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Cut and move: protein machinery for DNA processing in bacterial conjugation

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Conjugation is a paradigmatic example of horizontal or lateral gene transfer, whereby DNA is translocated between bacterial cells. It provides a route for the rapid acquisition of new genetic information. Increased antibiotic resistance among pathogens is a troubling consequence of this microbial capacity. DNA transfer across cell membranes requires a sophisticated molecular machinery that involves the participation of several proteins in DNA processing and replication, cell recruitment, and the transport of DNA and proteins from donor to recipient cells. Although bacterial conjugation was first reported in the 1940s, only now are we beginning to unravel the molecular mechanisms behind this process. In particular, structural biology is revealing the detailed molecular architecture of several of the pieces involved.

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Introduction

Mechanisms leading to lateral gene transfer in bacteria are classically categorized as transduction, transformation or conjugation [1–3]. Transduction occurs via bacteriophages, which can incorporate portions of the host bacterial DNA and introduce them into newly infected hosts. Transformation consists of the uptake of naked DNA from the environment. Finally, conjugation is the unidirectional transfer of single-stranded (ss) DNA (known as the T-strand) of conjugative plasmids (or chromosome-integrated conjugative elements) from a donor to a recipient cell by intimate cell-to-cell contact [3–6]. After transfer, the recipient becomes a transconjugant, possessing the capacity to start new rounds of conjugation. Through this highly efficient mechanism, a few conjugative-plasmid-harbouring cells within a strain can spread this information among the whole population within short timescales, thus enabling rapid dissemination of adaptive genes and infectious or antibiotic resistance factors. Studies of *Escherichia coli* strain K12 plasmid F led to the discovery of bacterial conjugation in the 1940s; this plasmid has since become a model for plasmid-encoded conjugation systems in Gram-negative bacteria [7,8]. Another example is the enterobacterial plasmid R388, which confers resistance to the antibiotics sulphonamide and trimethoprim [9]. Conjugative plasmids have also been found in several Gram-positive bacterial genera, such as *Streptococcus*, *Enterococcus* and *Staphylococcus* [10–12]. Conjugative-like DNA delivery further occurs between bacteria and eukaryotic plant and fungi cells. A well-known example is *Agrobacterium tumefaciens*, the etiological agent of crown gall disease, which transfers the tumour-causing plasmid pTi to plants [13].

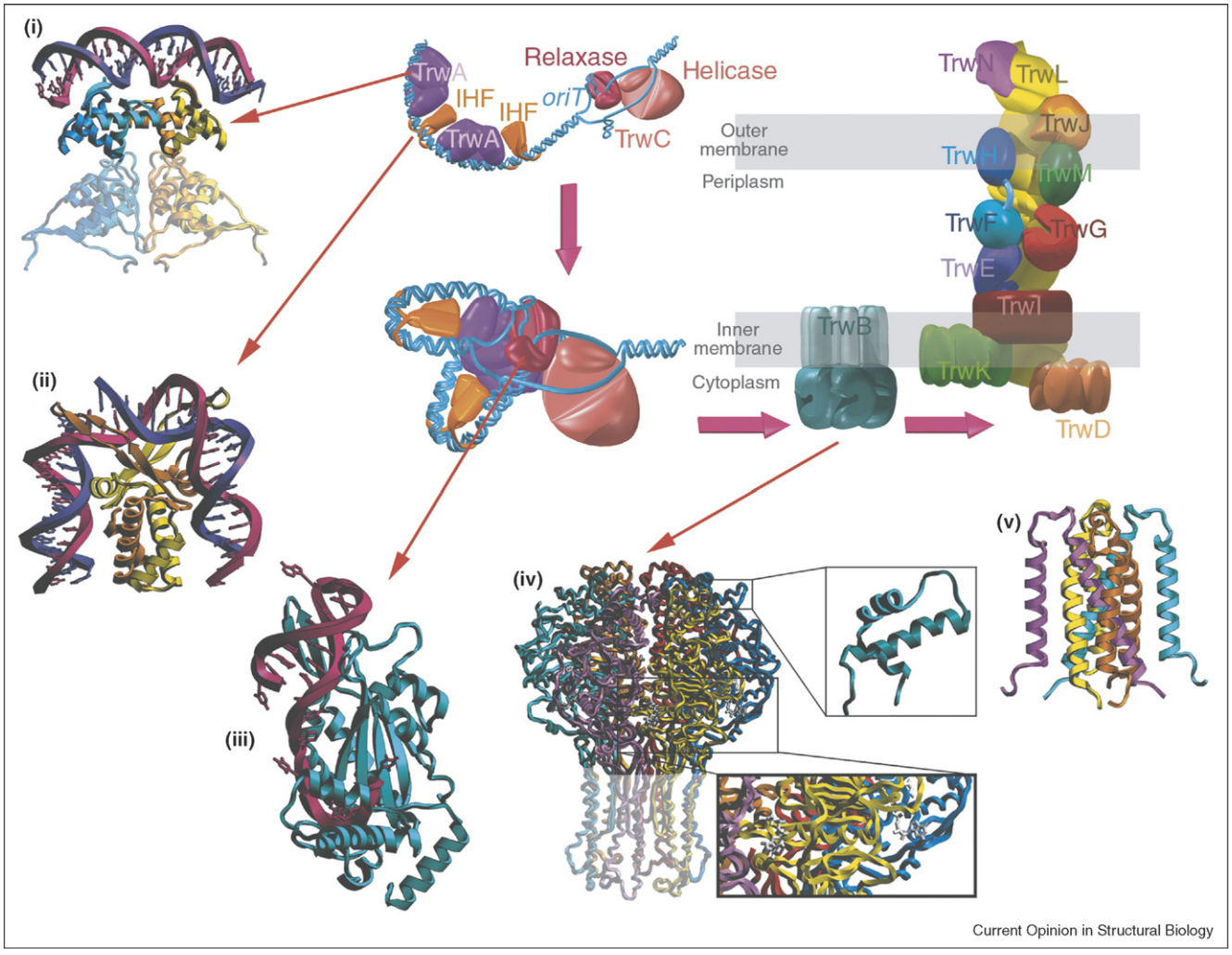
Most of the proteins engaged in conjugation are encoded by plasmid genes located in the *tra* (transfer) region, which includes the *mpf* (mating-pair formation) and *dtr* (DNA processing and transport) genes [14,15,16•]. *Dtr* encodes proteins responsible for the process in which the T-strand is prepared for transfer. It includes the formation of the relaxosome [17], a multicomponent nucleoprotein complex comprising an ATP-dependent relaxase/helicase, the T-strand, a transcriptional regulator and the host-encoded integration host factor (IHF), and its recruitment to the membrane transport pore (see Figure 1). *Mpf* encodes proteins that participate in pilus formation and assembly of a type IV secretion system (T4SS), a multiprotein organelle required for horizontal transfer through membranes in Gram-negative bacteria (for recent reviews, see [15,18–22]). Conjugation initiates when the pilus, anchored on the donor cell surface, binds to the surface of the recipient cell through its distal end and subsequently retracts to enable stable intercellular wall-to-wall contact. An unknown mating signal then triggers mobilisation of donor DNA, which leads to a site-specific nick in the plasmid T-strand. The relaxosome is subsequently coupled to the T4SS by T4CP, a *dtr*-encoded receptor or coupling protein ([22,23]; see Figure 1).

In recent years, structural biology has revealed the detailed molecular architecture of several of the pieces involved in the intricate scenario of conjugation. Here, we review the structures of proteins that participate in the first two stages of DNA transfer, namely processing and recruitment to the cell membrane.

Relaxase/helicase

A key player in the generation of the transferable T-strand is the relaxase/helicase, TrwC in the R388

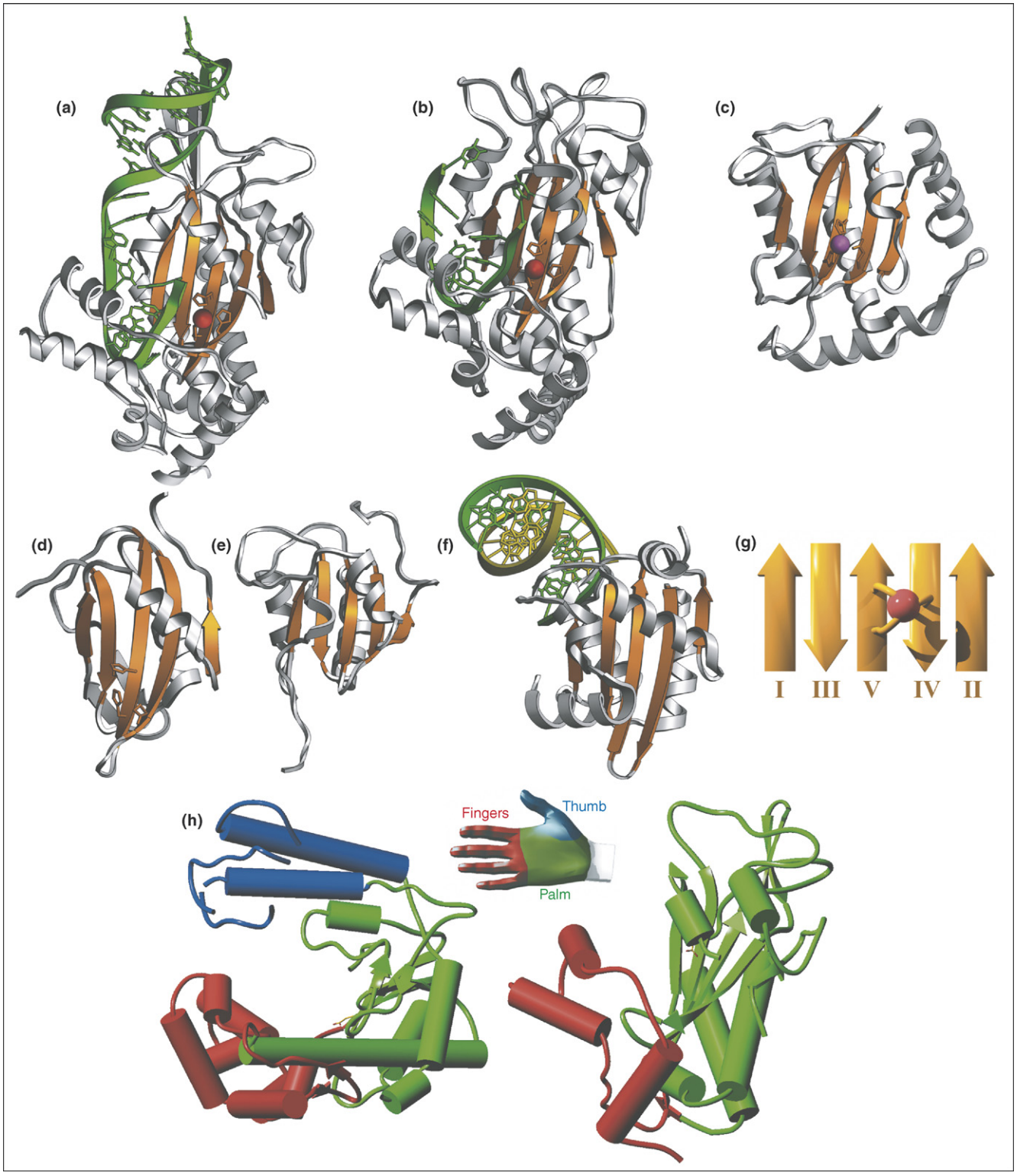
Figure 1



DNA processing and recruitment of the relaxosome to the T4SS in the *E. coli* plasmid R388 system [59]. Initially, the relaxosome is assembled from the transcriptional regulator TrwA, host-encoded IHF, relaxase/helicase TrwC and the plasmid dsDNA around *oriT* (upper centre). After nicking the T-strand DNA, the 5′ end remains attached to TrwC and the nucleoprotein complex is transported to the T4SS pore (proteins TrwD to TrwN in R388; see [22,54]) to be driven into the recipient cell. This operation is mediated by the T4CP (TrwB in this system), which is inserted into the cytoplasmic membrane. Richardson plots of the following structures are presented. **(i)** CopG dimer of dimers (blue/cyan and orange/yellow; PDB code 1b01) in complex with its cognate 19-bp dsDNA, as a model for TrwA. The dimerisation domain of TrwA bears distant sequence similarity to a region of *Bacillus licheniformis* β-lactamase (PDB code 4blm), which was used to tentatively model this domain (faded part). **(ii)** *E. coli* IHF heterodimer (yellow and orange ribbon) in complex with its cognate 35-bp dsDNA (magenta and violet), which forms a U-turn (PDB code 1ihf). **(iii)** TrwC relaxase domain (cyan ribbon) in complex with a 25-base DNA hairpin featuring the recognition sequence around *oriT* (PDB code 1qx0). **(iv)** T4CP TrwB hexamer in complex with an ATP analogue, showing each chain in one colour and the tentatively modelled transmembrane helices (faded part) (PDB code 1gl7). The insets depict the NTP-binding site of TrwB (lower inset; ATP analogue as white stick model) and the N-terminal domain of plasmid R1 TraM (upper inset; PDB code 1dp3), which is structurally similar to a region of the all-α domain of TrwB. **(v)** Tetrameric C-terminal domain of plasmid F TraM (PDB code 2g7o).

plasmid system and TraI in plasmid F. Initially described as helicase I in *E. coli*, this endonuclease has been termed 'relaxase' because it relaxes supercoiled plasmid double-stranded (ds) DNA by cleaving one of the strands. It participates in DNA mobilisation by nicking within the origin-of-transfer region (*oriT*) of the T-strand [23]. Subsequently, a catalytic tyrosine remains covalently attached to the 5′ end of the T-strand through a phosphotyrosyl linkage, while the 3′ end is released [24,25]. The relaxase/helicase then unwinds the dsDNA in an ATP-dependent manner and moves processively in a 5′ to 3′ direction on the displaced strand, while the intact strand serves as a template for complementary strand biosynthesis by host DNA polymerase III (donor conjugal DNA synthesis; [26]) using the rolling circle mechanism [27,28]. Studies of several conjugation systems indicate that the DNA-loaded relax-

Figure 2



TrwC relaxase domain and related proteins. **(a)** *E. coli* R388 TrwC relaxase domain Y18F mutant (white/orange) in complex with a 27-base ssDNA (green) that mimics the recognised *oriT* sequence (PDB code 2cdm). The central five-stranded antiparallel β sheet shared with other ORBD proteins is shown in orange. A water molecule occupies the metal site (red sphere) and is coordinated by three histidine residues (orange sticks) from two parallel strands. **(b)** *E. coli* F TraI in complex with 10-base ssDNA (PDB code 2a0i); colour coding as in (a). The red sphere indicates a metal ion assigned as Mg^{2+}. **(c)** Catalytic domain of the adeno-associated virus type 5 Rep protein (PDB code 1m55); ribbon colour coding as in (a).

ase/helicase pilots the passage of the T-strand through the T4SS into the recipient cell, where activity of this key protein has been detected [29–31].

The two main activities of the relaxase/helicase are localised in distinct domains; the relaxase (or transesterification) and helicase activities are ascribed to the N-terminal and C-terminal domains, respectively. The structure of the N-terminal domain of TrwC and TraI (Figures 1 and 2a,b) shows a two-layer α/β plate or open sandwich core comprising a central antiparallel five-stranded β sheet and two long helices on one face of the sheet [32••,33••]. The active site is located on the opposite face in an extended and narrow crevice, whose floor is paved by the β sheet and whose walls are shaped by two further α helices and loops. The active site pocket contains the catalytic tyrosine (Tyr18 in TrwC) responsible for T-strand DNA cleavage and a divalent metal ion, whose identity *in vivo* remains unclear. The metal ion is coordinated by two histidines (His161 and His163 in TrwC) embedded in the short consensus sequence HXH (single-letter amino acid code; X is any residue) and a further distal histidine (His150 in TrwC). These three metal ligands are positioned on two adjacent β strands of the sheet (Figure 2a,g). TrwC has been co-crystallised with a 25-base oligonucleotide encompassing the R388 *oriT* sequence upstream of the cleavage site [33••]. The DNA adopts a hairpin structure, mimicking one arm of the extruded cruciform of plasmid *oriT*, followed by a segment in an extended conformation and a sharp U-turn just before entering the active site pocket (Figure 1). The protein recognises this oligonucleotide through unique structural features, including the N-terminal methionine, which is trapped in a hydrophobic cage formed by the DNA bases of the U-turn. More recent studies of TraI and TrwC in complex with 10- and 27-base oligonucleotides, respectively (Figure 2a,b), reveal that the scissile phosphate is directly coordinated to the metal ion [34,35]. Accordingly, the latter could participate in catalysis, either by polarising the phosphate and facilitating nucleophilic attack by the catalytic tyrosine or by stabilising the pentacoordinate reaction intermediate [36].

The overall fold of the TrwC/TraI relaxase domain bears a structural resemblance to viral Rep proteins [36,37], the DNA-binding domain (DBD) of replication initiation protein E1 from papillomavirus [38] and the origin-of-replication DBD of SV40 large T-antigen [39] (Figure 2c–f). All these proteins bind to viral or plasmid DNA near the origin-of-replication (or transfer). They are grouped into the same topological family in the SCOP database [40], adopting the origin-of-replication binding domain (ORBD) fold. They have a central antiparallel five-stranded β sheet and two diagonally crossing 'back' helices in common (Figure 2g). However, the presence of other structural elements, in particular sequence- and structure-specific DNA-binding substructures, differs greatly and their functions vary considerably. Whereas Rep proteins and TrwC/TraI are transesterification enzymes and share the HXH motif for metal binding and the catalytic tyrosine (the third metal-binding residue may be either a histidine or a glutamate, see Figure 2a–d,g), the DBDs of E1 and the SV40 T-antigen have only a DNA-binding function and lack the catalytic residues and active site pocket (Figure 2e,f). Furthermore, the basic fold of the TrwC/TraI relaxase domain is more distantly related to that of DNA polymerases, although in the former only palm and finger subdomains are recognisable (Figure 2h). Superimposition of the relaxase domain and the Klenow fragment of DNA polymerase I [41] shows that the catalytic residues of relaxase in the palm subdomain spatially coincide with those of the polymerase, despite the differing functions of these proteins.

Transcriptional regulator

A protein named TrwA in plasmid R388 has a dual role: it enhances the relaxase/helicase activity of TrwC and represses the operon that jointly encodes TrwA, TrwB and TrwC within *dtr* [42]. The equivalent protein in the F system, TraY, has been shown to likewise enhance the relaxase/helicase activity of TraI and to regulate the expression of *tra* genes [43]. TrwA/TraY displays an N-terminal DBD, which recognises two palindromic sites near *oriT*, and a C-terminal dimerisation domain. In the absence of experimental structures, sequence analyses and alignments predict a ribbon-helix-helix motif within the DBD, as found in the Arc/MetJ/CopG superfamily of transcriptional repressors [44]. CopG is a small 45-residue transcriptional repressor that regulates plasmid replication and recognises cognate dsDNA as a dimer of dimers. A homology model of the DBD of TrwA/TraY based on the CopG crystal structure [45] is shown in Figure 1. Each dimer penetrates the major groove of DNA using an

(Figure Legend 2 Continued) The structure depicts a catalytic zinc ion (purple sphere) coordinated by two histidine residues from the HXH consensus sequence and an additional glutamate residue (orange sticks). **(d)** N-terminal DBD of the replication initiation apoprotein from tomato yellow leaf curl virus Sardinia; ribbon colour coding as in (a). The putative cation-binding residues [same as in (c)] are shown as orange sticks. **(e)** SV40 T-antigen DBD (PDB code 2tbd); ribbon colour coding as in (a). **(f)** Bovine papillomavirus replication initiation protein E1 in complex with a 21-bp dsDNA fragment (PDB code 1ksy). For clarity, only half of the dimeric protein–operator complex is shown; ribbon colour coding as in (a), with the second DNA strand depicted in yellow. **(g)** Topological scheme for the central five-stranded antiparallel β sheet found in ORBD proteins. Strand numbering order may vary between different ORBD proteins because circular permutation has occurred in some cases. Those family members harbouring a catalytic site (a–d) bind a divalent cation through two histidine residues embedded in a short HXH consensus sequence in the central strand (strand V in TrwC) and a histidine or glutamate residue from the strand adjacent to the right (strand IV in TrwC). **(h)** Overall topological similarity between DNA polymerase I (left) and TrwC (right), showing the domains characteristic of polymerases, namely the thumb, the palm and the fingers.

antiparallel β ribbon made up of the ribbon-helix-helix motifs of the constituent protomers. CopG curves its cognate DNA by more than 120° per protein tetramer [46]. TrwA/TraY could act similarly, thereby contributing to the changes in DNA bending required for proper relaxase activity within the relaxosome.

Integration host factor

IHF is a heterodimeric *E. coli* protein made up of two structurally equivalent ~10 kDa subunits that are ~30% identical in sequence. It recognises a specific DNA sequence and assists in many prokaryotic processes that require special DNA axis distortion, such as replication, transcriptional regulation and several site-specific recombination events. The protein dimerises through an N-terminal segment consisting of two consecutive α helices and a three-stranded antiparallel β sheet (Figure 1). Between the second and third of these strands, a DBD with the shape of a β ribbon is inserted and curls around the minor groove of a nicked 35-bp dsDNA [47]. This introduces a sharp bend into the double helix in the form of an ~160° U-turn. Most of the bending is due to two large kinks mediated by prolines at the tip of each β ribbon. Together with the transcriptional regulator, IHF contributes to producing the DNA curvature required for optimal relaxosome binding and T-strand DNA nicking [48].

Coupling protein

Transport of the relaxosome to the T4SS pore requires the assistance of a T4CP — an indispensable homohexameric nucleoside triphosphate (NTP)-dependent integral membrane protein [49••,50–53]. T4CPs include TrwB from R388, TraD from F, TraG from several other Gram-negative plasmids, archetypal VirD4 from *A. tumefaciens* pTi and related proteins [22,54]. The first 70 residues of TrwB form its transmembrane domain. The structure of the remaining 48 kDa cytosolic domain resembles an elongated orange segment (Figures 1 and 3a) [55]. It contains an all-α domain with seven helices facing the cytoplasm and an NTP-binding domain attached to the inner membrane. The latter domain mainly comprises a central twisted β sheet flanked by helices on both sides. Six TrwB protomers associate to produce a pole-flattened spherical particle of roughly 110 Å diameter. A central channel runs from the cytosolic pole shaped by the all-α domains to the membrane-proximal pole, ending at the pore formed by the transmembrane domains (Figure 1). The NTP-binding site of TrwB is positioned at the interface between vicinal monomers (Figure 1, iv, lower inset), is shallow and accessible to bulk solvent.

T4CPs belong to the RecA-like family of ATPases [40], which includes F_1 ATPase, part of the mitochondrial membrane proton-driven molecular machine for ATP synthesis [56]. Superposition of the NTP-binding domains of TrwB and F_1 ATPase indicates that these two domains are oriented similarly with respect to the membrane (Figure 3). Both proteins have a central channel, occupied in the ATP synthase by the rotary γ-subunit. In TrwB, the channel is 'empty' and ~20 Å wide along its entire length, except at the cytosolic pole, where it decreases in diameter to form a gate of ~8 Å. The membrane-proximal pole of TrwB is formed by β strands, whereas a fully helical domain forms the membrane-proximal pole of F_1 ATPase.

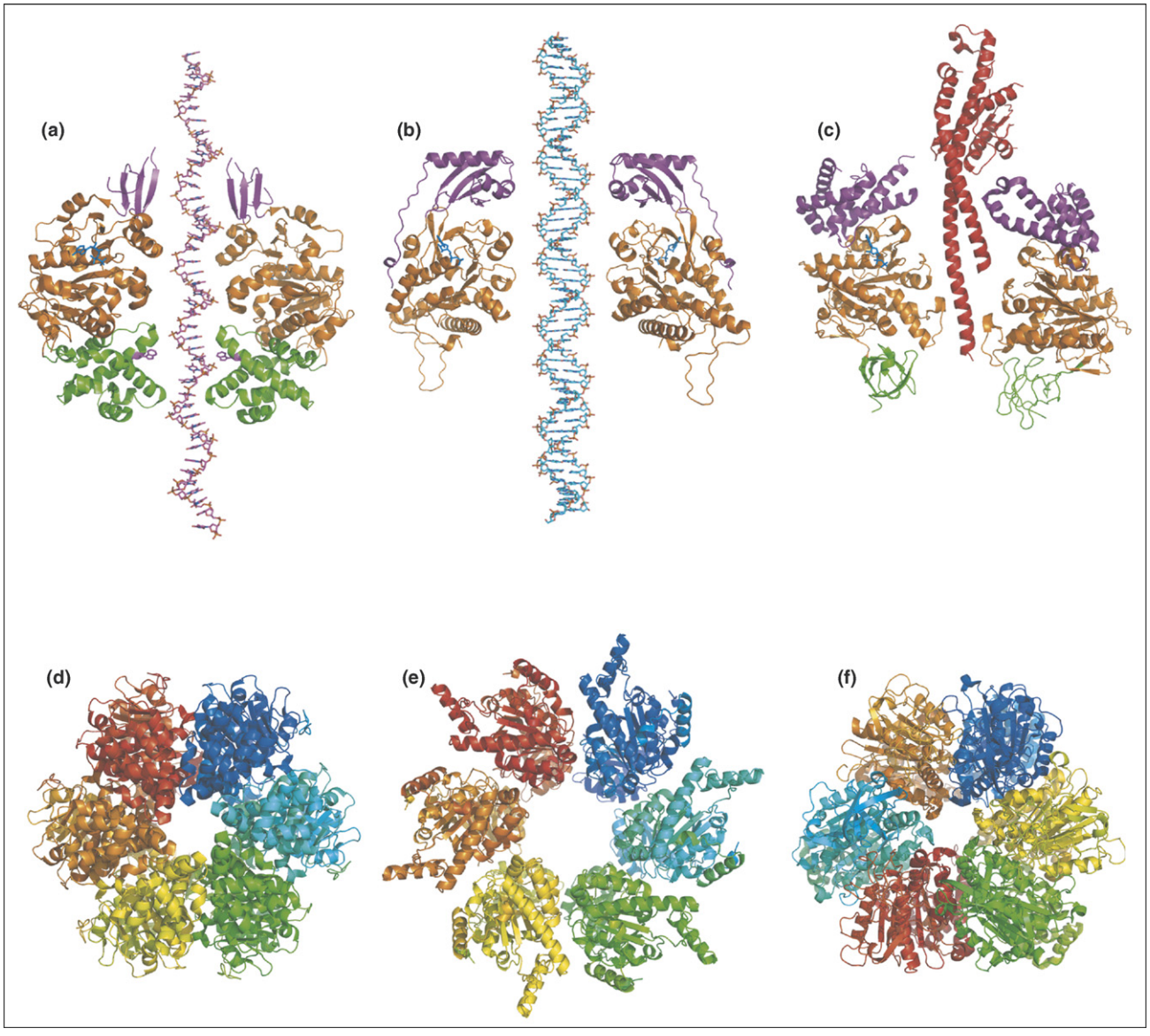
A very recent study of the dsDNA translocase FtsK [57••] indicated that this hexameric helicase is the closest structural relative to TrwB determined so far. FtsK, also a membrane protein, acts in the last stage of chromosomal segregation at the closing division septum in eubacteria. A three-dimensional X-ray structure of soluble FtsK shows that each monomer has three domains (α, β and γ). The β domain is the NTP-binding domain, which is similar to that of TrwB, whereas the α domain differs greatly from the part that corresponds topologically in TrwB (Figure 3). The γ domain is disordered in the FtsK crystal structure and thus is not observed. The FtsK hexamer has a central channel of ~30 Å, wider than that of TrwB and large enough to accommodate, in this case, a dsDNA helix, as shown by electron micrographs [57••].

Structures of several TrwB hexamers with phosphate or nucleotides bound to the active site have been reported to show small movements that propagate to the central channel [58]. De la Cruz and collaborators have recently demonstrated that substitution of Trp216, located in the channel wall and far from the active site (Figure 3a), by an alanine impairs conjugation and hinders ATP hydrolysis [49••]. The same authors also demonstrated that TrwB is a DNA-dependent ATPase. Although no direct image of any DNA–T4CP complex is available and no conclusive DNA tracking experiments have been performed to date, all data, in particular the structural homologies described above, suggest that the coupling protein is a molecular motor: a DNA translocase whose role is to pump ssDNA across the inner bacterial membrane during conjugal transfer.

Ancillary transfer protein

Several plasmids, such as F and R1, contain within their *tra* region a gene encoding protein TraM, which is required for T-strand processing complementing relaxase/helicase, IHF and the transcriptional regulator. Other plasmids, such as R388, lack a TraM orthologue [59]. TraM is a 14 kDa cytoplasmic DNA-binding protein that binds to three sites within *oriT* and, in this manner, represses its own gene promoter. It might be a constituent of the relaxosome and/or could signal to the cell that the mating process is completed; however, it is not essential for the nicking reaction. Other studies claim that TraM enhances relaxase activity and that it specifically interacts with the T4CP of plasmid R1, TraD [60]. If this were the case, TraM may link the relaxosome with the DNA

Figure 3



T4CP TrwB and other membrane proteins of the RecA-like family of ATPases: **(a,d)** conjugative T4CP TrwB (PDB code 1e9r); **(b,e)** FtsK DNA translocase (PDB code 2iuu); **(c,f)** F_1 ATPase (PDB code 1h8e). Upper panels (a–c) show two opposing monomers in a view perpendicular to the central channel, with the membrane side uppermost. Lower panels (d–f) show the corresponding hexamers viewed along the channel. In the upper figures, all nucleotide-binding or ATPase domains are oriented similarly and shown in orange, whereas the adjacent domains are in purple (membrane-proximal domain) or in green (membrane-distal domain). Bound nucleotides or nucleotide analogues are depicted as blue sticks. The γ-subunit of F_1 ATPase is shown in red (c). The ssDNA in (a) was modelled based on the 13-base ssDNA described in the central channel of the papillomavirus E1 hexameric helicase [67••]. The dsDNA shown in (b) is standard B-DNA, as built with TURBO (http://www.afmb.univ-mrs.fr/-TURBO-). TrwB residue Trp216, mutation of which impairs function, is shown in magenta (a). ATPase domains are depicted in equivalent orientations after their superimposition. Although all three molecules share a common ATPase domain (DALI Z-scores: TrwB/FtsK = 14.2, TrwB/F_1 ATPase = 9.2; http://www.ebi.ac.uk/dali), the adjacent domains have no structural relationship.

transfer apparatus [61–63]. This is noteworthy because whereas part of the TraD cytoplasmic domain has strong sequence similarity to TrwB, the C-terminal 140 amino acids of TraD, which interact with TraM, are not present in TrwB, whose R388 system, in turn, lacks a TraM orthologue [59,60]. A fragment of the 127-residue TraM protein from R1 comprising amino acids 2–56 retains the specific DNA recognition and binding capacity, and was studied by NMR [63]. The structure reveals three helical regions, the first two connected by a loop and the last two by a glycine residue (Figure 1, iv, upper inset). Interestingly, this N-terminal fragment of TraM has topological similarity to a region within the all-α domain of TrwB (Figure 1) [58]. A speculative hypothesis is that TrwB uses a DBD,

similar to that of TraM, embedded in its structure to contact the relaxosome. More recently, structural analysis of a C-terminal fragment encompassing the TraD-binding segment (residues 58–127) of TraM from plasmid F revealed that four protomers interact to form a compact eight-helix bundle (Figure 1, v). The N-terminal helices of each protomer interact to form a central, parallel four-stranded coiled coil, whereas each C-terminal helix packs in an antiparallel arrangement around the periphery of the structure [64••]. Oligomerisation produces a central shaft surrounded by the inner N-terminal helices, where four protonated glutamate residues provided by each of the protomers create a central solvent-mediated ring. Deprotonation of this acidic residue relaxes the TraM structure and this affects interactions with TraD [64••].

Conclusions

Bacterial conjugation, an early-discovered pathway for lateral gene transfer and the main process responsible for the spread of antibiotic resistance, has been a 'black box'. Structural biology is now making a dramatic contribution to unveiling the molecular machinery underlying this complicated protein–DNA transfer mechanism. Structural analyses of the components of the T4SS transport apparatus are currently underway (reviewed in [65•]). Also, the structures of the main players in DNA processing and membrane recruitment are being solved. Many questions remain to be answered and the results of the structural analyses will lead to new ones. For example, what is the structure of the helicase domain of the relaxase and how does it move processively along DNA? If the coupling protein is the DNA translocase, what is the role of the other T4SS ATPases also associated with the inner side of the membrane [66]? Some of them may be involved in protein transport (of the piloting relaxase?), but how is this compatible with the T-strand DNA being threaded through the hexameric coupling protein, which traverses the inner membrane? What are the precise mutual interactions of the components within the relaxosome and of those with the coupling protein? In addition to ingenious functional studies, the answer to these questions will require further structural efforts, including the determination of the structures of more protein–DNA and protein–protein–DNA complexes, and ultimately the relaxosome itself.

Acknowledgements

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