Structural and Molecular Biology of Type IV Secretion Systems

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Chapter 2

Abstract
Type IV secretion systems (T4SSs) are nanomachines that Gram-negative, Gram-positive bacteria and some archaea use to transport macromolecules across their membranes into bacterial or eukaryotic host targets or into the extracellular milieu. They are the most versatile secretion systems, being able to deliver both proteins or nucleoprotein complexes into targeted cells. By mediating conjugation and/or competence, T4SSs play important roles in determining bacterial genome plasticity and diversity; they also play a pivotal role in the spread of antibiotic resistance within bacterial populations. T4SSs are also used by human pathogens such as *Legionella pneumophila*, *Bordetella pertussis*, *Brucella* sp or *Helicobacter pylori* to sustain infection. Since they are essential virulence factors for these important pathogens, T4SSs might represent attractive targets for vaccines and therapeutics. The best characterized conjugative T4SSs of Gram-negative bacteria are composed of twelve components that are conserved across many T4SSs. In this chapter we will review our current structural knowledge on the T4SSs by describing the structures of the individual components and how they assemble into large macromolecular assemblies. With the combined efforts of X-ray crystallography, nuclear magnetic resonance and more recently electron microscopy, structural biology of the T4SS has made spectacular progress during the past fifteen years and has unraveled the properties of unique proteins and complexes that assemble dynamically in a highly sophisticated manner.

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1 Architectures and functions of type IV secretion systems

1.1 Classification and overview

Type IV secretion systems (T4SSs) are nanomachines that Gram-negative (G^-), Gram-positive (G^+) bacteria and some archaea produce to transport macromolecules across their membranes. The systems are used for a very diverse set of functions that include exchange of genetic material between bacterial species, acquisition of novel genetic material from the external milieu, delivery of nucleoprotein complexes or effector proteins into recipient cells (Wallden et al. 2010). The systems have raised considerable interest since the discovery of conjugation systems and their exploitation has launched the molecular biology "era". From a structural and mechanistic point of view, T4SSs represent a remarkably large and membrane-embedded dynamic macromolecular assembly. Although T4SSs share common features, they have been evolutionary tailored to tackle specific functions in each bacterium (Bhatty et al. 2013). T4SSs have been grouped into three functional categories according to the biological processes they mediate (Cascales and Christie 2003). The first group contains probably the most widely-distributed and the best characterized T4SSs of G^- bacteria. These systems operate in conjugation, being used to deliver single-stranded DNA and one or more proteins into bacterial or eukaryotic cells. Conjugative T4SSs are exemplified by the *Agrobacterium tumefaciens* VirB/D4 system or the pKM101, F, and R388 conjugative plasmids of *Escherichia coli*. Comprehensive and recent reviews have summarized our understanding of the structural biology of conjugation and can provide more details on this group of T4SS (Ilangovan et al. 2015; Chandran Darbari and Waksman 2015). The second group of T4SSs is used to deliver protein effectors into the cytosol of eukaryotic cells and promote bacterial colonization and survival. Examples are the *Legionella pneumophila* Dot/Icm system (Kubori and Nagai 2016; Nagai and Kubori 2011), *Brucella sp*. (Lacerda et al. 2013; O'Callaghan et al. 1999), or the *Helicobacter pylori cag* T4SS that injects the oncoprotein CagA (reviewed in (Backert et al. 2015)). The third group includes the remaining T4SSs with some that mediates DNA release and/or uptake from and to the extracellular medi-

um such as *H. pylori* ComB system or GGI system of *Neisseria gonorrhoeae*. Another T4SS, discovered in the plant pathogen *Xanthomonas* (Alegria et al. 2005), is not directly involved in infection like the second group of T4SS described above, but it secretes toxins in order to kill other G^- bacteria (Souza et al. 2015), and this makes difficult the assignment to one of these three groups. Finally, a genomic analysis proposed a novel group of T4SS that are present in the genomes of the G^+ bacteria of the genera *Streptococcus* (Zhang et al. 2012).

1.2 Composition of T4SSs

X-ray crystallography, nuclear magnetic resonance (NMR) and more recently electron microscopy have been successfully used to provide insights into the structure of T4SSs. Most of our structural knowledge on these secretion systems comes from studies of G^- conjugative T4SSs individual components and complexes or from their homologues in other systems (Table 1). Conjugative T4SSs are considered prototypical and are composed of 12 proteins, VirB1-11 and VirD4 based on *A. tumefaciens* VirB/D system nomenclature (Figure 2.1A) (Chandran Darbari and Waksman 2015). These proteins assemble into three interlinked compartments (Figure 2.1B): the inner membrane complex (IMC), the core complex (also named the outer membrane core complex) and the external pilus. Three ATPases (VirB4, VirB11 and VirD4) provides the energy for T4SS assembly and substrate transfer. The inner membrane complex is composed of VirB3, VirB4, VirB6 VirB8 and VirD4[Office1], each present in 12 copies, except VirD4 where only 4 copies could be counted. The core complex is made up of 14 copies each of VirB7, VirB9 and VirB10 and form a large channel in the periplasm with the VirB10 protein forming the outer membrane channel and connecting the outer and inner membranes. The transglycosylase VirB1 facilitates the insertion of the system into the periplasmic space by breaking parts of the peptidoglycan layer. Outside the bacterial envelope, the system is extended by a pilus consisting of VirB2 (major pilin) and VirB5 (minor pilin). This pilus can interact with other bacteria, with the extracellular medium or the host cell.

The diversity of the T4SS repertoire is remarkable. Some T4SS contain only homologues of VirB1, VirB4, VirB6 and VirD4 (e.g. the T4SS of *Streptococcus*) while the more complex can use

nearly 30 proteins (*cag*T4SS from *H. pylori*). In the latter, twelve proteins have been proposed to be VirB homologues (sometimes only functional homologues) but the system also encodes for fifteen proteins unique to *H. pylori* with several that are essential for *cag*T4SS function. The exact roles of these proteins are not always known but their presence reflects the adaptation of T4SS composition to the bacterial niche. The structures of several Cag proteins have been solved (Cendron and Zanotti 2011) and their contributions to the *cag*T4SS function has been recently reviewed (Merino et al. 2017). Along these lines, the Dot/IcM system of *L. pneumophila* is also markedly different from the prototypical VirB/D system. The Dot/Icm system encodes for 26 proteins and sequence similarity is found only between the proteins VirB10 and DotG, and VirB11 and DotB (Nagai and Kubori 2011). The *Brucella* T4SS has also an extra component VirB12 but its expression is not required for *Brucella* survival (Sun et al. 2005).

Conjugation systems of G^+ bacteria lack the core complex proteins VirB7, VirB9, VirB10 and the pilins VirB2 and VirB5 but the system is still competent for secretion (Alvarez-Martinez and Christie 2009). Homologues of VirD4, VirB4, VirB1 and of the inner membrane proteins VirB6 and VirB8 have been identified in the G^+ T4SS (Wisniewski and Rood 2017; Grohmann et al. 2016) (Figure 2.1). As for G^-, there is an important diversity in the composition of G^+ T4SSs and some, such as the one encoded by the pIP501 conjugative plasmid, present unique proteins that have no sequence similarity with VirB/D proteins (Figure 2.1[Office2][LT3]).

2 Structures of individual components

2.1 ATPases

Three ATPases are associated with T4SSs and power the secretion machinery: VirD4, VirB11 and VirB4. VirB4 is the only ubiquitous proteins and VirB11 proteins are not conserved in the T4SS of G^+ bacteria. Some G^+ bacteria conjugative plasmids encode for TraB, a FtsK-like ATPase that is essential to translocate DNA from one cell to the other (see Chapter 5 for more information).

VirB11

VirB11 belongs to the traffic ATPases family, required for energizing transport across the T4SS (Planet et al. 2001). The protein associates with the cytoplasmic side of the inner membrane and its ATPase activity is enhanced upon interactions with phospholipids (Rashkova et al. 1997). VirB11 is able to interact with VirB4 and this interaction might be involved in pilus biogenesis (Sagulenko et al. 2001). VirB11 interacts also with VirD4, this interaction might induce the transfer of the substrate (Ripoll-Rozada et al. 2013). The crystal structure of VirB11 from *H. pylori cag*T4SS (Cagα) showed that the protein is a hexamer of 100 Å in diameter and 50 Å in height (Figure 2.2A) (Yeo et al. 2000). The hexamer is composed of two rings. One ring is formed by the six N-terminal domains (NTDs) and the second by six RecA-like C-terminal domains (CTDs) with a short linker in between. The nucleotide-binding site (NBS) is located at the interface between these two domains (Figure 2.2A). The hexamer opens a central chamber of 50 Å diameter that is restricted at the CTD-ring to a 10 Å diameter hole. Another crystal structure of VirB11 from *B. suis* T4SS revealed that the organization of the hexameric assembly was conserved despite a large domain swap between the NTD and the CTD across subunits (Hare et al. 2006). The structure of Cagα in different nucleotide bound states have shown that VirB11 is a dynamic assembly regulated by ATP binding/hydrolysis (Savvides et al. 2003). In the absence of nucleotides, the NTD is disordered causing an open ring conformation. The binding of nucleotides to the NBS induces a modification of the NTD resulting in a closed ring conformation (Savvides et al. 2003). Cagα can also be regulated by HP1451, a protein unique to *H. pylori* that forms a complex with the hexamer and reduces the ATP hydrolysis activity of Cagα (Hare et al. 2007) although the conservation and importance of this regulatory mechanism is still not known. Finally, VirB11 was found as a potent target for ATPase inhibitors (Sayer et al. 2014) in order to block CagA secretion (Hilleringmann et al. 2006)[LT4].

VirD4

VirD4 are integral inner membrane proteins with two domains: a short N-terminal domain (NTD) anchored at the membrane and a large cytoplasmic domain. The protein is involved in substrate recruitment and transport but not in pilus biogenesis. In conjugative T4SS, VirD4 is a coupling protein (T4CP) that recruits the relaxosome (substrate) to deliver it to the T4SS channel for its injection across the membrane (Llosa et al. 2003). In *H. pylori*, Cagβ, a VirD4-like protein, interacts with the effector CagA and the chaperone CagF (Jurik et al. 2010) suggesting that Cagβ could play the role of a T4CP (Fischer 2011). However, some T4SS systems (such as those encoded by *Bordetella pertussis* or *Brucella spp*) can recruit substrates independently of a T4CP. The pIP501 conjugative plasmid from G^+ *Enterococcus sp.* encodes for a T4CP named TraJ. The protein has been shown to interact with the relaxase TraA and with others components of the conjugation system (Abajy et al. 2007). TraJ also has some sequence similarities to VirB4, including the typical Walker A and B of P-loop ATPases (Goessweiner-Mohr et al. 2013a). The N-terminal transmembrane domain of the VirD4 homologue TrwB is involved in the interaction with TrwE (VirB10 homologue) (de Paz et al. 2010) and *A. tumefaciens* VirD4 also interacts with VirB10 (Cascales et al. 2005) suggesting that VirD4 is connected to the T4SS core complex via the VirB10 protein.

The structure of the cytoplasmic domain of TrwB from *E. coli* plasmid R388 (TrwBΔN70) was solved by X-ray crystallography. The protein is a globular homohexamer of 110 Å in diameter and 90 Å in height with an orange like-shape (Gomis-Ruth et al. 2001). A central channel of 20 Å of diameter traverses the hexamer. At the cytoplasmic pole the channel is restricted to 8 Å of diameter (Figure 2.2B) but this size is sufficient for the passage of single-stranded DNA. The crystal structure revealed that TrwBΔN70 consists of two domains: a conserved nucleotide-binding domain (NBD) and a sequence variable all-α domain (AAD). The NBD is composed of a central parallel/antiparallel nine-stranded β-sheet flanked by eleven α-helices and contains the Walker A and B motifs (Figure 2.2C). The ATP binds the protein at the interface between two subunits. At the bottom of the NBD, the AAD domain is composed of seven α-helices. The AAD has a high structural similarity with the N-terminal domain of the recombinase XerD.

VirB4

VirB4 is the most conserved ATPase (Arechaga et al. 2008) and is required for T4SS function. The localization, topology and oligomerization of VirB4 are unclear and are probably T4SS-dependent. In *A. tumefaciens*, the protein has been localized at the inner membrane and anchored by transmembrane helices (Dang and Christie 1997) but might be a soluble component in other T4SSs. The protein can form monomers, dimers and hexamers (Arechaga et al. 2008; Dang et al. 1999; Durand et al. 2010). VirB4 plays a essential role in the T4SS and interacts with many T4SS components, including VirB3, VirB8, VirB10, VirB11 and VirD4 and also with the substrates (reviewed in (Fronzes et al. 2009a). A better understanding of VirB4 structure and localization in the T4SS came from the determination of an almost complete T4SS (see part 3 in this review) where it is observed as a hexamer. The VirB4 proteins consist of two domains, a NTD and a CTD, with the latter containing the NBS. The structure of the CTD of the VirB4-like protein from the G^+ *Thermoanaerobacter pseudethanolicus* bacteria ($TpsVirB4_{CTD}$) has been solved by X-ray crystallography (Wallden et al. 2012). This protein displayed low sequence identity with *A. tumefaciens* VirB4 (12 %). Interestingly the structure revealed that $TpsVirB4_{CTD}$ presents remarkable structural similarities with VirD4 proteins (Figure 2.2C). In particular, the NBS of $TpsVirB4_{CTD}$ displays a α/β RecA-fold with conserved Walker A and B motifs as well as a four helix bundle domain reminiscent of the AAD domain of VirD4.

2.2 Inner membrane

VirB3

Although early studies suggested that VirB3 could be a minor pilin, it becomes clearer now that VirB3 is part of the T4SS inner membrane complex (Low et al. 2014; Mossey et al. 2010). VirB3 is predicted to have two transmembrane domains of unknown function (Mossey et al. 2010). In *Campylobacter jejuni* and *H. pylori* VirB3 and VirB4 are produced as a single protein (Fronzes et al. 2009a). This suggests that the two proteins are part of the same func-

tional unit and that VirB3 might recruit VirB4 to the membrane and modulate its activity. No structural information is available for VirB3 proteins.

VirB6

Very limited information is available on the biochemistry of VirB6 proteins although the protein is essential for T4SS function. VirB6 is an inner membrane protein with a periplasmic N-terminus, five transmembrane domains (TM) and a cytoplasmic C-terminus (Jakubowski et al. 2004). The authors of this study have shown that a large periplasmic loop between TM2 and TM3 mediated the interaction with the substrate. TM3, 4 and 5 are necessary for the transfer of the substrate from VirB6 to VirB8. The N- and the C-terminal portions of the protein play a role in the transfer of the substrate to VirB2 and VirB9. The periplasmic domain of *Brucella* VirB6 was also found to interact with VirB8 (Villamil Giraldo et al. 2012) and VirB10 (Villamil Giraldo et al. 2015). VirB6-like proteins are present in G^+ bacteria such as the protein TraL of the pIP501 plasmid, which is predicted to have six TM domains (Goessweiner-Mohr et al. 2013a).

VirB8

VirB8 is an essential component of the inner membrane complex of G^- T4SS. It consists of a short cytoplasmic tail, a TM domain followed by a periplasmic CTD. Yeast two-hybrid studies have shown that the periplasmic domain of VirB8 interacts with VirB1, VirB4, VirB9 and VirB10 ((Ward et al. 2002; Das and Xie 2000). The structures of the periplasmic domains of many VirB8 have been solved (Table 1) (Sharifahmadian et al. 2017; Fercher et al. 2016; Casu et al. 2016; Kuroda et al. 2015; Gillespie et al. 2015; Goessweiner-Mohr et al. 2013b; Smith et al. 2012; Porter et al. 2012; Bailey et al. 2006; Terradot et al. 2005). These structures have a remarkably conserved nuclear transport factor 2 family (NTF-2) fold, consisting of a four-stranded antiparallel β-sheet flanked by five helices one side (Figure 2.3A). The protein forms dimers, but the dimerization interface might not be conserved throughout the

family (Casu et al. 2016). Interestingly, the structure of the *Legionella* DotI has revealed that the protein is a homologue of VirB8 in absence of sequence similarity, and forms a heterocomplex with its paralogue DotJ (Kuroda et al. 2015). The structures of VirB8 have also enabled the design and characterization of small molecules inhibitors that might represent a plausible way of disarming T4SSs in different bacterial species (Casu et al. 2016; Smith et al. 2012; Paschos et al. 2011).

The fold of VirB8 is conserved in the G^+ homologues TraM and TcpC from pI501 plasmid of *E. faecalis* and *Clostridium perfringens* respectively (Figure 2.3A) (Goessweiner-Mohr et al. 2013b; Porter et al. 2012). However, TraM is monomeric in solution but both TraM and TcpC form trimers in the crystals. Interestingly TcpC has two NTF-2 domains suggesting that these proteins function as multiple of dimers. In addition, this T4SS has a second homologue of VirB8, TraH, which is monomeric in solution (Fercher et al. 2016). As the G^- VirB8, TcpC and TraM are able to interact with others T4SS protein counterparts such the T4CP, VirB1 and VirB6 (Abajy et al. 2007; Steen et al. 2009). TraM is localized at the cell envelope and it was proposed that the protein may have a role in the scaffolding of the core complex or could be involved in adhesion to the host cell (Goessweiner-Mohr et al. 2013a).

2.3 Outer membrane and periplasm

VirB1

These proteins are muramidases that play a role in the machinery assembly (Hoppner et al. 2004) but are not essential for secretion (Chandran Darbari and Waksman 2015). The enzymatic activity of the protein breaks down the peptidoglycan layer but other portions might have additional roles (Zupan et al. 2007). By liberating the peptidoglycans, VirB1 facilitates the incorporation of the T4SS in the periplasmic space. In the G^+ T4SSs, the role of VirB1 might be played by TraG but the protein might have additional roles as it has both lytic transglycosylase and endopeptidase activities and can also interact with the translocation channel protein TraM (Arends et al. 2013).

VirB7 and VirB9

VirB7 are small lipoproteins that are anchored to the outer membrane of G^- T4SSs. The N-terminus is acetylated and inserted in the outer membrane while the remaining part of the VirB7 is periplasmic and interacts with VirB9. VirB7 has been found associated with the *A. tumefaciens* T-pilus and its homologue CagT was also detected around the base of *H. pylori cag*T4SS pili (Rohde et al. 2003). The VirB7 protein of the *Xanthomonas* T4SS contains an additional N0 domain that is otherwise present in the secretins of T2SSs and T3SSs (Souza et al. 2011). VirB9 is a periplasmic protein with two domains, the N-terminal (NTD) and C-terminal domains (CTD). The VirB9 and VirB7 form a tight complex and the interaction is essential for T4SS function (reviewed in (Chandran Darbari and Waksman 2015)). A structure of the $TraO_{CTD}$ (VirB9)/TraN (VirB7) complex was determined by NMR (Bayliss et al. 2007). $TraO_{CTD}$ adopts an immunoglobin-like β-sandwich fold of six β-strands with three additional β-strands forming an appendage protruding outside the sandwich (Figure 2.3B). A 3_{10} helix connects the first two strands of the sandwich. This fold is remarkably conserved in the CTD of CagX from *H. pylori cag*T4SS (Zhang et al. 2017) and VirB9 from the *Xanthomonas* T4SS (Coutinho Oliveira et al., 2016). The TraN protein wraps around and complement a β-strand of $TraO_{CTD}$ sandwich (Figure 2.3B, (Bayliss et al. 2007) and this binding mechanism is likely conserved in other systems (Oliveira et al. 2016).

VirB10

VirB10 is a remarkable protein since it traverses the entire bacterial envelope in G^- bacterial T4SS. The protein is composed of a N-terminal domain that contains a small cytoplasmic portion, a TM helix, a flexible part and a globular CTD. The protein has the central function in the T4SSs of bridging the different compartments by interacting with many proteins and is also able to transmit signals (Cascales and Christie 2004a). The first structural information on the protein came from the crystal structure of $VirB10_{CTD}$ from *H. pylori* ComB10 protein (Terradot et al. 2005). The structure revealed that the $VirB10_{CTD}$ consists of a modified β-barrel flanked by a α- helix.

Protruding outside the β-barrel is an "antenna" consisting of a helix-loop-helix motif. The fold of VirB10 was conserved in the structure of the VirB7/VirB9/VirB10 ternary complex (Figure 2.3B) (Chandran et al. 2009). More details will be provided on this complex in part 3.

2.4 Pilus protein(s)

VirB2

The major pilin VirB2 is initially produced as a propilin that is transported across the IM using the target signal peptide. There is a strong variability within the sequence of T4SS pilins and also in the way they are polymerized (reviewed recently in (Hospenthal et al. 2017)). F-type propilins are transacetylated and maturated while T-type pilins are cyclized after truncation. VirB2 from *A. tumefaciens* is also cyclic and contains a hydrophobic and hydrophilic region. The hydrophobic region could be responsible of the pilin-pilin interaction (Kerr and Christie 2010). The structure of the VirB2/TraA F-type pilus has been recently determined by cryo-electron microscopy (cryo-EM) (see part 3).

VirB5

VirB5 proteins are minor pilins since they are generally less abundant than VirB2. The crystal structure of VirB5 homologue TraC from *E. coli* plasmid pKM101 showed that the protein is composed of a three-helix bundle with a loose globular appendage consisting of four short helices (Figure 2.3C) (Yeo et al. 2003). TraC has been located at the tip of the pilus (Krall et al. 2002; Hapfelmeier et al. 2000) but 12 subunits of TrwJ, another VirB5 like protein, from *E. coli* R388 plasmid seems to be present in the stalk or/and inner membrane (Low et al. 2014) of the T4SS (see below). Interestingly, CagL the functional homologue of VirB5 in *H. pylori cag*T4SS is present on the pilus and interacts with the α5β1 integrin receptor but has a different fold (Barden et al. 2013)[LT5][Office6].

3 Structures of T4SS molecular assemblies

3.1 The core (or outer membrane) complex

The first breakthrough in assembling large sub-complexes of T4SSs came with the purification and characterization of the so-called "core complex" (CC) of the pKM101 T4SS. This ~1.1 MDa

complex consists of 14 copies of each VirB7, VirB9 and VirB10 protein. The structure was determined by negative stained electron microscopy (nsEM) at 15 Å (Fronzes et al. 2009b) and the resolution was later improved at 12.4 Å (Rivera-Calzada et al. 2013). The CC is a double-chambered cylinder of 185 Å diameter and height (Figure 2.4A). The cylindrical assembly channel is opened on the cytoplasmic side (55 Å diameter) and constricted at the extracellular side (10 Å). It displays an inner- and an outer layer (I-layer and O-layer, respectively) connected by thin linkers. The I- and O-layer connect respectively the inner and outer membranes. The crystal structure of the O-layer revealed 14 copies of a ternary complex consisting of VirB7, $VirB9_{CTD}$ and $VirB10_{CTD}$ (Chandran et al. 2009). In this complex, $VirB10_{CTD}$ forms the interior of the channel and is surrounded by $VirB9_{CTD}$ and full-length VirB7 (Figure 2.4A). Two remarkable features of VirB10 were identified in this crystal structure: a N-terminal lever arm that interacts with three adjacent VirB7/VirB9/VirB10 heterotrimers and the helical hairpin antennas of 14 subunits that assemble into a 32 Å diameter pore or "cap" at the outer membrane (Figure 2.4A). The I-layer consists of the N-terminal part of VirB9 and VirB10 and is inserted into the inner membrane. Another higher resolution structure of the CC after proteolysis obtained by cryo-EM (Rivera-Calzada et al. 2013) revealed that the I-layer inner wall is made of $VirB10_{NTD}$ subunits, which form 14 pillar-like structures. The outer wall is made of $VirB9_{NTD}$ and a tetradecamer of a computational model of $VirB9_{NTD}$ could be fitted into the I-layer density map (Figure 2.4A) (Rivera-Calzada et al. 2013). Inside the cylinder, a middle platform region formed by internal protuberance separates the I- and O-layers thereby delimiting two chambers. This platform adopts different conformations in the CC structures obtained and can be open (full-length complex) or closed (proteolytically truncated). A nsEM structure of the CC bound to VirB4 revealed that a monomer of the ATPase associated with the I-layer of the CC. This structure was also the first evidence that VirB4 could be positioned laterally relative to the CC (Wallden et al. 2012).

The CC of the *L. pneumophila* Dot/Icm T4SS has been visualized by EM *in situ* (Kubori et al. 2014). Mutational analysis and immunodetection determined that the CC contains DotC, DotD,

DotH, DotG and DotF. DotG has a sequence similarity with the C-terminal domain of the VirB10 and could form the central channel. DotC and DotD are lipoproteins. The CC is a ring-shape structure of 380 Å of diameter and the diameter of the channel is of around 80 Å. DotD and DotF are respectively an outer membrane protein and an inner membrane protein. DotC, DotD and DotH are essential for the formation of the ring-shaped structure. The proposed CC of *cag*T4SS has been isolated from *H. pylori* cells and visualized by nsEM (Frick-Cheng et al. 2016). At least five proteins were identified amongst which CagT, CagX, and CagY that are the VirB7, VirB9 and VirB10 homologues, respectively. In addition, Cag3 and CagM that have no homologues in other T4SSs were found essential to generate the assembly. This finding corroborates previous studies that found that CagX interacted with CagY (Busler et al. 2006) and with CagM (Kutter et al. 2008) and that Cag3 was part of the outer membrane (Pinto-Santini and Salama 2009). The *cag*CC forms two rings connected by fourteen spokes, thus with a similar symmetry than the one (14-fold) observed in all CC structures determined to date (Fronzes et al. 2009b; Chandran et al. 2009). The outer ring has a diameter of 410 Å and the central ring has a diameter of 190 Å. The exact composition of the rings and the spokes is not known but mutational analysis suggested that CagX and CagY associated with the inner ring and that Cag3 was part of the outer ring (Frick-Cheng et al. 2016).

3.2 The $T4SS_{3\text{-}10}$ complex

A massive leap in our understanding of the structural biology of T4SS came from the purification and reconstruction by nsEM at a resolution of 20 Å of a complex consisting of the proteins VirB3,4,5,6,7,8,9 and 10 ($T4SS_{3\text{-}10}$) from the R388 conjugative plasmid (Low et al. 2014). The 3.5 MDa complex traverses the entire bacterial envelope with a length of around 340 Å (Figure 2.4B). In addition to the core complex the structure reveals a number of additional compartments. The inner membrane complex (IMC) is positioned under the CC, with the two compartments connected by a thin structure, named stalk. The CC still displays a 14-fold symmetry and its dimensions are the same than those observed in previous EM and crystal structures. The O- and I-layers were unambiguously recognized and the crystal structure of the O-layer and $VirB9_{NTD}$ I-layer

could be fitted in the electron density map. Nonetheless, the fourteen $VirB10_{NTD}$ forming the lower part of the I-layer in the CC structure were not visible in the $T4SS_{3\text{-}10}$, suggesting that they could be unstructured or too flexible to be observed.

The IMC is a completely novel structure, much wider than the CC with a width of 255 Å. Two barrel-like densities were observed on each side and were clearly identified as VirB4 by immunogold labeling. The barrels have a diameter of 105 Å and a height of 134 Å and each show three tiers: the upper, middle and lower tiers (Figure 2.4B). The upper tier is partly or fully inserted into the IM. The crystal structure of $TpsVirB4_{CTD}$ could be fitted into the middle and lower tier as a dimer of trimers. The stalk connects the center of IMC to the I-layer of the CC (Figure 2.4B). This part of the T4SS is flexible and various orientations of the CC relative to the IMC could be observed on the electron micrographs. Above the inner membrane, a flat structure named "arch" connects the IMC to the stalk (Figure 2.4B). The exact composition of the arch, the stalk and the inner membrane complex is not entirely clear. An interesting finding is that except for the CC proteins that are present in 14 copies, the other proteins are in multiple of 12, *i.e* VirB3, VirB4, VirB5. VirB8 have 12 copies and VirB6 has 24 copies. It is likely that VirB3 and VirB6, that are membrane proteins, are components of the IMC along with the VirB4 TM domains. The stalk might be composed of the flexible NTDs of VirB10. VirB8 that has a periplasmic domain and a transmembrane domain might form the arches since the protein was found to interact with VirB10, VirB9 (Das and Xie 2000) and VirB4 (Ward et al. 2002).

Recently, the structure of the Dot/Icm T4SS has been visualized by *in situ* cryo-tomography in *L. pneumophila* (Ghosal et al. 2017). The structure presents some remarkable similarities with the $T4SS_{3\text{-}10}$ complex but is twice as wide and long. The Dot/Icm T4SS has also a cap domain located at the outer membrane and the proposed core complex is connected to the inner membrane by a stalk. The structure of the O-layer (Chandran et al. 2009) fits well with the dimension of the so-called Dot/Icm T4SS "cap" domain. In the cytoplasm and anchored at the inner membrane, four elongated densities could be observed. These four densities were proposed to be side view of the two VirB4 ATPases barrels identified in the $VirB_{3\text{-}}$

complex (Ghosal et al. 2017). Thus, all T4SSs appear to have a similar architecture with a 14-fold symmetrical CC mounted onto a 2-barreled IMC, with a stalk linking the CC and the IMC. Recently, a more complete IMC was formed by the addition of VirD4 (Redzej et al. 2017). In this structure, two VirD4 dimers are observed inbetween the two VirB4 barrels on each side of the IMC. VirD4 would eventually hexamerize upon substrate recruitment and thus a fully functional T4SS might include 4 hexameric ring ATPases, 2 VirB4s and 2 VirD4s.

3.3 Structure of the pilus

The best-characterized T4SS pili are the conjugative pili found in G^- bacteria. These pili are appendages that serve both for host cell adhesion and for DNA or effectors transfer (Hospenthal et al. 2017). Two main types of conjugative pilus have been described: the F-like and the IncP-like pilus. The F-like pili are between 2-20 µm long, have an external diameter of 85-95 Å and a central diameter of 2 nm. They are flexible and able to dynamically extend and retract (Clarke et al. 2008). The IncP-like pili measures 1 µm and is more rigid than the F-like pilus. The two types of pili are composed of repeating units of a single protein called the major pilin. The major pilin is expressed as pro-pilin with a signal peptide, which is cleaved after insertion in the inner membrane and undergoes an acetylation for the F-like pilin and cyclization for the IncP-like pilin. After maturation, subunits of pilin accumulate in the inner membrane (Hospenthal et al. 2017), where they are recruited during pilus biogenesis. Other proteins called minor pilins can be also present in the pilus, but in weak proportion and little is known about their integration into the pilus.

Pili of T4SS involved in bacterial pathogenesis (second functional group as defined in the introduction) are still poorly characterized and sometime their compositions are unclear. This is particularly the case for the *H. pylori cag*T4SS pilus. Non-regular sheathed pilus-like structures were observed in *H. pylori* cells (Tanaka et al. 2003; Rohde et al. 2003) and several Cag proteins were detected on or near the pilus such as CagY and CagT, homologues of VirB10 and VirB7, respectively (reviewed in (Terradot and Waksman 2011)). Pili were also identified in other studies where they appeared more regular (Shaffer et al. 2011). Surprisingly (and somewhat con-

fusingly), these pili were observed even after deletion of the VirB2 pilin homologue CagC or of the VirB10 homologue CagY (Johnson et al. 2014). Instead CagL, CagI, and CagH were required for pilus biogenesis and CagA injection (Shaffer et al. 2011). This study demonstrated that the three proteins interacted together and that CagH was important for the regulation of the size of the pilus (Shaffer et al. 2011). In addition, CagA was detected at the tip of the pilus where it could play a role in its delivery (Jimenez-Soto et al. 2009). Indeed, CagA but also CagY, CagL and CagI were found to interact with the host cell receptor integrin (reviewed in (Berge and Terradot 2017)). In G^+ bacteria the outer membrane complex and the pilins are absent but the bacteria adhere to the cell via adhesins. For example, the protein PrgB in pCF10 plasmid, which allows the contact with the cell, contains an integrin binding motif and a glucan-binding domain (Goessweiner-Mohr et al. 2013a).

A major step towards the understanding of T4SS pilus structures was reached with the determination of the structures of F-like pili encoded by the conjugation plasmids F and pED208 by cryo-EM at resolutions of 5.0 and 3.6 Å, respectively (Figure 2.5A) (Costa et al. 2016). The two pili have the same general architecture, *i.e* a five-start helical filament of the TraA pilin (Figure 2.5B). Interestingly the T4SS of Anaplasma phagocytophilum encodes five copies of the VirB2 subunit (Voth et al. 2012) and thus this five-fold helical assembly might be conserved in other types of T4SS pili. The pED208 TraA pilin forms an elongated two α-helix bundle that interacts with height adjacent subunits (Figure 2.5C). The loop between the α1 helix and α2 helix is exposed within the lumen, which is also consistent with prior suggestions that the α1-α2 loop might be involved in contacting the DNA as it passes through the pilus (Silverman 1997; Paiva et al. 1992). The N- and C-terminal ends are exposed outside the filament. This feature is also consistent with previous studies, which found that this region was accessible for phage attachment (Frost and Paranchych 1988). Importantly, the structure also revealed that pili are composed of protein-phospholipid units in a 1 to 1 ratio. Each pilus is composed of a main and pilus-specific phospholipid belonging to the phosphatidylglycerol family. In the pilus, one subunit of TraA engages five lipid molecules and one lipid interacts with five TraA subunits. The head group of lipids is ex-

posed into the lumen, making the surface inside the pilus moderately negative. Such an interface might promote the transport of the negatively charged single strand DNA (Costa et al. 2016).

4 Mechanism of substrate transport

Substrate translocation by T4SSs is still poorly understood (Chandran Darbari and Waksman 2015). The majority of T4SS produces a pilus and also transports substrate(s). There are several lines of evidence that pilus assembly and substrate translocation do not involve the same mechanism (Costa et al. 2015). For instance, these activities have been uncoupled in *A. tumefaciens* VirB/D4 system (Jakubowski et al. 2009) and in *H. pylori cag*T4SS (Shaffer et al. 2011). One hypothesis for pilus biogenesis includes the formation of a prepilus at or near the middle platform of the CC and that the stalk might act as a nucleation point for the pilus. VirB4 was found to interact with the VirB2 pilin and thus might be involved in pilus polymerization, possibly by extracting VirB2 subunits from the inner membrane (Hospenthal et al. 2017).

Mechanistic insights into substrate translocation by the Agrobacterium VirB/D4 T4SS have been suggested (Cascales and Christie 2004b). In this study, the route of a DNA substrate from the cytosol to the perisplamic CC and then the pilus was derived from a TrIP (Transfert DNA immunoprecipitation) assay. These experiments enabled to potentially monitor the sequential interactions of DNA with VirB proteins. The TC4P and the VirB11 proteins were identified as the cytosolic interacting partners of DNA substrate prior to translocation. The route then involves VirB6 and VirB8. It was found that VirB9 also interacts with the DNA but, based on the CC structure, it is not clear how VirB9, which is located on the external side of the CC, could interact with the substrate. VirB10 and VirB11 seem to play an important role in the functional selection for the T4SS: VirB11 as a traffic ATPase able to orchestrate substrate translocation versus pilus biogenesis and VirB10 as an energy sensor that bridges all the compartments (Cascales and Christie 2004a). Finally, the DNA interacts also with the major pilin VirB2 and this suggests that DNA is at some point in contact with the pilus. As in T3SSs, the T4SS pilus may indeed act as a conduit for the substrate and accordingly DNA has been visualized inside an F-like pilus (Chandran Darbari and Waksman 2015). In the case of conjugation, the relax-

ase is the main substrate and is covalently linked to the transferred DNA. The targeting of the relaxase occurs via a translocation signal present on the protein (Redzej et al. 2013). Recent structural insights have been obtained on the TraI relaxase and on the mechanism of DNA transport (Ilangovan et al. 2017). For more details on the relaxosome, the reader is referred to Chapter 4.

Several protein effectors translocated via the other T4SSs harbor a C-terminal translocation signal, which was first identified in the substrate proteins VirE2 and VirF from *A. tumefaciens* (Vergunst et al. 2000). This recognition motif of around 20 aa contained positively charge residues that are recognized by the VirB/D system (Vergunst et al. 2005). Similarly, a C-terminal secretion signal was found important for the translocation of RalF effector into macrophages by the Dot/Icm T4SS of *Legionella* (Nagai et al. 2005). For the *Bartonela henselae* T4SS, a bi-partite recognition motif was identified in the C-terminal portion of several effectors and these sequences were sufficient for efficient substrate translocation (Schulein et al. 2005). Some T4SS are used to deliver a single effector such as the *H. pylori* cagT4SS and *B. pertussis* Ptl, nevertheless the two systems deliver their respective effectors differently. In the case of the *H. pylori cag*T4SS, the translocation mechanism of CagA is still unclear. CagA also contains a C-terminal sequence with positive charges, but these are not essential for translocation (Schindele et al. 2016; Hohlfeld et al. 2006). The protein CagF, unique to the *cag*T4SS, interacts with multiple parts of the CagA protein with very high affinity and could be involved in its translocation (Bonsor et al. 2013). It might act as a chaperone that, together with the T4CP Cagβ, modifies the CagA structure, delivers it to the *cag*T4SS and facilitates its transport from the cytoplasm to the CC (Couturier et al. 2006; Pattis et al. 2007). Accordingly, the CC of the *cag*T4SS and CagA could be isolated using immuno-precipitation experiments directed against CagF (Frick-Cheng et al. 2016). It was found that several pilus-associated proteins might interact with the host cell receptor integrin including CagL, CagI, CagY and the substrate CagA (reviewed by (Berge and Terradot 2017)). All these interactions might reflect the need for the *cag*T4SS to use several steps to traverse the host cell membrane in order to inject CagA. A completely different mechanism takes place for the delivery of the *B.*

pertussis Ptl toxin. The holotoxin is first assembled in the periplasm and is then exported by a T4SS encoded by the ptl (pertussis toxin liberation) locus. The toxin is secreted into the extracellular milieu, but not injected across host-cell membranes. It was proposed that the protein PtlA may form a modified pilus-like structure that could act as a piston to push assembled Ptl toxin molecules out of the periplasm and across the bacterial outer membrane (Shrivastava and Miller 2009).

Some T4SSs might use two delivery mechanisms for different sets of substrates. In *Brucella* T4SS some effectors are translocated by the T4SS from the cytosol, but others contain characteristic signal peptides and thus might be translocated from the periplasm (Marchesini et al. 2016; Del Giudice et al. 2016; Dohmer et al. 2014; Myeni et al. 2013; de Jong et al. 2008). It was hypothesized that the periplasm may represent an "effector reservoir" selectively delivered into the host at different times in the process of bacterial replication/infection. The VirJ protein from *Brucella* was found to interact with effectors and with the VirB/D components VirB5 and VirB8 in the periplasm and was proposed to act as a periplasmic "sorting" chaperone (Del Giudice et al. 2016). A two-step process involving VirJ was also proposed for *A. tumefaciens* (Pantoja et al. 2002), but the protein was later found not required for T-DNA translocation (Cascales and Christie 2004b). This dual system seems to be particularly relevant for the Legionella Dot/Icm system, where two cytoplasmic proteins IcmS and IcmW interact with each other and form complexes with protein substrates (Cambronne and Roy 2007; Ninio et al. 2005). The effectors SidG and SidJ were found to have an internal region important for IcmSW-dependent translocation in addition to an IcmSW-independent C-terminal signal (described above) (Jeong et al. 2015; Cambronne and Roy 2007). It seems that the role of IcmSW complex is to target a subset of T4SS substrate to the DotL protein that could act as a T4CP (Kubori and Nagai 2016).

5 Concluding Remarks

During the past fifteen years, the structural biology of T4SS has made remarkable progress. Regarding the conjugative T4SS, we now have the structures of many individual components and the general architecture of the machinery across the bacterial envelope

has progressively come to light, owing to the outstanding development of electron microscopy. It seems that obtaining a complete, atomic view of a T4SS is now a feasible perspective. As seen in this Chapter, despite this substantial progress, a number of challenging questions and gaps remain to be addressed that will require concerted efforts in structural biology, molecular Biology, biochemistry, microbiology and cellular biology. Even for the best-characterized T4SS, the factors and molecular mechanisms regulating the hierarchical assembly of the machinery and of the pili are unknown. We are also still far from understanding how the machinery and pilus are utilized to deliver macromolecules from the bacteria cytoplasm to the recipient cell.

Furthermore, the architecture of the T4SSs of *Legionella*, *H. pylori* and other pathogenic bacteria are still much less understood than the conjugative system of *E. coli*, probably because of their increased complexity. Thus more efforts should be directed towards the study of these protein secretion nanomachines that have important medical impact. The first, low resolution assemblies already observed (Ghosal et al. 2017; Frick-Cheng et al. 2016) suggest that some magnificent discoveries are ahead of us and that the next years will see some exciting novel structures.

With the structural information gained, exploiting T4SS biology to prevent or reduce infection and/or antibiotic spread might become possible in the near future. Because T4SS systems are not essential for growth, these T4SS inhibitors could exert less selection pressure than bactericidal molecules (Ruer et al. 2015). The rational design of inhibitors that prevent T4SS assembly has already been initiated and will probably intensify with more detailed structural information becoming available. Alternatively, it is also possible that these systems might be engineered to deliver specific macromolecules for the benefit of human. Thus if many challenges are ahead, future years promise some exciting discoveries in the T4SS research and there is no doubt that Structural Biology will play a key role.

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Figure legends

Figure 2.1 General organization and architecture of T4SSs. A) Schematic view of the genetic organization of the VirB/D and the pI501 plasmid T4SSs. Homologous genes are represented by arrows with the same colour and unique genes are coloured in grey. B) Schematic representation of the assembly of the VirB/D from *A. tumefaciens* and schematic subunits of pI501 plasmid T4SS from *Enterococcus* sp. coloured as in A).

Figure 2.2 Structures of T4SS ATPases. A) Ribbon representation of the hexamer (left) and monomer (right) of the crystal structure of Cagα (VirB11) from *H. pylori* (PDB ID 1NLY). B) Ribbon representation of the hexamer of TrwB (VirD4) from *E. coli* R388 plasmid (PDB ID 1GL7). C) Ribbon representation of monomers of TrwB (left) from *E. coli* R388 plasmid and monomer of VirB4 (right) from *T. pseudethanolicus* (PDB ID 4AG5). Structures are displayed in the same orientation to illustrate their structural similarity.

Figure 2.3 Structures of T4SS components. A) Structural comparison of the VirB8 family. Ribbon representation of the crystal structures of VirB8 from *A. tumefaciens* (PDB ID 2CC3),DotI from *Legionella* (PDB ID 3WZ4) and TraM from *Enterococcus sp*. (PDB ID 4EC6). B) Structure of the O-layer ternary complex TraN (VirB7), TraO (VirB9), TraF (VirB10) (PDB ID 3JQO) from the pKM101 plasmid. C) Ribbon representation of the crystal structure of TraC (VirB5) from pKM101 plasmid (PDB ID 1R8I).

Figure 2.4. **Structures of T4SS assemblies.** A) top (left) and side (right) views of the cryo-EM structure of *E. coli* pKM101 plasmid core complex at a 12.4 Å resolution (EMD-2233). The crystal structure of the O-layer (PDB ID 3ZBI) and the model of the I-layer (PDB ID 2YPW) have been fitted into the map and coloured as in Figure 4. B) Two views of the nsEM structure of the $T4SS_{3\text{-}10}$ from the conjugative plasmid R388 from E. coli (EMD-2567) at a resolution of 20 Å. The different subcompartments identified are coloured according to Figure 2.

Figure 2.5. **Structure of the T4SS pilus from pED208 plasmid.** Side (A) and bottom (B) views of a surface representation of the F-

pilus from pED208 plasmid (PDB ID 5LEG). C) Ribbon representation of two TraA subunits bound to a phospholipid molecule.