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MicroReview

Relaxosome function and conjugation regulation in F-like plasmids – a structural biology perspective

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Summary

The *tra* operon of the prototypical F plasmid and its relatives enables transfer of a copy of the plasmid to other bacterial cells via the process of conjugation. Tra proteins assemble to form the transferosome, the transmembrane pore through which the DNA is transferred, and the relaxosome, a complex of DNA-binding proteins at the origin of DNA transfer. F-like plasmid conjugation is characterized by a high degree of plasmid specificity in the interactions of *tra* components, and is tightly regulated at the transcriptional, translational and post-translational levels. Over the past decade, X-ray crystallography of conjugative components has yielded insights into both specificity and regulatory mechanisms. Conjugation is repressed by FinO, an RNA chaperone which increases the lifetime of the small RNA, FinP. Recent work has resulted in a detailed model of FinO/FinP interactions and the discovery of a family of FinO-like RNA chaperones. Relaxosome components include TraI, a relaxase/helicase, and TraM, which mediates signalling between the transferosome and relaxosome for transfer initiation. The structures of TraI and TraM bound to *oriT* DNA reveal the basis of specific recognition of DNA for their cognate plasmid. Specificity also exists in TraI and TraM interactions with the transferosome protein TraD.

Introduction

Conjugation, a form of horizontal gene transfer between bacterial cells, is an important contributor to bacterial genetic diversity. 17% to 25% of the *Escherichia coli* genome is thought to originate from horizontal gene transfer (Narra and Ochman, 2006), which has recently shown to be responsible for protein family expansion in 88–98% of genes across eight genetically distant bacterial clades (Treangen and Rocha, 2011). In addition, conjugation mediates the transfer of genetic material between bacterial species (Gubbins *et al.*, 2005; Palmer *et al.*, 2010; Wozniak and Waldor, 2010).

Plasmids of the IncF incompatibility groups are relatively large, narrow host-range plasmids typically found in the *Enterobacteriaceae* family (Frost *et al.*, 1994; Mulec *et al.*, 2002). Examples include the prototypical F plasmid, and the R1, R100 and pED208 plasmids. Members of the F plasmid family are responsible for some of the earliest instances of antibiotic resistance, such as the emergence of multidrug-resistant *Shigella* in Japan in the mid-1950s (Watanabe, 1963) and F-like plasmids (many of them conjugative) continue to mediate a wide range of antibiotic resistance mechanisms in recent times (Conly, 2002; Strahilevitz *et al.*, 2009; Potron *et al.*, 2011). F-like replicons and portions of F-like *tra* systems are found in the majority of large virulence plasmids documented in *E. coli* and *Salmonella*, indicating a prominent role for F-like plasmids in their evolution (Ahmer *et al.*, 1999; Porwollik and McClelland, 2003; Chu and Chiu, 2006; Johnson and Nolan, 2009). The F-derived plasmid pOX38 is capable of transfer to *Salmonella*, *Klebsiella* and *Shigella* species (Mulec *et al.*, 2002), and evidence of horizontal propagation of transfer (*tra*) genes of the *E. coli* F plasmid have been found in a number of *Salmonella* strains (Boyd and Hartl, 1997).

The machinery of conjugation in F-like plasmids includes a DNA-processing complex (the relaxosome) that assembles on the plasmid's origin of transfer (*oriT*) and a type IV secretion system (the transferosome) through which the DNA is transferred (Lawley *et al.*, 2003) with a coupling protein acting as the link between the two complexes (de la Cruz *et al.*, 2009). Cell–cell contact is mediated via the pilus, following which the plasmid DNA is unwound and a single strand is transferred to the recipient cell.

Being energetically expensive, conjugation is usually tightly regulated and highly responsive to physiological

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and environmental stimuli. For instance, F plasmid transfer begins to decline in mid-exponential phase to undetectable levels in stationary phase, but is able to quickly become transfer positive when small amounts of glucose are added (Frost and Manchak, 1998). F plasmid transfer is regulated by a number of host factors that are sensitive to environmental cues. The regulation of conjugation in the IncF plasmids is one of the best studied in terms of mechanistic detail.

Over the last decade, macromolecular structures have become available that provide insight into regulation of conjugation at the atomic level. The structural biology of conjugative type IV secretion systems, the multi-protein pore complex spanning the inner and outer membranes that mediates substrate transfer, has been reviewed extensively (Schroder and Lanka, 2005; Juhas *et al*., 2008; Alvarez-Martinez and Christie, 2009; Llosa *et al*., 2009; Terradot and Waksman, 2011). This review provides an overview of recent developments in understanding the regulation of F-like plasmid conjugation based on the structural biology of relaxosome components, transferosome–relaxosome interactions, and fertility inhibition. An aspect of conjugation for which crystallography has been particularly illuminating is plasmid specificity of different conjugation systems, revealed by the plasmid-selective interactions that components of the conjugative machinery display for the proteins and DNA elements of their cognate plasmid.

An overview of the regulation of F plasmid conjugation

The F-like family of plasmids all contain a large, ~ 30 kb *tra* operon that encodes all the plasmid genes necessary for assembly of the conjugative pore and transfer of the plasmid (Frost *et al*., 1994; Lawley *et al*., 2003; Gubbins *et al*., 2005). Transcription of the *tra* operon is driven by a single promoter, P_Y, which is regulated by complex array of plasmid-encoded as well as host factors (Fig. 1A). Regulation of P_Y largely hinges on the plasmid-encoded transcription factor, TraJ, and the host-encoded transcription factor, ArcA. TraJ is itself subject to a complex regulatory network involving transcriptional, post-transcriptional and post-translational regulatory mechanisms.

Another key point of regulation is at the formation of the relaxosome, a large protein assembly centred on the multifunctional TraI protein. TraI specifically binds the plasmid *oriT* sequence, creating a single-strand nick at the plasmid *nic* site, and subsequently unwinding the plasmid to liberate the single transfer strand for conjugation (Fig. 1B). The relaxosome also appears to be critical for the direct recruitment of the plasmid to the conjugative pore through interactions between the DNA-binding protein, TraM, and the hexameric ring ATPase TraD, which likely forms the cytoplasmic entrance to the pore.

Regulation of P_J and P_Y transcription by environment-sensitive host factors

Transcription from P_Y, as well as the transcription of the major plasmid transcription factor, TraJ, depends on a number of host-encoded proteins which are sensitive to cellular conditions like nutrient availability and stress. Transcription from the *traJ* promoter, P_J, is controlled by the global transcription factors cyclic AMP receptor protein (Crp) (Harwood and Meynell, 1975; Starcic *et al*., 2003) and leucine-responsive regulatory protein (Lrp) (Starcic-Erjavec *et al*., 2003; Camacho and Casadesus, 2005; Camacho *et al*., 2005), as well as Dam-mediated DNA methylation (Camacho and Casadesus, 2005; Camacho *et al*., 2005). H-NS is a silencer of transcription from P_J, and P_Y, as well as P_M, the promoter for the *traM* gene (Will *et al*., 2004; Will and Frost, 2006a). It is thought that TraJ acts more as an 'anti-silencer' of P_Y expression rather than an activator (Frost and Koraimann, 2010), consistent with a model where transcriptional activators disrupt DNA bridges mediated by H-NS at promoters (Dorman and Kane, 2009). The crystal structure of *E. coli* H-NS (residues 1–83) bound to DNA revealed a superhelical structure proposed to be a scaffold for DNA condensation (Arold *et al*., 2010). The possibility that a large superhelical arrangement forms during H-NS silencing is intriguing in light of the requirement of P_M and P_J to be on the same fragment for silencing to occur (Will *et al*., 2004). The half-life of TraJ is the controlled by two protease systems in response to different environmental stimuli (Fig. 1A). HslVU (ClpYQ), an AAA+ ATPase, degrades TraJ when stimulated by the CpxAR stress response system (Lau-Wong *et al*., 2008). In response to elevated temperature, the GroEL protein chaperone is involved in repression of conjugation and *tra* gene expression through its ability to facilitate degradation of TraJ (Zahrl *et al*., 2007) (Fig. 1A).

In addition to TraJ, two other transcription factors, TraY and ArcA, are known to bind proximal to P_Y and regulate the *tra* operon (Inamoto and Ohtsubo, 1990; Nelson *et al*., 1993; Strohmaier *et al*., 1998; Rodriguez-Maillard *et al*., 2010). Activation of transcription at P_Y by TraJ may be sensitive to cell redox state (Arutyunov *et al*., 2011). TraY is a transcriptional regulator of P_y, exerting a positive or negative effect depending on the individual plasmid system (Silverman and Sholl, 1996; Taki *et al*., 1998). The general host transcription factor ArcA also regulates transcription of the *tra* operon via effects on P_Y (Silverman *et al*., 1991; Strohmaier *et al*., 1998; Serna *et al*., 2010) (Fig. 1A). In the R1 system, the TraM DNA-binding protein also appears to regulate P_Y, although this function could be an indirect effect due to the ability of TraM to mediate relaxosome formation (Polzleitner *et al*., 1997).

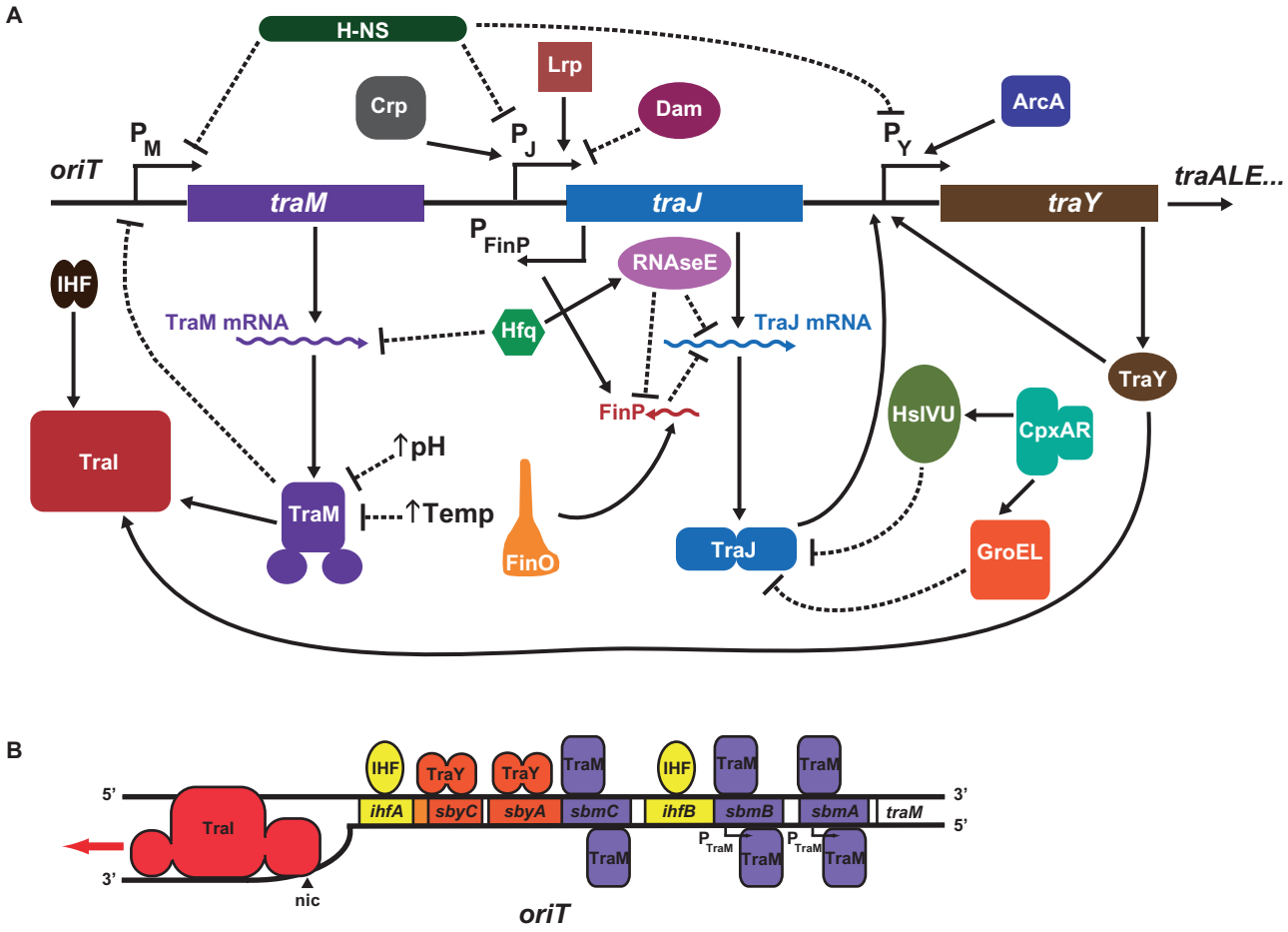


Fig. 1. *tra* operon regulation in F-like plasmids.
A. Overview of F plasmid *tra* operon regulatory factors. Positive regulatory effects are indicated by an arrow and solid lines, negative effects are indicated by a dash and dotted lines.
B. F plasmid *oriT* region with the binding sites for host and plasmid DNA-binding proteins indicated. The direction of TraI unwinding of DNA following cleavage at the *nic* site and covalent attachment to the 5′ end of DNA is indicated by a red arrow.

Post-transcriptional regulation of *traJ*

Following high-frequency transfer (HFT), where plasmids are spread rapidly via conjugation throughout the newly infected bacterial population, conjugation is repressed by the action of the FinO/FinP fertility inhibition system on *traJ* mRNA (Gubbins *et al.*, 2005). The FinO/FinP system acts by reducing the level of TraJ protein (Finnegan and Willetts, 1971; Frost *et al.*, 1989; 1994; Gubbins *et al.*, 2005). Translation of *traJ* mRNA is repressed by the 79 nt antisense RNA FinP, which is complementary to the 5′-UTR of *traJ* mRNA, and blocks its ribosome binding site (Frost *et al.*, 1994; Gubbins *et al.*, 2005). A myriad of small RNA species have been shown to play critical roles in regulation of plasmid transfer and replication (Brantl, 2007; Georg and Hess, 2011). Some work by directly blocking the *rbs* like FinP, including the *hok/sok* family of RNA toxin-antitoxin systems (Gerdes *et al.*, 1997), and the CopA/CopT system that regulates plasmid replication in the R1 plasmid (Nordstrom, 2006). A family of putative FinP structural homologues represented by PtaRNA1 is proposed to be part of a toxin–antitoxin pair due to it being frequently found antisense to the same putative toxin (Findeiss *et al.*, 2010)

Regulation of *traJ* mRNA by FinP critically depends on a plasmid encoded protein, FinO. FinO is an RNA chaperone that increases the lifetime of FinP by protecting it from degradation by RNase E (Jerome *et al.*, 1999), while enhancing duplex formation of FinP and *traJ* mRNA (van Biesen and Frost, 1994). The process of duplex formation has been shown to occur through a strand exchange mechanism (Arthur *et al.*, 2003) mediated by initial formation of a 'kissing complex' between complementary regions of the FinP and *traJ* mRNA stem loops (Gubbins *et al.*, 2003) (Fig. 2A).

Extensive work has been done to characterize the mechanism of FinO-chaperoned FinP–*traJ* mRNA interactions. The crystal structure of a proteolytically stable frag-

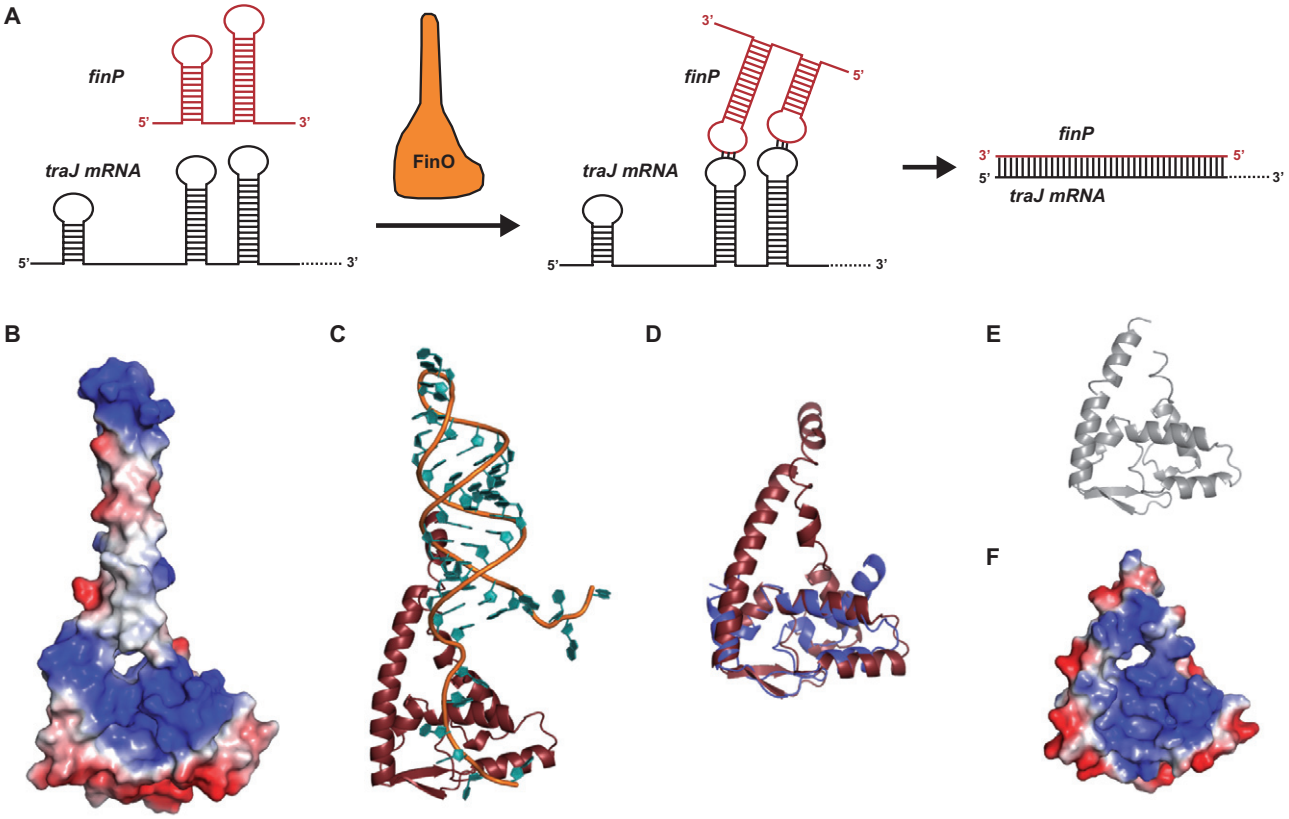


Fig. 2. Mechanism and structure of the FinO family RNA chaperones.
A. FinO/FinP fertility inhibition of F-like plasmids. FinO facilitates strand exchange and duplexing between FinP antisense RNA and the ribosome binding site of *traJ* mRNA. Initial contact between FinP and *traJ* mRNA is thought to occur by formation of a 'kissing complex' between complementary bases in the loops.
B. Electrostatic surface representation of the crystal structure of FinO$^{26–186}$.
C. HADDOCK model of FinP bound to FinO$^{45–186}$.
D. Structural alignment of FinO (red) with NMB1681 (blue).
E. Model of the ProQ FinO-like domain. Model was created with MODELER using the FinO structure as a template.
F. Electrostatic surface representation of the ProQ FinO-like domain.

ment of FinO$^{26–186}$ showed that it forms a novel, largely α-helical fold that is elongated due to a flexible N-terminal α-helix. It has two highly positively charged surfaces one at the top of the N-terminal helix, and the other covering one face of the core of the protein (Ghetu *et al.*, 2000) (Fig. 2B). The N-terminal helix is crucial for strand exchange, in particular the residue Trp36. The structured core of the protein, residues 45–186, was shown to bind RNA with high affinity but was unable to catalyse strand exchange (Ghetu *et al.*, 1999; Arthur *et al.*, 2003). Regions in closest contact with the RNA have been determined by cross-linking to be the large positively charged patch in the core of the protein, and the tip of the N-terminal helix (Ghetu *et al.*, 2002). RNase protection experiments reveal that the lower half of the SLII stem-loop and the 3′-tail single-stranded tail, are contacted by FinO in a manner that is dependent on the presence of a free 3′-hydroxyl (Arthur *et al.*, 2011). The RNA footprinting and cross-linking data, together with structural data from small-angle X-ray scattering (SAXS), were used as restraints in generating models for FinP–FinO interactions (Fig. 2C) (Arthur *et al.*, 2011). Based on the proximity of Trp36 to the RNA in the model, it has been proposed that Trp36 may form stacking interactions with the RNA bases following a conformational change.

Two other RNA chaperones with structural and functional similarities to FinO have been recently discovered, revealing that these proteins represent a wide-spread family of bacterial RNA chaperones. The crystal structure of the previously uncharacterized *Neisseria meningitidis* 1681 (NMB1681) is very similar to the core of FinO (Fig. 2D). NMB1681 also has significant RNA binding, strand-exchange and duplexing activities *in vitro* (Chaulk *et al.*, 2010). Remarkably, NMB1681 is able to partially restore conjugative repression to *finO*-deficient *E. coli in vivo* even though its ability to protect *FinP* from degradation is relatively weak (Chaulk *et al.*, 2010). Sequence alignments and proteolytic mapping have also suggested

that the N-terminal domain of *E. coli* ProQ, a regulator of the membrane transporter, ProP, is also related to FinO (Smith *et al.*, 2007) (Fig. 2E and F). In addition to the FinO-like domain, ProQ also contains an additional C-terminal domain predicted to have structural similarity to another RNA chaperone, Hfq. The ProQ FinO-like domain displays significant RNA binding activity, while the C-terminal Hfq-like domain has significant RNA strand exchange and duplexing activities *in vitro* (Chaulk *et al.*, 2011). The native RNA substrates of these two proteins have yet to be determined.

In addition, *traJ* mRNA is also regulated by Hfq, which specifically recognizes the 5′-UTR of *traJ* mRNA (Will and Frost, 2006b) and is known to interact with RNase E (Morita *et al.*, 2005) (Fig. 1A). Therefore, Hfq may enhance degradation of *traJ* mRNAs by bringing them in closer proximity to RNase E. Hfq may also mediate degradation of *traJ* mRNA via UtpR, a small RNA transcribed from outside the *tra* region that is complementary to the *traJ* mRNA promoter (Frost and Koraimann, 2010). A flexible C-terminal protrusion from the Hfq core has been shown to have a role in interactions with long RNAs (Beich-Frandsen *et al.*, 2011), and several crystal structures show that Hfq forms a hexameric Sm fold that binds RNA single strands along the central pore (Schumacher *et al.*, 2002; Link *et al.*, 2009; Sauer and Weichenrieder, 2011).

Overview of relaxosome function

The primary function of the relaxosome at *oriT* is to initiate nicking of plasmid DNA for transfer. The relaxosome is composed of several protein components including the plasmid encoded TraI, TraY and TraM, as well as the host factor, IHF. TraI is a bifunctional relaxase/helicase that recognizes the *nic* sequence within *oriT* and introduces a nick on the transfer strand that results in the covalent attachment of TraI to the 5′ end of the nick (Byrd and Matson, 1997). TraI then unwinds the DNA in a 5′ → 3′ direction and is transported into the recipient cell along with the transfer strand (Lang *et al.*, 2010; Dostal *et al.*, 2011). A minimum of 60 bases of single-stranded DNA around the *nic* site is necessary for this to occur efficiently (Csitkovits *et al.*, 2004). TraY is an accessory protein that binds to two sites at *oriT* and to the P_Y promoter (Nelson *et al.*, 1993; Luo *et al.*, 1994). IHF stimulates TraI nicking and helicase activities (Inamoto *et al.*, 1994; Howard *et al.*, 1995; Nelson *et al.*, 1995; Kupelwieser *et al.*, 1998; Karl *et al.*, 2001) and likely contributes to the three-dimensional structure of the relaxosome by inducing sharp DNA bends (Fig. 1B). The relaxosome is brought in close proximity to the transferosome through a key interaction between the transferosome ATPase TraD, and TraM, which binds to multiple sites near *oriT* (Disque-Kochem and Dreiseikelmann, 1997; Beranek *et al.*, 2004; Lu *et al.*, 2008). In general, these interactions selectively occur between proteins of the same plasmid; heterotypic interactions are much less stable. Single-stranded DNA is then transferred through the transferosome (Lawley *et al.*, 2003).

Structural insights into TraI function and plasmid specificity

TraI activity is modulated by several proteins and negative cooperativity between two domains for DNA binding. F plasmid TraI is a 192 kDa protein consisting of a relaxase domain (~ 1–306) (Byrd *et al.*, 2002), two putative RecD-like helicase folds (~ 303–844 and ~ 830–1473) (Dostal and Schildbach, 2010), and a C-terminal domain of unknown function (~ 1476–1756) that also appears to be required for F conjugation (Guogas *et al.*, 2009). A model of full-length TraI was constructed, using a SAXS envelope that shows that TraI has an elongated, conformation in solution (Cheng *et al.*, 2011). The relaxase domain cleaves at *nic* through nucleophilic attack by the Tyr16 hydroxyl. This tyrosine is part of a YY-$X_{5–6}$-YY motif (Tyr16, Tyr17, Tyr23 and Tyr24 in F TraI) that is largely conserved in the Mob_F family of conjugative relaxases (Byrd and Matson, 1997). Binding and nicking activity of the relaxase at *nic* is highly sequence specific, and therefore plasmid specific (Fekete and Frost, 2000; Stern and Schildbach, 2001; Harley and Schildbach, 2003; Gonzalez-Perez *et al.*, 2009).

Crystal structures have been solved for Mob_F class relaxases from three plasmids [F (Datta *et al.*, 2003), pCU1 (Nash *et al.*, 2010), R388 (Guasch *et al.*, 2003)], and one Mob_Q class relaxase from the plasmid R1162 (Garcillan-Barcia *et al.*, 2009). Although the structures represent multiple Inc groups (F plasmid -IncF, pCU1 -IncN, R388 -IncW and R1162 -IncQ), all structures share a conserved fold, consisting of a 5-stranded β-sheet, the 'palm', with a pair of long α-helices on one face and two largely α-helical domains on the DNA binding face The α-helical flap that closes over the bound DNA are the 'fingers' that becomes ordered upon binding (Larkin *et al.*, 2005) (Fig. 3A). Structures of relaxase–*nic* DNA complexes for F plasmid TraI and R388 plasmid TrwC have revealed that the relaxase binds to a single-stranded DNA U-turn stabilized by intramolecular contacts between the DNA bases (Guasch *et al.*, 2003; Larkin *et al.*, 2005). From the crystal structure of F TraI bound ssDNA, the key tyrosine for cleavage, Tyr16, is in good position to cleave the DNA phosphate backbone. Tyr17, which exhibits some functional redundancy with Tyr16, forms a hydrogen bond with Asp81, a residue important for transfer and cleavage (Larkin *et al.*, 2005; 2007). There is strong structural conservation of the HUH motif, a triple-histidine divalent cation co-ordination site (His146, His157 and His159)

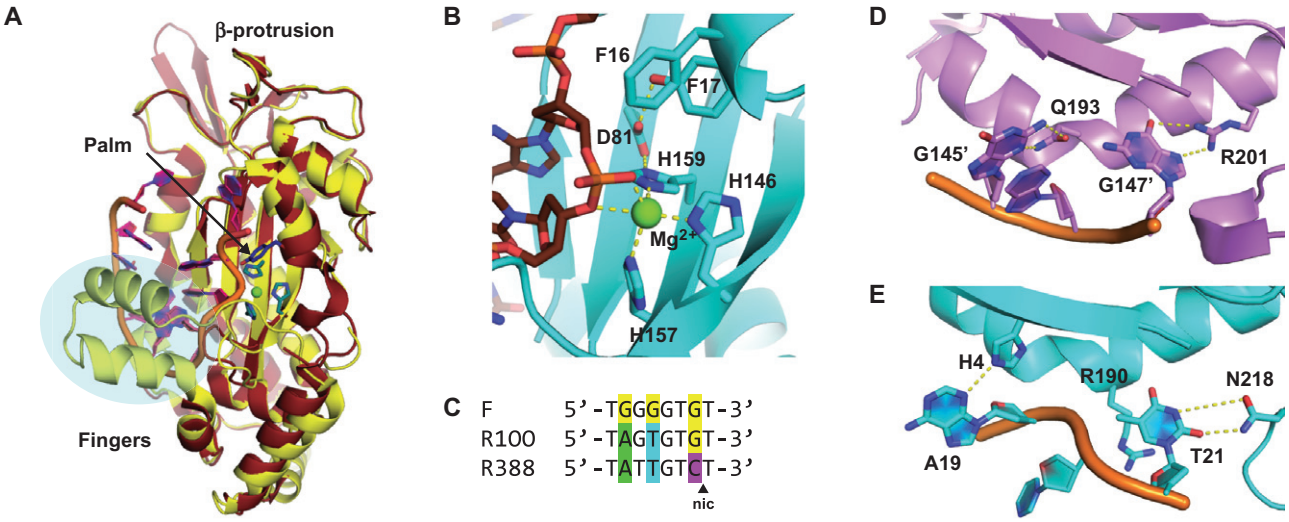


Fig. 3. Relaxase structure and DNA-binding specificity.
A. Alignment of crystal structures of F TraI in its apo-form (red) (PDB ID: 1P4D), and DNA-bound form (yellow) (PDB ID: 2A0I). *oriT* DNA from the TraI–DNA complex is shown in purple. F TraI H146, H157, H159 are shown in blue, and Y16 in dark blue. Mg^{2+} is shown as a green sphere.
B. F plasmid TraI catalytic tyrosines and metal co-ordination. Structure shown is the DNA-bound form of the catalytically inactive TraI-mutant Y16F (PDB ID: 2A0I). Key TraI residues important for *nic* cleavage are shown as sticks. DNA is shown in brown sticks, and Mg^{2+} is shown as a green sphere.
C. Sequence alignment of relaxase binding sites at *oriT* Bases with key roles in determining plasmid specificity for relaxase–*nic* DNA recognition are highlighted.
D. TraI–DNA interactions that determine plasmid specificity in the F plasmid system.
E. TrwC–DNA interactions that determine plasmid specificity in the R388 plasmid system.

in close proximity to the active-site tyrosines (Larkin *et al.*, 2005; Boer *et al.*, 2006) (Fig. 3B). The metal ion in the F TraI crystal structures has been assigned as Mg^{2+} (Larkin *et al.*, 2005; 2007; Lujan *et al.*, 2007), but the physiologically active metal ion of F TraI is not entirely resolved, as it is capable of significant nicking activity in the presence of Ca^{2+}, Mg^{2+} or Mn^{2+} (Larkin *et al.*, 2005; 2007).

The relaxases of F-like plasmids show a high level of binding specificity to the *nic* site of their cognate plasmids. Harley and Schildbach (2003) have shown that TraI of F and R100 plasmids bind to their cognate *nic* site three orders of magnitude more tightly than to the *nic* site of the non-cognate plasmid. This selectivity is largely due to the interactions of a non-conserved pair of amino acid residues, Gln193 and Arg201 in F TraI, and a pair of single-stranded bases at 145′ and 147′ (according to the base-numbering scheme of the *nic* site in Frost *et al.*, 1994) (Fig. 3C). The specificity of binding can be swapped to some extent between R100 and F by switching residues only at these positions (Harley and Schildbach, 2003). The crystal structure of F TraI bound to *nic* DNA bases 144′–153′ provides an explanation for the role of Gln193, Arg201, G145′ and G147′ in binding specificity. In addition to revealing hydrogen bonds between the DNA bases and the side-chains, Arg201 forms part of a pocket entered by G147′ (Larkin *et al.*, 2005) (Fig. 3D). Comparison between the structures of F TraI and R388 TrwC (Boer *et al.*, 2006) reveal the nature of specificity in relaxase-*oriT* DNA interaction between the two plasmid groups. None of the above-mentioned specificity determinants is conserved. Residues corresponding to that of F TraI Gln193 and Arg201, Thr189 and Asn197 of TrwC, are not appropriately positioned for interaction with bases in the R388 *nic* site corresponding to F 145′ and 147′. Instead, a hydrogen bond is formed between His4 and A19 and between Asn218 and T21. In addition, Arg190 forms a cation-pi stacking interaction with T21 (Fig. 3E). A further site of specific binding is at the position immediately 5′ to the *nic* site, which is T in R388 but is G in the *nic* sites of other F-like plasmids (Fig. 3C). It was predicted that Lys262 in TrwC, which interacts with the cognate T in the R388 *nic* site, would be precluded from interaction with guanine in the *nic* site of other F-like plasmids due to steric hindrance (Gonzalez-Perez *et al.*, 2009).

The TraI relaxase domain is followed by two helicase folds and a C-terminal domain that may interact with TraM. The C-terminal helicase fold is the functional helicase, whereas the N-terminal helicase fold functions as a binding domain for ssDNA (Haft *et al.*, 2006). Supporting this, the C-terminal fold but not the N-terminal fold contains a β-hairpin required for helicase activity homologous to *E. coli* RecD (Dostal and Schildbach, 2010). The crystal

structure of the region C-terminal to the helicase domain, consisting of residues 1476–1629 of F TraI, has been solved, revealing a novel fold. Although truncations in this region are very detrimental to conjugation, the precise function of this region is yet to be determined (Guogas *et al*., 2009).

Two binding sites for single-stranded DNA have been discovered on TraI, one in the relaxase domain, and the other in the N-terminal helicase domain. Several findings indicate that there is negative cooperativity in single-stranded DNA binding between the two domains. The isolated helicase domain exhibits greater unwinding activity than the full-length protein (Sut *et al*., 2009). Twice as much DNA as expected was required to reach binding saturation with the full-length protein, indicating that binding of the relaxase site interferes with binding to the helicase site (Dostal and Schildbach, 2010). High-affinity binding of the relaxase domain to the DNA hairpin formed by an inverted repeat 3′ to *nic* is hypothesized to act as a 'switch' between an inactive state to a helicase active state (Mihajlovic *et al*., 2009; Sut *et al*., 2009; Dostal and Schildbach, 2010).

The nature of TraI interactions with transferosome components still needs to be clarified. Direct interaction of coupling proteins with the relaxase has been reported in R388, RP4, and the RP4-mobilizable plasmids pBHR1 and pLV22a (Szpirer *et al*., 2000; Schroder *et al*., 2002; Llosa *et al*., 2003; Thomas and Hecht, 2007). Direct TraI–TraD interaction in F-like plasmids has yet to be demonstrated, although it has been suggested in a number of studies. TraI colocalizes with TraD in the membrane fraction when TraD is coexpressed (Dash *et al*., 1992). The TraD cytoplasmic domain stimulates the relaxase and helicase activities of TraI (Mihajlovic *et al*., 2009; Sut *et al*., 2009). TraI is transported to the recipient cell while it is attached to the transferred plasmid DNA (Lang *et al*., 2010; Dostal *et al*., 2011), therefore interaction with the conjugative pore is necessary at some point. Evidence suggests that interaction occurs in a sequence-specific manner through its translocation sequences. Residue Leu626 in the first translocation sequence of F TraI is essential for transfer (Lang *et al*., 2010). It has been hypothesized that there is a signalling conduit from TraD through $TraI^{1–992}$ for export or import of substrates through the T4SS (Lang *et al*., 2010; 2011). Whether TraI forms a relaxosome–transferosome bridge with TraD in F-like plasmids akin to the TraD–TraM interaction or affects TraI activity indirectly through DNA is unknown.

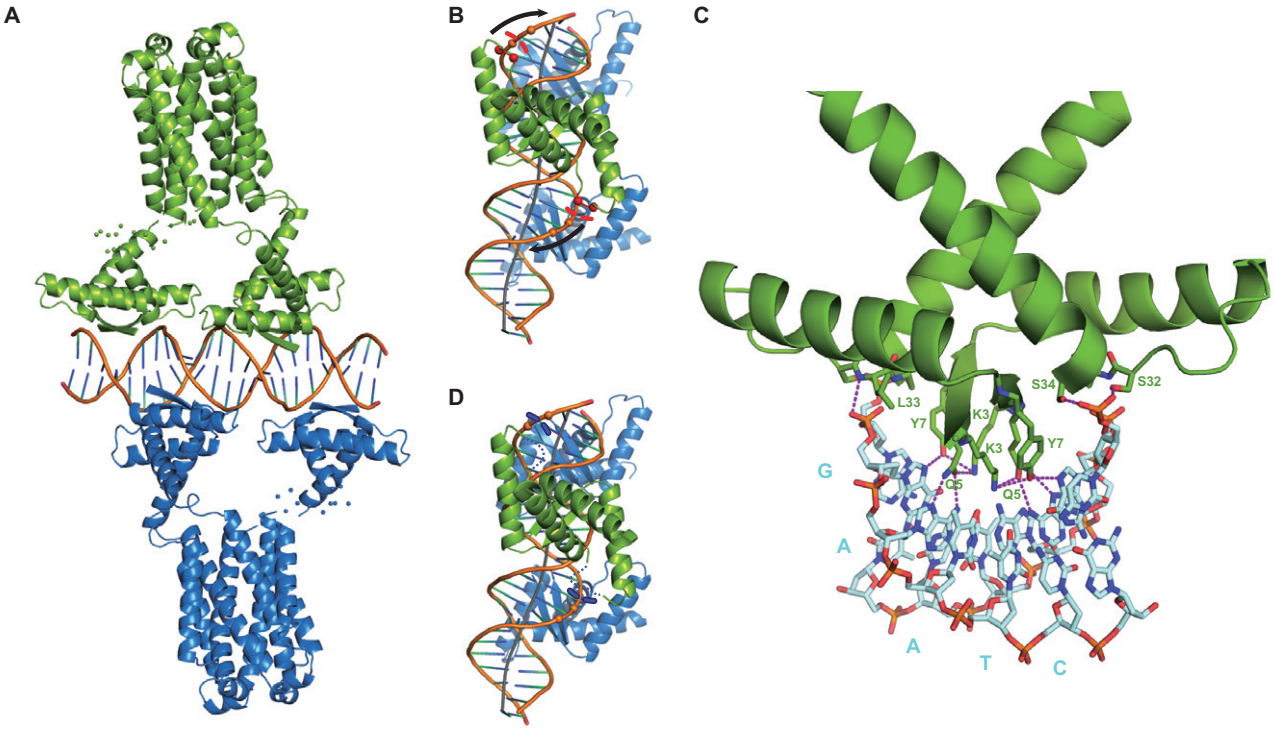
Structural insights into TraM autoregulation and plasmid specificity

TraM has multiple functions in the relaxosome and is essential for conjugation to occur. TraM stimulates DNA nicking and unwinding by the TraI relaxase/helicase and mediates relaxosome–transferosome contact. In addition, it autoregulates its own transcription and is sensitive to environmental conditions. F plasmid TraM binds to three sites at *oriT, sbmA, sbmB* and *sbmC* (Fig. 1B). Each site contains DNA-binding motifs which are specific to TraM of the cognate plasmid. Binding of TraM to these sites is cooperative, and the highest affinity binding site is *sbmA* (Fekete and Frost, 2002). *sbmA* and *sbmB* overlap with the TraM promoter P_M, such that TraM negatively regulates its expression when bound to these sites (Penfold *et al*., 1996) (Fig. 1A and B). Crystal structures are available which shed light on how TraM performs these functions and maintains plasmid specificity while interacting with other transfer machinery components.

TraM is a tetrameric protein consisting of a C-terminal tetramerization domain (Verdino *et al*., 1999; Miller and Schildbach, 2003) and an N-terminal dimerization and DNA-binding domain (Schwab *et al*., 1993; Kupelwieser *et al*., 1998; Miller and Schildbach, 2003; Lu *et al*., 2004). Oligomerization of TraM is essential for TraM function (Lu *et al*., 2004). The crystal structure of the C-terminal domain shows that it forms an α-helical bundle (Lu *et al*., 2006), and the crystal structure of full-length TraM bound to *sbmA* DNA shows that the N-terminal domains dimerize to form a ribbon–helix–helix (RHH) domain (Wong *et al*., 2011). RHH domains are a commonly used DNA-binding motif in prokaryotes (Schreiter and Drennan, 2007) and are widely distributed among the plasmid kingdom.

Many relaxosome accessory proteins are predicted to utilize RHH folds to contact DNA. These include a family represented by MbeC of the ColE1 plasmid (Varsaki *et al*., 2009), TraY of F (Bowie and Sauer, 1990; Lum and Schildbach, 1999) and TrwA of the R388 (Moncalian *et al*., 1997; Moncalian and de la Cruz, 2004). TraY of F-like plasmids regulate P_Y promoter activity (Silverman and Sholl, 1996; Taki *et al*., 1998) and stimulates the activity of TraI (Howard *et al*., 1995) when bound to its DNA sites. The RHH domain of TraY is believed to be encoded by two domains in tandem on a single chain, and bends the DNA by ~ 50° upon binding (Lum and Schildbach, 1999). Indeed, the structure of the relaxosome accessory protein VirC2 from the *Agrobacterium tumefaciens* T-DNA transfer system reveals a novel fold that mimics an RHH dimer within a single polypeptide chain (Lu *et al*., 2009)

The crystal structure of TraM of an F-like plasmid, pED208, in complex with a minimal *sbmA* site has been determined (Wong *et al*., 2011). Two TraM tetramers are bound to *sbmA* on opposite sides of the DNA double helix, with their N-terminal RHH domains in a staggered arrangement. Their cooperative binding to *sbmA* is mediated entirely through the DNA, as no protein–protein contacts are observed. Similar binding arrangements have



E

	β-ribbon	Binding motif	*sbmA* homologue	α1-α2 loop
F	KVNLY	G G A–CG–T C C	5'-GATACCGCTAGGGGCGCTGCTAGCGGTGCG-3' 3'-CTGTGGCGATCCCCGCGACGATCGCCACGC-5'	GAREKDV
R100	RVILY	TCCNNG	5'-TGATCCTAGAGGCGCTGCTAGGAGTGTCTTTC-3' 3'-ACTAGGATCTCCGCGACGATCCTCACAGAAAG-5'	GARDKDI
R1	KVQAY	GANTY	5'-CGAATTGACTCTAGATTCAATTCG-3' 3'-GCTTAACTGAGATCGAAGTTAAGC-5'	GAKSTDV
pED208	KIQTY	GANTC	5'-AGATTCGAATCTGGATTCGAATCC-3' 3'-TCTAAGCTTAGACCTAAGCTTAGC-5'	GIE--EA

Fig. 4. TraM binding to *sbmA*.
A. Crystal structure of two pED208 TraM tetramers cooperatively bound to *sbmA*. Dots indicate disordered regions of polypeptide chain linking the tetramerization and DNA binding RHH domains.
B. Kinking of *sbmA* DNA by the pED208 TraM α1–α2 loop. Acidic residues Glu29 and Glu30 are shown by red spheres. The DNA axis is shown by a grey line. Repulsion between the acidic residues and the DNA backbone is indicated with red curved lines, and the direction of kinking is indicated by arrows.
C. Interactions between the pED208 TraM RHH domain and GANTC-binding motif in *sbmA* DNA. Specific interactions between the N-terminal β-sheet and the major groove of the GANTC motif are indicated, as well as non-specific contacts between TraM and the DNA phosphate backbone.
D. Putative binding of F *sbmA* phosphate backbone by the F TraM α1–α2 loop. The basic loop is shown by a blue dotted line, with attraction between the loop and phosphate backbone indicated by blue curves.
E. Comparison of DNA-binding specificity determinants in F-like plasmids. Residues of the RHH β-sheet that contact DNA bases are boxed and are coloured-coded (basic – blue, hydrophobic – orange, Gln/Asn – yellow, Tyr – purple).

been observed in other bacterial transcription factors, including QacR (Schumacher *et al*., 2002), CgmR (Itou *et al*., 2010), IdeR (Pohl *et al*., 1999) and DtxR (White *et al*., 1998). Features of the binding mechanism are underwinding of the DNA to ~ 12 base pairs per turn and kinking of the DNA axis (Fig. 4A). Since the spacing between the two binding motifs bound by the same tetramer is 12 base pairs, underwinding positions the

binding motifs on the same side of the DNA helix. The kinking results from repulsion of the DNA phosphate backbone by the acidic α1–α2 loop (Fig. 4B). The mechanism of high-affinity binding is likely to be conserved among other F-like plasmids as a similar arrangement of binding motifs and binding mechanism is seen with the F, R1 and R100 *sbmA* sites (Geist and Brantl, 2008; Wong *et al*., 2011), (Fig. 4E).

The pED208 TraM–*sbmA* complex also reveals why the TraM–DNA interaction is an important plasmid specificity determinant. Alternating β-sheet residues of the RHH domain form specific contacts with the DNA bases of the pED208 GANTC-binding motifs, in particular hydrogen bonding of Gln5 and Tyr7 to the conserved adenine and guanine bases within the GANTC motif (Fig. 4C) (Wong *et al*., 2011). In comparison, the F *oriT* has a different and less well-defined consensus sequence, A(G/C)CG(G/C)T, and is 6 base pairs long instead of 5 (Fig. 4E). This provides an explanation for the observation that TraM proteins only mediate conjugation of their cognate plasmid, and not the transfer of other plasmids with a different TraM DNA binding specificity (Kupelwieser *et al*., 1998; Fekete and Frost, 2000; Lu *et al*., 2002; Wong *et al*., 2011). While the highly acidic α1–α2 loop of pED208 TraM repels the DNA backbone (Fig. 4C), the additional length and basic residues in the α1–α2 loop of F may form electrostatic interactions with the DNA backbone which are required for stable binding (Fig. 4D) (Wong *et al*., 2011).

Additional plasmid specificity occurs at the level of TraM interactions with the coupling protein TraD of the transferosome (Disque-Kochem and Dreiseikelmann, 1997; Beranek *et al*., 2004). This forms a physical tether between the transferosome and relaxasome which may be the conduit for signalling of cell–cell contact to the relaxosome. TraD is a hexameric ATPase of the FtsK/SpoIII family (Gomis-Ruth *et al*., 2001), consisting of an N-terminal membrane-spanning region and a C-terminal cytoplasmic domain that makes up the bulk of the protein (Frost *et al*., 1994). The conserved ATPase domain is followed by a C-terminal extension in F (Frost *et al*., 1994). TraD is able to bind to both single- and double-stranded DNA, with a preference for single-stranded DNA (Schroder *et al*., 2002). Structural and functional studies of the TraD orthologue from plasmid R388, TrwB, reveal a narrow channel within the TrwB ring through which the ssDNA must pass during conjugation (Gomis-Ruth *et al*., 2001).

Genetic studies have shown that the C-terminal 8 amino acids of TraD are sufficient to define specific interactions with its cognate TraM (Wong *et al*., 2011). The C-terminal 38 amino acids of TraD is sufficient for TraM binding (Beranek *et al*., 2004) TraM was shown to interact with TraD via its C-terminal domain, as a single mutation in this domain, K99E, abrogates TraM–TraD interaction without affecting autoregulation or tetramerization (Lu and Frost, 2005). The mechanism of this interaction was revealed at the atomic level by the crystal structure of the TraM C-terminal domain in complex with the last 7 amino acids of TraD. The highly acidic TraD peptide forms a β-turn and interacts with the largely basic cleft on TraM that includes Lys99. Especially critical for recognition is the C-terminal phenylalanine of TraD and its main chain carboxylate. The Phe side-chain fits into a hydrophobic pocket, while the C-terminal carboxylate is recognized by nearby positively charged residues Arg110 and Lys76 (Lu *et al*., 2008) (Fig. 5A and B). The structure of pED208 TraM has enabled modelling of TraM–TraD interactions in the pED208 system. The TraD binding groove is largely maintained in pED208, but differs in only a few residues within the last 8 amino acids of TraD. A charge swap at F Lys83 to pED208 Glu81, allow for discrimination between F and pED208 systems *in vivo* (Wong *et al*., 2011) (Fig. 5B).

The regions of TraD contacted by TraM are likely not restricted to the C-terminal tail. Full binding affinity and conjugative ability is only attained when the last 38 residues are intact (Beranek *et al*., 2004). Deletion of the last 8 amino acids in F results in at 10^3-fold decrease in F plasmid mobilization while truncation of the full C-terminal extension at residue 576 leads to an additional 10^2-fold decrease (Lu *et al*., 2008) (Fig. 5C). The C-terminal extension appears to mediate specificity in interactions between F TraD and its cognate relaxosome, while inhibiting transfer of other plasmids such as R388 and RSF1010 (Sastre *et al*., 1998).

A protein with an analogous function to TraM in the R388 plasmid is TrwA, a relaxosome component with a putative RHH-fold and a C-terminal tetramerization domain (Moncalian and de la Cruz, 2004). The N-terminal domain is the DNA-binding domain, and the C-terminal domain is a tetramerization domain that interacts with TrwB, the coupling protein of the R388 system (Llosa *et al*., 2003). It also functions as a negative transcriptional regulator of the *trw* operon and enhances activity of TrwC, the relaxase (Moncalian *et al*., 1997). The TrwA–TrwB interaction is more than simply a bridge between the relaxosome and transferosome, as TrwA affects the ATPase activity and oligomerization state of TrwB. In the absence of TrwA and DNA, TrwB is a monomer with weak ATPase activity. Both TrwA and DNA stimulate TrwB's ATPase activity and formation of TrwB hexamers (Tato *et al*., 2007). Whether this also occurs in the F plasmid has yet to be shown. However, evidence suggests that F TraD is largely dimeric *in vivo* in the absence of the F plasmid, but forms higher-order oligomers when F is present (Haft *et al*., 2007). This suggests that F plasmid proteins, possibly TraM, are required for hexamer

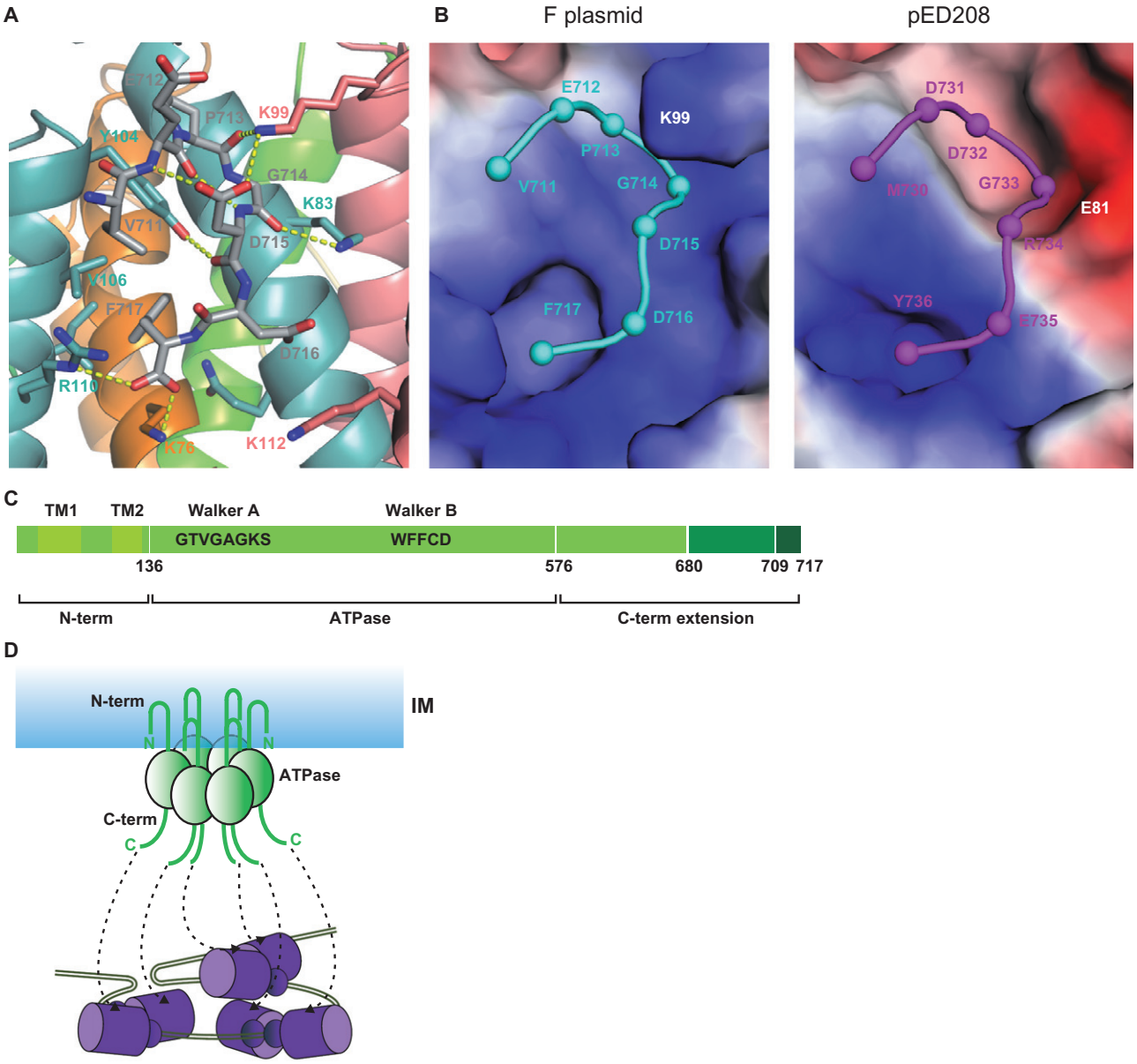


Fig. 5. TraM binding to TraD.
A. Detailed view of the structure of the F TraD C-terminal peptide (grey sticks) bound to the TraM C-terminal domain.
B. Comparison of TraM electrostatic surface in the F and pED208 TraD binding pockets. F TraD peptide is in the conformation observed in the crystal structure and the pED208 peptide is modelled based on the F TraD peptide.
C. Functional domains of F TraD. Residues known to bind TraM are shown in dark green. TM, transmembrane domain.
D. Model of TraM avidity effect in binding to TraD. IM, inner membrane. TraD is shown in green, and TraM in purple. TraM N-terminal domains are shown as ellipsoids, and TraM C-terminal domains are shown as cylinders. Multiple TraM tetramers are bound to three *sbmA* sites at *oriT* in a compact arrangement due to nucleosome-like DNA wrapping. The localized concentration of TraM tetramers facilitates interaction between multiple TraM binding sites and multiple TraD C-termini.

formation. The presence of multiple TraM sites may be required for an avidity effect, where multiple TraM tetramers bound to DNA are required for efficient binding to TraD (Fig. 5D).

Structural studies of the TraM tetramerization domain have also suggested a mechanism for the regulation of conjugation in response to increased pH or temperature (Lu *et al.*, 2006). The central helical bundle within the TraM tetramerization domain contains an unusual protonated glutamic acid (Glu88) packed in a fourfold symmetric arrangement. Basic pH and/or increased temperature result in its deprotonation, leading to decreased tetramer stability and reduced conjugation. Tetramerization is essential for interaction of TraM with TraD (Lu *et al.*,

2006). Thus, the deprotonation of Glu88 appears to be a direct mechanism by which conjugation can be repressed in non-optimal pH and temperature. This residue is conserved among the IncFI and FII plasmids F, R1 and R100, but is not in others like the IncFV plasmid pED208. It remains to be seen if TraM from pED208 or other plasmids exhibit the same pH and temperature-dependent stability.

Indirect evidence suggesting an interaction between TraM and the C-terminal domain of TraI has been reported by one group, but another group could not confirm the interaction (Ragonese *et al*., 2007; Guogas *et al*., 2009). TraM is known to stimulate nicking and unwinding activity of TraI (Sut *et al*., 2009). The ability of TraM to induce negative supercoils in plasmid DNA may be part of the mechanism of TraI transesterase stimulation (Mihajlovic *et al*., 2009). This activity is consistent with the unwinding of DNA observed in the TraM–*sbmA* crystal structure (Wong *et al*., 2011).

Towards an understanding of relaxosome architecture

The DNA topology-modifying effects of TraM binding and the DNA bending effects of TraY, IHF and other host transcriptional factors suggest a complex three-dimensional arrangement of proteins and DNA at the relaxosome. The distance and rotational orientation between relaxosome components on the DNA helix is crucial, as insertion of bases between IHF and TraY binding sites are poorly tolerated (Williams and Schildbach, 2007). The arrangement of relaxosome proteins also appears to be mediated by intrinsic and protein-induced DNA bends, as well as DNA unwinding by *tra* components. The IHF heterodimer induces a 160° bend when bound to the minor groove of DNA (Rice *et al*., 1996), and is likely a major contributor to a complex three-dimensional relaxosome conformation. The TraD homologue TrwB, as well as TraM, have been shown to induce negative supercoiling on plasmid DNA (Mihajlovic *et al*., 2009; Sut *et al*., 2009). TraM has been shown to aggregate non-specifically on DNA at high concentrations, and has been proposed to polymerize on the DNA to yield a nucleosome-like structure similar to TraK of the plasmid RP4 (Di Laurenzio *et al*., 1992; Ziegelin *et al*., 1992; Fekete and Frost, 2002). Electron microscopy of TraM on F DNA has indicated that TraM shortens the DNA but does not induce a significant bend, supporting this idea (Di Laurenzio *et al*., 1992; Fekete and Frost, 2002). The unwinding by TraM which is observed in the TraM–*sbmA* crystal structure would not yield unwinding of DNA to the extent of that observed in plasmids isolated from TraM-expressing cells (Mihajlovic *et al*., 2009). This would also support the idea that TraM aggregates on *oriT* DNA beyond its defined *sbm* sites. The presence of *sbmA*-like sites across various plasmids (Fig. 4E) indicates that this DNA element probably plays a key role in relaxosome function, perhaps serving as a nucleation point for TraM 'spreading' along plasmid DNA.

Conclusion

Study of the regulation of F plasmid conjugation paints a complex picture in which many plasmid-encoded and host factors work together at multiple levels to render transfer highly sensitive to diverse cellular stimuli. These factors include global regulatory proteins that control many other genes in the bacterial genome, as well as plasmid-encoded factors (Fig. 1A). The complexity of conjugative regulation and responsiveness to many environmental factors may be part of a mutual survival strategy for the plasmid and host cell. The repression of conjugation as nutrients are used up approaching stationary phase appears to be a strategy to avoid overtaxing the host cell in suboptimal conditions, as conjugation is an energetically demanding process (Frost and Manchak, 1998). However, additional factors may be involved, as conjugation of certain plasmids can be upregulated under unfavourable growth conditions such as low glucose for pRK100 (Starcic *et al*., 2003), and low oxygen for pSLT (Serna *et al*., 2010). In the case of pSLT, overall favourability of the conditions for growth may be what ultimately determines conjugation levels, which are driven to high levels in the nutrient-rich, microaerobic small intestine of mice (Garcia-Quintanilla *et al*., 2008).

Although the host acquires benefits from maintenance of conjugative plasmids such as antibiotic resistance and enhanced virulence, the plasmid is not without selfish tendencies. *In vitro, in vivo* and structural biology studies have shown a high level of plasmid specificity in relaxosome protein–*oriT* DNA interactions and relaxosome–transferosome protein interactions. The specific interactions between components of the conjugation machinery and their target plasmid DNAs allow transfer of only the cognate plasmid DNA and a limited number of related conjugative and mobilizable plasmids, so that the plasmid avoids taxing the cell by mediating the transfer of other plasmids apart from its own.

While structural studies of F conjugation have been extremely insightful in explaining the plasmid specificity of individual *tra* protein interactions with *oriT* DNA, how conjugative components and bacterial regulatory factors work together while bound to *oriT* is much less clear. In addition, structural information is not yet available for the key plasmid transcriptional regulatory proteins TraJ and TraY. DNA distortion appears to be critical for establishing these large multi-protein complexes. Many of the proteins involved, such as IHF and TraY, are known to significantly bend DNA upon binding. The DNA itself likely contains

significant intrinsic bends in and around the frequent AT tracts (Frost *et al*., 1994). In addition, the key plasmid-encoded factors TraM and TraI are known to unwind the DNA double helix, and evidence suggests that relaxosome components stimulate the transesterase and helicase activities of TraI in a mechanism that involves alterations in the structure of the DNA near *nic* (Mihajlovic *et al*., 2009; Sut *et al*., 2009). Further work is needed to elucidate the structural details of how conjugative components work in concert to control gene expression and mediate DNA transfer.

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