Structural basis of specific TraD–TraM recognition during F plasmid-mediated bacterial conjugation

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Summary

F plasmid-mediated bacterial conjugation requires interactions between a relaxosome component, TraM, and the coupling protein TraD, a hexameric ring ATPase that forms the cytoplasmic face of the conjugative pore. Here we present the crystal structure of the C-terminal tail of TraD bound to the TraM tetramerization domain, the first structural evidence of relaxosome-coupling protein interactions. The structure reveals the TraD C-terminal peptide bound to each of four symmetry-related grooves on the surface of the TraM tetramer. Extensive protein–protein interactions were observed between the two proteins. Mutational analysis indicates that these interactions are specific and required for efficient F conjugation *in vivo*. Our results suggest that specific interactions between the C-terminal tail of TraD and the TraM tetramerization domain might lead to more generalized interactions that stabilize the relaxosome-coupling protein complex in preparation for conjugative DNA transfer.

Introduction

The rapid acquisition of antibiotic resistance within bacteria is largely due to the ability of prokaryotes to share their DNA, not only within a single species, but also between unrelated species. One of the most important means of horizontal gene transfer is through a process termed conjugation, in which plasmid DNA is transferred from donor to recipient cell using a complex array of plasmid-encoded proteins which form a pore linking the two cells (Willetts and Wilkins, 1984; Lanka and Wilkins, 1995; Gomis-Ruth and Coll, 2006). Conjugation was first described for the F

plasmid, and this model system has revealed many of the key steps in the pathway (Frost *et al.*, 1994). F conjugation initiates with the recognition and binding of the recipient cell by the F-pilus, a helical protein polymer extruded from the donor cell. The pilus then retracts, bringing the two cells into intimate contact. The cytoplasmic spaces of the two cells are then linked via the conjugative pore (also known as the transferosome) that consists of a type IV secretion system (TFSS) spanning the cell envelope (Lawley *et al.*, 2003), which is connected to the coupling protein, e.g. TraD in the F conjugative system. At this point, a signal appears to be transmitted to the donor cell that leads to the recruitment of the plasmid to the cytoplasmic face of the coupling protein. In preparation for transfer, based on results for the R388 plasmid, the plasmid is nicked in a strand- and sequence-specific manner at *nic* within the origin of transfer (*oriT*) by the relaxase/helicase, TraI, and a single strand of the plasmid, covalently linked to TraI, is transferred to the recipient cell (Draper *et al.*, 2005).

Recruitment of the plasmid to the coupling protein remains one of the least well-understood aspects of conjugation. The F relaxosome normally resides at the cell centre or quarter centre, suggesting that there must be a mechanism for recruiting the plasmid to the randomly positioned conjugative pore at the cell membrane (Lawley *et al.*, 2002). This process is thought to involve the constituent proteins of the relaxosome, which, in addition to TraI, contains TraM, TraY and IHF that occupy binding sites within *oriT* (Kupelwieser *et al.*, 1998; Ragonese *et al.*, 2007). TraM is a tetrameric DNA-binding protein (Verdino *et al.*, 1999), which binds to three sites in F between *nic* and the *traM* promoter (Di Laurenzio *et al.*, 1992; Fekete and Frost, 2002). In F, there appear to be two stages in the recruitment process. The first involves a highly specific interaction between TraD and TraM (Disque-Kochem and Dreiseikelmann, 1997; Beranek *et al.*, 2004). The second stage involves further interactions between more extensive regions of TraM and TraD (Sastre *et al.*, 1998), presumably in preparation for the melting and unwinding of the DNA (Csitkovits *et al.*, 2004).

TraD is a member of the TraG family of coupling proteins that recruit the plasmid to the transferosome through interactions with components of the relaxosome. The

structure of the R388 orthologue, TrwB, has been determined revealing a sixfold symmetric ring ATPase structure with a central channel through which the single-stranded plasmid might be transferred, likely with the hydrolysis of ATP (Gomis-Ruth *et al.*, 2001; Tato *et al.*, 2005). TraD contains an additional C-terminal domain not found in TrwB which is required for the efficient transfer of F (Sastre *et al.*, 1998). This C-terminal region directly contacts the C-terminal tetramerization domain of TraM (Beranek *et al.*, 2004; Lu and Frost, 2005). TraD has been shown to be critical for plasmid transfer, but not for pilus expression and assembly, consistent with the hypothesis that TraD–TraM interaction is responsible for the recruitment of the plasmid to the conjugative pore (Beranek *et al.*, 2004).

Previous evidence suggests that residue 99 (Lys99) in the TraM tetramerization domain is involved in interacting with TraD and the last 38 residues of TraD interact with TraM (Beranek *et al.*, 2004; Lu and Frost, 2005); however, it was not clear how TraD specifically recognizes TraM to recruit the relaxosome complex to the conjugation machinery. Here we report the crystal structure of the C-terminal 10–12 residues of TraD bound to the TraM tetramerization domain, which reveals specific interactions between TraD and TraM. TraD–TraM interactions involve hydrophobic stacking of side-chains between the two proteins, as well as electrostatic complementarity that hinges on the availability of the free carboxylate at the C-terminus of the TraD polypeptide chain. Mutational analysis and mating assays demonstrate the importance of key amino acid contacts for plasmid conjugation.

Results

Crystallization and overall structure of the TraD–TraM complex

Previous work had identified the C-terminal 38 residues of TraD, and the C-terminal TraM tetramerization domain as critical interacting domains within the TraD–TraM complex (Beranek *et al.*, 2004; Lu and Frost, 2005). We expressed and purified the C-terminal 73 amino acids of TraD (TraD[645–717]), as well as the TraM tetramerization domain (TraM[58–127]), which had been previously crystallized (Lu *et al.*, 2006). The crystals of the complex belong to space group R3 while crystals of unliganded TraM[58–127] belong to space group I4. The complex structure was determined by molecular replacement and refined to final resolution of 2.55 Å (Table 1). The crystallographic asymmetric unit contains two TraD–TraM complexes, each consisting of a TraM tetramer bound by four TraD peptides, arranged in a fourfold symmetric fashion around the outside of the tetramer (Fig. 1A and B). One TraD chain contains the last eight amino acids of TraD while each of the other seven

Table 1. Data collection and refinement statistics.

|	
Data collection	
Space group	R3
Cell dimensions	
a, b, c (Å)	142.25, 142.25, 70.95
α, β, γ (°)	90, 90, 120
Wavelength (Å)	1.1159
Resolution (Å)	2.55
R_{sym}	0.061 (0.387)
$I/\sigma I$	22.7 (4.0)
Redundancy	5.2 (4.2)
Completeness (%)	99.95 (100.0)
Refinement	
Resolution (Å)	46.5–2.55
Number of reflections	16 609
R_{work}/R_{free} (%)	21.8/25.7
Number of protein atoms in asymmetric unit	4456
Number of water molecules	12
B-factor (overall)	49.8
Bond angle RMSD (°)	1.06
Bond length RMSD (Å)	0.011

Values in parentheses are for the highest resolution shell.

chains contains the last seven residues of TraD. MALDI-TOF mass spectrometry of the TraM[58–127]–TraD[645–717] crystal content indicated that the crystal contained peptides with molecular weights of 1080.1 and 1365.2 Da, corresponding to the tryptic fragments that contain C-terminal 10 and 12 residues of TraD respectively (Fig. 2C). The proteolysis of TraD[645–717] could be due to trace amount of trypsin present in purified TraM[58–127] because trypsin was used to cleave off the His$_6$-tag from the trypsin-resistant TraM[58–127] during purification (Lu *et al.*, 2006).

The electron density of the TraD peptides was validated by prime-and-switch phasing using the final refined structure as a starting model (Terwilliger, 2004). The resulting phases have a figure of merit of 0.56 and bias ratio of 1.01, indicating the elimination of the model bias from the prime-and-switch map (Fig. 1C). The TraD C-terminal peptide binds to a pocket on each of the four symmetry-related faces of the TraM tetramer (Fig. 2A and B). The peptide adopts a type II β-turn with Pro and Gly residues found at i+1 and i+2 positions. The β-turn structure is further stabilized by hydrogen bond interaction between the side-chain of Asp715 and the main chain of Glu712. The conformation of each of the eight independently determined TraD peptides is nearly identical, with a root mean square deviation (RMSD) for C$_\alpha$ atoms of ~0.3 Å.

Detailed TraD–TraM interactions

The TraD peptide binds into a pocket composed of residues from three of the four TraM protomers (Fig. 2A and B). The centre of the pocket is hydrophobic (including
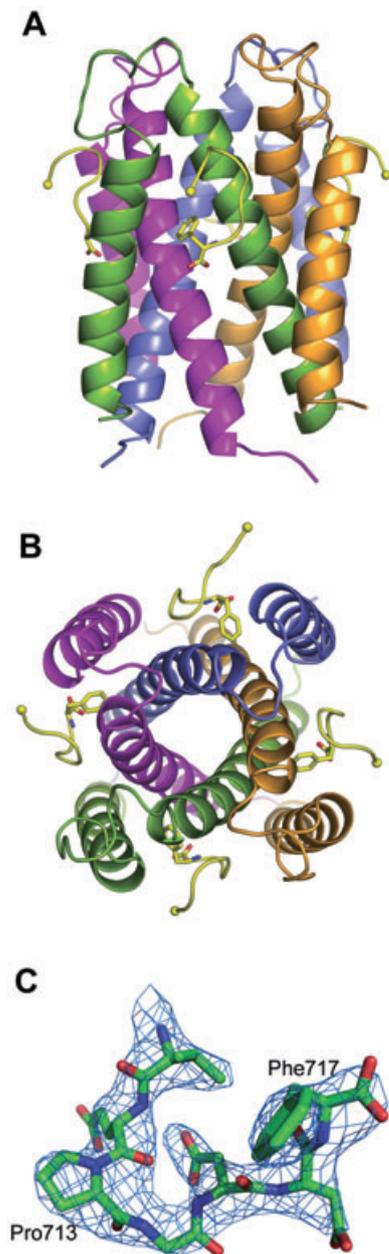
Fig. 1. Structure of the TraD C-terminal tail bound to the tetramerization domain of TraM.
A. Overview of the TraD–TraM complex. The four TraM protomers that constitute the tetramerization domain are coloured green, blue, magenta and orange. The four TraD peptides are in yellow. The N-terminus of each TraD peptide is indicated by a sphere, and the C-terminal phenylalanine residue is shown in sticks.
B. As in (A), showing an orthogonal view down the fourfold symmetry axis of the complex.
C. The electron density of the TraD peptide bound to TraM. The electron density is contoured at 1.0 σ to 2.55 Å resolution, phased by the prime-and-switch method to remove model bias. The final refined model of the peptide is shown.
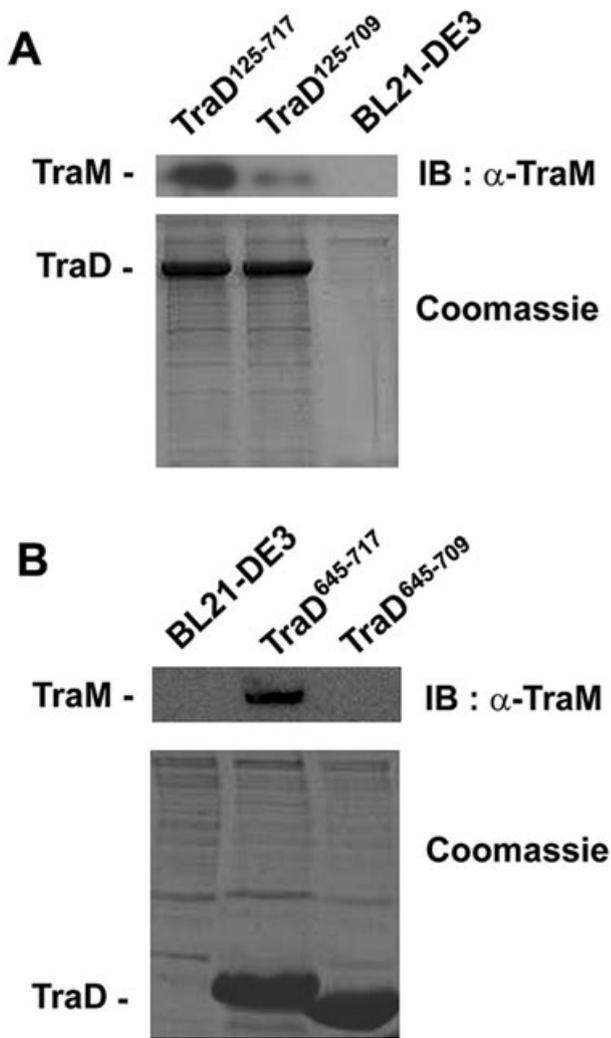
TraM residues Leu85, Val106 and Ile109) and binds the side-chain of the C-terminal residue of TraD, Phe717. Further hydrophobic interactions are made by Val711 and Pro713, which largely pack against Tyr102 of TraM. Surrounding the hydrophobic pocket are a number of positively charged residues in TraM – Lys76, Lys83, Lys99, Lys112 and Arg110, which interact with an equal number of carboxylate groups in TraD. Most striking is the direct recognition of the C-terminal carboxylate of TraD by both the guanidinium group of Arg110 and the amino group of Lys76, both of which are within hydrogen bonding distance to the carboxylate. Negatively charged TraD residues Glu712, Asp715 and Asp716 also make electrostatic interactions with TraM residues, although none of these interactions are close enough (< 3.5 Å) to be considered true hydrogen bond/salt bridging interactions.

Comparisons of TraM and TraD from other F-like plasmids suggest the TraD–TraM interactions observed in F are likely conserved in other members of the F plasmid family. We analysed the sequences of TraM and TraD from two plasmids that have diverged significantly from F: pKPN3 isolated from *Klebsiella pneumoniae*, and pED208 from *Salmonella typhimurium* (Fig. 2C). The residues important for TraD–TraM interactions in the C-terminal tail of TraD are largely conserved. The glycine residue which is important for the β-turn is conserved in all three sequences, as is an aromatic residue (Phe or Tyr) at the end of the chain. In addition, each of the tails is overall highly acidic. In TraM, the positively charged residues that surround the TraD binding site are less well conserved, except at position 76, which is either a lysine (F and pKPN3) or arginine (pED208). This residue makes the closest approach to the TraD C-terminal carboxylate in the crystal structure. Residue 102 is a conserved planar aromatic (Tyr or Trp). In the TraD–TraM structure, this residue forms extensive hydrophobic contacts with the TraD β-turn. Thus, while the similarities of TraD and TraM in these plasmids suggest a similar mode of interaction, the differences are significant and may provide a degree of plasmid specificity.

Comparison with the structure of unliganded TraM

Overall, the structure of the TraM tetramer is nearly identical to that observed in free TraM, with an RMSD based on all C_α atoms of 0.65 Å. In particular, the TraD binding pocket is almost unchanged between the free and bound forms, with the exception of the side-chains of Lys76 and Arg110, which rotate in the complex with TraD to interact with the TraD C-terminal carboxylate (Fig. 3). The free TraM tetramerization domain was previously shown to be metastable (Miller and Schildbach, 2003), largely due to the packing of four symmetry-related, protonated glutamic acid side-chains (Glu88) at the centre of the TraM helical

Fig. 2. Detailed analysis of TraD–TraM interactions.
A. TraM and TraD are coloured as in Fig. 1. Hydrogen bonding and electrostatic interactions < 3.5 Å are indicated by dotted bonds.
B. Same view as in (A), with TraM displayed as an electrostatic surface.
C. Sequence alignment of TraM and TraD homologues from F, pKPN3 and pED208. Top: alignment of the amino acid sequences of the TraM C-terminal tetramerization domain from F, pKPN3 and pED208 (GenBank Accession No. BAA97941, YP_001338610 and AAM90702 respectively); secondary structural elements were obtained from the X-ray structure of TraM[58–127]–TraD tail. The *a* and *d* positions of the heptad repeats in the N-terminal helices of TraM forming the central coiled coils are highlighted. Bottom: alignment of the amino acid sequences of the TraD C-terminal tails from F, pKPN3 and pED208 (GenBank Accession No. BAA97972, YP_001338644 and AAM90726 respectively). The underlined sequence is observed in the crystal structure of TraD–TraM complex, and arrows indicate sites of proteolysis.

bundle (Lu *et al.*, 2006). These residues are packed in a similar manner within the TraD–TraM complex, and are within hydrogen bonding distance of one another, likely in a protonated state. As Glu88 and the TraD-binding pocket are at the same end of the TraM tetramer, deprotonation-induced destabilization of the TraM tetramer will certainly affect the neighbouring TraD-binding pocket as previously suggested (Lu *et al.*, 2006)

The C-terminal tail of TraD is necessary for efficient interactions with TraM in solution

We used Ni-NTA pull-down assays to test the importance of the C-terminal eight amino acids of TraD for interactions with TraM in solution (Lu and Frost, 2005; Lu *et al.*, 2006). His$_6$-tagged TraD cytoplasmic domain, either full length (TraD[125–717]) or lacking the C-terminal eight-residue tail (TraD[125–709]), was bound to Ni-NTA agarose and used to

Fig. 3. Comparison of the structure of TraD–TraM complex with the unliganded TraM structure. Unliganded TraM is coloured yellow. In the complex, TraM is coloured blue and TraD is coloured green.

Fig. 4. Pull-down assays to determine TraM interaction with TraD or its C-terminal truncated mutant.
A. Ni-NTA agarose beads pre-incubated with a TraD-free cell lysate or a cell lysate containing His$_6$-tagged TraD$^{125-717}$ or His$_6$-TraD$^{125-709}$ were used to pull down TraM. TraM was visualized by Western blot using anti-TraM antibody (top); and TraD was visualized by Coomassie blue staining of the gel (bottom).
B. Pull-down experiments as in (A) with His$_6$-TraD$^{645-717}$ or His$_6$-TraD$^{645-709}$.

pull down purified full-length TraM. The results show that while TraD$^{125-717}$ interacts with TraM in this assay, interaction with TraD$^{125-709}$ is much weaker when equivalent amounts of protein are used (Fig. 4A). Pull-down experiments also demonstrated an interaction between the His$_6$-tagged crystallization construct (TraD$^{645-717}$) and TraM, while a similar fragment lacking the C-terminal eight-residue tail (TraD$^{645-709}$) did not bind TraM (Fig. 4B). Taken together, these results indicate that the C-terminal region of TraD is sufficient for interactions with TraM in solution, and that the last eight residues of TraD is particularly critical for efficient interaction with TraM.

TraM interactions with the C-terminal tail of TraD is necessary for efficient conjugation *in vivo*

To understand the importance of the C-terminal tail of TraD for conjugation, we tested the ability of TraD mutants to complement conjugation of a plasmid derived from F, pOX38-D411, in which *traD* is knocked out (Table 2). Deletion of the C-terminal 141 amino acids of TraD (TraD^{576*}) resulted in a 10^4-fold decrease in conjugation efficiency, compared with wild-type TraD, consistent with previous results (Sastre *et al.*, 1998). This deletion removes the entire C-terminal tail but leaves intact the ATPase domain. Deletion of just the C-terminal eight amino acids (TraD^{709*}) resulted in a 10^3-fold decrease in conjugation compared with the wild-type control, indicating that this region is critical for the normal function of the C-terminal tail in initiating contact with TraM.

To identify the relative importance of different residues in the TraD tail to conjugation, a subset of the residues within TraD$^{709-717}$ were individually mutated and assessed in the mating assay (Table 2). Mutation of Phe717, which constitutes the hydrophobic core of the TraD portion of the interface (Fig. 2A and B), to alanine yielded the largest effect with a 10^4-fold reduction in mating efficiency. Several negatively charged residues in TraD make long-range electrostatic interactions with positively charged residues in TraM. We mutated two of these residues, Glu712 and Asp715, to Lys to test the importance of these interactions. Only TraD^{D715K} showed a significant loss in conjugation (~10-fold), whereas TraD^{E712K} yielded no significant change in conjugation efficiency. TraD Asp715 stabilizes the TraD β-turn through a hydrogen bond

Table 2. Effect of TraD mutations on F conjugation.

TraD[a]	–	wt	D576*	E709*	E712K	D715K	F717A	*718G
Mating efficiency[b]	$< 5 \times 10^{-7}$	1	5×10^{-5}	2×10^{-3}	4	9×10^{-2}	2×10^{-4}	9×10^{-4}

a. The dash '–' represents vector control. 'wt' represents wild type. The asterisk '*' represents a stop codon. Mutants are named after their codon changes.
b. Determined by assaying donor ability of cells containing pOX38-D411 and pJMTraD or one of its mutant derivatives. Mating efficiency is normalized to that of the wild-type TraD.

Table 3. Effect of TraM mutations on F conjugation and *traM* promoter repression.[a]

TraM or its mutant[b]	–	wt	K76E	R110E	K99E	V106A	N5D
Mating efficiency[c]	$< 1 \times 10^{-6}$	1	1×10^{-5}	3×10^{-5}	$< 1 \times 10^{-6}$	5×10^{-4}	$< 1 \times 10^{-6}$
LacZ activity (MU)	45.0 ± 3.1	7.9 ± 0.6	7.8 ± 0.5	7.7 ± 0.6	8.0 ± 0.3	8.8 ± 0.6	44 ± 2.1

a. The promoter repression ability of TraM or its mutants is represented by the β-galactosidase (LacZ) activity assayed by using equivalent numbers of cells containing pOX38-MK3, pACPM24fs::*lacZ* and pRFM200 or one of its derivatives.
b. Wild-type TraM or its mutants were expressed by pRFM200 or its derivatives respectively. The dash '–' represents vector control. 'wt' represents wild type. Mutants are named after their codon changes.
c. Determined by assaying donor ability of cells containing pOX38-MK3, pACPM24fs::*lacZ* and pRFM200 or one of its mutant derivatives. Mating efficiency is normalized to that of the cells expressing wild-type TraM from pRFM200.

between the carboxylate of Asp715 and the main chain amide of Glu712, which may explain the sensitivity of Asp715 to mutation compared with Glu712. The only TraD carboxylate that makes close contact to positively charged groups in TraM is the C-terminal carboxylate, which contacts both TraM Lys76 and Arg110. To test the importance of this group, we created a mutant with an additional glycine residue appended to the TraD C-terminus (TraD*[718G]). This mutation reduced conjugation efficiency by 10^3-fold, similar to the TraD[F717A] and TraD[709*] mutations. This result demonstrates the importance of the C-terminal carboxylate in TraM–TraD interactions during F conjugation.

TraM[K99E] was initially identified in a screen for random *traM* mutations that greatly decreased conjugation efficiency (Lu *et al.*, 2003). Unlike the other mutants isolated in this screen, the TraM[K99E] mutation did not disrupt tetramerization, DNA binding activity or its ability to repress the *traM* promoter, but specifically abrogated interactions with TraD *in vitro* (Lu and Frost, 2005). To further determine the importance of TraM interactions with the TraD tail during F conjugation, we generated mutations in TraM Lys76 and Arg110, two residues involved in electrostatic interactions with the C-terminal carboxylate. Both TraM K76E and R110E mutants have similar characteristics of the TraM K99E mutant (Table 3). They are highly defective for F conjugation but repressed their native promoter at levels comparable to wild-type TraM, indicating normal tetramerization and DNA binding ability (Lu *et al.*, 2004; Lu and Frost, 2005). TraM Val106 is part of the hydrophobic pocket that accommodates the side-chain of TraD Phe717. A previously obtained TraM mutation at this posi-

tion, V106A, results in a decrease in F conjugation but no defect in promoter repression. As a control, the TraM mutant N5D that affects its DNA binding ability (Lu *et al.*, 2004) is defective for F conjugation and autoregulation at a level comparable to the TraM⁻ vector control. These results further indicate that the TraM–TraD tail interaction is required for F conjugation. Mating efficiencies presented in Table 3 were obtained using a pRFM200 TraM expression system, which gives TraM protein levels similar to those expressed by the native F plasmid (Lu *et al.*, 2003). Therefore, the conjugation levels are lower overall than those presented in Tables 2 and 4, which were obtained using pBAD overexpression system, which gives higher levels of protein and is therefore less sensitive to mutation.

Suppression of the TraM[K99E] conjugation defect by a compensatory mutation in TraD

The structure of the TraM–TraD complex clearly explains why TraM K99E is highly defective for F conjugation. TraM Lys99 lies over the TraD C-terminal tail, hydrogen bonding with the main chain carbonyl of Pro713 of TraD, and making long-range electrostatic interactions with Glu712 and Asp715. Thus the lysine to glutamate substitution at residue 99 would be expected to disrupt this hydrogen bonding interaction and result in electrostatic repulsions with Glu712 and Asp715. We reasoned that TraM[K99E] might be rescued by compensatory mutations in TraD that introduce positively charged side-chains at either residue 712 or 715. To test this hypothesis, we generated an F derivative plasmid, pOX38-DM, which is deficient for both TraM and TraD, and tested the ability of TraM and TraD

Table 4. Compensatory mutations in TraD rescue TraM[K99E].

| TraM[a] | – | – | wt | wt | wt | wt | K99E | K99E | K99E | K99E |
TraD[a]	–	wt	–	wt	E712K	D715K	wt	E712K	D715K	E712K D715K
Mating efficiency[b]	$< 1 \times 10^{-7}$	$< 1 \times 10^{-7}$	$< 1 \times 10^{-7}$	1	4×10^{-1}	5×10^{-3}	3×10^{-5}	1×10^{-3}	7×10^{-5}	2×10^{-4}

a. The dash '–' represents vector control. 'wt' represents wild type. Mutants are named after their codon changes.
b. Determined by assaying donor ability of cells containing pOX38-DM, pJMACTraM or pJMACTraM[K99E], and pJMTraD or one of its mutant derivatives. Mating efficiency is normalized to that of the cells containing wild-type TraD and TraM.

mutants, either alone or in combination, to facilitate the conjugation of pOX38-DM when supplied *in trans* (Table 4). As previously shown (Lu and Frost, 2005), TraM^{K99E} results in a dramatic, 10^5-fold reduction in conjugation efficiency compared with wild-type TraM in cells expressing wild-type TraD. This defect is significantly rescued (~10^2-fold) by a compensatory mutation TraD^{E712K}. As TraD^{E712K} does not significantly alter conjugation when coexpressed with wild-type TraM, this result strongly suggests that electrostatic repulsions between TraM and TraD are responsible for the conjugation defect in TraM^{K99E}/TraD^{wt} cells, and that electrostatic interactions between TraM Lys99 and TraD Glu712 stabilize the interaction of the wild-type proteins. In contrast, TraD^{D715K} alone did not rescue TraM^{K99E}, perhaps because this side-chain also stabilizes the β-turn in the TraD tail.

Discussion

Relaxosome–transferosome interaction, through which DNA is brought to the membrane-bound TFSS for conjugative transfer, is a key step during bacterial conjugation. This interaction provides a potential pathway for the elusive mating signal, originating from donor–recipient contact, to reach the cytosolic relaxosome. In the F plasmid-mediated conjugation system, a relaxosome component, TraM, specifically interacts with the coupling protein TraD of the type IV secretion machinery (Disque-Kochem and Dreiseikelmann, 1997). The K99E mutation that specifically reduces the binding affinity of TraM for TraD also decreases the efficiency of F conjugation (Lu and Frost, 2005), suggesting that the TraD–TraM interaction is important for F conjugation. Previous results have shown that the C-terminal 38 residues of TraD are necessary and sufficient to bind TraM (Beranek *et al.*, 2004) although not as efficiently as intact TraD. In this work, we presented the crystal structure of the C-terminal 10–12 residues of TraD bound to the C-terminal tetramerization domain of TraM (TraM$^{58–127}$), which is the first structural evidence of specific interactions between a relaxosome and its cognate transferosome in a bacterial conjugation system.

In vivo mating assays indicated that deletion of the C-terminal eight-residue tail of TraD resulted in a 500-fold decrease in the mating efficiency of the F plasmid (Table 2), whereas the naturally occurring fertility inhibition system (FinOP) decreases the mating efficiency of the F or F-like plasmids by approximately 100-fold (Lu *et al.*, 2002). Point mutations potentially affecting interactions between TraD and TraM decreased the efficiency of F conjugation (Table 2). Structure-based second-site suppressor experiments showed that a mutation in TraD (E712K) could rescue the TraM^{K99E} conjugation defect by over 30-fold (Table 4). These results demonstrate that the TraD–TraM interaction visualized in our crystal structure is involved in F conjugation. A residual level of mating efficiency was observed in the F conjugation system with a deletion of the very C-terminal eight residues of TraD (Table 2), suggesting that other regions of TraD might also interact with TraM or other components of the relaxosome to achieve a basal level of DNA transfer in the absence of the C-terminal tail.

As TraD exists as a hexameric, membrane-anchored ring, and the TraM tetramer binds to multiple sites on plasmid DNA (Di Laurenzio *et al.*, 1992; Fekete and Frost, 2002; Haft *et al.*, 2007), multivalent contacts between the TraD hexamer and multiple TraM tetramers on DNA likely stabilize this complex *in vivo.* The structure of the TraM–TraD complex indicates that as many as four TraD C-terminal tails can simultaneously bind a single TraM tetramer. However, constraints imposed by the other domains of these proteins, their oligomerization, interactions with DNA, and perhaps other components of the conjugation machinery will undoubtedly influence the stoichiometry and overall architecture of the complex. The lack of stable secondary structure in TraD$^{645–717}$ as determined by circular dichroism spectroscopy (J. Lu and J.N.M. Glover, unpubl. obs.) may provide a degree of flexibility that is important to initiate the formation of this oligomeric protein–DNA complex.

Many TFSS in Gram-negative bacteria have a membrane-bound SpoIIIE/FtsK-like ATPase that recognizes an unstructured C-terminal signal sequence of a type IV secretion substrate to direct substrate translocation. For example, *Agrobacterium tumefaciens* VirF has a C-terminal unstructured tail containing several arginine residues required for translocation (Vergunst *et al.*, 2005). RalF of the *Legionella pneumophila* Dot/Icm system has an unstructured C-terminal signal sequence with a Leu residue at the −3 position critical for translocation (Nagai *et al.*, 2005). A substrate of TFSS-like ESX-1/Snm secretion system in *Mycobacterium tuberculosis*, CFP-10, has an unstructured tail with a C-terminal phenylalanine residue essential for interacting with a SpoIIIE/FtsK-like ATPase Rv3871 during translocation (Renshaw *et al.*, 2005; Champion *et al.*, 2006). Although in the F system it is the TraD ATPase that bears the flexible tail that is recognized by the substrate TraM–DNA complex, it appears that a common feature in TFSS is the recognition of a flexible C-terminal peptide tail for substrate recruitment and translocation.

Altogether, the structural, biochemical and genetic data presented here reveal a novel mechanism of substrate recruitment by the type IV secretion apparatus in F-like conjugation systems. TraD appears to recruit the relaxosome through initial interactions with TraM through the flexible C-terminal tail. Our study provides structural details on how a flexible C-terminal tail from the coupling

protein TraD specifically interacts with the relaxosome component TraM. This knowledge will help to understand how other signal sequences specifically interact with their corresponding coupling proteins for translocation. Moreover, these conserved interactions provide a new target for development of novel agents which could control the spread of multidrug resistance mediated by the conjugation of F-like R-factors.

Experimental procedures

Bacterial growth media and strains

Cells were grown in LB (Luria–Bertani) broth or on LB solid medium containing appropriate antibiotics. Antibiotics were used at the following final concentrations: ampicillin (Amp), 50 μg ml^{-1}; kanamycin (Km), 25 μg ml^{-1}; spectinomycin (Spc), 100 μg ml^{-1}; chloramphenicol (Cm), 50 μg ml^{-1}; streptomycin (Str), 200 μg ml^{-1}; and nalidixic acid (Nal), 20 μg ml^{-1}. The following *Escherichia coli* strains were used: XK1200 [F$^-$ Nal^r Δ*lacU124* Δ(*nadA aroG gal attLbio gyrA*)] (Moore *et al.*, 1987), MC4100 (F$^-$, Sm^r Δ*lacU169*, *araD139*, *rpsL150*, *relA1*, *ptsF*, *rbs*, *flbB5301*) (Sauter *et al.*, 1992), DH5α [Δ*lacU169* (Φ80d*lacZ*Δ*M15*) *supE44 hsdR17 recA1 endA1 gyrA96* (Nal^r) *thi-1 relA1*] (Hanahan, 1983), and BL21(DE3) [F$^-$*dcm ompT hsdS* (r$_B^-$ m$_B^-$) *gal* λ(DE3)] (Stratagene).

Plasmids and plasmid construction

All the plasmids and oligonucleotides used in this work are listed in Table 5. Plasmid pJLTraD$^{645–717}$, which encodes the C-terminal 73 residues (645–717) of TraD, was constructed by ligating the EcoRI–BamHI fragment of pT7-7 to the EcoRI–BamHI fragment of DNA amplified from pOX38-Km using JLU261 and JLU207 as primers. pJMACTraM and pJMACTraM^{K99E} were constructed by ligating the EcoRI–ScaI fragment from pACYC184 to the EcoRI–ScaI fragments from pRFM200 and pJLM105 respectively. pJMTraD^{front} was constructed by ligating the EcoRI–PstI fragment from pNLK5 to the EcoRI–PstI fragment of pBAD24. The 3′ half of TraD was generated by PCR product using the primers LFR120 and JMA66, which was then blunt-end cloned into pTOPO (Invitrogen). The corresponding PstI–HindIII fragment in pTOPO was ligated to pJMTraD^{front} digested with the same enzymes, to generate pJMTraD. The PCR product amplified from pBAD24-TraD using the forward primer JWG01 and the reverse primer JWG02 was blunt-end cloned using a TOPO kit (Invitrogen). The corresponding NdeI–EcoRI fragment was ligated into the NdeI–EcoRI fragment of pT7-7, resulting in pJWTraD$^{125–717}$. pJWTraD$^{125–709}$ was constructed similarly, except using the reverse primer JWG03.

The *traM–traD* double mutant derivative of pOX38-Km was generated by inserting a chloramphenicol (Cm) cassette in the *traM* gene of pOX38-D411. Briefly, the *traM* gene amplified from pRS27 using primers LFR28 and RW168 was cloned into the TOPO vector. The *traM* gene was subsequently disrupted by insertion of a SmaI fragment containing a Cm cassette from pUC4CIXX at the HincII site. The disrupted *traM* gene was PCR-amplified using primers LFR28

and RW168 and was transformed into *E. coli* strain DY330 containing pOX38-D411 for recombination according to the previously described procedure (Yu *et al.*, 2000). DY330 cells containing the double mutant pOX38-DM were selected on media containing chloramphenicol and kanamycin. DNA sequencing verified that pOX38-DM contains a *traM* gene disrupted by a Cm cassette.

Protein purification

TraM$^{58–127}$ was overexpressed and purified as previously described (Lu *et al.*, 2006). TraD$^{645–717}$ was overexpressed from plasmid pJLTraD$^{645–717}$ (Table 5) and was purified similarly except that His$_6$-tag was cleaved off by AcTEV protease (Invitrogen) instead of Trypsin. Full-length TraM was purified as previously described (Lu *et al.*, 2004).

Crystallization and data collection

TraD$^{645–717}$ and TraM$^{58–127}$ were concentrated to 15 mg ml^{-1} in 50 mM Tris, pH 7.5, and were mixed together in a 1.25–1.5:1 ratio. Crystals were obtained using the hanging drop vapour diffusion technique with 1 μl of protein mixture mixed with 1 μl of well solution containing 30% PEG 2000, 100 mM Tris pH 8.5 and 200 mM sodium acetate at 25°C for 2 weeks. Crystals were soaked in 80% (v/v) well solution plus 20% glycerol (v/v) for 20 min prior to flash freezing in liquid nitrogen. A native data set was collected to 2.55 Å from a TraM$^{58–127}$–TraD$^{645–717}$ co-crystal at beamline 8.3.1 at Advanced Light Source, Lawrence Berkeley National Laboratory. Data collection statistics are described in Table 1.

Structure determination

The TraM$^{58–127}$–TraD$^{645–717}$ structure was solved by molecular replacement using the TraM$^{58–127}$ structure (Lu *et al.*, 2006) as the search model in MOLREP (Vagin and Teplyakov, 2000). Amino acids corresponding to the C-terminal residues of TraD were manually built into electron density using Coot (Emsley and Cowtan, 2004). Manual model building and iterative cycles of refinement in REFMAC (Murshudov *et al.*, 1997) with eightfold NCS restraints were used to complete and refine the model (Table 1). 96.3% of residues fall within the most favoured regions of the Ramachandran plot with the remaining in the additionally allowed regions. The molecular structure figures were prepared by using PyMOL (DeLano Scientific, http://www.sourceforge.net). PDB accession number: Atomic coordinates and structure factors (PDB ID: 3S8A) have been deposited in the Protein Data Bank (www.pdb.org).

Structure-based mutagenesis

Similar to the construction of pJMTraD, TraD C-terminal mutations were generated through PCR amplification of the 3′ half of *traD* using primer LFR120 and mutagenesis primers listed in Table 5. These PCR products were ligated to the 5′ half of *traD* in pJMTraD^{front} to form different *traD* mutants.

Site-directed mutagenesis in *traM* by overlap extension was performed as described previously (Lu *et al.*, 2006). The

Table 5. Plasmids and oligonucleotides.[a]

Plasmid and oligos	Description and references
pACYC184	Tc[r]; cloning vector (Chang and Cohen, 1978)
pACPM24fs::*lacZ*	pACYC184 with a P$_{traM}$-*lacZ* fusion for promoter repression assays (Lu *et al.*, 2003)
pBAD24	Amp[r]; cloning vector (Guzman *et al.*, 1995)
pJLM105	pT7-4 with an F *traM* mutant, *K99E* (Lu and Frost, 2005)
pJLTraD$^{645–717}$	pT7-7 with an insertion expressing His$_6$-tagged TraD$^{645–717}$; this work
pJLTraD$^{645–709}$	pT7-7 with an insertion expressing His$_6$-tagged TraD$^{645–709}$; this work
pJMACTraM	pACYC184 with *traM* from pRFM200; this work.
pJMACTraM^{K99E}	pACYC184 with *traM* mutant K99E from pJLM105; this work
pJMTraD	pBAD24-traD^{front} with *traD* from pNLK5; this work
pJMTraD^{D576*}	pBAD24 with a *traD* mutant encoding first 576 residues; this work
pJMTraD^{D715K}	pBAD24 with a *traD* mutant D715K; this work
pJMTraD^{E709*}	pBAD24 with a *traD* mutant missing the last eight residues; this work
pJMTraD^{E712K}	pBAD24 with a *traD* mutant E712K; this work
pJMTraD$^{E712K+D715K}$	pBAD24 with a *traD* double mutant D715K plus D715K; this work
pJMTraD^{F717A}	pBAD24 with a *traD* mutant F717A; this work
pJMTraD^{front}	pBAD24 with the front half of *traD* from pNLK5
pJMTraD^{*718G}	pBAD24 with a *traD* mutant with an extra C-terminal glycine codon; this work
pJWTraD$^{125–717}$	pT7-7 with a partial *traD* encoding residues 125–717; this work
pJWTraD$^{125–709}$	pT7-7 with a partial *traD* encoding residues 125–709; this work
pNLK5	pBAD18 with F *traD* (Lee *et al.*, 1999)
pOX38-D411	Km^{r}Cm^{r}Tra$^+$FinO$^-$TraD$^-$; a *traD*-deficient F derivative (Maneewannakul *et al.*, 1996)
pOX38-DM	Km^{r}Cm^{r}Tra$^+$FinO$^-$TraD$^-$TraM$^-$; this work
pOX38-Km	Km^{r}Cm^{r}Tra$^+$FinO$^-$; an F plasmid derivative (Chandler and Galas, 1983)
pRFM200	pT7-5 with an F BstBI–BglII fragment from *traM* to P$_{finP}$ (Lu *et al.*, 2003)
pRS27	pSC101 with an F fragment from *oriT* to *traV* (Achtman *et al.*, 1978)
pT7-7	Amp[r]; T$_7$ promoter-based expression vector (Tabor and Richardson, 1985)
pUC4CIXX	Derivative of pUC4KIXX (Gibco/BRL) carrying a Cm resistance cassette (Maneewannakul *et al.*, 1992)
JLU261	TAGAATTCACCATCACCATCACCATGAGAACCTGTACTTCCAAGGGATCGA GCAGGAGCTGAAAAT G
JLU207	TGGGGATCCTGAGAATTGAAGACTGGAG
JLU284	GCTTCTTGAATGCGTTGTAGAAACACAATCATCAGTAGCGAAAATTTTGGG
JLU284rev	GTGTTTCTACAACGCATTCAAGAAGCAATTTATTAAACTCAG
JLU285	GGTTGAAGATATCGAGGAGAAGGTATCATCTGAGATGG
JLU285rev	CCTTCTCCTCGATATCTTCAACCATATTGGCATATTCAAAC
JMA66	AAGCTTCAGAAATCATCTCCCGGC
JMA67	CAAGCTTACTCCCCGCGCTCCCGG; for TraD^{D576*}
JMA68	CAAGCTTAGTCACGCGGAATAAACCTC; for TraD^{E709*}
JMA70	AAGCTTCATCCGAAATCATCTCCCGGCTCAAC; for TraD^{*718G}
JMA71	AAGCTTCAGGCATCA TCTCCCGGCTTCAAC; for TraD^{F717A}
JMA72	AAGCTTCAGAAATCT TTTCCCGGCTCAACA TC; for TraD^{D715K}
JMA73	AAGCTTCAGAAATCA TCTCCCGGCTTAACA TCC TC; for TraD^{E712K}
JMA75	AAGCTTCAGAAATCT TTTCCCGGCTTAACATCC TC; for TraD$^{E712K+D715K}$
JWG01	CATATGCATCATCAT CATCATCATGAAAATCTTTATTTTCAAGGTGGATCCATTACCTTCTTTGTTGTCTCCTGG
JWG02	CGGAATTCTTAGAAATCATCTCCGGGCTC AAC
JWG03	GCGAATTCTTACTCCCCGCGCTCCCGGTG
LFR120	CGTATCTGATGCGTAATGACC
LFR28	CGAATTCGTCCCTGTTTGCATTATGA
RW168	TTTCCAGCAGATCTATTTGACGAGCA

a. Mutants are named after their codon changes. The asterisk '*' represents a stop codon.

primer pairs used for introducing point mutations into *traM* are JLU284 and JLU284rev for K76E, and JLU285 and JLU285rev for R110E (Table 5).

Pull-down assays to determine TraD–TraM interaction

Cells pelleted from 75 ml of culture (at ~2.0 OD$_{600}$) expressing His$_6$-TraD$^{125–717}$ or His$_6$-TraD$^{125–709}$ were lysed by sonication in 15 ml of Buffer A (20 mM Tris, 50 mM NaH$_2$PO$_4$ pH 7.5, 8 M Urea, 300 mM NaCl, 10% glycerol, 1 mM DTT) with one Complete Mini EDTA-free protease inhibitor tablet. After centrifugation at 17 000 *g* for 40 min, 500 µl of supernatant was added to 30 µl of Ni-NTA beads and incubated at room temperature on shaker for 2 h. After washing with 300 µl of Buffer A once and 1 ml of Buffer B (same as Buffer A except without urea) three times, beads were incubated in 250 µl of Buffer B with 2 µg of purified TraM and 5 µg of BSA on a shaker at room temperature for 1 h. Beads were washed with 1 ml of Buffer B three times. TraD was eluted from beads with 100 µl of Buffer C (250 mM imidazole in Buffer B). Ten microlitres of eluate was taken for SDS-PAGE gel, and proteins were visualized by Coomassie blue staining or Western blot as previously described (Penfold *et al.*, 1996). Pull-down assays with cells expressing His$_6$-TraD$^{645–717}$ or His$_6$-TraD$^{645–709}$ were performed similarly except that Buffer A did not contain urea.

Mating assays

Donor cells and recipient cells were grown to mid-exponential phase at 37°C. For donor cells containing pBAD-derived plasmids, arabinose was added to 0.05% to donor cells for induction of 1 h prior to mating. Mating assays were performed as previously described (Lu *et al.*, 2002). Mating efficiency is calculated as the number of transconjugants divided by the number of donors. Each assay was repeated three to four times and the averaged values are reported. Standard deviations of all mating assays were within one log unit.

Promoter repression assays

The native promoter repression ability of TraM or its mutants was determined as previously described (Lu *et al.*, 2003) and reported as Miller units (MU).

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