Insights into the regulation of F and ColE1 plasmid transfer

by

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Abstract

Multidrug resistant bacteria pose a threat to human health and economic burden for the healthcare system. Transmission of antibiotic resistance genes can occur by the conjugative transfer of the F plasmid between bacteria. The transferosome forms a channel linking the two cells together, while formation of the relaxosome at the *oriT* nicks and unwinds DNA for transfer. This thesis explores the interaction of the hexameric coupling protein and transferosome component, TraD, with TraM, the DNA binding protein of the relaxosome. Via mating assays, we determined that TraD-TraM interaction does not depend on specific sequences upstream of the terminal 8 residues within the TraD C-terminal domain (CTD). Rather, this region of the TraD CTD functions as a flexible unstructured tether linking the ATPase domain to the terminal 8 residues. These tethers must be more than 44 residues long in order to make multiple simultaneous contacts with TraM and form a stable TraD-TraM complex through avidity effects.

Mobilizable plasmids, such as ColE1 encode the relaxase, but lack genes encoding coupling proteins and components for mating pair formation. For this reason, their transmission relies on the presence of conjugative plasmids to encode the necessary conjugation machinery. Expression constructs were made to further structurally and functionally characterize the ColE1-encoded relaxase, MbeA and accessory protein MbeC, which together with MbeB form the relaxosome. EMSA indicated that MbeC binds double-stranded *oriT* ColE1 DNA in a non-specific manner.

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List of Abbreviations

ATP	adenosine triphosphate
BME	2-mercaptoethanol
bp	base pair
BSA	bovine serum albumin
CTD	C-terminal domain
dsDNA	double stranded DNA
DTT	dithiothreitol
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
EMSA	electrophoretic mobility shift assay
F	Fertility factor
GST	glutathione S-transferase
h	hour
HGT	horizontal gene transfer
IHF	integration host factor
Inc	Incompatibility group
IPTG	isopropyl-β-D-thio-galactoside
IR	inverted repeat
ITC	isothermal titration calorimetry
mg	milligram
min	minutes
mL	milliliter
mob	mobilizable region of ColE1
mM	millimolar
MW	molecular weight
Ni-NTA	nickel nitrilotriacetic acid
oriT	origin of transfer
PCR	polymerase chain reaction
PEG	polyethylene glycol

RHH	ribbon-helix-helix
SAXS	small angle X-ray scattering
SDS-Page	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Sbm	specific binding site of the TraM protein
ssDNA	single stranded DNA
T4SS	type IV secretion system
μL	microlitre
V	volt

Chapter 1
Introduction

1.1 Diversifying the bacterial genome

The diversification and evolution of the bacterial genome is largely a result of mechanisms of horizontal gene transfer (HGT) (Ochman *et al.*, 2000) such as bacterial transformation, transduction, and conjugation. With increasing availability of sequences of bacterial genomes, it has been determined that *Escherichia coli* has acquired 17% to 25% of its DNA through HGT (Lawrence & Ochman, 1998, Lawrence & Ochman, 2002).

Transduction entails the transmission of genetic information via bacteria-infecting viruses, bacteriophages (Narra & Ochman, 2006) introducing viral or foreign bacterial genes. In this process, the bacteriophage must recognize receptors on the bacterial cell, thus limiting the host range (Ochman *et al*., 2000, Narra & Ochman, 2006, Mazodier & Davies, 1991). Transformation is the uptake of naked DNA found in the environment by competent bacteria (Thomas & Nielsen, 2005, Mazodier & Davies, 1991). Following its uptake, DNA is then processed and incorporated into the chromosome via recombination (Thomas & Nielsen, 2005) allowing bacteria to acquire genetic material from distantly related species (Narra & Ochman, 2006).

Bacterial conjugation is the unidirectional transfer of DNA from donor to recipient cells in direct contact (Heinemann & Sprague, 1989). Within Gram-negative bacteria, donor cells extend pili to recipient cells to form mating pairs. A multi-protein transferosome assembles at the donor membrane forming the conjugative pore and linking the cytoplasms of the cells. The relaxosome, a nucleoprotein complex, forms at the *oriT* of the plasmid to be transferred to the recipient. The relaxosome initiates and processes the DNA for conjugative transfer. The transfer of the relaxosome-plasmid complex to the conjugative pore is triggered by an as yet uncharacterized signal; ultimately ssDNA is transported to the recipient cell. Within the transconjugant, ssDNA

recircularizes and a complementary strand is synthesized. Recombination of the transferred DNA into the chromosome can also occur resulting in an Hfr strain and allowing for future conjugative transfer of chromosomal sequences into recipient cells (Jalajajkumari & Manning, 1989, Ochman *et al*., 2000). Bacterial conjugation can also propagate transposons and integrated conjugative elements.

1.1.1 Impact of Bacterial Conjugation

HGT can provide a survival advantage for Gram positive and negative bacteria. Plasmids contain genes that allow bacteria to withstand environmental pressures, the ability to metabolize new substrates, encode virulence factors, and confer resistance against antibiotics (Finley *et al*., 2013). Moreover, conjugation is not a process limited to bacteria. Transmission of broad and narrow host range plasmids from *E.coli* to *Saccharomyces cerevisiae* has been reported (Heinemann & Sprague, 1989). Genetic information transferred from *Agrobacterium tumefacians* to plants is an indication that HGT also occurs between kingdoms (Zupan & Zambryski, 1995).

The conjugative transfer of plasmids conferring resistance to antibiotics has partly contributed to the emergence of multidrug resistant bacteria. The first reported cases of multi-drug resistant bacteria occurred among Shigella in the 1950s due to the transmission of F like plasmids bearing antibiotic resistance genes against sulfonamide, streptomycin, and tetracycline (Watanabe, 1963). Currently, methicillin-resistant *Staphylococcus aureus* (MRSA) is a worldwide public health issue affecting those in the hospital and community (Goetghebeur *et al*., 2006). A predictive study estimated that the health care cost in Canada for 2010 to manage MRSA will amount to $129 million (Goetghebeur *et al*., 2006).

1.2 F Plasmid Conjugation

1.2.1 F Plasmid background

The first account of bacterial conjugation was described in 1946 where it was proposed that "cell fusion" must take place for the transfer of genetic information between bacteria (Lederberg & Tatum, 1946). Bacterial sex was initially attributed to the role of the 100-kb F plasmid (Frost *et al*., 1994). This plasmid was originally identified in *E.coli*; it exists as a single copy plasmid in the family, *Enterobacteriaceae* (Sastre *et al*., 1998), and is a member of the IncFI incompatibility group (Frost *et al.*, 1994). These plasmids have been extensively used as models in the mechanistic study of conjugation. IncFI plasmids are generally large and are found within a narrow host range (Mulec *et al*., 2001). The F plasmid is transferred between *E.coli* and other *Enterobacteriaceae* (Guiney, 1982). The 33.3 kb transfer region, *tra*, is driven by a single promoter , P_Y (Wong *et al.*, 2012) , and contains all the necessary genes involved in conjugative transport of the F plasmid (Frost *et al.*, 1994. Willets and Wilkins, 1984). Plasmids of other incompatibility groups such as R1 and R100 of IncFII and pED208 of IncFV share homology with *tra* of F (Frost *et al.*, 1994). Mutations introduced into F *tra* can be compensated by R100 *tra* genes, indicating a high degree of homology between the two plasmid systems (Otsubo *et al*., 1970. Achtman *et al*., 1972).

1.2.1.1 Cell-cell contact

Bacterial conjugation begins with pilus formation extending from the F plasmid bearing donor cell (F+) to the recipient (F-). Pilus formation occurs under conditions of high glucose (Frost & Manchak, 1998). In Gram-negative bacteria, the (F+) cell assembles an extracellular F pilus via a Type IV Secretion System (T4SS), that spans the cytoplasmic membrane, periplasmic space, and outer membrane of the cell (Thomas & Nielsen, 2005. Cruz *et al*., 2009. Haft *et al*., 2007). The T4SS is a translocation system known to export proteins, effector molecules, and genetic material in bacteria (Christie, 2001).

Sixteen *tra*-encoded proteins are involved in pilus formation (Firth *et al*, 2006). The F pilus has a cylindrical shape with a helical arrangement of subunits, F pilin

(Brinton, 1971, Date *et al*. 1977), forming a diameter of 8 nm and a central channel of 1 nm radius (Marvin & Folkhard, 1986). The specific contact between two cells is termed as the mating pair (Frost *et al.*, 1994), although contact can be made with up to three recipient cells at a time. Retraction of the pilus occurs by depolymerization and brings the two cells into close proximity (Durrenberger *et al*., 1991). Pilus formation in the F plasmid system is similar to the mechanism in the Ti plasmid system of *Agrobacterium tumefaciens* (Frost *et al.*, 1994).

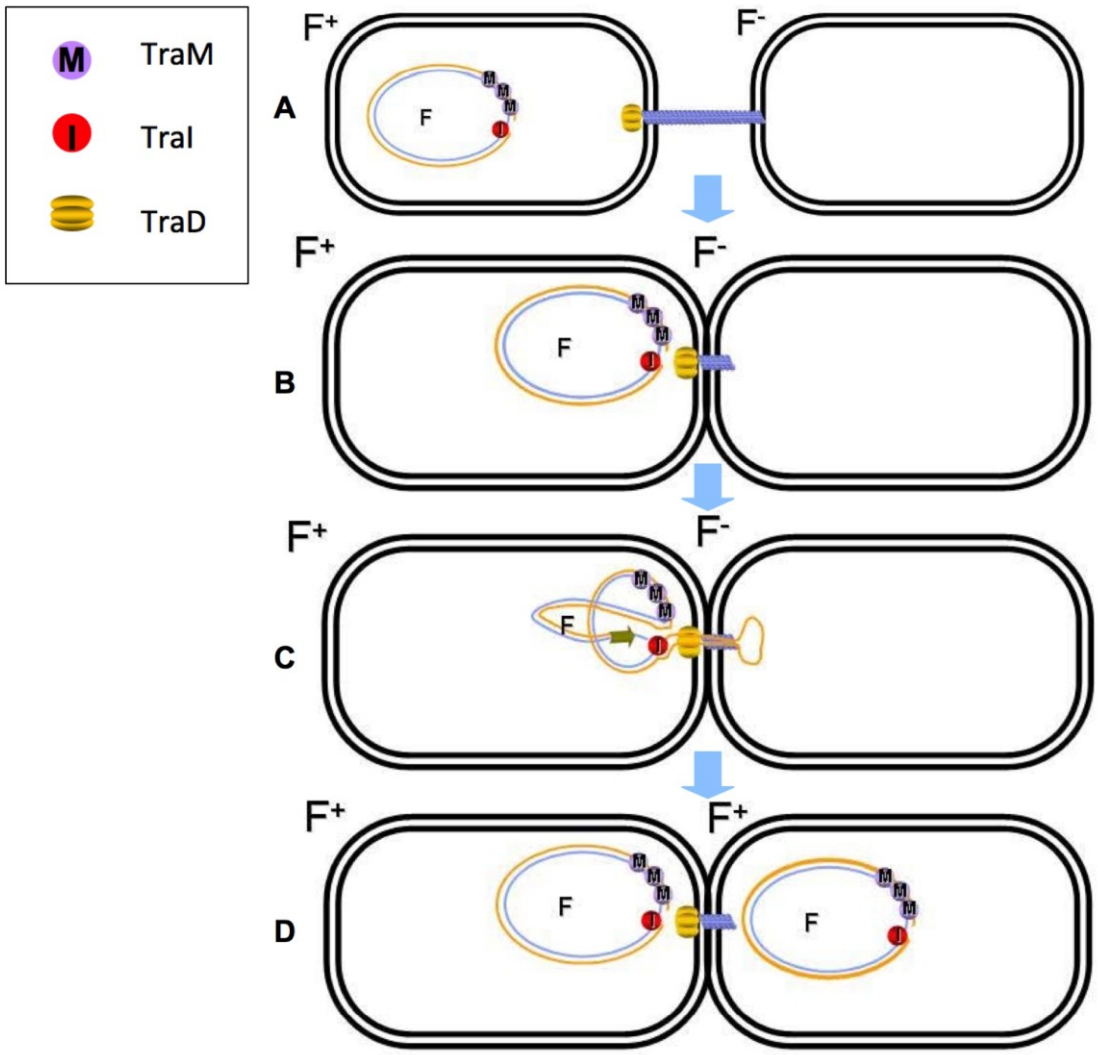


Figure 1.1 Overview of Bacterial Conjugation. A simplified diagram that emphasizes the role of TraD and TraM, that is the focus of the thesis. (A) Mating pair and relaxosome formation (B) Pilus retraction, conjugative pore formation, and recruitment of relaxosome to pore (C) F plasmid nicking and unwinding. ssDNA transferred to recipient (F-) via rolling circle replication (D) Recircularization and synthesis of complementary strand. Roles of these proteins are described in the following sections.

1.2.1.2 Relaxosome formation and DNA processing

The relaxosome is a nucleoprotein complex consisting of *tra*-encoded proteins bound to the *oriT* which initiate nicking and unwinding of DNA for conjugative transfer (Wong *et al.*, 2012). The relaxosome specifically consists of proteins TraI , TraY, TraM, and host encoded protein, IHF.

TraI is a 192 kDa bifunctional protein functioning as a relaxase and helicase within the relaxosome (Howard *et al*., 1995). It is a member of the DNA helicase superfamily (Yoshioka *et al*., 1990). TraI first catalyzes a strand specific phosphodiester bond cleavage at the *nic* site and bonds covalently at the 5' end of the ssDNA via its N-terminal helicase domain (Dostal *et al*., 2011, Lang *et al*., 2010). TraI depends on ATP hydrolysis to unwind DNA in a 5'→3' direction (Lahue & Matson, 1988). The helicase fold of TraI closest to the C-terminal domain may interact with TraM (Ragonese *et al*., 2007).

TraY is a 17,000 MW (Lahue & Matson, 1989) accessory protein found within the relaxaosome complex. It binds near the *nic* site, *sbyA*, and the promoter P_Y (Lahue & Matson, 1989, Nelson *et al*., 1993. Wong *et al*., 2012) inducing a 50° bending of the DNA (Luo *et al*., 1994). TraY directs TraI to *oriT* (Ragonese *et al*., 2007) and enhances the nicking reaction at the *nic* site by TraI (Inamoto *et al*., 1994). TraY may interact with TraM based on the location of its binding sites on DNA (Laurenzio *et al*., 1992).

IHF is a histone-like heterodimer (Drlica & Rouviere-Yaniv, 1987), which binds to two sites within the oriT introducing a 140° turn (Yang & Nash, 1989). IHF has a high affinity for the site near *nic* and a lower affinity to a site found between the TraM binding sites *sbmB* and *sbmC* (Tsai *et al*., 1990). IHF is essential for the nicking reaction (Inamoto *et al.*, 1994). IHF and TraY bind to the *oriT* first and direct TraI binding at the DNA (Howard *et al*., 1995). TraY and IHF bound to *oriT* allow for TraI to be able to recognize the *nic* site more effectively and to catalyze the nicking reaction (Nelson *et al*., 1995).

TraM binds as a tetramer to 3 sites at the *oriT*: *sbmA, sbmB*, and *sbmC* (Laurenzio *et al.*, 1992). TraM binding to *oriT* confers a level of specificity with its cognate plasmid (Wong *et al*., 2011). Upon the formation of a mating bridge, it has been

postulated that TraM plays a role in sending a signal to transfer the plasmid bound relaxosome towards the conjugative pore (Frost *et al.*, 1994).

1.2.1.3 Transferosome formation and DNA transfer

The transferosome is a large transmembrane multiprotein complex at the donor cytoplasmic membrane, mediating a link between the cytoplasms of the mating pair. The hexameric ATPase, TraD, forms the cytoplasmic face of the transferosome (Frost *et al*., 1994). In the presence of an as yet unknown mating signal the relaxosome bound plasmid moves towards and interacts with the conjugative pore. TraD interacts with TraM, coupling the interaction between the transferosome and relaxosome (Beranek *et al*., 2004).

TraI also functions as a relaxase to unwind the duplex DNA following strand nicking. One DNA strand enters the recipient cell in a 5'→ 3' direction with the nick site entering first (Lahue & Matson, 1988). The ssDNA is covalently bound to TraI and is transported through the transferosome with the 5' end leading. Movement of ssDNA from donor to recipient is similar to rolling circle replication (Lanka & Wilkins, 1995). Within the recipient cell TraI re-ligates the nicked DNA, thus terminating bacterial conjugation. This is followed by the synthesis of the complementary strand in the donor and transconjugant. Both cells are now defined as F+ (Frost *et al.*, 1994).

1.2.2 TraD

TraD is a 81.7kDa (Jalajakumari & Manning, 1989) coupling protein of the TraG family (Lessl *et al*., 1992) localized to the cytoplasmic membrane (Panicker & Minkley, 1992, Jalajakumari & Manning, 1989). F TraD is homologous to other coupling proteins encoded by conjugative plasmids of Gram negative bacteria, such as TraD of R100, TraG of RP4 (IncP), TrwB of R388 (IncW) and VirD4 of the Ti plasmid system in *Agrobacterium tumefaciens* (Lessl *et al.*, 1992, Frost *et al.*, 1994, Sastre *et al.*, 1998). Both TraD and TrwB are members of the FtsK/SpoIIIE coupling protein superfamily (Llosa *et al*., 2002). Homology is also found between F TraD and TraK of plasmid pSK41 of staphylococci (Firth *et al*., 1993).

Coupling proteins typically contain two N-terminal transmembrane spanning regions and a cytoplasmic C-terminal region (Figure 1.2) (Llosa *et al.*, 2002, Lee *et al.*, 1999). Conserved Walker motifs in the cytoplasmic domain suggest that ATP hydrolysis is required for the transfer of DNA through the T4SS (Llosa *et al.*, 2002). These nucleotide binding sites are found at residues 192-199 and 421-426 in F TraD (Frost *et al.*, 1994).

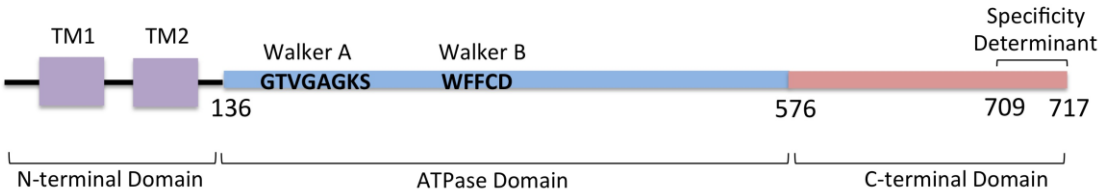


Figure 1.2 Schematic of F TraD. Adapted from Wong *et al.* (2012) Relaxosome function and conjugation regulation in F-like plasmids – a structural biology perspective. *Mol Microbiol*. 85, 602-617.

It is proposed that TraD and related coupling proteins, TraG and TrwB, also bind ssDNA during their transport from donor to recipient (Llosa *et al.*, 2002, Schroder & Lanka, 2003). F TraD binds non-specifically to DNA on a cellulose matrix (Panicker & Minkley, 1992), having a higher affinity for ssDNA than for dsDNA (Schroder *et al.*, 2002). Likewise, the soluble domain of TrwB non-specifically binds ssDNA and dsDNA (Moncalian *et al*., 1999). Similarly, TraG binds DNA non-specifically (Schroder *et al.*, 2002).

C-terminal cytoplasmic fragments of R388 TrwB form a hexameric structure with a central channel as shown by X-ray crystallography (Gomis-Ruth *et al*., 2001). TrwB resembles the ring structure of F0/F1-ATPase (Gomis-Ruth *et al.*, 2001), but fails to exhibit ATPase activity *in vitro* (Schroder & Lanka, 2003). The TrwB structure can

provide insights into the roles that coupling proteins play during conjugation. It is important to note that TraD has a flexible unstructured C-terminal tail (645-717) following the conserved ATPase domain (Lu *et al*., 2008), that is not present in TrwB (Sastre *et al.*, 1998).

Crosslinking and genetic studies show that TraD forms a homodimer in the membrane via residues in the transmembrane and cytoplasmic domains. Interestingly, it is suggested that the presence of F plasmid encoded proteins facilitate the oligomerization of TraD into higher order complexes (Haft *et al*., 2007). Likewise, the transmembrane regions of TraG or TrwB are responsible for their *in vitro* oligomerization (Schroder & Lanka, 2003). Oligomerization of coupling proteins is required for the translocation of protein TraI or plasmid R1 (Lang *et al*., 2014).

The crystal structure of TrwB suggests that F TraD also forms hexameric ring that forms the entrance of the conjugative pore at the cytoplasmic membrane (Wong *et al.*, 2012). Genetic studies have shown that TraD mutants do not permit the transmission of DNA from the donor to recipient, but allow for mating pair formation (Lu & Frost, 2005), interaction with the relaxosome (Jalajakumari & Manning, 1989), and DNA processing (Firth *et al*, 2006). Moreover, TraD does not play a role in nicking (Everett & Willetts, 1980) or unwinding of DNA (Kingsman & Willetts, 1978).

1.2.3 TraM

TraM is a 14.5 kDa cytoplasmic protein (Laurenzio *et al.*, 1992) that forms homotetramers (Verdino *et al*., 1999, Miller & Shildbach, 2003, Lu & Frost, 2005) and binds to the *oriT* of the F plasmid, thereby mediating its interaction with the conjugative pore. DNase I footprinting experiments reveal that F TraM binds at three specific sites, *sbmA*, *sbmB*, and *sbmC*, that span a 200 bp segment within the F *oriT* (Laurenzio *et al.*, 1992, Fekete & Frost, 2002). F TraM has preferential binding for one strand within a DNA duplex (Laurenzio *et al.*, 1992). Moreover, *sbmA* is the high affinity binding site (Wong *et al*., 2011), while *sbmC* exhibits low binding affinity for F TraM (Laurenzio *et al.*, 1992).

Two F TraM tetramers cooperatively bind the cognate *sbmA* duplex DNA as indicated by multiangle laser light scattering (MALLS) and EMSA (Wong *et al*., 2011).

EMSA further reveals that an 8 molar equivalent of pED208 TraM is essential to a form a stable complex with its *sbmA* DNA (Wong *et al*., 2011). Tetramerization of TraM occurs at the C-terminal domain and is required for high affinity binding to DNA (Wong *et al*., 2011) as isolated N-terminal domains of pED208 TraM bind its *sbmA* DNA with 500 fold reduced affinity.

The crystal structure of pED208 TraM bound to its cognate *sbmA* DNA shows that the N-terminal RHH (ribbon helix-helix) of each tetramer dimerizes to form antiparallel β-sheets, which make specific contacts in the major groove. Moreover, two pED208 TraM tetramers bind GANTC nucleotide motifs of *sbmA* in a staggered arrangement. A single tetramer binds on one side of the DNA unwinding the DNA to align the other GANTC for binding to the second tetramer. The complex is formed by coordinated binding of TraM tetramers at the DNA without any contacts between tetramers (Wong *et al*., 2011) (Figure 1.3).

The RHH motif at the N-terminal domain confers allelic specificity as shown by significantly reduced mating efficiency of TraM-deficient F plasmid derivative by R100 TraM (Wong *el al*., 2011). TraM makes specific contacts with the GANTC motifs of pED208 via its RHH motif. F *oriT* has consensus sequences that are 6 bp long and are not as conserved as pED208 TraD. This is supported by experiments showing TraM having highest binding specificity to its cognate plasmid (Harley & Schildbach, 2003).

Other functions of TraM during conjugation entail the sensing of mating pair formation and signaling for the transfer of the plasmid bound relaxosome to the transferosome (Frost *et al.*, 1994). TraM also enhances the nicking reaction, but is not essential for nicking or conjugative pore formation (Howard *et al*., 1995).

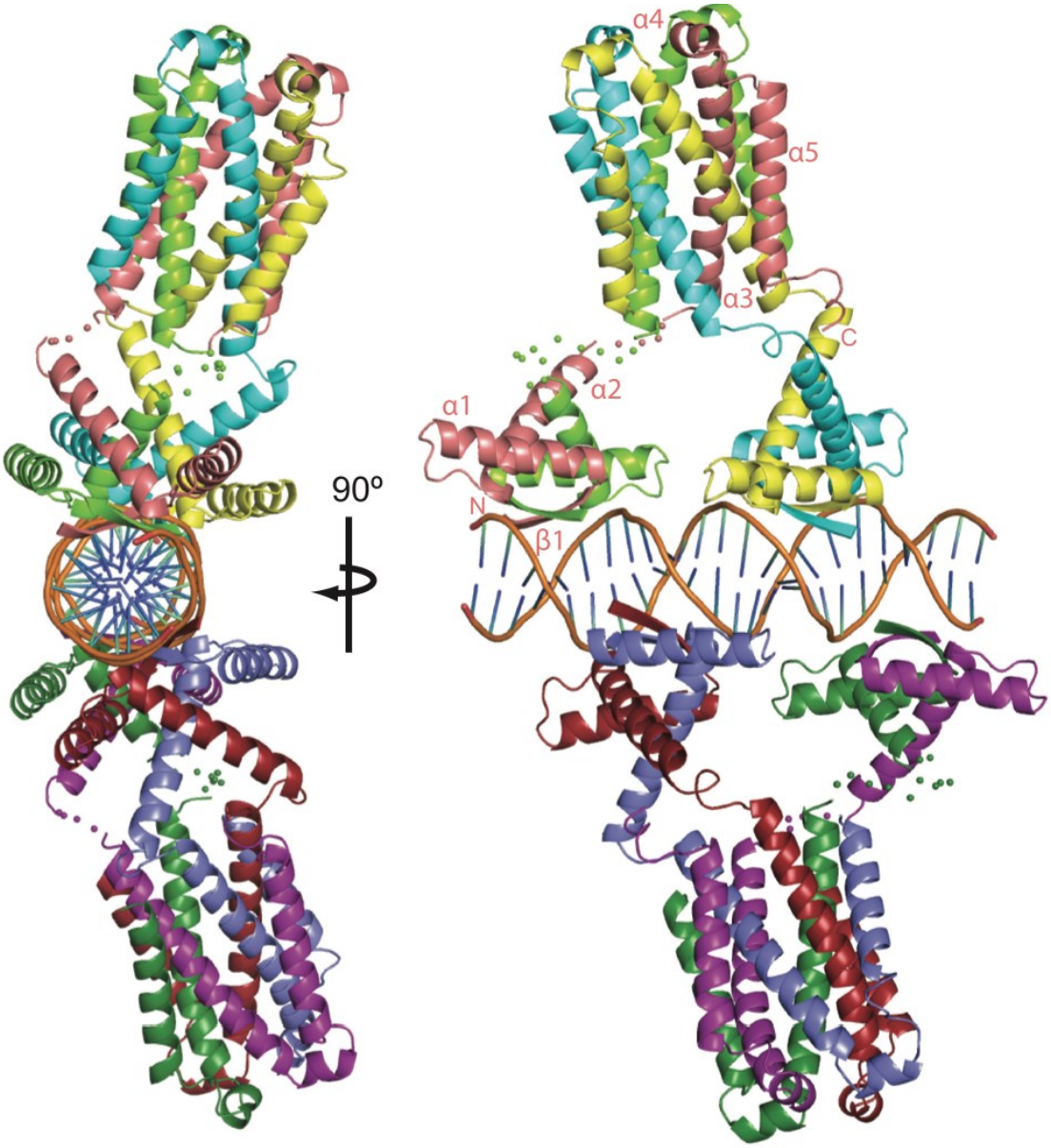


Figure 1.3 Crystal structure of pED208 TraM tetramers bound to *sbmA* DNA.
Adapted from Wong *et al.*, (2011) *Nucleic Acids Research 39*, 6775-6788.

1.2.4 Role of TraD-TraM interactions in conjugation

Although TraM has been characterized as a cytoplasmic protein, traces have been found in the inner membrane suggesting potential interactions with integral membrane proteins (Laurenzio *et al.*, 1992). Moreover it has been postulated that the binding of TraM to its low affinity site, *sbmC*, in the *oriT* signals for transferring of the plasmid bound relaxosome to the conjugative pore (Laurenzio *et al.*, 1992).

The first *in vitro* TraD-TraM interactions were reported by Disque-Kochem and Dreiseikelmann in 1997 via overlay assays and affinity chromatography (binding of TraD to TraM immobilized on a column, and the coelution of the two proteins) suggesting relaxosome interactions at the inner membrane (Disque-Kochem & Dreiseikelmann, 1997). Similarly, studies of other coupling proteins indicate their *in vitro* interaction with relaxosome components. TraG coupling protein of IncP plasmid RP4 binds TraI while the R388 coupling protein interacts with TrwA (Beranek *et al*., 2004).

EMSA studies revealed the interaction of the R1 TraD cytoplasmic region with TraM bound to its cognate *oriT*. Overlay assays and ELISA further identified that the 38 C-terminal residues of R1 TraD are responsible for interaction with full length R1 TraM. In addition, mating assays validated the *in vivo* interaction of TraD and TraM within the R1 plasmid system (Beranek *et al*., 2004).

Similar studies have highlighted the importance of the C-terminal region of TraD in interaction with the relaxosome. Truncation of the terminal 140 residues in F TraD reduces conjugative transfer of the F plasmid. However, this F TraD variant allows for more efficient conjugative transfer (1000 fold higher) of R388 plasmid than full length F TraD. A truncation of the 37 terminal residues of F TraD produces the same mating efficiencies of both F and R388 plasmid. These data suggest that the full length TraD C-terminal tail hinders interaction with relaxosomes of the non-cognate plasmid. This also implies that the C terminal tail-relaxosome interaction is important for plasmid specificity. The authors further suggested that the F TraD C-terminal tail, which is not present in other coupling proteins, such as TrwB, confers specificity for interaction with the F system relaxosome in particular (Sastre *et al.*, 1998).

Additional studies have elucidated important residues in the C-terminal tetramerization domain of F TraM, which interact with the CTD of F TraD.

A mutation in the C-terminal domain of TraM, K99E, reduces the affinity for TraD whereas the truncation of the TraM C-terminal residues (79-127) abolishes the binding to TraD as indicated by affinity chromatography and co-immunoprecipitation (Lu & Frost, 2005).

More recently, the crystal structure of a F TraD peptide (10-12 residues) bound to a hydrophobic pocket in the F TraM tetramerization domain has been solved. Up to four peptides bind to a single TraM tetramer. The Phe of the peptide interacts in a hydrophobic pocket while the C-terminal carboxylate makes electrostatic interactions with positively charged residues, Arg110 and Lys76 in TraM. Truncation of TraD CTD following the ATPase domain reduces mating efficiency by 10^{-5} fold. Truncation of the last 8 residues in TraD significantly reduces interaction with TraM as indicated by pull-down assays. This is further confirmed by the reduction in mating efficiency by 10^{-3} fold when the last 8 residues of TraD are absent (Lu *et al*., 2008). Subsequent studies entailed interchanging components from different plasmid systems to show specificity of interactions. The terminal 8 residues of TraD confer plasmid specificity as indicated by use of chimeric TraM and TraD in mating assays (Wong *et al*., 2011) (Figure1.4).

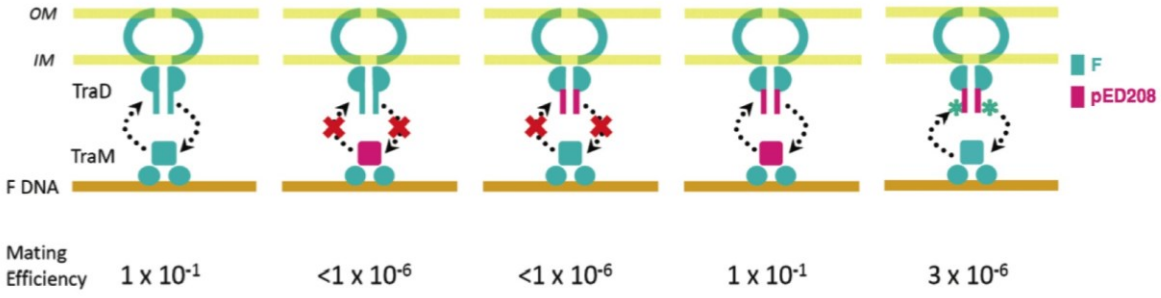


Figure 1.4 Terminal 8 residues of F TraD determine plasmid specificity. Mating assay was carried out using chimeric TraD and TraM from F and F-like plasmid, pED208. Adapted from Wong *et al.*, (2011) *Nucleic Acids Research* 39, 6775-6788.

1.3 Conjugation of mobilizable plasmid ColE1

1.3.1 Mobilizable Plasmids

Bacterial plasmids can be categorized according to their ability to independently mobilize during conjugation. Mobilizable plasmids contain genes encoding the relaxase, but lack those encoding coupling proteins and components for mating pair formation (Llosa *et al.*, 2002). For this reason, their transmission to recipient cells depends on the presence of conjugative/self-transmissible plasmids to encode the necessary conjugation machinery. The lack of genes present in mobilizable plasmids explains their small size (<15 kb) and high copy numbers. In contrast, conjugative plasmids are found in lower copy numbers but are larger, greater than 30 kb (Garcillian-Barcia *et al*., 2009).

Of the many mobilizable plasmids such as RSF1010, pMV158, and CloDF13 (Francia *et al.*, 2004), ColE1 serves as a model. ColE1 is 6650 bp long, found in both Gram positive and negative bacteria, (Francia *et al*., 2004) and is a member of the ColE1 superfamily (Garcillian-Barcia *et al*., 2009). *E.coli* containing this plasmid have immunity against the toxic channel-forming protein, colicin E1, that is encoded on ColE1 (Clewell & Helinski, 1969, Bazaral & Helinski, 1968). This plasmid confers a survival advantage in the environment as the toxic colicin can kill nearby bacteria. Within a laboratory setting, this plasmid has been extensively used in vector technology (Francia *et al*., 2004).

ColE1 can be mobilized to a broad host range. It is effectively mobilized by F and R conjugative plasmids (Veltkamp & Stuitje, 1981), as well as conjugative plasmids found in groups IncF, IncI, IncP, and IncW (Warren *et al*., 1979, Laurenzio *et al.*, 1992). Non-mobilizable plasmids containing the *mob* (mobilizable) region were efficiently mobilized by the groups IncFI and IncIα, indicating the necessity of this region for conjugation (Warren *et al*., 1979).

The *mob* region encodes relaxosome proteins, which bind the ColE1 *oriT*. The mobilization of ColE1 does not rely on the presence of TraM, TraD, and TraI of the F plasmid (Laurenzio *et al.*, 1992, Sastre *et al*., 1998). Studies have shown that weak interactions occur between F-TraM and oriT of the ColE1 plasmid (Laurenzio *et al.*, 1992).

1.3.2 ColE1 background

The study of the Colicinogenic factor E1 (ColE1) plasmid began with its isolation from *E.coli* as a supercoiled circular DNA molecule with a MW of $4.2x10^6$ (Bazaral & Helinksi, 1968). Later studies reported the isolated complex to be composed of three proteins at MW of 11,000, 16,000, and 60,000 tightly bound to DNA (Clewell & Helinski, 1969, Lovett & Helinski, 1975). This complex can form in the absence of a conjugative plasmid (Clewell & Helinski, 1969). Induction of the complex from a supercoiled to an "open circular" state or "relaxation complex" (Clewell & Helinksi, 1969, Blair & Helinski, 1975) by SDS showed only the 60,000 MW protein covalently bound to the 5' end of the single nicked strand (Guiney & Helinski, 1975, Blair & Helinski, 1975). This ssDNA-protein complex is translocated with the 5' end leading into the recipient cell, similar to the transmission of conjugative plasmids (Warren *et al*., 1978). It has been previously hypothesized that this plasmid is transferred via passive diffusion or recombination into the conjugative plasmid. Rather, ColE1 moves independently in an active manner utilizing the machinery encoded by the conjugative plasmid (Warren *et al*., 1978).

1.3.3 Gene Products of the *mob* region

There are 5 genes, *mbeA, mbeB, mbeC, mbeD, mbeE*, within the mobilizable (*mob*) region (Francia *et al*., 2004). The mob region contains overlapping ORFs such that both *mbeB* and *mbeD* overlap *mbeA* (Varsaki *et al*., 2009) (Figure 1.5). The structures and functional properties of the mob proteins have not been well characterized. MbeE is not essential for ColE1 mobilization (Boyd *et al.*, 1989). MbeD is proposed as an entry exclusion protein localized to the inner membrane via an N-terminal alpha helix (Yamada *et al*., 1995). It has been shown that MbeA, MbeB, and MbeC are components of the relaxosome, which bind and process DNA at the *oriT*. MbeA, MbeB, MbeC, and MbeD are necessary for the mobilization of the ColE1 plasmid.

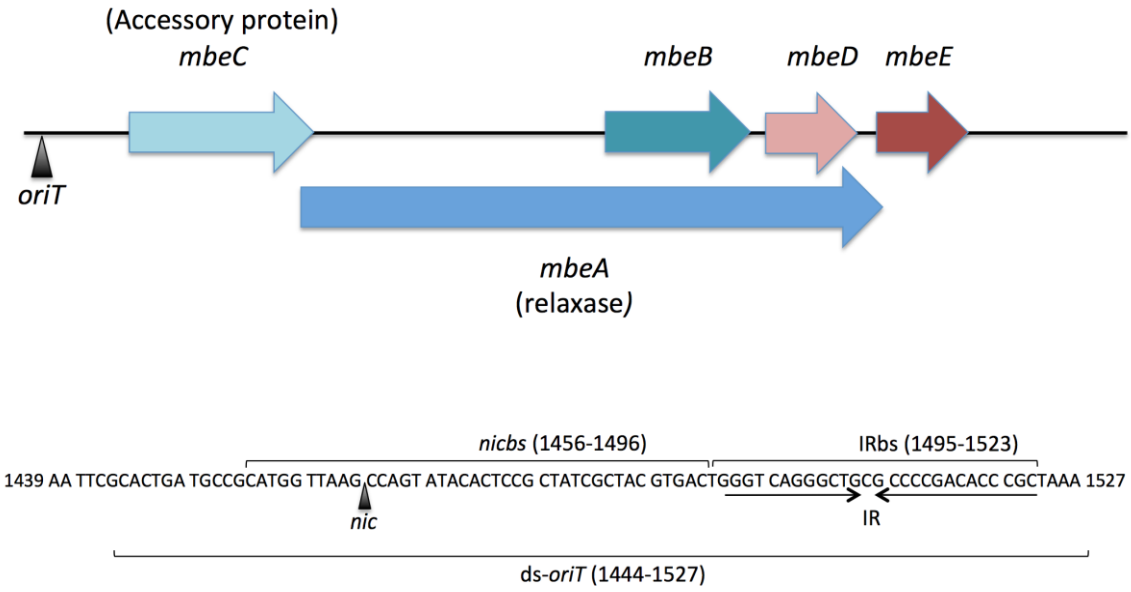


Figure 1.5 Mobilization region (*mob*) and *oriT* of ColE1. Adapted from Varsaki *et al.*, (2009) MbeC unveils an extended ribbon-helix-helix family of nicking accessory proteins. *Journal of Bacteriology* 191, 1446-1455.

1.3.3.1 MbeC

MbeC is 12.9 kDa protein (Lovett & Helinski, 1975) found within the relaxosome. It is characterized as an accessory protein that is functionally similar to F TraY and R388 TrwA. (Varsaki *et al*., 2009). TraY binds dsDNA via its RHH motifs and TrwA is a member of the RHH family (Schreiter & Drennan, 2007).

Limited trypsin digestion of MbeC reveals 2 stable domains. An RHH motif is present in the 5.5 kDa N-terminal fragment. β-sheets of RHH motifs contain alternating hydrophobic and hydrophilic residues known to bind DNA in the major groove (Schreiter & Drennan, 2007). Mutation of arginine within the β-sheet abolishes MbeC binding to dsDNA as indicated by EMSA and significantly reduces mating efficiency (Varsaki *et al*., 2009). MbeC purifies as a dimer, which is typical of proteins containing RHH motifs as they dimerize at this region (Varsaki *et al*., 2009). The C-terminal 9.4 kDa fragment contains 2 tandem alpha helices, and highly conserved NLNQ and HEN motifs (Varsaki

et al., 2009). MbeC can also be classified as a member of the MOB_{HEN} family of accessory proteins (Varsaki *et al*., 2009).

Mating assays have indicated that the presence of *oriT* (Figure 1.5) is required for transfer of ColE1. Truncation of this region to 41 bp (*nicbs*) reduces mating efficiency by 100 fold. EMSA corroborates the finding that MbeC does not bind the inverted repeat (IR). In addition, MbeC does not bind ss *oriT* DNA (Varsaki *et al*., 2009). In vitro binding assays suggest that MbeC binds ds*oriT* in a specific manner in the presence of nonspecific or specific competitors (Varsaki *et al*., 2009).

1.3.3.2 MbeA

MbeA is a 57 kDa protein and the relaxase of the ColE1 relaxosome. Relaxases in the ColE1 superfamily contain 3 conserved motifs. Motif I contains Ser, while Motif II bears the acidic residues Glu or Asp. Motif III contains either a HHH or HEN motif. MbeA is a member of the MOB_{HEN} clade within the MOBp plasmid family. (Garcillian-Barcia *et al.*, 2009). Motifs I and III form the catalytic centre for the relaxase function of MbeA (Varsaki *et al*., 2003).

In addition, MbeC interacts with MbeA in a 1:1 ratio as shown by isothermal titration calorimetry (ITC). MbeC enhances the affinity of MbeA for *nic* containing ssDNA (Varsaki *et al*., 2012). Bacteria two-hybrid experiments elucidated that the C-terminal region of MbeC interacts with the N-terminal portion of MbeA (Varsaki *et al*., 2012). It is predicted that the HEN motif in the C-terminal region of MbeA binds ssDNA, while the N terminal fragment makes contact with MbeC, possibly at the NLNQ motif (Varsaki *et al*., 2012).

1.4 Thesis Objectives

This thesis investigates the functional interactions of proteins involved in bacterial conjugation. Within the F plasmid system, TraD-TraM interaction is essential for the recruitment of conjugative plasmids to the transferosome. Chapter 3 defines the role of additional regions in the TraD C-terminal domain (CTD) with respect to TraM interaction and conjugation. In addition, TraD regions that are non-essential for conjugation can be deleted in order to overexpress a construct with reduced flexibility that is thus more amenable to crystallization. This may be a useful approach in the structural analysis of TraD as there are no crystal structures currently available. Chapter 4 details the structural and functional analysis of newly characterized mob ColE1 proteins. Structural studies of MbeC were pursued through crystallization. MbeC-DNA interaction and identification of a minimal DNA binding site were investigated using EMSA. The crystallization of a protein can be induced in the presence of a binding partner or ligand, such as DNA. Attempts were made to co-express MbeA and MbeC in a complex, as well as to purify MbeB.

Chapter 2

Materials and Methods

2.1 Materials

Kits

QIAprep Spin Miniprep Kit (Qiagen)

QIAquick PCR purification Kit (Qiagen)

QIAquick Nucleotide Removal Kit (Qiagen)

Miniprep Plasmid Purification Kit (Truin Science)

BCA Protein Assay (Thermo Scientific)

Enzymes

Restriction enzymes BamH1, EcoR1, PstI, HindIII (Invitrogen)

Platinum Pfx DNA Polymerase (Invitrogen)

AcTEV protease (Invitrogen)

Halt Protease Inhibitor Cocktail (Thermo Scientific)

Medium

Luria-Bertani (LB) broth Miller (Fisher Scientific)

Difco LB Agar (Fischer Scientific)

Antibiotics

Ampicillin Sodium Salt (Invitrogen)

Kanamycin Sulphate (Invitrogen)

Spectinomycin (Sigma)

Chloramphenicol (Fischer Scientific)

Crystallography Suites

Nextal Classics I and II (Qiagen)

Wizard I and II (Emerald Biosystems)

Table 1. Solutions

Lysis Buffer	50 mM Phosphate, 300 mM NaCl, 10 mM imidazole, pH 7.0
Wash Buffer	50 mM Phosphate, 300 mM NaCl, 20 mM imidazole, pH 7.0
Elution Buffers	50 mM Phosphate, 300 mM NaCl, 50, 100, 150, 200, 300, or 500 mM imidazole, pH 7.2
Buffer A	50 mM Phosphate (0.5 ml of 1M DTT per 1 L), pH 7.2
Buffer B	50 mM Phosphate, 1 M NaCl (0.5 ml of 1M DTT per 1 L), pH 7.2
Buffer C	50 mM Phosphate, 300 mM NaCl (0.5 ml of 1M DTT per 1 L), pH 7.2
Storage Buffer	0.5 M Ammonium acetate
SSC Buffer	0.15 M NaCl, 0.015 M Sodium citrate, pH 7.0
5X TBE Buffer	450 mM Tris-borate, 10 mM EDTA, pH 8.0
Annealing Buffer	10 mM Tris, 50 mM NaCl, pH 7.5
EMSA Binding Buffer	20 mM Tris-HCl, 50 mM NaCl, 1 nM BSA, pH 7.5

Table 2. Cloning Primers for ColE1 *mob* region

$mbeA_{FWD}$	GGCGGCATAGGATCCATGATAGTTAAATTTCATGCCAGGGGAAAAGGTGGT
$mbeA_{REV}$	GGCGGCATAGAATTCTCACCATCCCAGCGAAGGGCC
$mbeB_{FWD}$	GGCGGCATAGGATCCATGAGCAATCTTTTGCAGACGGGCGCGGG
$mbeB_{REV}$	GGCGGCATAGAATTCTCACTCCTTCAGATGTACCCACTCTTT
$mbeC_{FWD}$	GGCGGCATAGGATCCGAGAACCTTTACTTCCAGGGCATGATACCGATGAAACGCGAGAGG
$mbeC_{RV}$	GGCGGCGAATTCTTAACTATCATCCCGCGCCCCCTG

Table 3. Annealed DNA substrates for EMSA

$nicbs_{FWD}$	CATGGTTAAGCCAGTATACACTCCGCTATCGCTACGTGAC
$nicbs_{REV}$	GTCACGTAGCGATAGCGGAGTGTATACTGGCTTAACCATG

Table 4. Site directed mutagenesis primers

F $TraD_{578\text{-}598FWD}$	CATGGCCAGCCTCTTCGAACC
F $TraD_{578\text{-}598\ REV}$	GAAGAGGCTGGCCATGATGTCACGCGGAATAAACTCCG
F $TraD_{599\text{-}619FWD}$	GAACAGCCGCAACAGCCG
F $TraD_{599\text{-}619REV}$	GTTGCGGCTGTTCCTGACGACCTTCTGCTTCCC
F $TraD_{620\text{-}640FWD}$	CTGCCACCCGGGATCAG
F $TraD_{620\text{-}640REV}$	CCCCTGCCGGAACAGCCTGAGTCACGTCTTCTCC
F $TraD_{641\text{-}661FWD}$	CTGCCACCCGGGATCAG
F $TraD_{641\text{-}661REV}$	CCCGGGTGGCAGATTCACACCTGAATCTGACTTCTTATCG TTG
F $TraD_{662\text{-}682FWD}$	CAGGAAAATCATCCGGACATCCAG
F $TraD_{662\text{-}682REV}$	GGATGATTTTCCTGTTGCTGTTCCATCTCTTCTTCC GG
F $TraD_{683\text{-}703FWD}$	CACCGGGAGCGCGGG
F $TraD_{683\text{-}703REV}$	GCGCTCCCGGTGTTGCCATGCCTCATAAGCGGC
$TraD_{632\text{-}653FWD}$	GAAGAAGAGATGGAACAGCAACTGC
$TraD_{632\text{-}653REV}$	GCTGTTCCATCTCTTCTTCATCGTTGATGGCAGGAGACAC
$TraD_{621\text{-}664FWD}$	ATCAGTGAATCCGGTGAAGTGGT G
$TraD_{621\text{-}664REV}$	CCACCACTTCACCGGATTCACTGATCTGTTCAGCCTGAGT CACGTC
$TraD_{610\text{-}675FWD}$	GCTTATGAGGCATGGCAACAGG
$TraD_{610\text{-}675REV}$	GCCATGCCTCATAAGCAACCTCCGGGACATCCGG
$TraD_{599\text{-}686FWD}$	GACATCCAGCAGCAGATGCAG
$TraD_{599\text{-}686REV}$	CTGCTGCTGGATGTCCATCTGACGACCTTCTGCTTCCCTTG C GGCAAG
$TraD_{588\text{-}697REV}$	GCCGCCAAGCTTTCAGAAATCATCTCCCGGCTCAACATCC TCCCCGCGCTCCCGGTGCACATTAATGTTCACTACGGCAC TCAGACGG
$TraD_{577\text{-}709REV}$	GCCGCCAAGCTTTCAGAAATCATCTCCCGGCTCAACATCG AT GTCACGCGGAATAAACTC
$JLU262_{FWD}$	GCCATCCGTTACCTGCAGG
$JLU269_{REV}$	ATATATAAGCTTCAGAAATCATCTCCCGGCTCAAC

2.2 Methods

2.2.1 Cloning

Cloning and overexpression of MbeC was performed by Jun Lu. *mbeC* was cloned into pt7-7 vector to be expressed as a His_6-tagged fusion protein. This construct was purified and used for crystallization trials and EMSA.

Mob genes, *mbeA*, *mbeB*, and *mbeC* were PCR amplified using primers indicated in Table 2. Reactions were catalyzed by Pfx using ColE1 plasmid as a template. *mbeC* was also cloned into pACYC Duet-1 (Novagen) vector to be expressed as a His_6-tagged protein. *mbeB* and *mbeA* were cloned into vector pGEX6P-1(GE Healthcare) to be expressed as GST-fusion proteins. All proteins were cloned using restriction enzymes BamH1 and EcoR1. Vectors containing *mbeA*, *mbeB*, and *mbeC* were propagated using DH5α *Escherichia coli* cells (Invitrogen). Subsequently, vectors containing *mbeC* and *mbeA* were co-transformed into BL-21 Gold *E. coli* cells for overexpression. Co-transformation efficiency was low and only a couple colonies were obtained as three different selection antibiotics were used. Sequencing of constructs indicated that random mutations had occurred in *mbeA*, thus overexpression of MbeA and MbeC was not further pursued.

pGEX6P-1 containing *mbeB* was transformed into BL-21 Gold Cells for overexpression.

2.2.2 Site Directed Mutagenesis

Site directed mutagenesis was used to introduce internal deletions into the CTD of F TraD. Two rounds of PCR were performed using the primers noted in Table 4.

The polymerase chain reaction to generate the DNA fragment upstream of the deletion region consisted of primer $JLU262_{FWD}$ combined with reverse primers $F\ TraD_{578\text{-}598REV}$, $F\ TraD_{599\text{-}619REV}$, $F\ TraD_{620\text{-}640REV}$, $F\ TraD_{641\text{-}661REV}$, $F\ TraD_{662\text{-}682REV}$ or $F\ TraD_{683\text{-}703REV}$. To generate a DNA fragment with an overhang down stream of the deleted region, primer $JLU269_{REV}$ was combined with $F\ TraD_{578\text{-}598FWD}$, $F\ TraD_{599\text{-}619FWD}$, $F\ TraD_{620\text{-}640FWD}$, $F\ TraD_{641\text{-}661FWD}$, $F\ TraD_{662\text{-}682FWD}$, or $F\ TraD_{683\text{-}703FWD}$. The appropriate pair of PCR

products obtained from these two reactions were then combined in the subsequent round of PCR where they annealed to form the template with an internal deletion. TraD mutants with 22, 44, 66, 88, 110, and 132 residue deletions were generated in a similar fashion. All proteins were cloned using restriction enzymes PstI and HindIII and cloned into vector pBAD24 (Amp^r).

2.2.3 Expression and purification

2.2.3.1 GST-MbeB Expression

A 200 mL starter culture of GST-MbeB was grown at 37°C to an A600 of 0.6 and tested for overexpression in various conditions. LB media was supplemented with 100mg/L of ampicillin as BL21-DE3 clones are ampicillin resistant. Increasing concentrations (0.2 mM, 0.5mM and 1mM) of IPTG were used to induce cultures. Following induction, cultures were further grown at 20°C or 37°C. Samples were obtained from cultures 2h post induction and after overnight 16h growth. Overexpression was assessed via SDS PAGE.

2.2.3.2 MbeC-His_6 expression and purification

Colonies of BL21-DE3 MbeC-His_6 clones from ampicillin-glucose LB agar plates were inoculated in 1.5 L of LB supplement with 100 mg/L of ampicillin. Cells were grown at 37°C with vigorous shaking and induced with 0.1 mM IPTG at an A600 of 0.6. Following induction, cells were grown overnight for 16h at 20°C and harvested via centrifugation.

The frozen pellet of BL21-DE3 MbeC-His_6 clones was suspended in 100 mL of Lysis Buffer containing 1:300 of Halt Protease Inhibitor Cocktail (Thermo Scientific) and 1:2000 of βMe (Sigma). Five rounds of 30s sonication with 30s break was applied to the suspension on ice. The cell lysate was centrifuged at 14,000 rpm for 1 hour at 4°C. The supernatant was loaded on Ni-NTA agarose (Qiagen) pre-equilibrated with Lysis Buffer and subsequently washed with Wash Buffer. The protein that bound to the column was then eluted with 4 mL of Elution Buffer containing increasing concentrations of imidazole (50mM, 100 mM, 150 mM, 200 mM, 300 mM, 500 mM). Fractions were run

on SDS-PAGE and visualized by Coomassie blue staining to assess the presence and purity of protein. The purest fractions were pooled and separated by cation exchange chromatography (SP Sepharose Fast Flow, GE Healthcare Life Science), using Buffers A and B. Fractions containing protein were pooled, concentrated, and separated via size exclusion chromatography (Hiload 26/60 Superdex 75 prep grade) using Buffer C. Fractions containing protein were identified via SDS PAGE, pooled, and incubated with AcTEV protease (1:200 v/v) at room temperature overnight to cleave His_6 tag. The digested sample was run over the Ni-NTA column where flow through was collected, spin concentrated, buffer exchanged with Storage Buffer. SDS PAGE was used to confirm the cleavage of His_6 tag and concentration of MbeC was measured by Pierce BCA Assay Kit (Thermo Scientific).

2.2.4 Crystallization trials

Crystallization was carried out using sitting drop INTELLI-PLATE 96-3 (Hampton Research) trays. Reservoirs were filled with 70 μL of crystallization conditions. Sitting drops consisted of 1 μL of concentrated MbeC protein (1.9 μg/μL) and 1 μL of crystallization condition obtained from reservoir. Crystal trays were incubated at room temperature or 4°C and observed approximately at 1, 2, and 3 weeks of growth.

2.2.5 DNA Annealing and labeling

The DNA oligos (Table 3) *nicbsf* and *nicbsr* were purchased from Integrated DNA Technologies. Equal amounts of complementary strands were combined in 1X annealing buffer and incubated in a 100°C water bath, that slowly cooled to room temperature overnight. Annealed DNA was labeled using radioactive γ-P32 ATP (PerkinElmer).

2.2.6 Electrophoretic mobility shift assay (EMSA)

Increasing amounts of MbeC was incubated with 0.1 μM ^{32}P-radiolabeled dsDNA, *nicbs*, in Binding Buffer for 30 min at room temperature. To test for specific binding, MbeC was incubated with 0.1 μg of poly(dI-dC) (Roche) for 30 mins prior to incubation with *nicbs*. A 10 μL reaction contains a final concentration of 20mM Tris-

HCl (pH 7.5), 50 mM NaCl, 1 nM BSA, 0.1 μM *nicbs*, with varying concentrations of MbeC. Prior to loading the 10 μL reactions, the 15% native polyacrylamide minigel was pre-run for 1hr in TBE at 4°C. After the gel was run for 4 h at 5A at 4°C, it was exposed to a phosphor screen which was scanned using a Typhoon PhosphorImager (GE Healthcare) at a resolution of 200 microns.

2.2.7 Mating assay

Prior to running the mating assay, F TraD-deficient donor *E.coli* cells, POXD411 (Km^r) were rendered competent following 3 washes with 0.1 M $CaCl_2$. Donor cells were transformed with vector pBAD24 (Amp^r) containing full length F TraD or mutant F TraD (Figure 3.11A, Figure 3.12 A). pOXD411 cells transformed with the empty vector were used as a negative control. TraD mutants missing the entire CTD (D576*) or the last 8 residues (E709*) were reported to have low mating efficiencies and therefore were used as a type of negative control. ED24 *E.coli* ($Spec^r$) were used as the recipient strain. The mating assay tested for the transmission of F plasmid, that is deficient in TraD (Km^r).

Donor and recipient cells were streaked out on the appropriate antibiotic plates the afternoon before the mating assay. A single colony of ED24 and pOXD411 cells were grown in LB containing no antibiotics and 1:1000 of Km and Amp respectively. Cultures were grown to mid-exponential phase at 37°C. Fifty uL of pOXD411 and 200 uL of ED24 culture was aliquoted into 1 mL of pre-warmed LB and inverted several times. Tubes were incubated at 37°C and immediately put on ice after 30 minutes. Cultures were vortexed to break mating pairs and kept on ice to prevent further conjugation. Ice cold sodium chloride sodium citrate (SSC) buffer was used to make 5 serial 10-fold dilutions up to 10^{-5}. Ten uL drops were plated onto Km/Amp and Spec/Km plates and allowed to dry. Plates were incubated at 37°C overnight.

Mating efficiency was calculated as number of transconjugant colonies divided by donor colonies. Mating efficiency was normalized against the WT mating efficiency. The assay was repeated three times, and an average value of all assays was reported.

Chapter 3

Role of TraD in F Plasmid Conjugation

3.1 Introduction

It has been well established that an interaction between the relaxosome and transferosome is essential for the conjugative transfer of the plasmid from the donor to the recipient cell. Numerous studies discussed in the introduction elucidate the role of TraD CTD-TraM interaction in mating efficiency and allelic specificity. There are two proposed stages in TraD-TraM interaction. Evidence shows that the first stage of TraD-TraM interaction entails the binding of the terminal 8 residues of TraD in the hydrophobic pocket located in the tetramerization domain of TraM (Lu *et al*., 2008). It is hypothesized that the second stage involves extensive interaction between the remainder of the TraD CTD and TraM (Sastre *et al*., 1998). In this chapter we aim to characterize the role of additional regions in the F TraD CTD and their role in conjugative transfer of the F plasmid.

Interestingly, a paradox exists regarding TraD-TraM interaction. Pull down assays using lysates of TraD and purified TraM show that the terminal 8 residues of TraD are essential for binding to TraM (Lu *et al*., 2008). However, isolated terminal TraD peptides only have weak interactions with the TraM tetramerization domain in solution (Lu *et al*., 2008). Together these experiments indicate a low affinity between a single TraD peptide and the TraM tetramerization domain. We hypothesize that the TraD CTD could make multiple simultaneous contacts with TraM and the affinity between each contact is enhanced through an avidity effect. An avidity effect specifically occurs when a protein makes multivalent contacts with its binding partner resulting in interactions that are stronger and more stable than the individual isolated contacts. We hypothesize that TraD is a hexamer on the basis of the crystal structure of its orthologue (Gomis-Ruth *et al.*, 2001), TrwB, and therefore a single TraD hexamer could provide 6 C-terminal tails that could each interact with a single TraM tetramer.

There could be an array of stoichiometric combinations that make up the TraD-TraM complex. X-ray crystallography has shown that one TraM tetramer can bind up to 4 TraD CTD peptides (Lu *et al*., 2008). Although this complex has been observed in vitro, we do not know if a similar complex exists within the bacterial cell. It is unknown if a single TraD hexamer contributes 4 C-terminal tails to occupy all binding pockets of a TraM tetramer.

The fact that TraM can bind to multiple DNA sites (*sbmA, sbmB, smbC*) on the F plasmid (Laurenzio *et al.*, 1992) further complicates our understanding of TraD-TraM interactions. Binding assays show that two TraM tetramers cooperatively bind *sbmA* on either side of the DNA (Wong *el al*., 2011). However, the mode of TraM binding at the other sites is not understood. TraD C-terminal tails may occupy all 4 binding pockets of a TraM tetramer bound at a single DNA site, such as *sbmA, sbmB, or sbmC*. Alternatively, TraD may simultaneously contact 2 TraM tetramers bound at a single DNA site such as *sbmA*. Interaction between TraD and TraM could be more broad, such that TraD C-terminal tails could contact TraM tetramers bound at two or three different DNA sites.

We took a genetic approach to better understand the role and function of the TraD CTD in TraM interaction. We aimed to investigate the following:

1) The function of the region upstream of the terminal 8 residues in the CTD via deletion of highly conserved sequences

2) The multivalent interaction between TraD-TraM, via shortening the CTD and consequently limiting the number of potential interactions in the TraD-TraM complex

Specifically, we used a mating assay to test the ability of various TraD mutants to restore the conjugative transfer of TraD deficient F plasmids. While this assay directly indicates the effect of TraD mutants on conjugation, it also indirectly tests TraD-TraM interaction.

F TraD deficient donor cells, pOXD411 (Km^r), were transformed with vector pBAD24 (Amp^r) containing full length F TraD or various mutant F TraD (Figure 3.1). ED24 *E.coli* ($Spec^r$) will be the recipient cell. Following the growth of cells to mid-exponential phase, donors and recipients will be incubated together at 37°C to allow for mating pair formation and conjugative transfer of the TraD deficient F plasmid. After incubation for 30 min, vigorous vortexing will break mating pairs and incubation on ice

will prevent further conjugation. Serial dilutions will be made of the culture solution and spot dropped on selection plates containing two antibiotics. Donors will be selected for using Amp/Kan plates as the TraD-deficient F plasmid of pOXD411 confers Kanamycin resistance and pBAD24 vector carries an Ampicillin resistant gene. Transconjugants will be selected for using Spec/Kan plates as the ED24 cells are Spectinomycin resistant and the transferred F plasmid is Kanamycin resistant. The mating efficiency will be calculated as the number of transconjugant colonies divided by donor colonies.

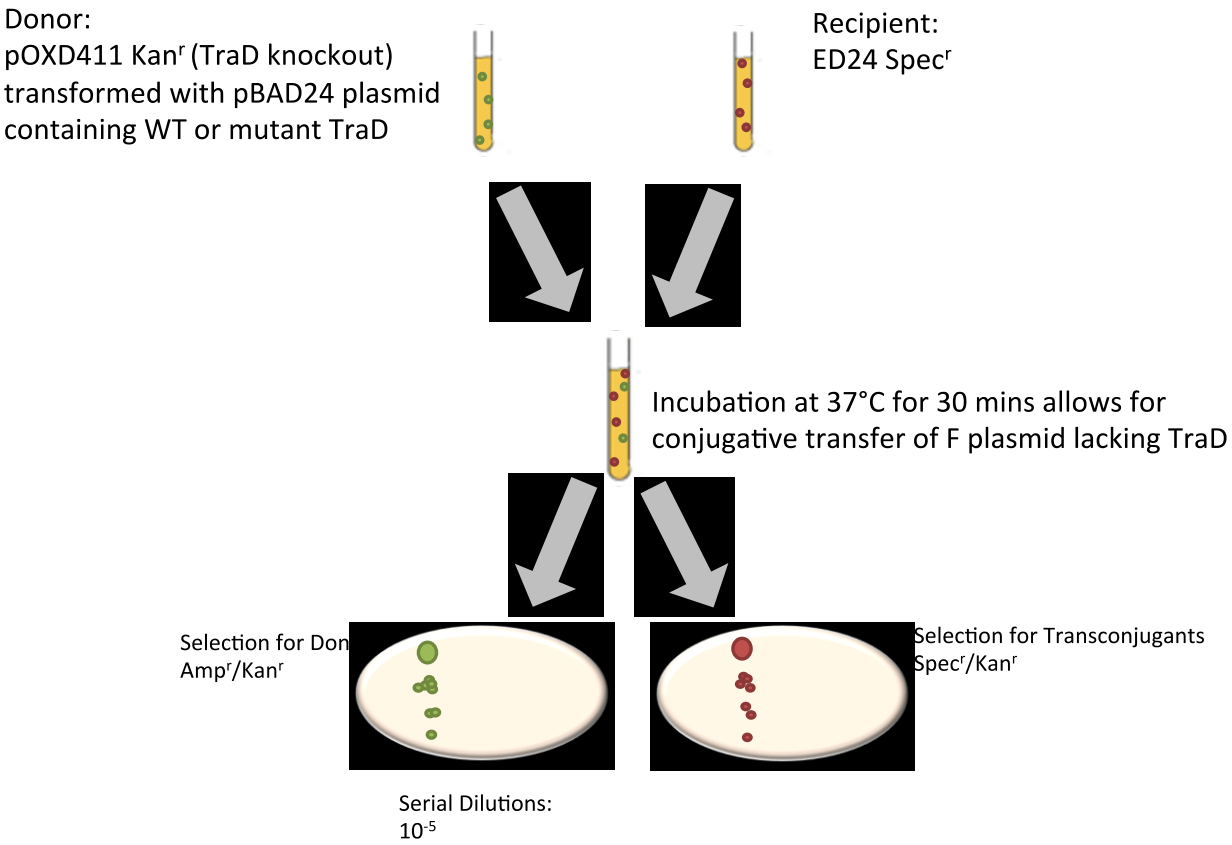


Figure 3.1 Mating assay to test the ability of TraD mutants to complement conjugative transfer of TraD deficient F plasmid.

3.2.1 Sequence alignments of F-TraD CTD

As the terminal 8 residues of the TraD CTD confer plasmid specificity (Wong *el al*., 2011), we aimed to further explore this region in other F-like plasmids. Of the selected plasmids, the pED208 sequence differs the most (Figure 3.2). Boxed areas in Figure 3.2 show that pED208 has substituted residues of a similar size and charge. Phe is typically found at the terminus of TraD, whereas Tyr is present in pED208. The terminal 8 residue segment of pED208 is highly acidic like other F-like plasmids, but contains Asp and Glu in different positions.

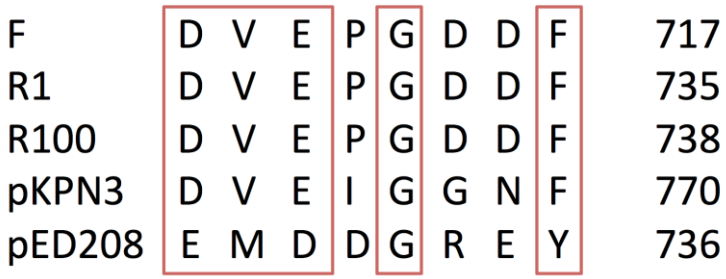


Figure 3.2 Sequence alignment of terminal 8 residues of TraD in F and F like plasmids.

Since the CTD is essential for the conjugative transfer of the F plasmid (Lu *et al*., 2008), we also aimed to identify possible regions or motifs beyond the terminal 8 residues, that may play a role in conjugation. A BLAST of the F TraD CTD identified 934 orthologues. Every 4th sequence of this list between 30-80% sequence identity was selected and partial sequences were omitted. In the end, 70 sequences were aligned and used to construct a sequence logo with a range of 800 residues.

This more extensive sequence alignment reveals that the 8 terminal residues are not as conserved as previously thought (Figure 3.3).

Figure 3.3 Sequence logo of TraD terminal 8 residues.

The terminal aromatic residue, Phe717, of F TraD makes hydrophobic stacking interactions in the binding pocket of TraM (Lu *et al*., 2008). Phe is the predominant terminal residue in our group of 70 TraD sequences. However, other residues of similar size (Tyr), and ones that are hydrophobic (Ile and Val) also occur. Additional hydrophobic residues, Val711 and Pro713, of F TraD make interactions with Tyr102 of TraM (Lu *et al*., 2008). These residues are not highly conserved as indicated by residue position 794 and 796 in the sequence logo.

In F TraD, Glu712, Asp 715, and Asp 716 contribute to long distance electrostatic interactions (Lu *et al*., 2008). Glu in position 795 appears to be common to the majority of TraD proteins within our list. Asp is found in a third of the proteins analyzed as indicated by residue position 798 and 799. Interestingly, residues with positive charges or different sizes are found among other TraD proteins.

Gly714 of F TraD contributes to the β-turn structure (Lu *et al*., 2008) and appears to be highly conserved (position 797) among 70 systematically chosen TraD. The presence of an Asp may contribute to an alternative structure within the terminal 8 residues in other TraD proteins.

The heterogeneity of the TraD terminal 8 residues further supports its role in plasmid specificity. This sequence logo can help to explain why TraD from one strain cannot support conjugation within other strains. Specific interactions between TraD and

TraM occur in order to effectively bring the plasmid bound relaxosome to the conjugative pore (Wong *et al*., 2011).

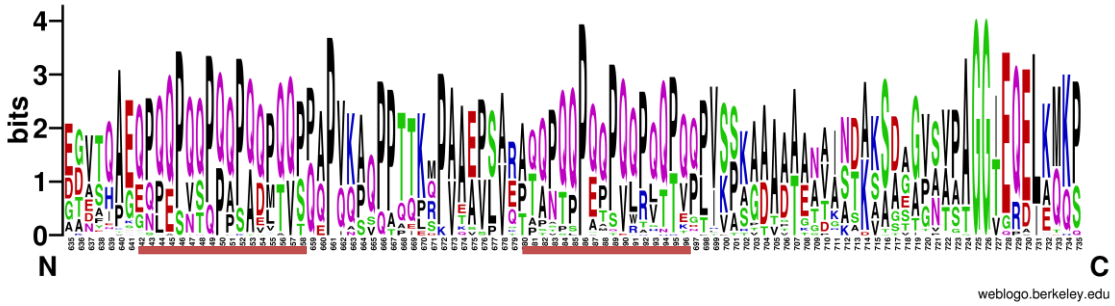


Figure 3.4 Sequence logo of TraD residues 635-735. PQQ repeats are underlined in red.

The repetition of the PQQ motif is variable among the selected TraD orthologues and contributes to different lengths of the CTD (Figure 3.4). A single PQQ motif is found in F TraD while 9 tandem repeats exist in R100 TraD, which have been noted by Yoshioka *et al*. The function of this motif is not known, although our lab has previously shown that deletion of the single PQQ motif of F TraD or insertion of multiple PQQ motifs have no effect on conjugation (Jun Lu, unpublished results).

Numerous other motifs throughout the sequence logo appear to be conserved. Figure 3.5 shows the region closest to the ATPase domain to have highly conserved residues. A single elongated letter implies that this residue was consistently aligned in our list of 70 TraD sequences. A TQGGQ motif (underlined) is found among all our selected sequences (Figure 3.5).

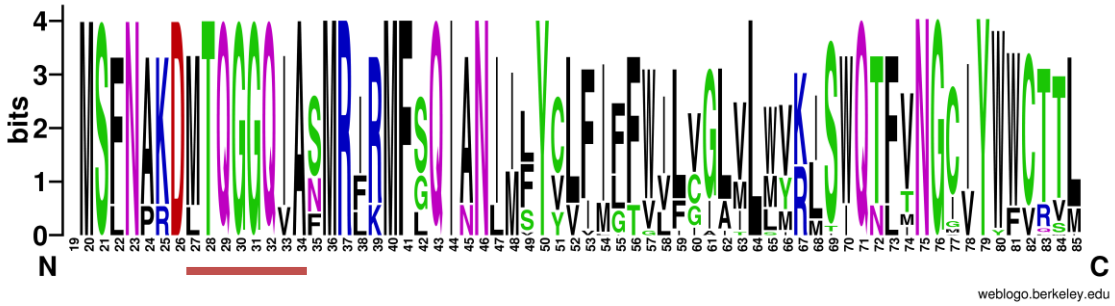


Figure 3.5 Sequence logo of TraD residues 19-85. Red line indicates highly conserved residues near the N-terminal domain

Other regions of high conservation are shown below and underlined in red.

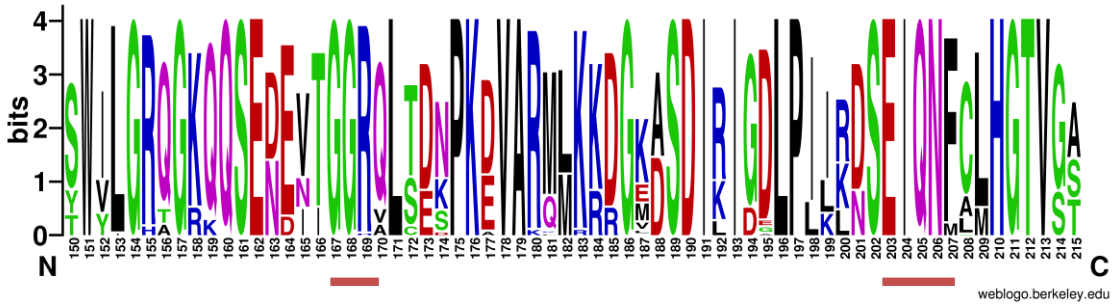


Figure 3.6 Sequence logo of TraD Residues 150-215.

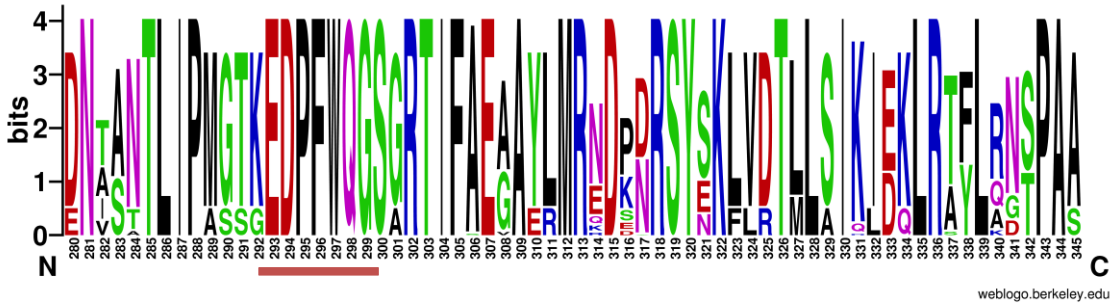


Figure 3.7 Sequence logo of TraD Residue 280-345.

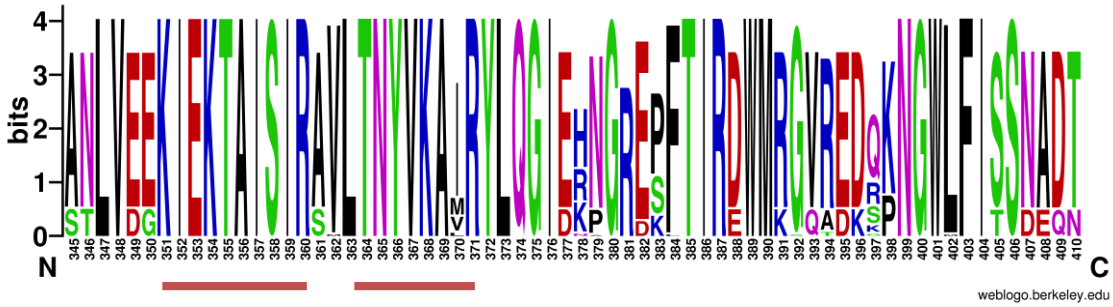


Figure 3.8 Sequence logo of TraD Residues 345-410.

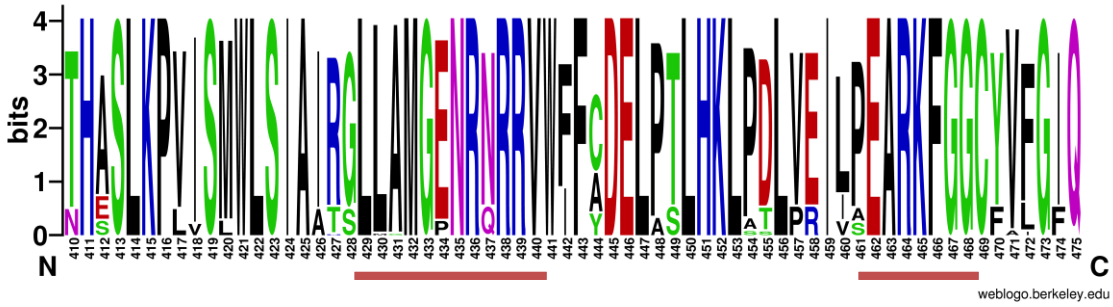


Figure 3.9 Sequence logo of TraD Residues 410-465.

To investigate the nature of the F TraD CTD, a hydrophobicity profile was constructed according to a scale developed by Kyte and Doolittle (Kyte & Doolittle, 1982). A high proportion of hydrophobic residues suggests that a protein will pack within its secondary structure, while a protein predominately composed of hydrophilic residues will likely be unstructured. The plot below shows the majority of residues of the F TraD CTD have a negative score, indicating their hydrophilic nature.

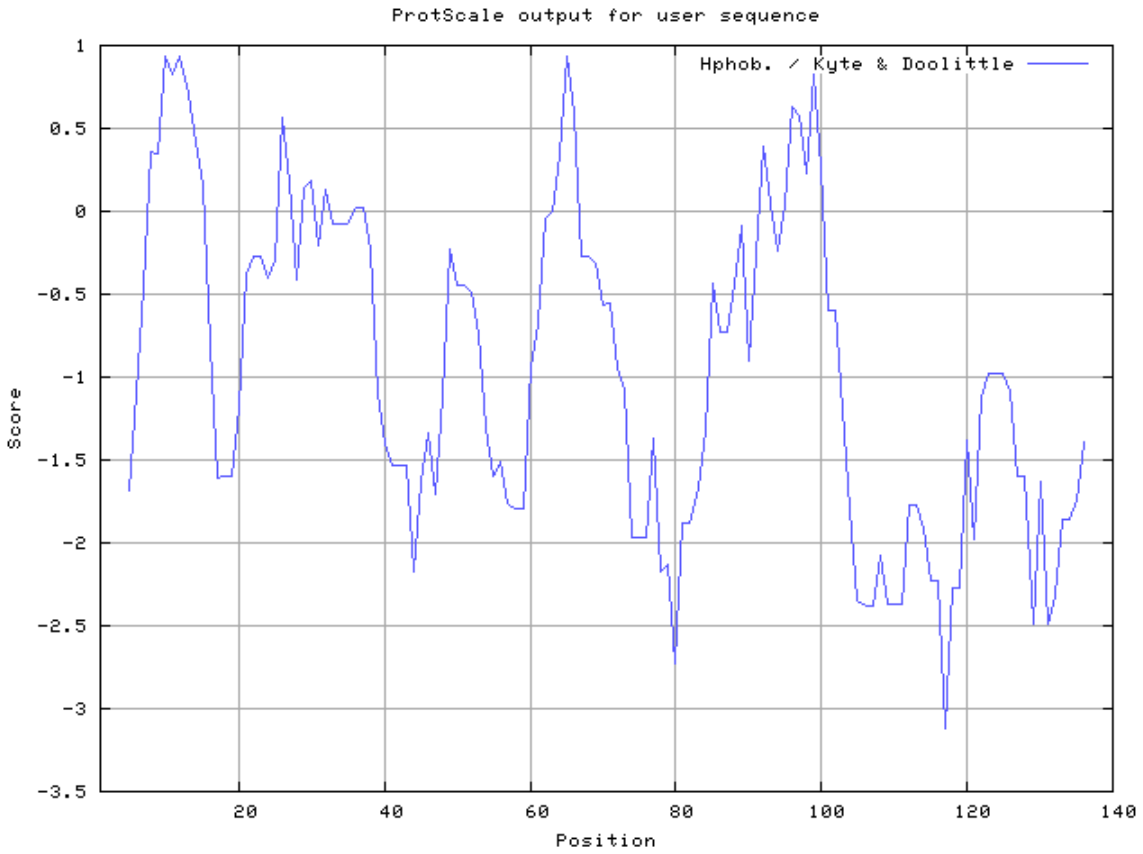


Figure 3.10 Hydrophobicity plot indicates that the F TraD CTD is unstructured.

3.2.2 Cassette deletions of F TraD CTD do not significantly impair conjugation

Extensive sequence alignments and sequence logos revealed conserved motifs dispersed upstream of the terminal 8 residues in TraD. To test whether these motifs play an essential role in conjugation, consecutive cassette deletions were introduced into the CTD of F TraD. Each deletion was 21 residues long keeping the length of the CTD constant. Mating assays were then used to test the ability of these constructs to rescue F plasmid conjugation in *E.coli* deficient of TraD.

The extent to which mating efficiency is reduced gives an indication of how important the deleted sequence is for the conjugative transfer of F plasmid. Of all the cassette deletions, the one closest to the ATPase domain, F $TraD_{578\text{-}598}$, had the greatest effect on reducing mating efficiency. F $TraD_{578\text{-}598}$ had a 3.0 $x10^{-2}$ fold mating efficiency relative to wild type (Figure 3.11). The adjacent downstream deletion, F $TraD_{599\text{-}619}$, reduced conjugative transfer of F plasmid by 2.0 $x10^{-1}$ fold. Deletion of the PQQ motifs, which are absent in mutant F $TraD_{620\text{-}640}$ only reduced mating efficiency by a 5.6 $x10^{-1}$ fold average. As previously reported, deletion of the entire CTD, F TraD D_{576*}, had a 5 $x10^{-5}$ fold reduction on conjugative transfer, while deletion of the last 9 residues, F $TraD_{E709}$*, had a 2 $x10^{-3}$ fold decrease (Lu *et al*., 2008). Our findings were similar; F $TraD_{D576*}$ reduced mating efficiency by 1.1 $x10^{-5}$ fold, and F $TraD_{E709*}$ by 4.4 $x10^{-4}$ fold.

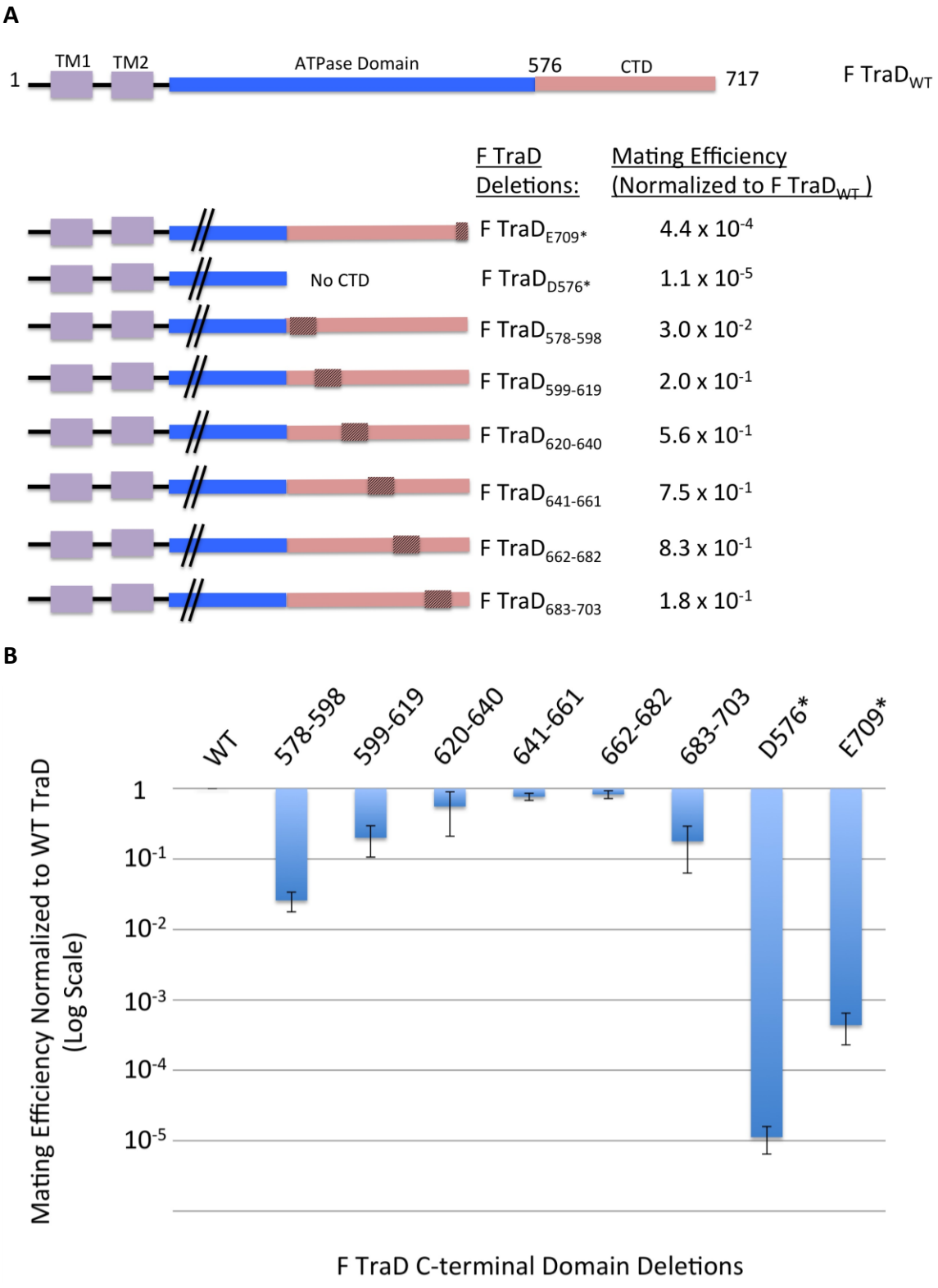


Figure 3.11 Effect of consecutive cassette deletions in F TraD on conjugation.
(A) and (B) show 2 representations of constructs and mating efficiencies.

3.2.3 Deletion of extended regions of F TraD CTD impairs conjugation

Our previous mating assays revealed that deleting highly conserved regions of the TraD CTD, did not negatively affect mating efficiency as much as truncations of the entire CTD or the 8 terminal residues. Moreover, deletion of regions with low sequence conservation, such as the PQQ motifs, had less of an effect on mating efficiency. On the basis of these results, we aimed to test whether the length of the TraD CTD is an important factor in TraD-TraM interaction. We hypothesize that multiple TraD C-terminal tails simultaneously contact TraM resulting in a stable complex through avidity effects. We postulate that the C-terminal tails must be of a minimal length to mediate formation of such a complex.

Deletions of increasing length were introduced such that the first deletion was 22 residues long at the center of the CTD (Figure 3.11). The largest deletion was 132 residues leaving only the terminal 8 residues, the specificity determinant, bound to the ATPase domain. The mating assay was repeated using these truncated TraD constructs. The conjugative transfer of F plasmid using TraD with CTD deletions of 22 ($TraD_{632-653}$), 44 ($TraD_{621-664}$), and 66 ($_{TraD610-675}$) residues long had a slight decrease in mating efficiency of 3.9 $x10^{-1}$, 7.1 x 10^{-1}, and 4.1 $x10^{-1}$ fold respectively relative to wildtype. Mating efficiency was increasingly impaired when the CTD was truncated to 88 residues ($TraD_{599-686}$) as indicated by a 9.0 x 10^{-2} fold reduction compared to wildtype. A deletion of 110 ($TraD_{588-697}$) residues further impairs conjugation by 5.7 x 10^{-3} fold. The largest cassette deletion was 132 residues ($TraD_{577-709}$) long and had the greatest effect on mating efficiency (5.0 $x10^{-5}$ fold relative to wildtype). This construct had a lower mating efficiency than when the 8 terminal residues are truncated.

Overall, these mating assays suggest that the CTD must be of a minimal length to allow for efficient conjugative transfer of the F plasmid. Additional truncation of the TraD CTD further impairs conjugation despite the presence of the terminal 8 residues.

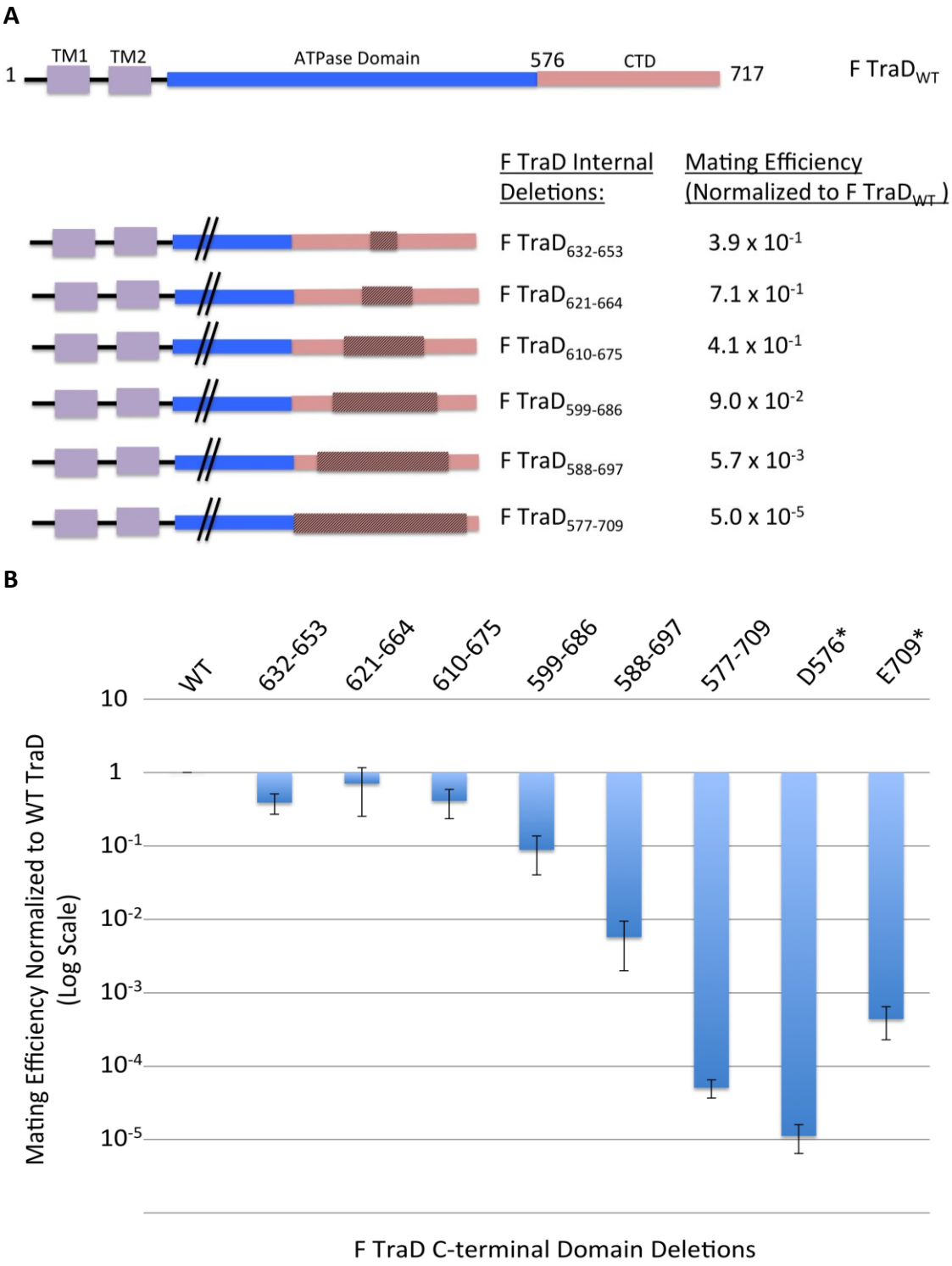


Figure 3.12 Effect of increasing larger cassette deletions in F TraD on conjugation.

3.3 Discussion

Our sequence alignments and sequence logos revealed several highly conserved motifs dispersed upstream of the terminal 8 residues in the TraD CTD. However, deletion of these regions using sequential 21 residue cassettes had little effect on mating efficiency (Figure 3.11). This suggests that the sequence upstream of the terminal 8 residues in the F TraD CTD is not critical for TraD-TraM interaction. Mating assays using these TraD constructs reduced conjugation at most by 10^{-2} fold relative to wildtype, whereas truncation of the entire CTD domain or deletion of the last 8 residues had a 10^{-5} and 10^{-4} fold reduction respectively.

Of the 21 residue cassette deletions, construct F $TraD_{578\text{-}598}$, resulted in the greatest reduction of F plasmid conjugative transfer. This deleted region may play a role in folding and stabilizing the adjacent ATPase domain, and its deletion could impair ATPase activity. Consequently, ssDNA may not be effectively pumped through the conjugative pore into the recipient cell. Our understanding of ATPase domain boundaries is based on the sequence alignment of F TraD with its orthologue, TrwB. Residue 507 indicates both the end of the ATPase domain and protein in TrwB, whereas F TraD contains an additional flexible CTD. The ATPase domain of F TraD may extend beyond the aligned region with TrwB, and explain the negative impact on conjugation efficiency.

Deletion of the region containing PQQ repeats (construct F $TraD_{620\text{-}640}$) had a minimal effect on conjugation. Our lab has previously shown that a deletion of a single PQQ repeat in F TraD or the insertion of multiple repeats does not negatively impact conjugation (unpublished results by the Glover lab). This expansion of PQQ repeats in TraD orthologues may haven arisen as a result of replication slippage, such that the DNA polymerase is released from the template strand upon encountering a tandem repeat. The polymerase reassembles upstream of the tandem repeat, which was previously replicated, and integrates the same sequence in the daughter strand, resulting in its expansion. The expansion of the PQQ repeat may not impair bacteria protein function. Rather, we postulate that these tandem repeats may take on a unique secondary structure that contribute to the flexibility of the C-terminal tail of TraD. Flexibility and extension of the TraD tail may be critical for its orientation and placement in the TraM binding pocket.

In the subsequent set of mating assays we aimed to determine whether the region between the ATPase domain and the terminal 8 residues of F TraD functions as a flexible tether. We hypothesize that the tether may take on numerous conformations, extend, and allow hexameric TraD to make multiple simultaneous contacts with TraM (Figure 3.13). CTD linker lengths of 110 ($TraD_{632-653}$), 88 ($TraD_{621-664}$), and 66 ($TraD_{610-675}$) residues all have a 10^{-1} fold mating efficiency relative to wildtype. A graded effect in mating efficiency is observed when the linker length is shorter than 44 residues ($TraD_{599-686}$). Further shortening of the linker, increasingly impairs conjugation as indicated by constructs $TraD_{588-697}$ and $TraD_{577-709}$.

We propose that the region upstream of the terminal 8 residues in the TraD CTD does not contain sequences or motifs critical for TraD-TraM interaction. Rather, this region may function as a flexible linker, which positions and orients the terminal 8 residues in the TraM binding pocket. The length of the linker is critical if the TraD tails must make multiple simultaneous contacts with one or more TraM tetramers to form a stable complex through avidity effects (Figure 3.13).

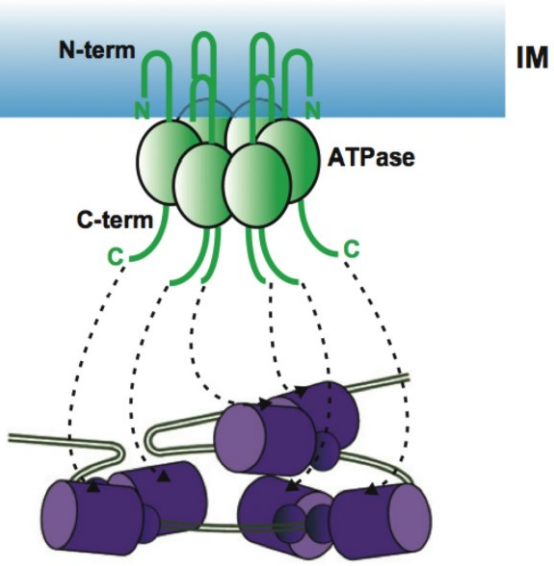


Figure 3.13 Model of avidity. Possible interactions of hexameric membrane bound TraD (green) with TraM tetramers (purple) bound at *sbmA, sbmB*, and *sbmC* DNA sites. Adapted from Wong *et al*., 2012.

We hypothesize that a stable interaction between TraD and TraM is a result of avidity. Avidity is defined as the accumulated affinities when a protein makes multiple contacts with its binding partner. This multivalent interaction results in stronger and more stable interactions between the two proteins than their individual contacts. Low affinity has been reported between isolated terminal TraD peptides and the TraM tetramerization domain in solution (Lu *et al*., 2008). However, a stable interaction occurs between the lysates of TraD and purified TraM as shown by pull down assays (Lu *et al*., 2008), suggesting that TraD integrated in the bacterial membrane and possibly in its hexameric state strongly binds TraM. According to our avidity model, the multiple contacts made by TraD tails with TraM, contribute to the stability of the TraD-TraM complex.

To represent our findings, we constructed a model using the crystal structure of TrwB (Gomis-Ruth *et al.*, 2001), a hexameric orthologue of TraD. TrwB is an inner membrane integral coupling protein (Cabezon & de la Cruz, 2005) with a sequence identity of 29% to F TraD. It is important to note that TrwB does not contain the flexible CTD found in F TraD. For this reason, we estimated the radius of gyration (R_G) (Figure 3.14) of the F TraD CTD to indicate where in space this flexible tether may exist. The formula below describes the random coil behavior of chemically denatured proteins and can be applied to the flexible unstructured CTD region.

$$R_G = R_0 R^v$$

Figure 3.14 Formula to determine the radius of gyration, R_G. R_0 is the constant related to persistence in length or stiffness of the polymer, 2.08 ± 0.19Å. v is the scaling factor depending on the quality of solvent, 0.6. R_o and v are obtained experimentally through Small angle X-ray Scattering (SAXS). R is the number of residues in the coil (Fitzkee & Rose, 2004).

The R_G corresponds to the radius of the spheres located at the terminal alpha carbon of the ATPase domain of TrwB (Figures 3.15, 3.16, 3.17). The radius of the sphere indicates the average distance from one end of the CTD to the other. The sphere is a representation of one end of the CTD sweeping out in space, while the other end serves as a pivot point.

As R_G indicates an average length of the CTD, smaller and larger radii can also exist. Full extension of the flexible CTD, a rare conformation, would result in a large R_G, whereas a more folded conformation of the CTD would reduce the R_G.

Through modeling, we interpret that the F TraM-*sbmA* DNA complex could interact with hexameric F TraD in two ways. The orientation of *sbmA* DNA could be such that a single TraM interacts with TraD (Figure 3.17A) and up to 2 neighbouring C-terminal tails of TraD bind TraM. Another conformation could involve 2 TraM hexamers simultaneously binding opposite sides of TraD (Figure 3.17B) and up to 4 C-terminal tails interacting with TraM. In the figures below, a TraD peptide (shown in red) is included to show the location of the binding pocket in the TraM tetramerization domain. The overlap of the spheres, which represent the TraD CTD, with the binding pocket of TraM shows potential interaction between the two proteins.

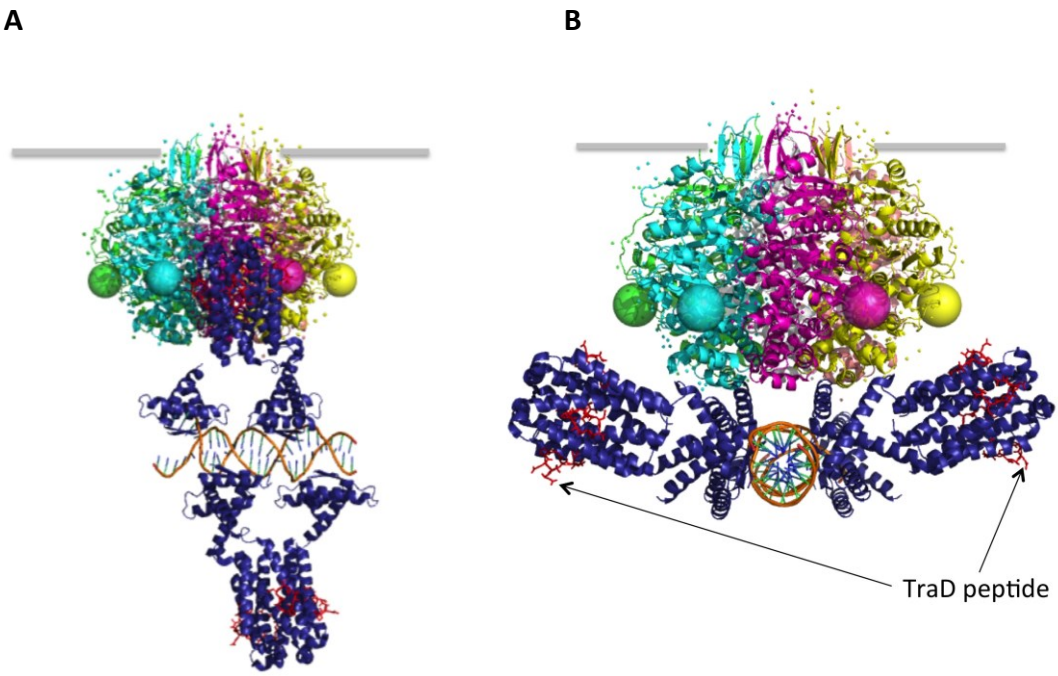


Figure 3.15 Model of TraM bound to TraD construct without a linker in the CTD. R_G spheres representing the F TraD CTD at C-terminal end of crystal structure of TrwB (Gomis-Ruth *et al.*, 2001). (A) and (B) display two possible modes of interaction. Mating efficiency of construct ($TraD_{577-709}$) is 5.0 x10^{-5} (relative to wildtype) and R_G (radius of spheres) is 7 Å. Inner bacterial membrane is indicated by grey line.

A B

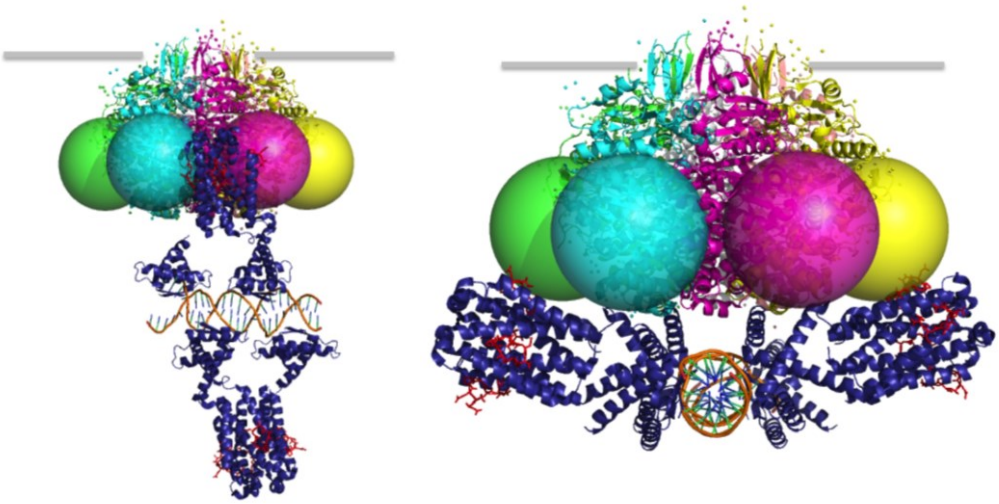


Figure 3.16 Model of TraM bound to TraD construct with a 44 residue linker.
Mating efficiency: 9.0 x 10^{-2} (relative to wildtype) and the R_G is 22 Å.

A B

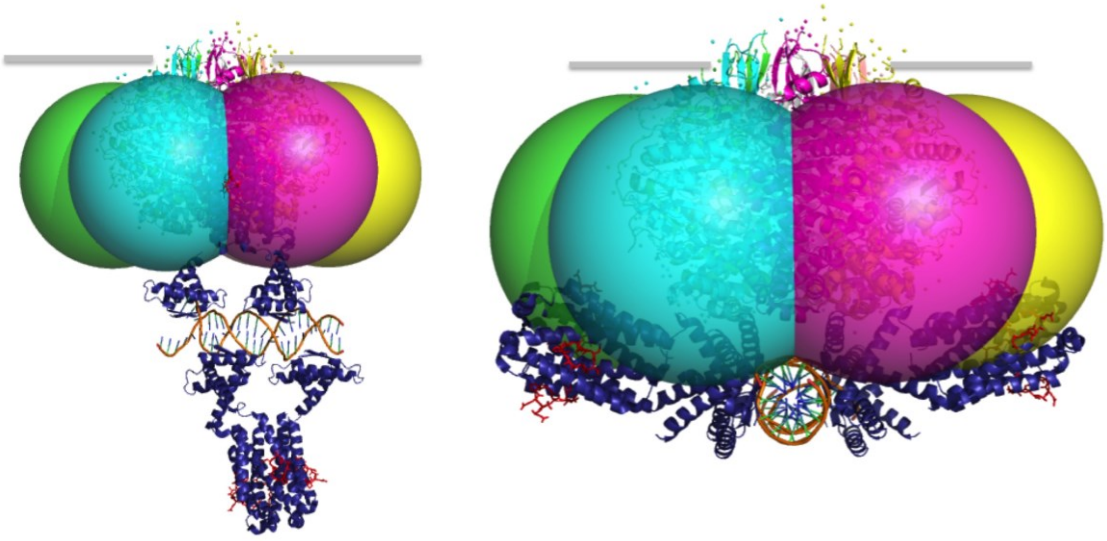


Figure 3.17 Model of TraM bound to WT TraD containing a 110 residue linker.
Mating efficiency is 1 (relative to wildtype) and the R_G: 41 Å.

We interpret that the 44 residue-long CTD linker of F TraD (construct $TraD_{599-686}$) in Figure 3.16 cannot interact effectively or reach all possible binding sites on the TraM tetramers. Such a complex would have reduced avidity leading to weak TraD-TraM interaction and consequently impair the ability to maintain the relaxosome at the conjugative pore. This would hinder conjugative transfer of the plasmid as indicated by the 10^{-2} fold reduction in mating efficiency. A comparison between Figures 3.16 and 3.17 shows the reduction of overlap between the R_G sphere, representing the F TraD CTD, and the binding site on TraM. As the R_G is an average value, extended conformations of the CTD are possible, which allow for more efficient interactions between the terminal 8 residues of TraD with TraM. However, it is important to note that these extended conformations are not energetically favorable, and do not occur frequently.

As the length of the CTD linker is reduced, fewer contacts are formed between TraD and TraM. We interpret that the stability of the complex relies on binding affinity between a single C-terminal tail and TraM rather than the cumulative effects of avidity. Reduction of the linker length to 22 residues (construct $TraD_{588-697}$) further places physical constraints on the number of simultaneous interactions made between TraD and TraM.

F TraD CTD consisting of only the terminal residues (construct $TraD_{577-709}$) has a reduction in mating efficiency by 10^{-5} fold compared to wildtype. We infer that there are several factors contributing to the poor ability of this TraD construct to complement F plasmid transfer. Our model (Figure 3.15) illustrates that the short CTD prevents multiple interactions with the TraM tetramer thereby abolishing any avidity. Moreover, the folded upstream regions of TraD may not sterically allow for the terminal 8 residues to be deeply positioned in the binding pocket of TraM. It has been previously reported that the C-terminal carboxylate group TraD forms ion pairs with Arg110 and Lys76 of TraM. The addition of Gly at the C-terminus of TraD significantly reduces conjugation by 9×10^{-4}, indicating the importance of this interaction (Lu *et al*., 2008). In a conformation where the terminal 8 residues of TraD are not deeply positioned in the TraM binding pocket, hydrophobic stacking interactions would be limited. The terminal Phe of TraD interacts with the Leu85, Val106, and Ile 109 located at the center of the TraM binding pocket.

Other hydrophobic interactions found at Val711 and Pro713 of TraD may be weakened (Lu *et al*., 2008). We interpret that contact between a single CTD of this TraD construct does not bind tightly to the binding pocket in TraM, consequently leading to a highly unstable transferosome-relaxosome complex.

We also acknowledge this TraD construct ($TraD_{577-709}$) may have impaired ATPase activity, thus affecting conjugative transfer. Our understanding of the TraD ATPase domain is based on sequence alignment with TrwB. The ATPase domain is located at the N-terminal region of TrwB. The rest of the alignment constitutes the CTD of TraD. It may be possible that the ATPase domain of F TraD extends beyond residue 576. The low mating efficiency could be a result of impaired ATPase activity, steric effects, and lack of avidity effects.

Mating efficiency is unaffected when the TraD CTD linker length is 110 ($TraD_{632-653}$), 88 ($TraD_{621-664}$), or 66 ($TraD_{610-675}$) residues long. Circular dichroism spectroscopy has shown that the CTD lacks a stable secondary structure (Lu *et al*., 2008). Likewise, our hydrophobicity plot has indicated the highly unstructured nature of the CTD of F TraD. We postulate that the highly flexible region found upstream of the terminal 8 residues allows the CTD to take on numerous conformations and accommodate variations in linker length longer than 66 residues. A CTD linker of 44 residues (construct $TraD_{599-686}$) limits the number of potential simultaneous contacts between TraD and TraM and in turn reduces the stability of the complex according to our avidity model. Furthermore, extensive truncation of the CTD linker (construct $TraD_{588-697}$) impairs the stability of the TraD-TraM complex due to lack of avidity effects and the inability of the TraD terminal 8 residues to make contacts deep within the TraM binding pocket. Overall, we infer that a TraD C-terminal linker between 44 and 66 residues long allows for the formation of a stable TraD-TraM complex, and therefore mediates effective interaction between the transferosome and relaxosome. Lastly, our mating assays suggest that this tether in TraD can be truncated to 66 residues, thus reducing some flexibility in this region and increasing the likelihood for crystal formation. Co-crystallization of TraD and DNA bound-TraM would further our understanding of protein interactions in this complex.

Chapter 4

Characterization of ColE1 relaxosome components

4.1 Introduction

ColE1 is one of the model systems for the class of mobilizable plasmids. Mobilizable plasmids do not encode all necessary machinery for conjugation, and therefore rely on the presence of conjugative plasmids to encode coupling proteins and proteins of the transferosome. ColE1 is transmitted by a broad range of plasmids and serves as another way that bacteria can acquire new genes.

Despite the discovery of the ColE1 plasmid in the 1960's few studies have elucidated the functional and structural characteristics of proteins encoded by the mob region, which are essential for ColE1 transmission (Heinemann & Sprague, 1989). MbeA, MbeB, and MbeC are understood to be components of the relaxosome. MbeA is the relaxase, which catalyzes the nicking reaction, while MbeC is the DNA-binding accessory protein. The function of the 19.5 kDa MbeB is unknown (Lovett & Helinski, 1975).

We hypothesize that MbeC could be playing a TraM-like role, by possibly recruiting the mobilizable plasmid to the conjugative pore and mediating contact between the plasmid and transferosome. MbeC may serve as an accessory protein to the relaxase, specifically by stimulating cleavage and unwinding of the ColE1 DNA. We aim to create expression constructs and purify MbeC to use in structural studies involving X-ray crystallography. Building upon previous studies, we aim to further understand MbeC interaction at the *oriT* of ColE1 using binding assays such as EMSA (Electrophoretic mobility shift assay). We will attempt to overexpress, purify, and crystallize MbeB as there have been no studies reporting on the structural and functional characteristics of MbeB.

4.2 Results

4.2.1 MbeC purification

MbeC-His_6 had low solubility during several stages of purification. Precipitation of MbeC-His_6 occurred during its elution from the Ni-NTA column under 300 mM and 500mM imidazole Elution Buffer conditions (Figure 4.1). The protein eluted off the cation exchange column at an NaCl concentration of 500mM (Figure 4.2). In subsequent protein preps the concentration of NaCl in the Wash Buffer was increased to 500mM to improve the solubility of MbeC-His_6. Protein containing fractions collected from the size exclusion column reveal that MbeC-His_6 behaves as a dimer under our buffer conditions. Digestion of MbeC-His_6 with AcTEV protease resulted in an approximately 1 kDa shift downwards indicating successful cleavage of the His-tag (Figure 4.4). Buffer exchange of MbeC into 0.5 M ammonium acetate Storage Buffer allowed us to attain a protein concentration of 1.9 mg/mL, whereas 10% glycerol, 0.5 M ammonium acetate allowed for 4 mg/mL.

MbeC crystallization trials

Crystallization trials were carried out using the following kits: Nextal Classics I and II (Qiagen), Wizard I and II (Emerald Biosystems). These suites were selected because of the wide spectrum of crystallization conditions as well as our lab's past experiences of growing crystals.

Crystals were found in a number of conditions:

Table 4.1 MbeC crystallization conditions

Suite	Well	Temperature	Composition
Classics I	A3	20°C	0.2 M Magnesium chloride, 0.1 M Tris pH 8.5, and 3.4 M 1,6 hexanediol
Classics I	G4	4°C	0.2 M Magnesium chloride, 0.1 M HEPES sodium salt pH 7.5, 30% (v/v) PEG 400
Classics I	H4	4°C	0.2 M Magnesium chloride, 0.1 M Tris HCl pH 8.5, 30% (v/v) PEG 4000
Wizard I and II	A1	20°C	CHES pH 9.5 , 20% (w/v) PEG 8000
Wizard I and II	E8	20°C	0.2 M Sodium chloride, 0.1 M Na/K phosphate pH 6.2, 10% (w/v) PEG 8000

Via the in house X-ray source, we determined that the composition of crystals found in wells A3, G4, H4 of Classics I Suite to be salt. The possible reason for salt crystal formation, likely magnesium phosphate, under this condition may be due to the presence of residual phosphate from inadequate buffer exchange. MbeC was purified again with an increased number of buffer exchange steps and this crystal formation did not occur.

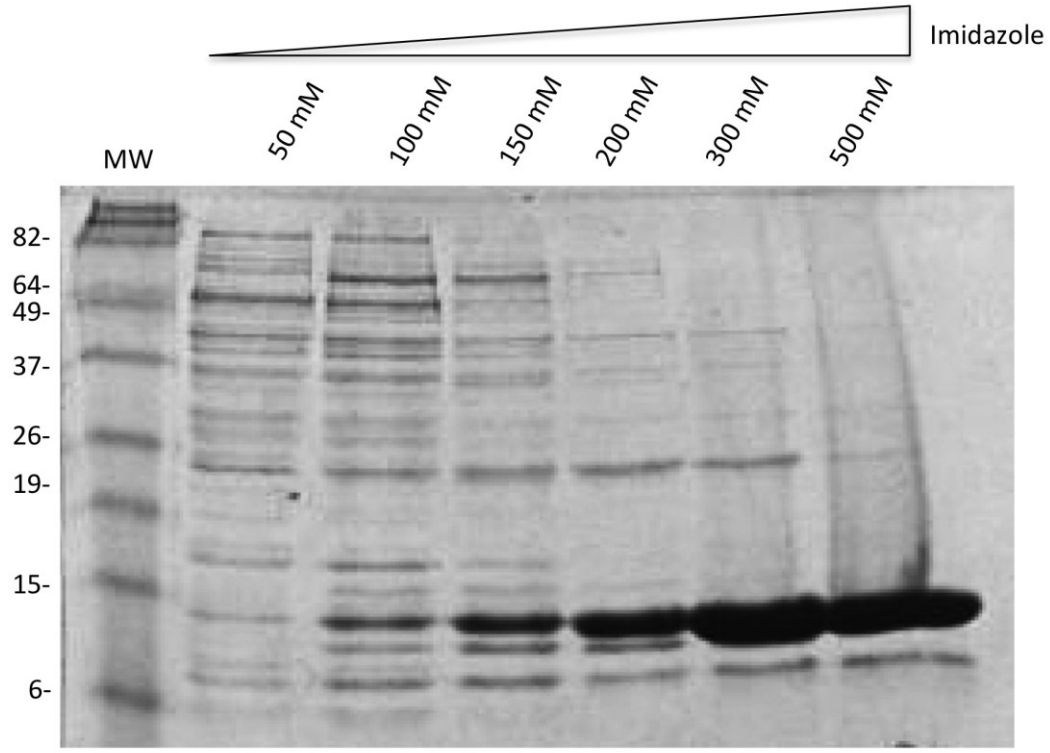


Figure 4.1 SDS-PAGE of MbeC-His$_6$ fractions eluted off Ni-NTA column. MbeC-His$_6$ was eluted off column with increasing concentrations of imidazole (50, 100, 150, 200, 300, 500 mM). 200, 300, and 500 mM fractions were pooled and subsequently separated by cation exchange.

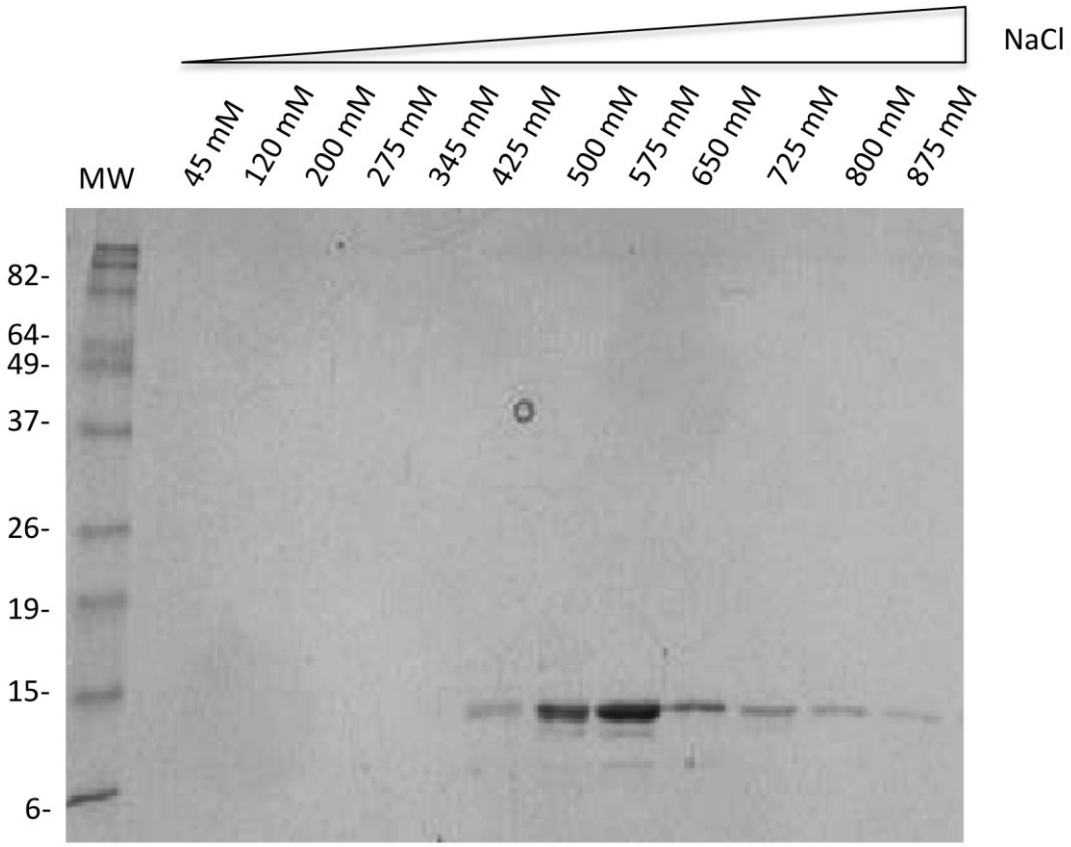


Figure 4.2 SDS-PAGE of MbeC-His$_6$ fractions eluted off cation exchange column. Peak fractions were obtained at approximately 500-575 mM of NaCl.

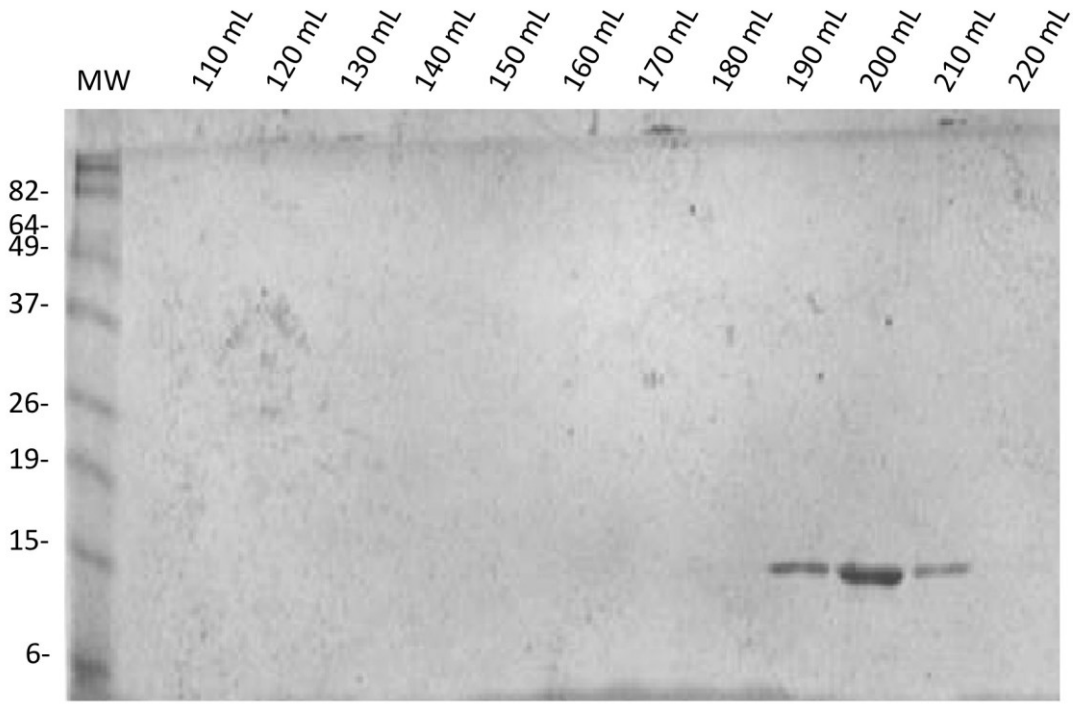


Figure 4.3 SDS-PAGE of MbeC-His_6 fractions eluted off size exclusion column.

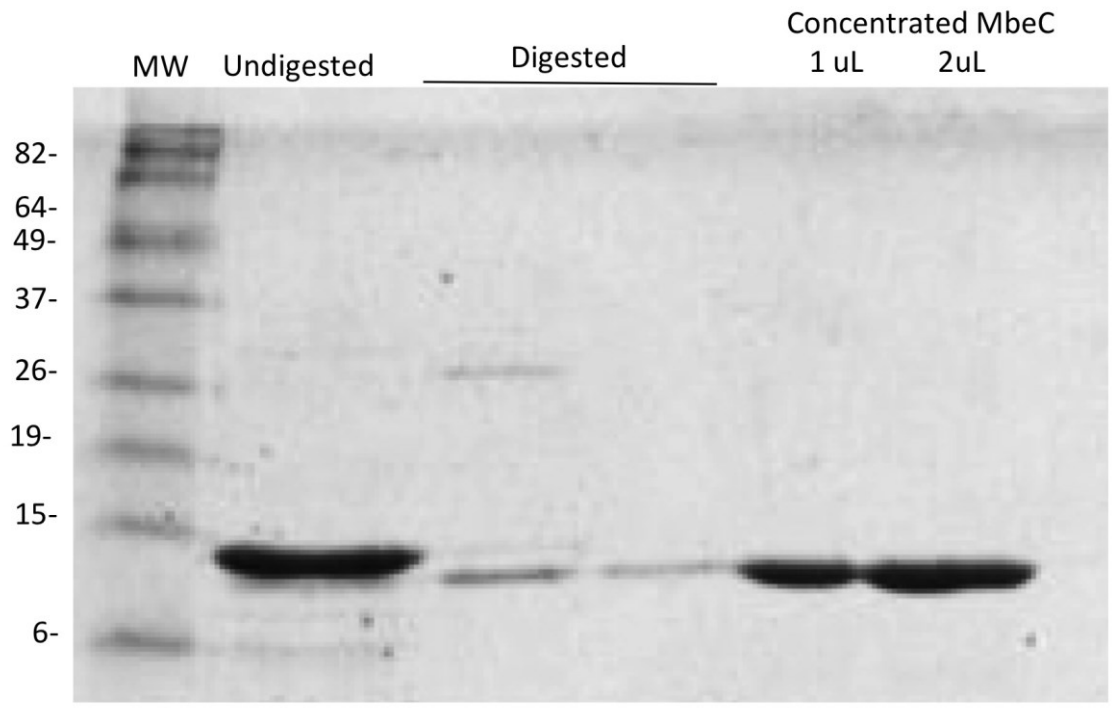


Figure 4.4 SDS-PAGE of MbeC fractions eluted off Ni-NTA column following cleavage of His tag. MbeC was digested using AcTEV protease.

4.2.3 EMSA with MbeC

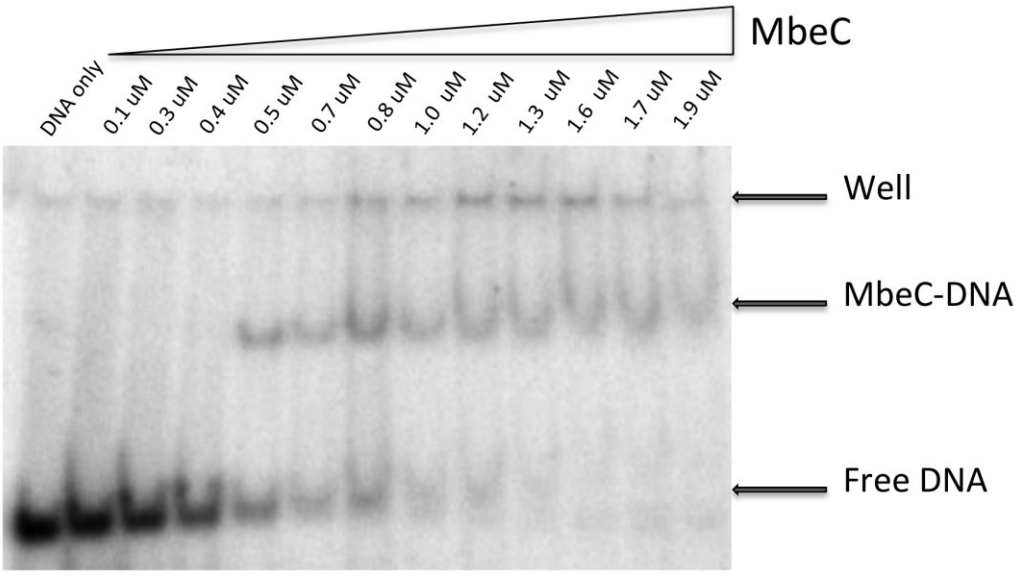
It has been previously reported that MbeC-His_6 binds to ds ColE1 *oriT* (1444-1527) and *nicbs* region (1456-1496) using EMSA (Varsaki *et al*., 2009). We aimed to determine the minimal length of *nicbs* DNA to which MbeC binds. As previous attempts to crystallize isolated MbeC were unsuccessful, we rationalized that the presence of a binding partner, such as DNA, may induce crystallization. To pursue this strategy, the DNA binding partner must be of minimal length to minimize flexibility.

Shifting of radiolabeled dsDNA, *nicbs*, begins at approximately 0.5uM of MbeC (Figure 4.5A). Varsaki *et al*. chose a broad range of MbeC concentrations that did not completely shift the *oriT* bands and that resulted in *oriT*-MbeC aggregate formation within the well (Varsaki *et al*., 2009). We titrated in MbeC using a finer gradient to identify the concentration of MbeC that causes a complete shift. Likewise, we observed significant aggregation of MbeC-*nicbs* complex in the well at concentrations of 0.8 uM of MbeC. Aggregation was also suspected at MbeC concentrations, which caused a shift in

the *nicbs* bands. Unbound DNA appeared as dark dense bands. It is expected that non-shifted and shifted DNA will have a combined density similar to unbound DNA. This was not observed in our EMSA gels and suggests that protein-DNA aggregation may have occurred in the wells.

Varsaki *et al*. showed specificity of MbeC binding to ds *oriT* (Varsaki *et al*., 2009). The presence of unlabeled sonicated salmon sperm, a nonspecific competitor, did not affect binding between ds *oriT* and MbeC. Likewise, titration of unlabeled *oriT*, a specific inhibitor, resulted in unshifted DNA species. To further test that MbeC binds ds *nicbs* specifically, and not DNA of any sequence, we added poly (dI-dC•dI-dC) as a non specific competitor. Poly (dI-dC•dI-dC) is a synthetic double stranded polymer consisting of alternating anionic deoxyinosinic-deoxycytidylic bases in each strand. It is typically used to bind proteins in crude lysates (Promega). MbeC bound poly (dI-dC•dI-dC) as indicated by a lack of shifted *nicbs* bands (Figure 4.5B). As MbeC binds ds *nicbs* DNA non specifically, we did not pursue further studies to determine the minimal DNA binding sequence.

A



B

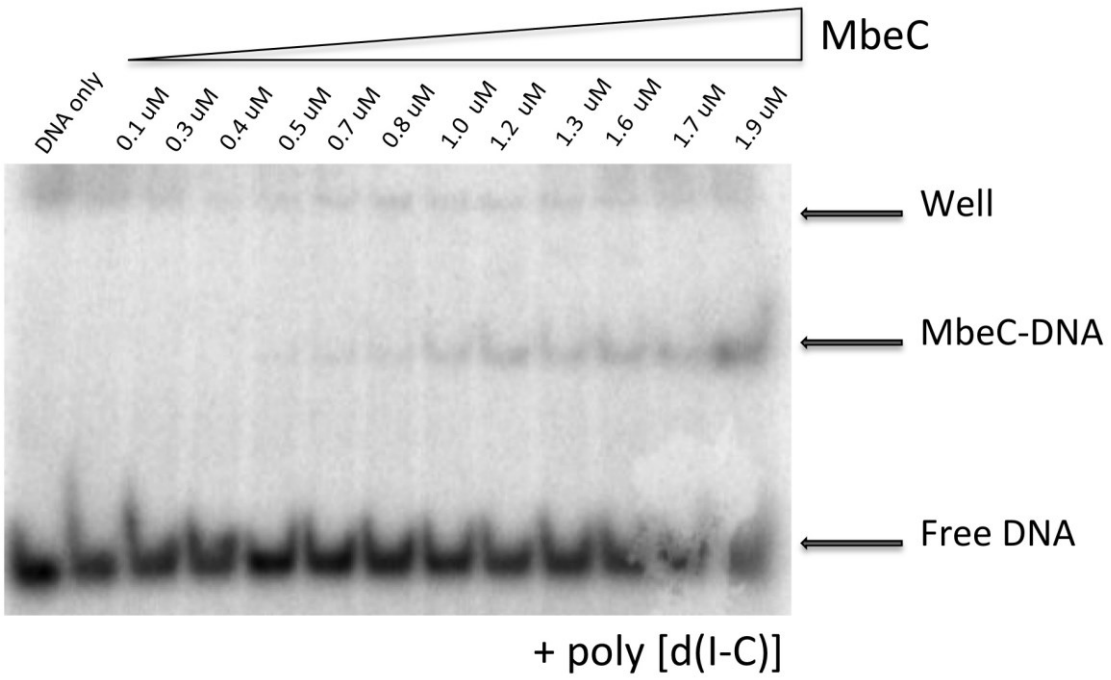


Figure 4.5 EMSA of ds *nicbs* and MbeC. (A) Increasing concentrations of MbeC added to 0.1 uM ^{32}P-radiolabeled ds *nicbs* (B) Addition of a non-specific competitor, poly dI-dC.

4.2.4 MbeA and MbeC co-expression

Isothermal titration calorimetry experiments indicate the *in vitro* interaction of MbeA and MbeC in a 1:1 ratio (Varsaki *et al*., 2012) Moreover, bacteria two hybrid assays validate this interaction *in vivo* (Varsaki *et al*., 2012) . We thought to co-express MbeA and MbeC for the following reasons. MbeC in complex with MbeA may improve the solubility of MbeC and consequently will increase the yield during purification. MbeA may play a role in directing MbeC to a specific sequence within the *nicbs*, and therefore can be used in EMSA.

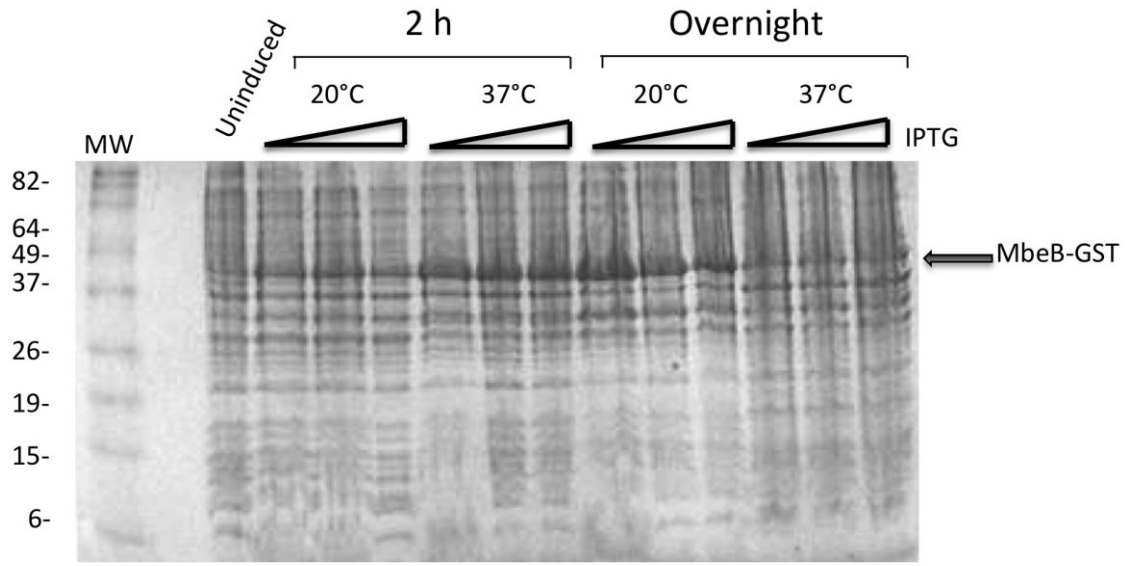
Few colonies were obtained following co-transformation of BL21-Gold *E.coli* (Kan^r) with pGEX6P-1 (Amp^r) containing *mbeC* and pACYC Duet-1 (Cm^r) containing

mbeA. Low transformation efficiency may be explained by the presence of 3 antibiotics in the agar used for selection. Sequencing of MbeA indicated mutations of several residues in key regions known to be involved in catalyzing the nicking reaction. The mutations altered the hydrophobicity of the residue and may have affected the functioning of MbeA. For this reason we did not pursue further experiments to study the MbeA-MbeC complex.

4.2.5 MbeB expression

MbeB was GST tagged to improve the solubility of the protein (Smith, 1988). Following induction of culture at an A600 of 0.6 with 0.2 mM IPTG, cells was grown overnight at 20°C (Figure 4.6A). The sonicated cell lysate was centrifuged and the supernatant was assessed via SDS PAGE. MbeB-GST was not soluble (Figure 4.6B). This construct most likely precipitated into the pellet as there was none present in the supernatant. The supernatant was subsequently run over a glutathione column. Lack of bands present at 45 kDa further indicated the extremely low solubility of MbeB-GST.

A



B

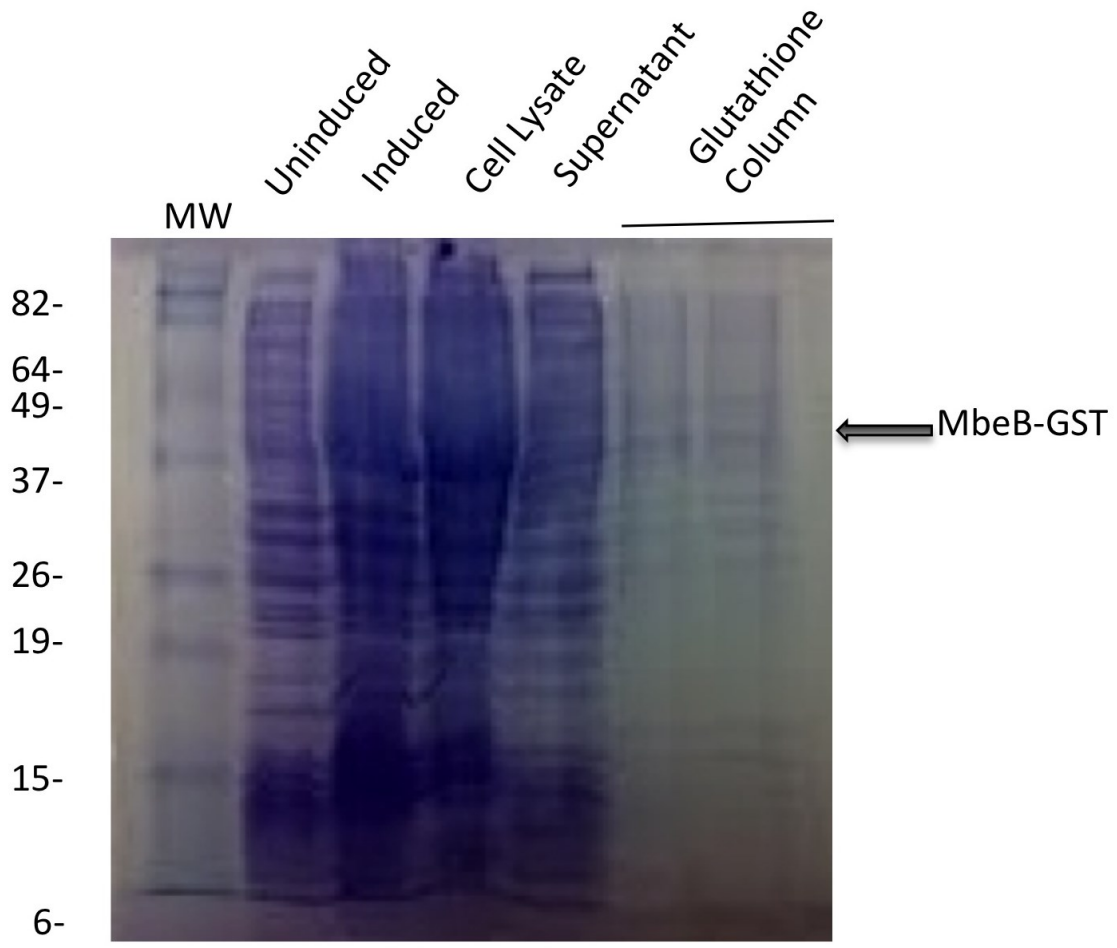


Figure 4.6 Overexpression and purification of MbeB. (A) Testing for MbeB overexpression at various temperatures and time points post induction. IPTG concentrations of 0.1mM, 0.2mM, and 0.5mM. (B) Testing of MbeB presence during stages of protein purification.

4.3 Discussion

Mobilizable plasmids, such as ColE1, cannot independently transfer from recipient to donor cell during conjugation. ColE1 only encodes proteins of the relaxosome and thus must coexist with a conjugative plasmid, which can encode and assemble the transferosome. The interaction of the ColE1 relaxosome with the conjugative plasmid-encoded transferosome is not well understood. X-ray

crystallography and functional assays such as EMSA could possibly provide insights into the structure of relaxosome components and possible mechanism of ColE1 transmission.

Relaxosome component, MbeC is a DNA binding protein with two stable domains. RHH motifs exist in the N-terminal region and EMSA indicates its ability to bind dsDNA at the *oriT* of the ColE1 plasmid (Varsaki *et al*., 2009). The C-terminal region of MbeC has been shown to interact with the N-terminal region of MbeA (Varsaki *et al*., 2012). We sought to purify MbeC for further characterization.

At numerous stages during the purification, MbeC exhibited low solubility and consequently resulted in a low yield of protein (approximately 1 mg per 1.5 L of culture). Poor folding may have contributed to the tendency of MbeC to precipitate in various buffers used in our purification. To improve yield, the salt concentration in the Lysis Buffer was increased from 300mM to 500mM on the basis of peak fractions obtained from cation chromatography. This modification prevented precipitation in the early stages of purification. Moreover, the Storage Buffer was supplemented with 10% glycerol, which allowed us to obtain a protein concentration of 4 mg/mL (2 mg per 1.5 L of culture). By increasing the salt concentration in the Lysis Buffer and adding glycerol to the Storage buffer, we were able to stabilize the protein and increase protein yield.

MbeC binds to *nicbs* with low affinity as shown by our studies and by those of Varsaki *et al*. Our EMSA showed that MbeC aggregates and forms non-specific interactions with *nicbs* DNA which contrasts previous findings (Varsaki *et al*., 2009). It is possible that MbeC was not properly folded and this affected its ability to make specific contacts with DNA. MbeC-DNA interaction may alternatively depend on DNA length or conformation, rather than specific sequences in the DNA.

There have yet to be experiments that characterize the structure and function of MbeB. Early studies have isolated the relaxosome complex, which contained the 19kD MbeB protein (Clewell & Helinski, 1969, Lovett & Helinski, 1975). In our attempts to purify MbeB, we have found this protein to be highly insoluble, even when expressed as a GST fusion protein. The expression of MbeA, MbeB, MbeC in a complex may be a promising approach to improve solubility and analysis via X-ray crystallography can provide insight into protein-protein interaction.

Chapter 5

Conclusion

5.1 F TraD-TraM interactions

Relaxosome-transferosome interaction is a necessary step for the conjugative transfer of plasmids containing a variety of genes, such as antibiotic resistance. Numerous studies have pointed to the interaction between TraM and the CTD of TraD as the link between the relaxosome and transferosome complexes. Moreover, an interaction between the terminal 8 residues of TraD and with the TraM tetramer is essential for the conjugative transfer of the F plasmid (Lu *et al*., 2008).

One of the goals of this research was to explore the role and function of residues upstream of the terminal 8 residues in the F TraD CTD. To accomplish this, we generated internal deletions in the CTD to investigate the effects on TraM interaction and consequently on conjugative transfer of the F plasmid. Based on the mating assays, we propose that the membrane bound hexameric TraD coupling protein makes multiple simultaneous contacts via its CTD with one or more TraM tetramers bound at the *oriT*. The sequence upstream of the terminal 8 residues in the CTD of TraD does not seem to play a significant role in TraD-TraM interaction. Instead, we postulate that the region between the ATPase domain and terminal 8 residues serves as a flexible tether, which helps to position and orient the terminal 8 residues in the TraM binding pocket. We think that the TraD-TraM complex is stabilized through avidity effects, whereby multiple contacts between the proteins strengthen the overall interaction. For this to occur, the tether must be more than 44 residues long to allow the terminal 8 residues to reach the TraM binding sites and that multiple simultaneous contacts are formed in TraD-TraM complex.

It is unknown what the exact stoichiometry of proteins in the TraD -TraM complex is. Although the crystal structure of TraM reveals 4 potential sites in the tetramerization domain to which the CTD of TraD can bind to (Lu *et al*., 2008), it is unclear whether such an interaction occurs under cellular conditions. Likewise, it is not

known whether all 6 CTDs of the TraD hexamer are interacting with TraM tetramers. It has been reported that two TraM tetramers bind on either face of the *sbmA* DNA in a cooperative manner (Wong *el al*., 2011). Armed with this information, we propose two possible modes of interaction (Figure 3.17A vs. Figure 3.17B) between TraD and *sbmA*-bound TraM. If we assume that TraD-TraM interaction is stable during transfer, and that the DNA must be transferred through the central pore of the hexamer, then this suggests that movement of ssDNA into the recipient cell is highly effective.

In order to determine which of our two models is correct, we can alter *sbmA* in the F plasmid such that the binding sites for F TraM and pED208 TraM are present on the plasmid in an alternating fashion; this results in the specific binding of a F TraM tetramer to one side of *sbmA* and pED208 tetramer on the other (Figure 5.1). Previous studies have shown that this chimeric *sbmA* binds F and pED208 TraM in a 1:1 molar ratio (Wong *el al*., 2011). The interaction between TraD and TraM confers allelic specificity, such that F TraD does not interact with the tetramerization domain of pED208 TraM as shown by poor mating efficiency (Wong *el al*., 2011)(Figure 1.4). If a single TraM tetramer interacts with TraD (Figure 3.17A) then the presence of chimeric sbmA should have no effect on mating efficiency. However, if 2 TraM tetramers bind on opposite sides of TraD (Figure 3.17B), then fewer contacts are formed between F TraD and TraM, thereby destabilizing transferosome-relaxosome interaction, and consequently reducing mating efficiency.

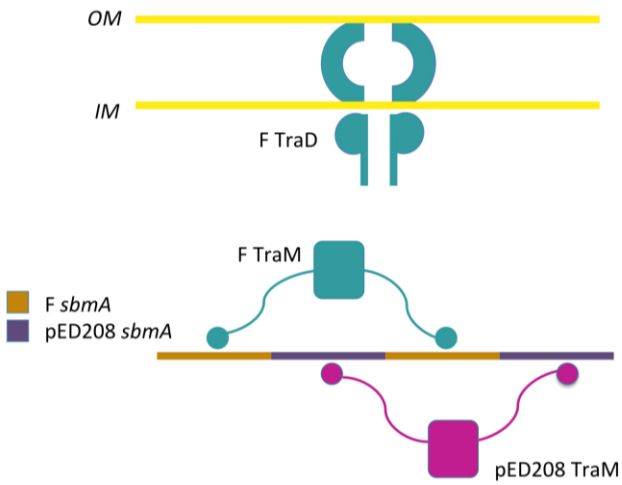


Figure 5.1 Schematic of F TraM and pED208 TraM bound to chimeric sbmA DNA. F TraD is embedded in the inner membrane (IM) and the transferosome spans the IM and outer membrane (OM).

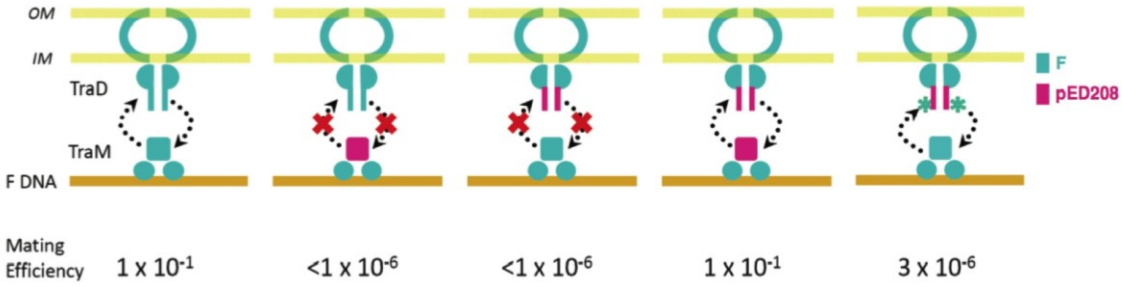


Figure 1.4 The terminal 8 residues of F TraD determine plasmid specificity. The mating assay was carried out using chimeric TraD and TraM from F and F-like plasmid, pED208. Adapted from Wong *et al.*, (2011) *Nucleic Acids Research* 39, 6775-6788.

Our understanding and interpretation of the mating assays carried out focused on the interaction between F TraD and F TraM bound to its cognate *sbmA* DNA. TraM tetramers also bind the *sbmB* and *sbmC* sites (Figure 5.2) (Laurenzio *et al.*, 1992), however the mode of interaction is unknown.

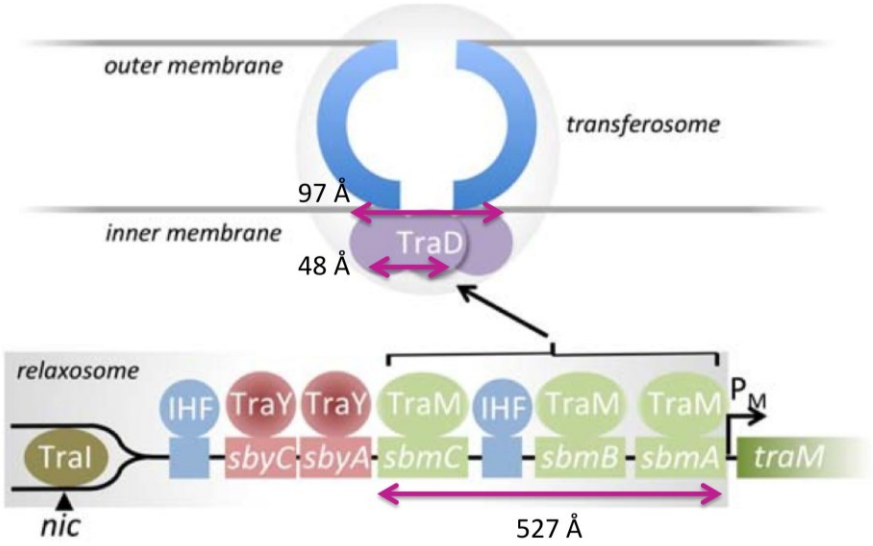


Figure 5.2 Relaxosome component binding sites. 48 Å indicates an approximate distance between neighbouring TraD C-terminal tails, while 97 Å is an approximate distance between C-terminal tails found on opposite sides of the TraD hexamer. 527 Å corresponds to the extended region of DNA spanning from *sbmC* to *sbmA* assuming linear DNA.

IHF binding sites exist between *sbmC* and *sbmB*, as well as approximately 30 bases upstream of the *sbmC* site (Frost *et al.*, 1994). *sbmA, sbmB* and *sbmC* span a region of 155 bp or 527Å if the DNA is in an extended linear conformation. The approximate distance between the ends of two neighboring TraD CTD is 48 Å. In the case that TraD interacts with TraM bound to all three binding sites on the DNA, it becomes clear that IHF proteins could play an important role in wrapping the DNA to bring TraM tetramers in close proximity to each other. IHF induces 140° turns (Yang & Nash, 1989) in DNA and likely helps TraD to form multiple simultaneous interactions with TraM. Additional mating assays can be carried out to understand if TraD interacts with TraM bound to *sbmA, sbmB*, and/or *sbmC*. Deletion of single or multiple TraM binding sites in the presence of TraD constructs with minimal-length linkers can indicate specific TraM interactions essential for conjugation.

5.2 ColE1

The mobilizable ColE1 plasmid was discovered in the late 1960's and has been used in the development of vector technology. However, few studies have characterized the proteins of the *mob* region, a region essential for the transfer of the ColE1 plasmid between bacteria. The low solubility of MbeC was problematic in its study via EMSA. We showed that isolated MbeC makes non-specific interaction with the *nic* site within the *oriT*. Moreover, MbeB could not be purified due to extremely low solubility despite its fusion to a GST tag. Early studies have isolated the ColE1 plasmid in complex with mob proteins, MbeA, MbeB, and MbeC. For this reason, co-expression of these proteins could be an alternative approach in their functional and structural analysis.

As the overexpression and purification of proteins is not always successful, in vivo experiments, such as a bacterial two hybrid system, can be used to test for an interaction between MbeB and the other components of the ColE1 relaxosome. In this assay MbeB will serve as the bait protein fused to the DNA binding protein fragment of a transcription factor, while components of the relaxosome (MbeA or MbeC) are the prey protein fused to the activation domain fragment of the transcription factor. Interaction between the prey and bait protein leads to the transcription of a reporter gene. In addition, extensive sequence analysis of MbeB could be used to identify regions and motifs and provide clues about its function.

In conclusion, we have yet to establish a detailed understanding of the functional and structural characteristics of *mob* encoded proteins of the ColE1 plasmid. Given the low solubility and difficulties in purifying the mob proteins, future studies should entail *in vivo* experiments to better understand the function of the ColE1 relaxosome components, MbeA, MbeB, and MbeC. Future research should also focus on the interaction between the ColE1 relaxosome and the conjugative plasmid encoded coupling proteins and transferosome, to further understand how this plasmid is transferred to the recipient cell.

Chapter 6

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