Review

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Structural biology of the Gram-negative bacterial conjugation systems

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Conjugation, the process by which plasmid DNA is transferred from one bacterium to another, is mediated by type IV secretion systems (T4SSs). T4SSs are versatile systems that can transport not only DNA, but also toxins and effector proteins. Conjugative T4SSs comprise 12 proteins named VirB1–11 and VirD4 that assemble into a large membrane-spanning exporting machine. Before being transported, the DNA substrate is first processed on the cytoplasmic side by a complex called the relaxosome. The substrate is then targeted to the T4SS for export into a recipient cell. In this review, we describe the recent progress made in the structural biology of both the relaxosome and the T4SS.

Type IV secretion in bacteria

Bacterial conjugation was first described by Joshua Lederberg and Edward L Tatum in 1946 as bacterial sexual reproduction [1]. Conjugation systems mediate DNA transfer between cells of the same species [2]; however, this process has also been shown to occur between cells of different bacterial species and even from prokaryotic to eukaryotic cells [3]. Conjugation contributes significantly to horizontal gene transfer and bacterial genome plasticity. It is at least in part responsible for the rise of multidrug-resistant bacteria that pose a major threat to human health [4–8].

The process of conjugation involves three distinct consecutive biochemical subprocesses; substrate processing, substrate recruitment, and substrate transfer. DNA processing and recruitment occurs in the cytoplasm and a trans-membrane secretion apparatus is then required for the transport of processed DNA from one bacterial cell to another. The secretion system involved in conjugation is called the T4SS, one of six major classes of secretion system in Gram-negative bacteria. This T4SS is a large nanomachine spanning the inner and outer membranes of Gram-negative bacteria and can functionally be classified into three categories. The first includes T4SS involved in DNA conjugation and the second is responsible for secreting effector proteins and toxins into mammalian cells. The latter are found in bacterial pathogens, such as *Legionella pneumophila*, *Brucella suis*, and *Helicobacter pylori* [9–11]. The third category of T4SS includes those that mediate DNA release and uptake, and have been found in *H. pylori* and *Neisseria gonorrhoeae* [1].

The initiation of conjugation occurs in the cytoplasm, where a region within the DNA called origin of transfer (*oriT*) is processed via the formation of a multi protein–DNA complex called the relaxosome. The protein relaxase forms the crucial part of the complex and various accessory proteins may be necessary for efficient recognition of *oriT* [12]. A coupling protein (T4CP) then recruits the relaxase–DNA complex to a T4SS responsible for the translocation of the DNA from the donor to the recipient cell. While structural studies have predominantly focused on Gram-negative systems, interest in understanding such systems in Gram-positive organisms is growing. In this review, we describe the structure and mechanism of the Gram-negative bacterial conjugation system, including recent attempts to determine the structures of large T4SS and relaxosome subcomplexes.

The T4SS

T4SS constituent proteins can be divided into three classes, based on the role of the individual proteins: the translocation channel proteins, the pilus proteins, and the ATPases that supply the power required for the system to function.

The translocation channel proteins

The translocation channel is the major structural feature of the system and comprises proteins VirB3, 6, 7, 8, 9, and 10. VirB3 has two transmembrane segments (TMSs) with both the N- and C-terminal ends in the cytoplasm. In some species, such as *Campylobacter jejuni*, this protein exists as a fusion protein with VirB4 [13]. VirB3 is the least-characterized system component and requires other VirB proteins, such as VirB4, 6, 7, and 8 for stability [14,15]. VirB6 is the most hydrophobic protein in the system and locates in the inner membrane of the bacteria. Biochemical and genetic analysis of this protein suggest a periplasmic N terminus and cytoplasmic C terminus with five TMSs and a large insertion loop termed the 'P2 loop' between TMS2 and TMS3 [16].

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VirB7 is a small monotopic membrane protein that acts as a pilotin, assisting insertion of the assembled T4SS channel into the outer membrane [17]. The protein has a distinct signal peptide II (SPII) consensus recognition sequence where the cysteine is acylated through two fatty acids and connected via a thioester-linked diglyceride [18]. VirB8, a bitopic inner membrane protein, has a cytoplasmic N-terminal domain (NTD), a transmembrane helix, and a large periplasmic domain. Crystal structures of VirB8 periplasmic domain from *Agrobacterium tumefaciens* (Figure 1A – orange) and *B. suis* (Figure 1A – pink) show a common fold that belongs to the nuclear transport factor 2 (NTF2) fold family, with a central β sheet juxtaposed by α helices that also form a dimer interface [19–21].

VirB9 is a periplasmic protein with two domains, the C-terminal domain ($VirB9_{CTD}$), which binds to VirB7 and has a structural role as an integral part of the so-called 'core' or outer membrane complex (see below for details), and the NTD ($VirB9_{NTD}$), which is involved in substrate selectivity [22,23]. Nuclear magnetic resonance (NMR) and X-ray crystal analysis of $VirB9_{CTD}$ bound to VirB7 show a compact β-sandwich fold around which VirB7 wraps (Figure 2A,B) [23,24].

VirB10 belongs to the unique class of proteins spanning both the inner and outer membranes and forms a pore in the outer membrane. This protein is known to interact with the ATPases in the inner membrane [25]. This suggests that the protein has a signal transmission and/or energy transfer role where ATP-driven conformational changes within the ATPases impact on the outer membrane translocation pore to facilitate substrate transport [23]. A crystal structure of the conserved periplasmic CTD of ComB10, a VirB10 homolog from *H. pylori*, revealed a unique β-barrel arrangement that was further validated by the structure of the TraN–$TraO_{CTD}$–$TraE_{CTD}$ complex, the pKM101 plasmid-encoded homologs of VirB7, $VirB9_{CTD}$, and $VirB10_{CTD}$, respectively (Figure 2B) [20,23]. Two parts of TraE/$VirB10_{CTD}$ stand out in this complex structure: (i) a double-helical bundle protruding out (also termed 'antennae projection') and (ii) a long linker called the 'lever arm', which is a major participant in the protein–protein interaction network stabilizing the outer membrane complex (see below for details).

Pilus components

Pili are extracellular tubular structures that are involved in DNA transfer into the recipient cell. Morphologically, two types of pilus are observed within the T4SS, an F-type pilus, which is long and flexible, and a P-type pilus, which is short and rigid [2]. Cryo-electron microscopy (cyro-EM) analysis of the F-plasmid pilus has shown the presence of a lumen, which is approximately 30 Å in diameter, suggesting passage of single-stranded DNA and unfolded but not folded protein [26]. The pilus comprises two VirB proteins, VirB2 and VirB5, the major and minor pilus components (or pilins), respectively. The VirB2 protein from *A. tumefaciens* and TrbC/VirB2 from the RP4 conjugative plasmid (IncP) undergoes post-translational modification, where the signal peptide is cleaved followed by the fusion of the N- and C-terminal ends resulting in the cyclization of the peptide [27,28]. F-like pilins have not been shown to undergo cyclization, but are acetylated before insertion into the inner membrane [29,30]. In *A. tumefaciens*, VirB5 localizes to the tip of the pilus and mutational analysis on a VirB5 homolog from the pKM101 plasmid system, TraC, also suggests that VirB5 serves as an adhesin [31,32]. The crystal structure of TraC/VirB5 from the pKM101 plasmid shows an elongated helical structure, with three helices forming an elongated backbone structure and four helices forming an appendage (Figure 1A – blue) [32]. VirB1 is a lytic transglycosylase protein. VirB1 functions in the periplasm, where it is processed into two parts, the N-terminal part with muramidase activity and a C-terminal part that is secreted outside the cell and appears to be involved in pilus biogenesis [33,34].

The T4SS ATPases

The T4SS has three ATPases that power the system: VirB4, VirB11, and VirD4. VirB4 is a ubiquitous protein conserved throughout Gram-negative and Gram-positive conjugative systems [25,35,36]. It is an inner membrane-associated protein with a complex interaction profile involving VirB3, 6, 7, and 8 [14,15,37]. This protein has two domains, a less-conserved NTD, and a highly conserved C-terminal domain (CTD) containing the ATPase activity [37,38]. When alone, the protein predominantly exists as a dimer; however, monomeric, trimeric, and hexameric forms have been observed [39–41].

The crystal structure of the VirB4 CTD has been solved from a Gram-positive thermophilic bacterium *Thermoanaerobacter pseudethanolicus* (Figure 1B – cyan), which showed striking resemblances to VirD4 despite low sequence similarity (12%) [41]. This structure shows two subdomains, an ATP-binding site containing a RecA-like α/β domain, and an α-helical domain formed of four helices. A second structure with bound ADP confirms the nucleotide-binding site (NBS) to the predicted Walker A and B motifs [41]. EM studies carried out on the TrwK/VirB4 *Escherichia coli* R388 T4SS homolog have shown that the hexameric form of VirB4 has a three-tiered ring structure [42]. A similar three-tiered ring structure was also observed for VirB4 in the recent EM structure of a large R388 plasmid-encoded T4SS subcomplex containing the VirB3, 4, 5, 6, 7, 8, 9, and 10 proteins (Figure 3A) [43]. However, in the latter complex, the VirB4 hexamer also showed a distinctive trimer of dimers arrangement.

VirB11 belongs to the family of 'traffic' ATPases and is indispensible in most T4SSs, with the F plasmid-like T4SS being a notable exception [44]. It localizes mainly to the inner membrane of Gram-negative bacteria and its ATPase activity is enhanced upon interactions with phospholipids [45,46]. VirB11 interacts with VirB4 and VirD4 and it has been proposed that the interaction with VirB4 might induce the T4SS to produce a pilus (pilus biogenesis functional mode), while interaction with VirD4 might induce the system to engage with the substrate (substrate-transfer functional mode) [47]. EM studies revealed hexameric forms of VirB11 [48]. The X-ray crystal structure of the VirB11 homolog from the *Cag* pathogenicity island of *H. pylori* also revealed a hexamer with distinct N- and C-terminal domains and a NBS at the interface of the two domains (Figure 1B – green) [49]. The subunit arrangement within