilm and motility.

4.1 From motility to initial adhesion

Planktonic *V. cholerae* are motile cells and their motility is ensured by a single polar flagellum. The flagellum is composed of three major structural components: i) the basal body, ii) the hook and iii) the filament (for detailed structure: [165]). Structurally, the flagellum is closely related to the T3SS and therefore has been referred as fT3SS (flagellum Type-3 Secretion System) [166]. The main proteins secreted through the fT3SS are the flagellins. In *V. cholerae*, five different flagellins (FlaA-E) encoded on two chromosomally distinct loci (*flaAC and flaEDB)* have been described. From these 5 flagellins, only FlaA is essential for the motility [167]. Recently, an elegant study from Dongre *and coll*. demonstrated that the cytotoxin MakA is also secreted through the fT3SS [168]. This toxin is involved in colonization and virulence in zebrafish and *C. elegans* infection models and represents the first characterization of a toxin secreted through the fT3SS in *V. cholerae* [168].

V. cholerae motility and chemotaxis are important for the virulence in the human gut [169]. They are necessary for the bacteria to travel from the lumen to the epithelial cells, where the virulence factors are secreted after attachment of the bacteria. Motility also plays a key role in biofilm formation on surfaces as an essential element of the initial adhesion of the bacteria. On abiotic surfaces, *V. cholerae*'s adhesion involves MSHA to "scan the surface" [170]. The flagellum is responsible for the

bacterial rotation on the surface of the support, which allows MSHA to reach a spot of high affinity. This adhesion is signaled through the flagellum rotor and results in an inhibition of motility pathways and the secretion of the *Vibrio* polysaccharides (VPS) [171]. Two other T4Ps are implicated in bacterial adhesion; ChiRP and TCP (see T4SS section). Initial adhesion to biotic surface is promoted by GbpA, which is secreted through the T2SS and induces mucin secretion [21].

Additionally, FrhA (hemagglutinin) and CraA (adhesin) secreted through the T1SS are involved in adhesion and biofilm formation (see T1SS section). The expression of both *frhA* and *craA* is controlled by a c-di-GMP-dependent regulatory system [108]. In addition, *frhA* is regulated by the flagella regulation pathways, reinforcing the role of flagella in the initial stages of biofilm formation [106].

4.2 Biofilm maturation

Once attached, *Vibrio* starts secreting VPS, which represent more than 50% of matrix composition. VPS polymers are secreted throughout the biofilm production and are mostly composed of [→4)-α-L-Gul*p*NAcAGly3OAc-(1→4)-β-D-Glc*p*-(1→4)-α-d-Glc*p*-(1→4)-α-D-Gal*p*-(1→] subunits. A variant representing around 20% of VPS consists on the same pattern except for the α-D-Glc*p* moiety that is replaced by α-D-Glc*p*NAc [172]. The VPS biogenesis and export systems are encoded on two clusters: VpsI and VpsII, encoding VpsA-K and VpsL-Q, respectively [173, 174]. Among the 18 *vps* genes, 15 of them induced highly impaired biofilm formation in *V. cholerae* when suppressed [174]. Recently, a model has been proposed for the production and secretion of VPS. In this model, the VPS are synthesized by formation and polymerization of individual subunits. The polymers are then transferred across the outer membrane through VpsM/N [175]. This system is tightly controlled by the tyrosine phosphoregulatory system VpsO/VpsU, which controls the level of phosphorylation of the C-terminal tyrosine-cluster of VpsO. High level of phosphorylation results in VpsO oligomer dissociation and VPS production reduction, whereas low level of VpsO phosphorylation results in high oligomerization and increased VPS production [175].

Shortly after VPS secretion has been initiated, the sequential secretion of the 3 major biofilm matrix proteins through the T2SS occurs. The first matrix protein to be secreted is RbmA, followed by Bap1 and RbmC [176]. More specifically, RmbC has a role in maintaining and stabilizing the biofilm [177]. A study using mutants lacking RbmC and its homolog Bap1 showed a change of colonial morphology and the loss of biofilm formation capacity [177, 178]. On the other hand, RbmA controls the structure of the biofilm [9, 179].

Growth of the biofilm is ensured by two different processes: (i) the bacteria inside the matrix are dividing inside an envelope formed by the VPS, RbmC and Bap1 [176] and (ii) new bacteria are recruited inside the biofilm. This recruitment requires the cleavage of the N-terminal domain of RbmA by PrtV. Once cleaved, RbmA can bind bacterial cells that are not producing VPS (planktonic) and recruits them into the biofilm [180]. Since MV have been observed in the biofilm matrix and PrtV can be associated to the surface of the MV, it is possible that MV play an important role in biofilm maturation in *V. cholerae* [181]. In addition, the association of Bap1 to the MV in specific conditions is likely to lead to the adhesion of the MV to the surface and to the exopolysaccharides [181]. Three other proteins with no role in biofilm formation and adhesion have been identified in biofilm preparation, *i.e.* the hemolysin HylA, HA/P and ChiA-2 [182].

Besides VPS, proteins and MV, a significant amount of eDNA is entrapped in the biofilm matrix. The roles of eDNA in bacterial physiology have been reviewed

elsewhere and include nutrient source, horizontal gene transfer and adherence [183]. Recently, a role in the tridimensional matrix structure of the biofilm in *V. cholerae* has been reported [184]. The eDNA inside the biofilm most probably results from cell autolysis and MV secretion [185].

4.3 Biofilm dispersion and detachment

Biofilm dispersal is a complex process by which bacteria actively succeed to evade biofilm matrix [186]. Conversely to adhesion and biofilm maturation, little is known about the dispersion process of *V. cholerae* biofilms. It requires specific environmental signals, which trigger the quorum sensing and the general stress response pathways [187], matrix degradation and motility resumption [188]. The matrix degradation requires RbmB, an extracellular polysaccharide lyase that digests the VPS, and LapG, a periplasmic protease that cleaves the adhesins FrhA and CraA located at the outer membrane [188]. Under substrate specific conditions, the extracellular protease HA/P also participates in biofilm dispersion by degrading the mucin [189]. Finally, secreted nucleases such as Dns and Xds have a role in biofilm dispersion by cleaving the eDNA present in the matrix [185]. The motility resumption requires the ability to switch the flagella rotation from counterclockwise to clockwise direction mediated by CheY3 independently of chemotaxis [188].

5. Conclusion

Over the last decades, numerous studies have focused on the secreted molecules and secretion systems used by *V. cholerae* to deliver extracellular effectors. Various roles have been assigned to the secreted molecules especially regarding the host colonization and virulence, and the environmental survival and persistence, denoting their importance in *V. cholerae*'s pathogenesis and life cycle. Additionally, the redundancy of some functions carried by multiple secreted effectors testifies of their importance. With gene acquisition, MGE, strain sequencing and the emergence of more efficient technologies, it is most likely that additional secreted effectors and secretion systems will be identified and characterized.

The recent characterization of the MakA toxin secretion through the fT3SS [168] and the numerous studies on the T6SS since its discovery 15 years ago [68] clearly demonstrate that there is still work to do on the secretome and secretion systems in *V. cholerae*. The secretion mechanism of some extracellular proteins - with characterized functions - remains to be determined. It is the case of ChxA, Ace, RbmB and the DNAse Dns.

The regulation of the secretion systems and their cargo molecules is a complex process. It involves numerous regulators that can be activated or repressed depending on the detection of specific intracellular and extracellular signals. So far, most of the studies aiming to decipher the regulation pathways have been performed under laboratory conditions. The featuring of conditions that characterize the intestinal environment before and during diarrhea, including the peristaltic movement, anaerobia, the presence of the microbiota, water efflux and high osmolarity, is likely to modify *V. cholerae* secretion in terms of regulation and nature, abundance and activity of the secreted molecules. Therefore, it would be highly beneficial to study the secretion mechanisms, the secreted molecules and their regulation in models that are closer to the physiological conditions encountered in the host, such as *ex vivo* devices. Understanding the regulation and the mechanisms of colonization, virulence and resistance in these physiological conditions is crucial for the development of treatments and vaccines against *V. cholerae*.

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