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Analysis of thioredoxin folds in proteins from plasmid F and R27

by

Trevor Charles Elton

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Abstract

F and R27 are conjugative plasmids of enteric bacteria belonging to the IncF and IncHI1 plasmid incompatibility groups, respectively. Two genes of the F transfer region, *traF* and *trbB*, and three genes of the R27 transfer region, *trhF, dsbC* and *htdT,* are predicted to encode periplasmic proteins containing a C-terminal thioredoxin fold. Mating assays using dithiothreitol (DTT) and *dsbA* null mutants indicate that conjugation is sensitive to the cellular redox state. Mutational analysis combined with complementation studies have revealed that $TrbB_F$, $DsbC_{R27}$, and $HtdT_{R27}$ contain a functional C-X-X-C active site in their thioredoxin folds and that they could act as disulfide-bond isomerases. $TrhF_{R27}$, which contains a C-X-X-C motif, and $TraF_F$, which does not, apparently have a function not directly related to disulfide bond formation. Mutational analysis has shown that $TraF_F$ is essential for conjugation and that $TrhF_{R27}$ variants can partially complement a $traF_F$ null mutation.

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Table of Contents

Chapter Page

List of Tables

Table Page

List of Figures

Figure Page

List of Abbreviations

α	alpha
β	beta
γ	gamma
λ	lamda
Δ	delta or deletion
Φ	phi
aa	amino acid
Amp	ampicillin
ANT	anthranilate
AP	alkaline phosphatase
ATP	adenosine triphosphate
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
bp	base pair
BPTI	bovine pancreatic trypsin inhibitor
cag	cytotoxin associated genes
CAR	carbazole
CAT	chloramphenicol acetyltransferase
CD	conserved domain
Cm	chloramphenicol
C-X-X-C	cysteine-any amino acid-any amino acid-cysteine
Da	daltons
DNA	deoxyribonucleic acid
Dsb	disulfide bond
DTT	dithiothreitol
F Factor	fertility factor, also F plasmid
Flm	F leading region maintenance locus
GAPDH	glyceraldehyde-3-phosphate
GFP	green fluorescent protein
GST	glutathione-S-transferase
Hfr	high frequency of recombination
Hig	host inhibition of growth
hok	host killing
Htd	H transfer determinants
ICE	integrative and conjugative element

IM	inner membrane
Inc	incompatibility
IPTG	isopropyl-β-D-thiogalactopyranoside
IS	insertion sequence
kan	kanamycin resistance cassette
Km	kanamycin
kbp	kilobase pair
kDa	kilodaltons
Km	kanamycin
Lac	lactose
LB	Luria-Bertani medium
Lep	leader peptidase
MBP	maltose binding protein
MBSU	Molecular Biology Services Unit
MCS	multiple cloning site
Mep	metallopeptidase
min	minutes
Mpf	mating pair formation
MPS	mating pair stabilization
MQ	milli-q (double distilled and deionized)
NADPH	nicotinamide adenine dinucleotide phosphate
Nal	nalidixic acid
OM	outer membrane
OMP	outer membrane protein
ORF	open reading frame
ori	origin
PBS	phosphate buffered saline
Pef	plasmid-encoded fimbriae
PhoA	alkaline phosphatase
psi	plasmid SOS inhibition
PSSM	position-sensitive scoring matrix
Ptl	*Bordetella pertussis* toxin secretion system
RBS	ribosomal binding site
Rep	replicon
SDS	sodium dodecyl sulfate
sec	seconds

Sm	streptomycin
sok	suppression of killing
Sop	stability of plasmid
Spv	*Salmonella* plasmid virulence
SSC	saline sodium citrate or standard saline citrate
Su	sulphonamide
T4SS	type IV secretion system
Tc	tetracycline
TCA	tricarboxylic acid
Tdi	temperature-dependent instability
Te^R	tellurite resistance
Tm	trimethoprim
Tn	transposon
Tra	transfer
Trb	transfer (Tra subset)
Trh	transfer (incH)
Trw	transfer (incW)
Trx	thioredoxin
Tsg	temperature-sensitive growth
Vir	virulence
XP	5-bromo-4-chloro-3-indolyl phosphate

Chapter 1

General Introduction

Bacterial conjugation is a fundamental mechanism for horizontal gene transfer that facilitates the transmission of genetic material, such as antibiotic resistance and other virulence factors, within and between bacterial species (Wilkins and Frost, 2001). The existence of pathogenic bacterial strains that cannot be treated by standard antibiotic regimens is one of the severe consequences of the spread of antibiotic resistance genes by conjugation. Two well studied conjugative transfer systems are the F plasmid of *Escherichia coli* and the IncHI plasmid, R27, of *Salmonella typhi*.

Although the F plasmid has been studied for nearly 60 years, the process of DNA transfer across the donor cell envelope during conjugation is still poorly understood. The activities of the cytoplasmic transfer proteins that are involved in F DNA regulation and processing are comprehended more thoroughly than the cell envelope transfer proteins that comprise its type IV secretion system (T4SS). Although the DNA regulatory machinery of R27 may vary considerably from the F plasmid, both encode T4SS with established homology to the T-DNA transport system (VirB) of the Ti plasmid of *Agrobacterium tumefaciens* (Lawley *et al.*, 2003). Therefore, homology of several F or R27 transfer proteins to core components of other T4SS at least allows comparison to experiments done in other such plasmids. In contrast, the auxiliary transfer proteins specific to both F- and H-like plasmids are much less well characterized and yet play a vital role in pilus assembly and the unique conjugation requirements of these systems.

In recent years a number of plasmids with transfer operons comparable to F or R27 have been sequenced. The use of bioinformatics has allowed numerous comparisons that provide a starting point for experiments to determine the roles of these transfer proteins. Since the focus of this thesis is on periplasmic components of the F and R27 plasmid trans-envelope complexes, the F- and H-like plasmids as well as their secretion system components will be discussed. Given the predicted roles of the transfer proteins of interest to this thesis, the mechanisms of disulfide bond formation and isomerization will also be presented.

1.1 Classification of the F plasmid

The fertility (F) factor was first described from the observation that sexual recombination can occur in nutritional *E. coli* K-12 mutants through the exchange of genetic material via a cell fusion (Lederberg and Tatum, 1946). Using streptomycin for bacterial strain selection, William Hayes concluded that bacterial mating depended on the continued viability of one parent cell only and consequently that mating was unidirectional (Hayes, 1952). Fertility, the ability to act as a donor, was illustrated to be a genetic trait; the donor strain was denoted as F^+ and the recipient strain as F^-. Although F was demonstrated to integrate in the bacterial chromosome as Hfr (high-frequency of recombination) donors (Cavalli *et al.*, 1953), F was also deduced to be a circular episome or plasmid able to replicate autonomously (Jacob and Wollman, 1958).

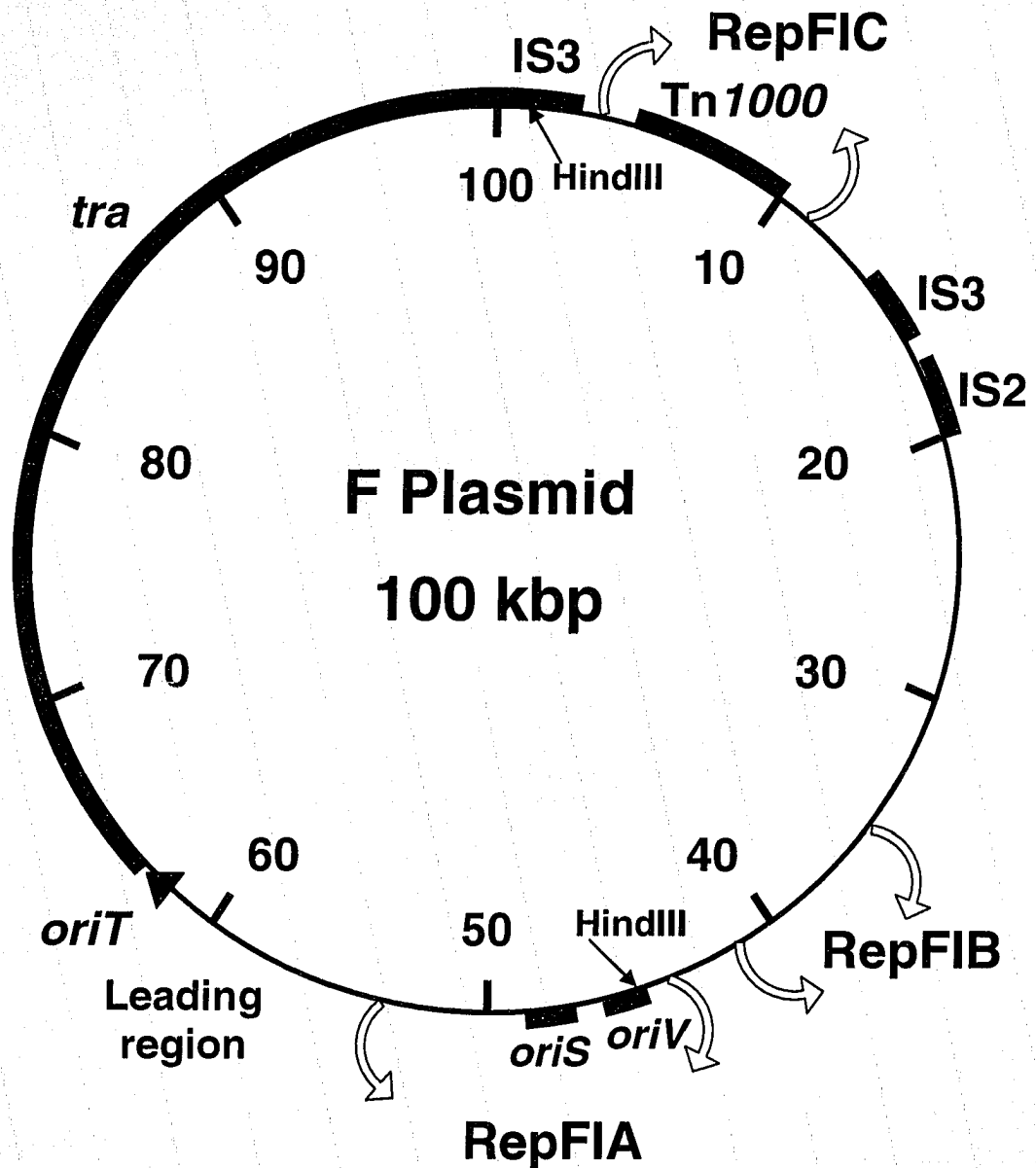
Based on the morphological and serological similarities amongst the sex pili of different gram-negative conjugative plasmids (Lawn *et al.*, 1967), the F plasmid became the model system for a series of similar F-like plasmids. A later classification placed plasmids in the same incompatibility (Inc) group when they cannot stably co-exist in the same host (Datta, 1975). According to this classification, F plasmid belongs to the IncFI group, one of seven or eight sub-divisions of the IncF group. Furthermore, there are now more than 20 other incompatibility groups classified (Manwaring, 2001).

1.1.1 General organization of the F plasmid

The F plasmid is a large circular double-stranded DNA plasmid that is generally independent of the host chromosome (Figure 1.1; Accession number = NC_002483). The main features of the F plasmid include its three replicons, the leading region, the transfer region, and a large region that encodes genes not required for conjugation or plasmid replication (Gubbins *et al.*, 2005; Lawley *et al.*, 2004).

The F plasmid contains three separate replicons: RepFIA, RepFIB and RepFIC. Replication and partitioning of the F factor are respectively controlled through origins of replication (*oriS* and *oriV*) and the *sopABC* partitioning region found in RepFIA. The *oriS* origin supports unidirectional replication and is essential for proper F plasmid maintenance whereas an additional origin, *oriV*, is for bidirectional replication (Lane, 1981). The *sopABC* partitioning system ensures plasmid segregation into daughter cells at division. A centromere, *sopC*, consists of twelve 43 base pair (bp) repeats that are

Figure 1.1. Physical map of the F plasmid. Numbers on the interior of the circle represent size coordinates in kbp; the exact size of the F plasmid is 99 159 bp. Two HindIII restriction endonuclease sites are indicated because this is where the 55 Kbp pOX38 plasmid is derived. The origins of transfer (*oriT*) and replication (*oriS* and *-V*) are indicated in green (a green arrow is used for *oriT* to note the direction of transfer), the transfer region (*tra*) is designated in red, and insertional elements (IS2, IS3, and Tn*1000*) are shown in blue. The locations of the replicons (RepFIA, -B, and –C) are marked by the broad white arrows. The leading region is also noted in the bottom left hand side.



bound by the SopB protein in an interaction thought to pair or handcuff F plasmids (Hanai *et al.*, 1996; Mori *et al.*, 1986). The formation of this nucleoprotein partition complex is required for the stable maintenance of the low-copy F plasmid in *E. coli* (Biek and Shi, 1994). Fluorescence *in situ* hybridization experiments revealed that F plasmid derivatives carrying the *sopABC* region have a mid-cell location in new daughter cells but later migrate to the quarter-cell position, whereas F plasmid derivatives without the *sopABC* region are randomly distributed in the cell (Niki and Hiraga, 1997). The polar location of SopB, as visualized by fluorescence-phase microscopy, suggests that its localization forces F plasmid partitioning (Kim and Wang, 1998). Both the replication and partitioning mechanisms ensure that the F plasmid is maintained at 1 to 2 copies per cell (Collins and Pritchard, 1973; Frame and Bishop, 1971).

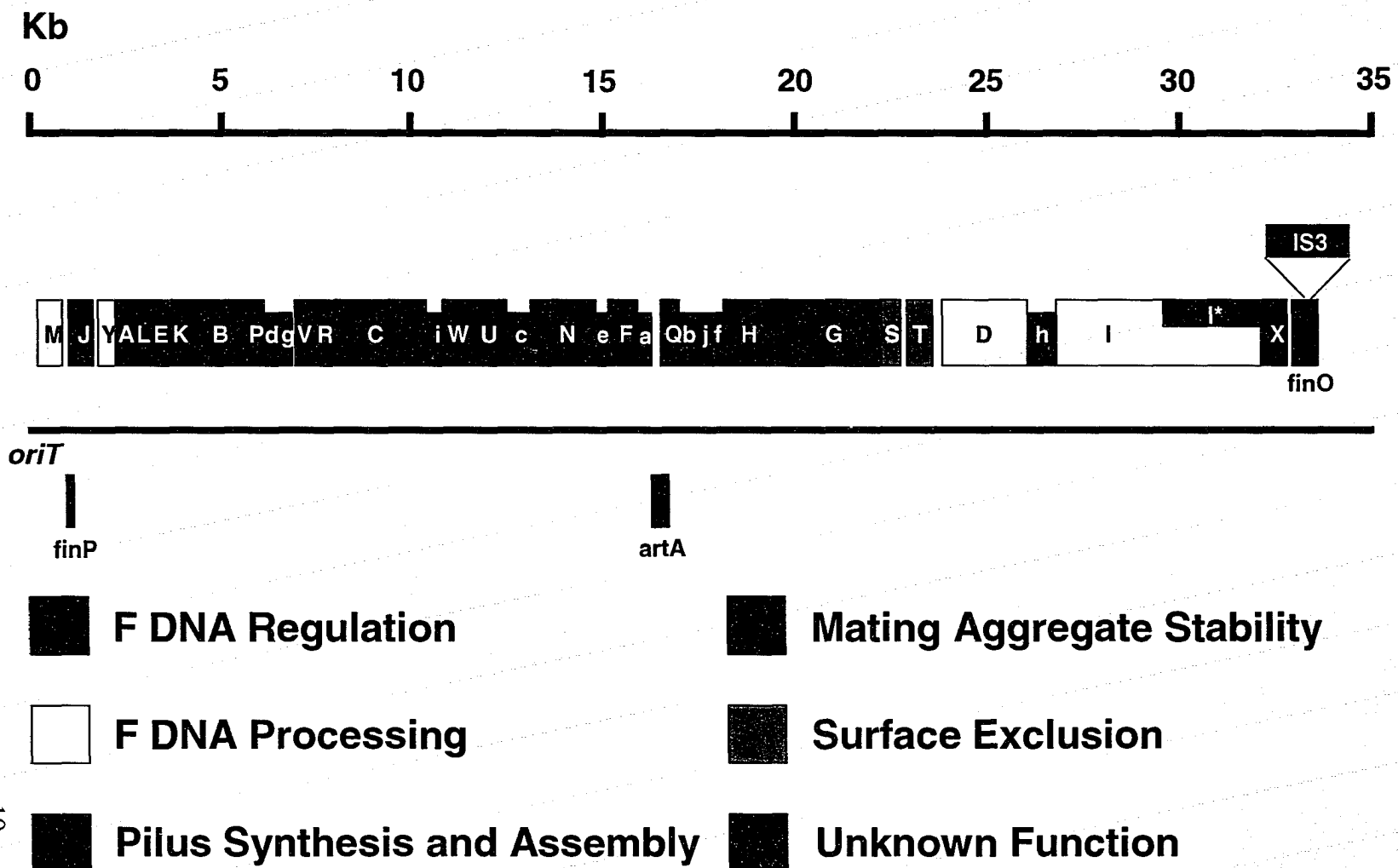
The 13.2 kbp leading region is the first segment of F plasmid DNA to penetrate the recipient cell. It extends from *oriT*, a special origin of transfer for conjugation, to the adjoining RepFIA replication region (Ray and Skurray, 1983). The considerable sequence conservation of this region amongst related plasmids has suggested that it encodes a number of genes important for plasmid establishment and maintenance in the recipient cell (Manwaring *et al.*, 1999). The first gene of the leading region, *orf169*, is believed to encode a transglycosylase capable of degrading the peptidoglycan layer (Bayer *et al.*, 1995). Mutations in *orf169* do not affect F plasmid transfer (Loh *et al.*, 1989), but mutations in the homologous *virB1* exhibit severely attenuated virulence (Berger and Christie, 1994). Such an enzyme could aid in DNA transfer through the

recipient cell envelope or in the establishment of the T4SS through the peptidoglycan. Other important genes in the leading region include *psiB*, *ssb*, and the *flmABC* locus. The F leading region maintenance locus (*flm*) encodes a post-segregational killing mechanism, which is regulated by antisense RNA and prevents the generation of F plasmid-free cells (Gerdes *et al.*, 1997). The *flm* locus shares 95% sequence identity to another series of post-segregational killer genes known as the *hok/sok* locus of plasmid R1 (Gerdes *et al.*, 1990). The plasmid SOS response inhibitor (PsiB) and the single stranded DNA binding protein (Ssb) are only transiently expressed during recipient cell DNA transfer (Bailone *et al.*, 1988; Kolodkin *et al.*, 1983). Their role could include protecting the transferred single-stranded plasmid in the recipient cell by arresting the SOS response until complementary strand DNA synthesis can occur.

The 33.3-kb F plasmid transfer region encodes for all the proteins needed for conjugation (Figure 1.2). Three main transcripts are produced from *traM*, *traJ*, and the multi-cistronic operon controlled by the major P_Y promoter; two minor transcripts are produced from *finP* and *artA*. TraJ positively controls expression of the transfer region from the P_Y promoter (Willetts, 1977), whereas FinP negatively regulates TraJ expression in the presence of FinO (van Biesen *et al.*, 1993). However, the *finO* gene is disrupted by an insertional element, and consequently *traJ* is constitutively expressed (Yoshioka *et al.*, 1987). With the exception of some regulatory factors, most of the transfer genes involved in functions such as DNA metabolism, pilus assembly, mating aggregate stability, and surface exclusion are transcribed on a single mRNA (Willetts, 1977).

Figure 1.2. Linear map of the F transfer region. The upper line indicates the length of the fragment (33 Kbp). Genes are presented as coloured boxes so as to indicate their general function: F DNA regulation (pink), F DNA processing (yellow), pilus synthesis and assembly (blue), mating aggregate stability (red), surface exclusion (orange), and unknown function (violet). The capital letters refer to *tra* genes while the lowercase letters refer to *trb* genes. Transcripts found in the anti-*tra* orientation are placed below the central black line. The site of the IS3 insertion is shown in black.

Linear Map of the F Transfer Region



1.1.2 Other F-like plasmids

Unlike the relatively innocuous F plasmid, several of the F-like plasmids are known to carry antibiotic resistance determinants or other virulence factors. For the purposes of this thesis, the plasmids mentioned below will be referred to as F-like because this study is based primarily upon the organization of the transfer operons and the degree of amino acid similarity amongst the conjugative protein homologues. The most recently sequenced F-like plasmids include: R100 (Anthony *et al.*, 1999), pED208 (Lu *et al.*, 2002), pSLT (McClelland *et al.*, 2001), and pYJ016 (Chen *et al.*, 2003).

The R100 (100 kbp) conjugative plasmid is from the IncFII incompatibility group and carries multiple drug resistance (Womble and Rownd, 1988; Accession number = NC_002134). More commonly, the derepressed mutant, R100-1, is used for experimentation. Although F and R100-1 are closely related, R100-1 varies from F in several important ways: its pili are serologically distinct, R100-1$^+$ cells exhibit reduced sensitivity to F-specific phages, R100-1$^+$ cells do not require the outer membrane protein, OmpA, in the recipient cell envelope for efficient conjugation, and R100-1 plasmids specify different surface and entry exclusion systems (Anthony *et al.*, 1999). Although the organization of the R100 *oriT* region is similar to that of F (Finlay *et al.*, 1986), plasmid specificity can be delineated by the ability of the DNA metabolism proteins, TraI, -M, and -Y, to bind at their cognate sites within *oriT* of their respective systems (Fekete and Frost, 2000).

pED208 (90 kbp) is a transfer-derepressed mutant of the IncFV plasmid F_O *lac* (Finlay *et al.*, 1986), which was originally identified in *Salmonella typhi* (Falkow and Baron, 1962). The organization of pED208 transfer region is quite similar to the F transfer operon although most pED208 *tra* proteins share only approximately 45% amino acid identity (Accession number = AF411480; Lu *et al.*, 2002). All required F transfer proteins have homologues within the pED208 transfer operon except for the F-pilin chaperone, TraQ (Lu *et al.*, 2002). Interestingly, pED208 is multi-piliated (approximately 20 pili per cell) (Armstrong *et al.*, 1980) due to an IS2 element situated in its *traY* gene, whereas the other derepressed F-like plasmids usually assemble 1 to 2 pili (Frost et al., 1985).

Most *Salmonella typhimurium* isolates (88%) carry a 90 kbp plasmid known as pSLT (Accession number = NC_003277; Ahmer *et al.*, 1999). Its ability to self-mobilize has been attributed to the numerous homologues of F *tra* genes. In addition, pSLT contains an 8 kbp region known as the *Salmonella* plasmid virulence (*spv*) locus (Guiney *et al.*, 1994). This virulence region is believed to enhance bacterial growth during the systemic phase of typhoid-like disease in mice (Gulig *et al.*, 1993). An additional locus, the plasmid-encoded fimbriae (*pef*) region, is associated with bacterial adhesion to intestinal epithelial cells (Baumler *et al.*, 1998).

pYJ016 is a 48.5 kbp plasmid isolated from *Vibrio vulnificus*, a causative agent of severe seafood-related infections in humans (Chen *et al.*, 2003). This plasmid contains several

conjugative genes (*traA, -B, -C, -D, -E, -F, -G, -H, -I, -K, -L, -N, -U, -W, -Y, trbB, -C*) that are similar to the F plasmid in terms of sequence and operon organization. However, no homologues to *traJ, -M, -P, -Q, -V,* or *traX* have yet been identified on pYJ016. Despite these differences, pYJ016 has been shown to conjugate with other vibrio strains (Chen *et al.*, 2003).

1.2 IncH Plasmids.

The persistence of typhoid fever can be greatly attributed to the appearance of multiple antibiotic-resistant *Salmonella typhi* (Sherburne *et al.*, 2000). This antibiotic resistance (chloramphenicol, ampicillin, trimethoprim, sulfonamides, and tetracycline) is often plasmid-mediated and in these cases is always encoded by plasmids of incompatibility group H (IncH) (Rowe *et al.*, 1990). Plasmids of the H incompatibility group (IncH) were first characterized after an outbreak of chloramphenicol-resistant typhoid fever in Mexico in 1972 (Anderson and Smith, 1972). Since then, *Salmonella typhi* containing IncHI plasmids have caused epidemics in India, Vietnam, and Thailand (Smith, 1974).

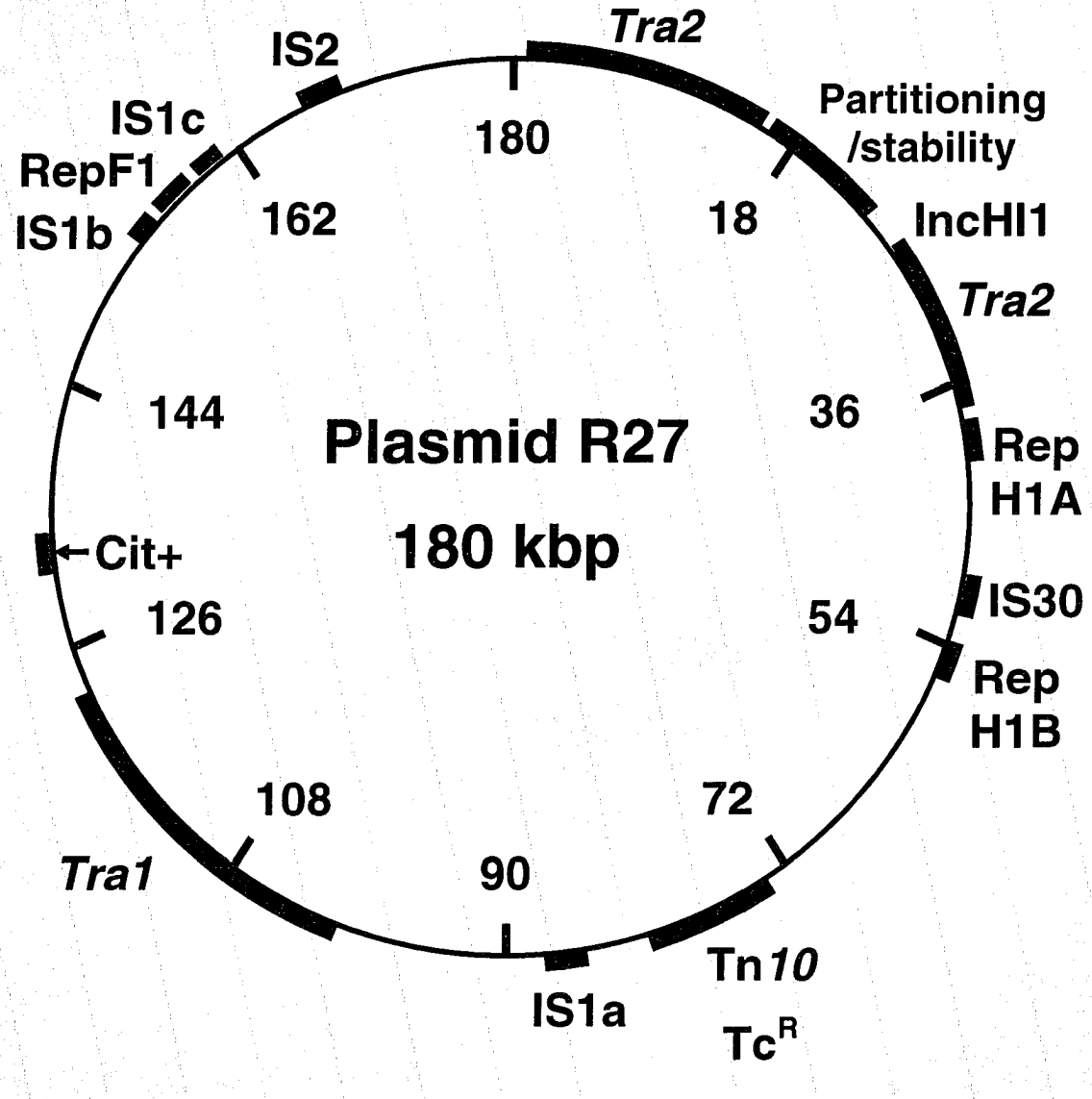
The large (>150 kbp), conjugative H plasmids can be divided into two subgroups: HI and HII. The IncHI group was further subdivided into HI1 to HI3 when restriction enzyme patterns from representative members of these different subgroups indicated limited DNA homology (Whiteley and Taylor, 1983). However, analysis of the complete sequences of HI1 plasmids R27 and pHCM1 with HI2 plasmid R478 revealed a high degree of sequence similarity (Gilmour *et al.*, 2004). The validity of the IncHI

subgrouping is verified in terms of their incompatibility classification. For example, the one-way incompatibility observed between F and HI1 plasmids is a result of a RepFIB replicon in F and HI1 plasmids but not HI2 plasmids (Taylor *et al.*, 1985). Most IncHI plasmids are low copy number plasmids that transfer at low levels, carry antibiotic resistance cassettes, and are found in human pathogens (Taylor, 1989). Also, the thermosensitive nature of conjugation in IncHI1 plasmids is illustrated by its optimal DNA transfer between 22 and 30°C and negligible transfer at 37°C (Taylor and Levine, 1980). These plasmids also exhibit a slow pilus polymerization or maturation (20 min) (Maher *et al.*, 1993) when compared to the quick F pilus growth rate (15 sec) (Novotny *et al.*, 1969). The transfer operons of IncHI1 plasmids are evolutionarily related to the IncP-like DNA and pilin processing systems, whereas the T4SS is of the IncF lineage (Lawley *et al.*, 2003). The IncHII group plasmids are compatible with IncHI plasmids but appear to transfer without any thermosensitivity and at higher levels than IncHI plasmids (Bradley *et al.*, 1982).

1.2.1 The IncH1 plasmid R27

R27 is a large (180 kb), conjugative plasmid often associated with *S. typhi* but capable of transfer between members of the *Enterobacteriaceae* (Figure 1.3; Maher and Taylor, 1993). R27 contains two replicons, RepHI1A or RepHI1B, which are required for proper maintenance of the plasmid (Gabant *et al.*, 1993) although the RepHI1A replicon can independently support plasmid replication (Newnham and Taylor, 1994). An additional partial IncFIA replicon, RepFIA, is implicated in the one-way incompatibility with the F

Figure 1.3. Physical map of the R27 plasmid. Numbers on the interior of the circle represent size coordinates in kbp; the exact size of plasmid R27 is 180 461 bp. The two distinct regions responsible for conjugative transfer are designated Tra1 and Tra2 (red). The Tra2 region is interrupted by a segment coding for plasmid partitioning and stability (black), as well as a segment responsible for IncH incompatibility (yellow). R27 contains two IncHI1-specific replicons and a partial IncFIA replicon, which are denoted in green. The insertional elements (IS1a, -b, -c, IS2, and IS30) are shown in blue while the Tn*10* transposon is shown in pink since it encodes the tetracycline resistance determinant of R27. A citrate utilization determinant is depicted in orange.



plasmid (Gabant *et al.*, 1993). R27 contains two distinct transfer regions, Tra1 and Tra2, which are required for DNA transfer as determined by restriction analysis and transposon mutagenesis (Taylor *et al.*, 1985). The Tra1 region contains the origin of transfer and encodes many of the genes involved in DNA processing (Lawley *et al.*, 2002). Furthermore, in the opposite orientation, there are three Mpf genes with homologies to the F-like plasmids (*trhF*, *trhH* and *trhG*) as well as a *dsbC* gene. The Tra2 region encodes genes required for pilus assembly and mating pair formation (Taylor *et al.*, 1999). The organization of the Tra2 region more closely resembles the F plasmid transfer region, and the similarity amongst their gene products implies that the R27 Tra2 region is ancestrally related to the F *tra* region (Rooker *et al.*, 1999). Unlike the F transfer region, the Tra2 region is interrupted by an intergenic region involved in IncHI1 incompatibility, a series of partitioning genes, and a conjugation regulator, *htdA* (Gabant *et al.*, 1993; Whelan *et al.*, 1994). R27 also carries a citrate utilization determinant (Taylor and Brose, 1986) and a tetracycline resistance determinant that is encoded by a resident Tn*10* transposon (Sherburne *et al.*, 2000). A similar but larger IncHI1 resistance plasmid, pHCM1 (218 kbp), has also been sequenced (Parkhill *et al.*, 2001). Despite the fact that pHCM1 was isolated more than 30 years after R27, these two plasmids bear approximately 99% sequence identity over a 168 kbp segment (Parkhill *et al.*, 2001). The plasmid size difference is attributed to additional drug resistance cassettes acquired by pHCM1 (Gilmour *et al.*, 2004).

1.2.2 Other H-like plasmids

The sequencing of several plasmids and conjugative elements in the last few years has revealed transfer regions with conservation, both within the individual genes and in the overall gene order, to R27. These conjugative elements include: R478 (Gilmour *et al.*, 2004), pNL1 (Romine *et al.*, 1999), R391 (Böltner *et al.*, 2002), SXT (Beaber *et al.*, 2002), Rts1 (Murata *et al.*, 2002) and pCAR1 (Maeda *et al.*, 2003). It is important to note that these H-like type IV secretion systems, which carry antibiotic resistance cassettes and numerous catabolic and degradative enzymes, are found in a variety of gram-negative bacteria from oceans to subsurface sediments across the world.

R478 is a large, conjugative plasmid that was isolated from *Serratia marcescens* (Medeiros and O'Brien, 1969). R478 was originally classified as an IncS plasmid but was later re-classifed as an IncHI2 plasmid based on entry exclusion and incompatibility studies (Taylor and Grant, 1977). The recent sequencing of this 274 kbp plasmid has revealed that approximately 49% of its 295 predicted genes are highly similar to those of R27 and pHCM1 (Gilmour *et al.*, 2004). It is established that these three plasmids (R27, pHCM1 and R478) share common core determinants, and it is speculated that their plasmid evolution arose from the gain or loss of genes from this backbone (Gilmour *et al.*, 2004). The medical significance of this plasmid is seen in its resistance to tetracycline, chloramphenicol, kanamycin, and several heavy metals. Notably, R478 also contains a tellurite resistance region (*ter*)(Te^R) (Whelan *et al.*, 1995), which is a

characteristic marker of all IncHII plasmids and all IncHI2 plasmids except R476b (Hou and Taylor, 1994; Walter and Taylor, 1992).

pNL1 has recently been identified as a plasmid of *Sphingomonas aromaticivorans* F199, a strain isolated from deep subsurface sediments in South Carolina (Balkwill, 1988). The sequencing of this 184 kbp plasmid has led researchers to predict that over 42% of its open reading frames (ORFs) are involved in the catabolism or transport of aromatic compounds (Romine *et al.*, 1999). Such ORFs are localized to 15 aromatic catabolic gene clusters, which contain genes known to encode enzymes associated with the degradation of biphenyl, naphthalene, *m*-xylene, and *p*-cresol (Romine *et al.*, 1999). Several resolvase, excisionase and intron-associated maturase genes are found in the replication region of pNL1, suggesting that this plasmid could be integrated into or excised from the chromosome (Romine *et al.*, 1999). pNL1 can be mobilized to other *Sphingomonas* species, and several ORFs, localized in 3 gene clusters, are predicted to be associated with conjugative transfer (Romine *et al.*, 1999).

SXT is an integrative and conjugative element (ICE) that carries multiple antibiotic resistance cassettes (Su^r Tm^r Sm^r) (Waldor *et al.*, 1996). ICEs differ from conjugative plasmids in that they lack an autonomously replicating extrachromosomal form (Burrus *et al.*, 2002). However, a nonreplicative extrachromosomal form of SXT, which is dependent on the site-specific recombinase, Int, is believed to be required for DNA transfer (Hochhut and Waldor, 1999). This element was first detected in the O139

serogroup of *Vibrio cholerae*, the causative agent of the severe diarrheal disease cholera, but has now been isolated from nearly all clinical *V. cholerae* isolates in India (Hochhut *et al.*, 2001). The complete 100 kbp sequence of SXT has revealed a chimera of transposon-related antibiotic resistance genes dispersed among a variety of plasmid- and phage-related genes (Beaber *et al.*, 2002). The SXT genes involved in conjugative transfer, as determined by one-step chromosomal gene inactivation, resemble the F-type IV secretion genes of R27 (Beaber *et al.*, 2002). The regulators of DNA transfer are encoded on two SXT loci, designated *setC* and *setD*, whose gene products show homology to the flagellar regulators FlhC and FlhD, respectively (Beaber *et al.*, 2002). SXT can be transferred via conjugation to a variety of gram-negative organisms, and it can mediate the mobilization of some plasmids in an Hfr-dependent manner (Hochhut *et al.*, 2000).

R391, an 89 kbp ICE, is the prototype of the conjugative IncJ incompatibility group (Böltner *et al.*, 2002). R391 was originally isolated from a South African *Providencia rettgeri* strain and was believed to be a plasmid bearing kanamycin resistance (Coetzee *et al.*, 1972). R391 has subsequently been shown to be integrated into the bacterial chromosome by employing a phage-like integration mechanism (Böltner *et al.*, 2002). Other similar IncJ elements have been isolated from other parts of the world: *Vibrio cholerae* (pJY1; phenotype, Cm^r Sm^r Su^r) from the Philippines (Yokota and Kuwahara, 1972), *Proteus vulgaris* (R997; phenotype, Ap^r Sm^r Su^r) from India (Matthew *et al.*, 1979), and *Shewanella putrefaciens* (pMERPH; mercury resistance) from the United

Kingdom (Peters *et al.*, 1991). R391 contains four conjugative gene clusters that share strong sequence similarity with SXT (Böltner *et al.*, 2002).

Rts1, a 217 kbp conjugative plasmid, is the prototype for the IncT incompatibility group (Coetzee *et al.*, 1972). Rts1 was originally isolated from a *Proteus vulgaris* strain and was determined to be a low-copy number kanamycin resistance plasmid (Terawaki *et al.*, 1967). This large plasmid regulates its replication (DiJoseph *et al.*, 1973), conjugative transfer (Terawaki *et al.*, 1967) and host cell growth (Terawaki *et al.*, 1968) in a temperature-sensitive fashion. Replication of Rts1 and its host cell growth are stable at 37°C but are inhibited at 42°C, whereas conjugation is efficient at 25°C but not at 37°C. The *tsg* (temperature-sensitive growth) and *hig* (host inhibition of growth) loci are responsible for the temperature-sensitive effect on host cell growth. An AT-rich DNA sequence within the *tsg* locus rather than a gene product is thought to be responsible for this phenotype (Mochida *et al.*, 1991). The *hig* locus encodes the HigB toxin and the HigA antitoxin proteins that mediate postsegregation killing at the non-permissive temperature (Tian *et al.*, 2001). Another locus, *tdi* (temperature-dependent instability), is believed to be responsible for the temperature sensitivity of Rts1 replication although this system remains to be characterized (Okawa *et al.*, 1985). Furthermore, no molecular mechanism has been identified that can be attributed to the thermosensitive nature of Rts1 conjugation. However, the complete genomic sequence of Rts1 has revealed the organization of this conjugation system, whose genes are clustered in two distinct

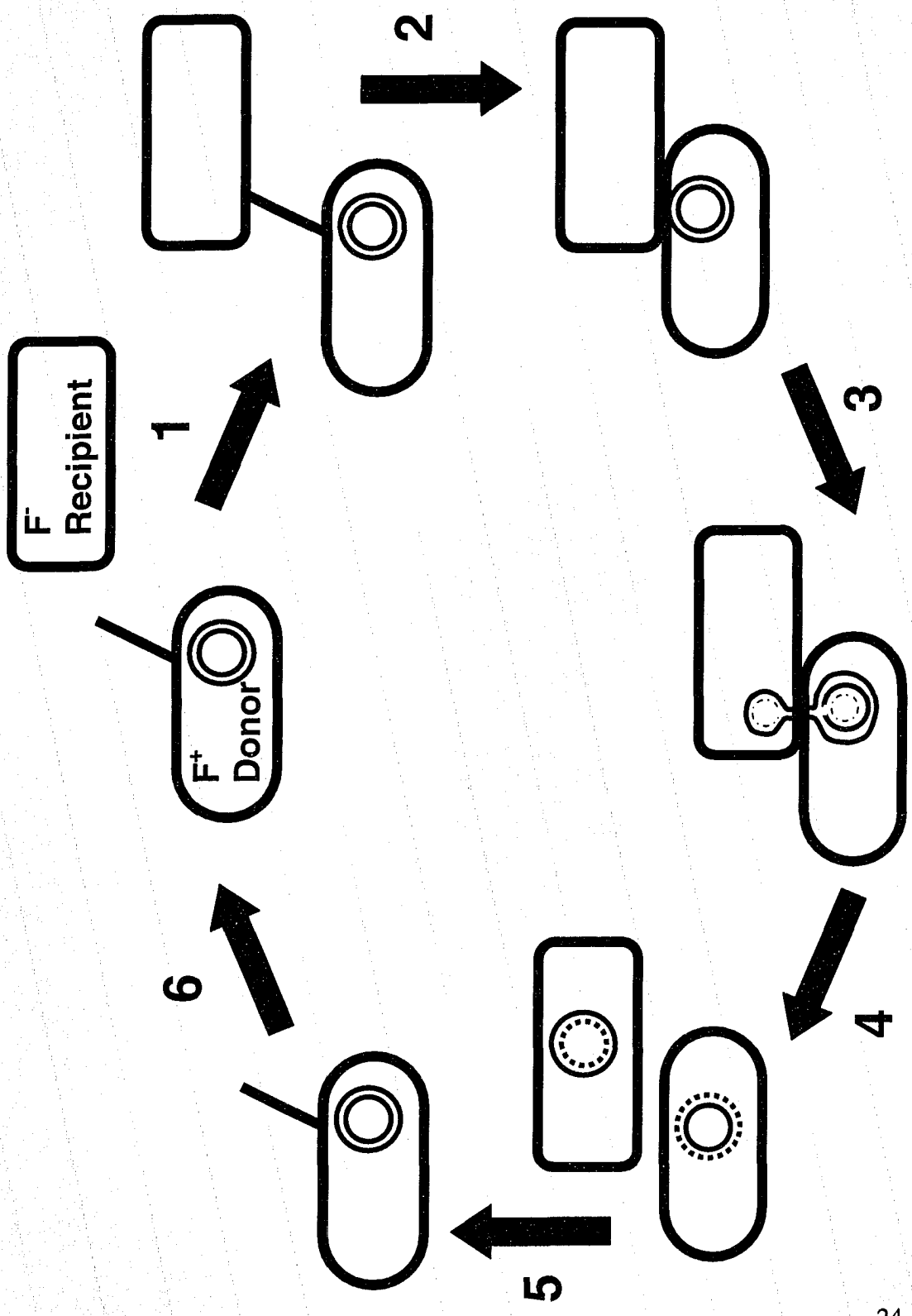
transfer regions (Murata *et al.*, 2002) and show similarity to the transfer genes of plasmid R27.

pCAR1 is a 199 kbp conjugative plasmid isolated from *Pseudomonas resinovorans* that belongs to the IncP-7 incompatibility group (Maeda *et al.*, 2003; Dennis, 2005). This plasmid offers potential for bioremediation of dioxin contamination and much work has been done to elucidate the function of its *car* and *ant* degradative operons. Carbazole (CAR), a mutagenic and toxic pollutant derived from coal tar, can be utilized as the sole carbon and nitrogen source by *Pseudomonas* strains containing pCAR1 (Ouchiyama *et al.*, 1993). The *car* operon of pCAR1 is responsible for the degradation of CAR and other dioxins to anthranilate (Habe *et al.*, 2001; Ouchiyama *et al.*, 1993). Subsequently, anthranilate can be processed to intermediates of the TCA cycles by the gene products of the *ant* operon (Nojiri *et al.*, 2001). In addition to this catabolic system, a potential tellurite resistance cassette, the *klaABC* locus, has been identified (Maeda *et al.*, 2003). Although DNA transfer of pCAR1 has not been demonstrated, numerous predicted gene products show homology (20-45% identity) to the Trh proteins of plasmid R27, suggesting that pCAR1 has conjugative capabilities (Maeda *et al.*, 2003).

1.3 Mechanism of F conjugation

Bacterial conjugation is a fundamental mechanism for horizontal gene transfer that facilitates the transmission of genetic material, such as antibiotic resistance and other virulence factors, within and between bacterial species (Figure 1.4; Gubbins *et al.*, 2005;

Figure 1.4. Schematic diagram of the F conjugation cycle. The recipient cell is represented as a rounded rectangle while the donor cell is represented as an oval. Plasmid DNA is indicated by the red circles, and the F pilus is signified as the blue line extending from donor cells. The basic steps are as follows: 1) pilus binding to the recipient cell, 2) pilus retraction and initial cell contact, 3) mating pair stabilization and DNA transfer initiation, 4) mating pair disaggregation, 5) second strand DNA synthesis in both cells, and 6) recipient cell becomes a donor cell and the process can repeat with a new recipient cell.



Wilkins and Frost, 2001). It involves the transfer of single-stranded DNA from a donor to a recipient cell that has established close contact via mating pair formation (Mpf). Although the F plasmid of *E. coli* remains a paradigm for this process, conjugative plasmids have been reported in a variety of species, including many bacterial pathogens (Lawley *et al.*, 2003). More recently, conjugation has been considered to belong to the type IV secretion family based on the similarities between the proteins involved in both processes (Cascales and Christie, 2003).

Mpf involves a complex apparatus spanning the donor cell envelope that assembles the conjugative pilus. This filamentous appendage interacts with the recipient cell and retracts by depolymerization into the donor cell thereby allowing intimate wall-to-wall contact during mating pair stabilization (MPS) (Achtman *et al.*, 1977). Without the intimate contact between the mating cells, conjugation would be easily confused with competence, the process of importing extracellular DNA. Achtman (1975) ascertained that DNA transfer is initiated by Mpf. A mating signal triggers the nicking reaction at *oriT* so that a single strand of 5' to 3' DNA can be transported into the recipient cell (Ihler and Rupp, 1969). However, the mechanism by which the conjugative plasmid is transferred across both bacterial envelopes remains poorly understood (Cascales and Christie, 2004). Once transfer has occurred, chromosomally-derived enzymes would then be responsible for the re-circularization and second-strand DNA synthesis necessary to reform the complete F plasmid (Kingsman and Willetts, 1978). Consequently, there are now two F^+ cells capable of repeating this process.

1.4 Conserved F and H transfer protein of the type IV secretion system

The type IV secretion systems are protein complexes that span the bacterial cell envelope and mediate the translocation of DNA, protein substrates, or virulence factors into recipient or host cells (Lawley *et al*, 2003). The gram-negative and -positive bacterial conjugation systems are included in this secretion family along with other macromolecular transfer complexes that deliver virulence factors to eukaryotic cells (Ding *et al.*, 2003). For instance, *Agrobacterium tumefaciens*, the causative agent of Crown gall disease, is able to transfer oncogenic T-DNA and effector molecules to plant cell nuclei (Christie, 1997), whereas *Helicobacter pylori*, a causative agent of peptic ulcers, uses a related system for the translocation of its CagA protein to mammalian cells (Odenbreit *et al.*, 2000). Despite the variance in application, several homologous core components comprise the backbone of these T4SS. The F *tra* and R27 *trh* region encodes eight of the 10 highly conserved (core) gene products of T4SS including Tra/Trh A, -L, -E, -K, -B, -V, -C, and -G (N-terminal region), which correspond to VirB2, -B3, -B5, -B9, -B10, -B7, -B4, and -B6 respectively, of the *A. tumefaciens* T4SS exemplified by the Ti plasmid (Table 1.1; Lawley *et al.*, 2003).

1.4.1 F- and H-like propilin processing

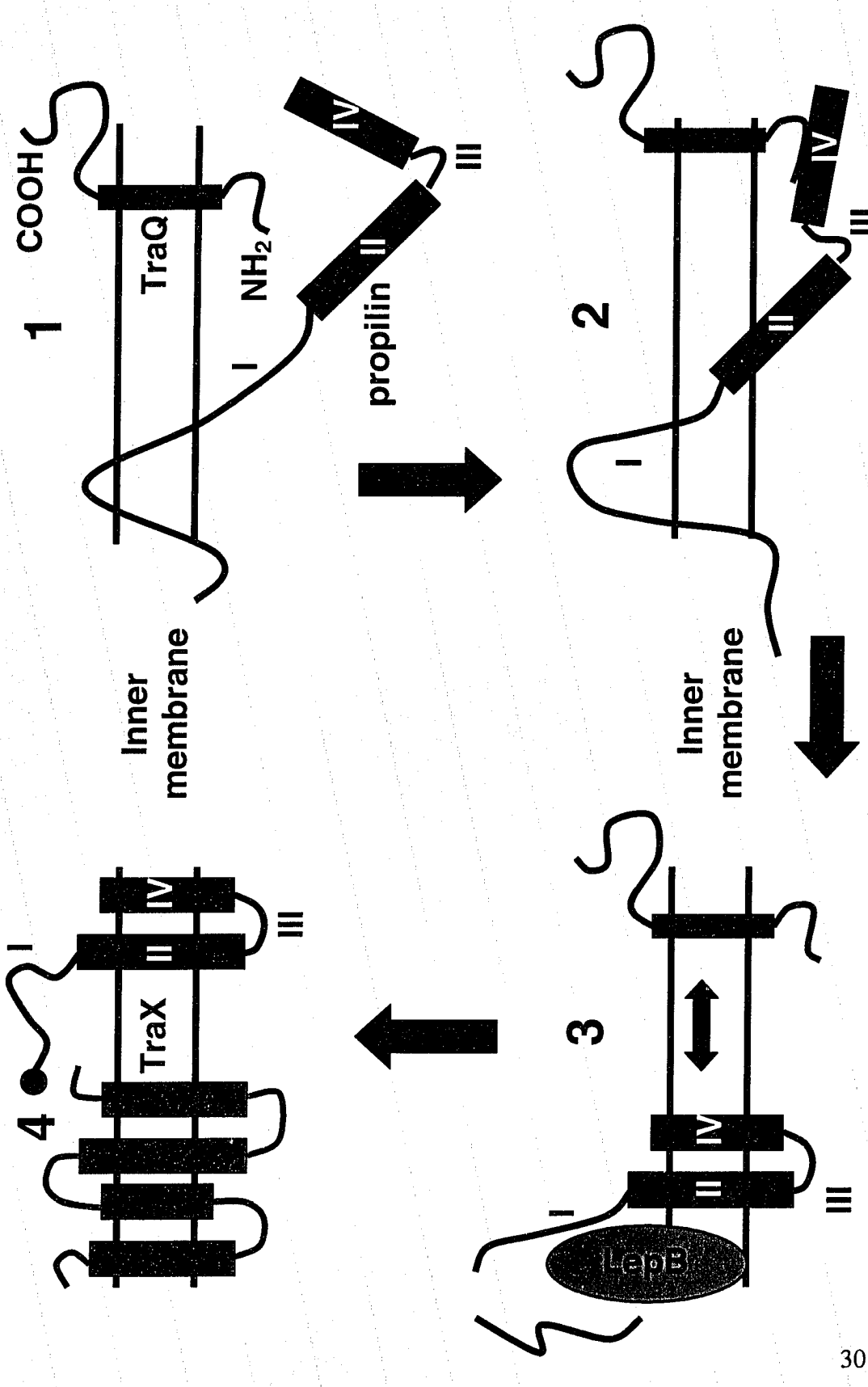
The propilin subunits from F- and H-like T4SS have similar sizes of 112 to 128 aa although the pilin subunits themselves vary drastically in sequence. Sequence analysis predicted $TraA_F$ to be a 12.8 kDa propilin protein with an unusually long 51 amino acids signal sequence (Frost *et al.*, 1984). Signal peptidase cleavage would result in a 70 aa

Table 1.1 Similarities amongst Type IV secretion systems based upon sequence homology and functional equivalence

Plasmid Group	IncF (F)	IncH (R27)	IncP (RP4)	IncW (R388)	IncN (pKM101)	VirB (Ti)	Ptl	LvhB
Proteins	TraA	TrhA	TrbC	TrwL	TraM	VirB2	PtlA	LvhB2
	TraL	TrhL	TrbD	TrwM	TraA	VirB3	PtlB	LvhB3
	TraC	TrhC	TrbE	TrwK	TraB	VirB4	PtlC	LvhB4
	TraE	TrhE	TrbJ	TrwJ	TraC	VirB5		LvhB5
	TraG	TrhG	TrbL	TrwI	TraD	VirB6	PtlD	LvhB6
	TraV	TrhV	TrbK	TrwH	TraN	VirB7	PtlI	LvhB7
			TrbF	TrwG	TraE	VirB8	PtlE	LvhB8
	TraK	TrhK	TrbG	TrwF	TraO	VirB9	PtlF	LvhB9
	TraB	TrhB	TrbI	TrwE	TraF	VirB10	PtlG	LvhB10
			TrbB	TrwD	TraG	VirB11	PtlH	LvhB11

long polypeptide whose molecular weight at 7.2 kDa corresponds closely to F pilin (Frost *et al.*, 1984). Removal of the signal sequence itself is a function of the host leader peptidase, LepB (Figure 1.5; Majdalani *et al.*, 1996). However, $traA_F$ alone in *E. coli* cannot produce mature F pilin, but requires the chaperone-like inner membrane protein, $TraQ_F$ (Figure 1.5; Moore *et al.*, 1982), which is only expressed by plasmids closely related to F. The hydropathy profile of F-pilin predicted its structure to be an integral membrane protein with two membrane-traversing domains (Frost *et al.*, 1994), while the N- and C-termini would be positioned in the periplasm. Circular dichroism studies indicate that F-pilin is largely α-helical in character (Armstrong *et al.*, 1980). TraA-PhoA fusion proteins determined that the hydrophobic C-terminal end of $TraA_F$, Domain IV, was necessary for preventing misfolding or rapid aggregation of the other three domains of $TraA_F$ (Paiva and Silverman, 1996). Yeast two-hybrid analysis confirmed that Domain IV of $TraA_F$ binds to $TraQ_F$, which suggested that $TraQ_F$ could prevent $TraA_F$ misfolding and subsequent degradation as well as assisting its entry into the membrane (Figure 1.5; Harris *et al.*, 1999). However, the expression of $TraA_F$, $TraQ_F$ and LepB alone cannot produce the wild-type acetylated F pilin polypeptide. Two monoclonal antibodies were used to determine the acetylating transfer protein: JEL92, which recognizes an internal pilin epitope, and JEL93, which recognizes the acetylated N-terminal pilin epitope (Frost *et al.*, 1986). Western blot analysis of individual Hfr strain deletion mutants demonstrated that $TraX_F$ was necessary to acetylate the pilin (Figure 1.5; Maneewannakul *et al.*, 1995; Moore *et al.*, 1993); however no direct function of this additional processing has been assigned.

Figure 1.5. Schematic diagram of F-pilin processing in the inner membrane. This figure is adapted from Harris *et al* (1999). TraQ is shown in red with its C-terminus in the periplasm and its N-terminus in the cytoplasm. Propilin is shown in blue as four domains entirely in the cytoplasm. Both proteins have boxed portions that signify potential transmembrane domains. (1) The signal sequence of propilin permits its passage into the inner membrane. (2) Domain IV of propilin interacts with the N-terminus of TraQ so as to stabilize its structure. (3) Domains II and IV of propilin are inserted into the inner membrane while the signal sequence adjacent to the periplasmic domain I is cleaved by LepB. (4) TraX facilitates acetylation (green circle) of the N-terminal end of pilin to complete its maturation.



H-like pilin subunits are believed to undergo an alternative processing reaction similar to the C-terminal cleavage and subsequent cyclization of RP4 pilin (IncP) by the peptidase/cyclase $TraF_P$ (Eisenbrandt *et al.*, 1999). The H-like propilin subunits from R27, Rts1, R391, SXT and pNL1 share greater similarity with these P-like pilins (Lawley *et al.*, 2003); furthermore, these H-like transfer regions also encode a putative peptidase/cyclase, $TraF_P$.

1.4.2 F- and H-like core transfer proteins of the type IV secretion system

The remaining 7 core transfer proteins expressed from the F plasmid transfer region are implicated in pilus synthesis. Presumably, these products interact with one another to remove F pilin subunits from the inner membrane, polymerize them, and extend the F pilus from the cell surface. Point mutations in *traL, -E, -K, -B,- V,- C* and the N-terminal portion of *traG* prevent F-pilus synthesis and DNA transfer, but have no apparent effect on the maturation of F pilin (Moore *et al.*, 1981). Therefore, the products of these genes are probably involved in the assembly of the pilus from its pilin subunits (Laine, 1985; Moore, 1981). These F proteins have been further subdivided into proteins involved in pilus tip formation (TraL, -E, -K, -C, -G) or in pilus extension (TraV and TraB) as measured by the M13K07 transducing phage assay (Anthony *et al.*, 1999).

$TraC_F$, a 99 kDa transfer protein, is homologous with the 87 kDa VirB4 protein (Taylor *et al.*, 1999). Homologues of this protein group contain Walker A and B motifs that

provide energy for pilus assembly (Cao and Saier, 2001; Rabel *et al.*, 2003). $TraC_F$ is known as a pilus assembly protein because F*lactraC* amber mutants are resistant to RNA and DNA F-specific bacteriophages yet are still capable of processing F propilin (Maneewannakul *et al.*, 1987). Later, a temperature-sensitive mutation, F*lacprotraC1044,* was shown to differ from these nonsense *traC*$_F$ mutations by permitting filamentous bacteriophage infection and by forming mating aggregates with normal recipient cells while still preventing pilus formation (Schandel *et al.*, 1987). A more sensitive assay, the M13K07 technique, confirmed the *traC1044* mutation to be completely sensitive to this phage (Anthony *et al.*, 1999). Consequently, the pilus tip is thought to be formed at the cell surface prior to the assembly of the remaining F pilin in an extended conformation that would grow out from the cell surface through the action of $TraC_F$. Although $TraC_F$ is predicted to be a peripheral inner membrane (IM) protein, $TraC_F$ localizes to the cytoplasm of F^- cells, suggesting that other IM transfer proteins may interact with the hydrophobic domains of $TraC_F$ (Schandel *et al.*, 1992). In this regard, $TrhC_{R27}$ was shown to form inner membrane complexes as detected by GFP-fusions with $TrhC_{R27}$ (Gilmour *et al.*, 2001). This protein complex formation depended on the presence of $TrhB_{R27}$, -E_{R27} and specifically $TrhL_{R27}$, implying their direct or an indirect interaction with $TrhC_{R27}$ or amongst themselves (Gilmour *et al.*, 2001). Furthermore, the R27 T4SS proteins TrhF, -G, -H, -N, -U, and –V are also required for the assembly of the TrhC-associated protein complex as measured by GFP-foci formation, which suggests that this protein complex extends throughout the cell envelope (Gilmour *et al.*, 2004).

$TraE_F$, an IM protein consisting of 188 amino acids (Achtman *et al.*, 1979), is predicted to contain a single N-terminal transmembrane domain and a large hydrophilic periplasmic domain (Frost *et al.*, 1984). A *traE18* mutation (Achtman *et al.*, 1972) has been shown to affect pilus tip formation (Anthony *et al.*, 1999). Interestingly, $TraE_F$ is homologous to the 220 aa VirB5, which is believed to act as a minor T-pilus component (Schmidt-Eisenlohr *et al.*, 1999).

$TraL_F$ (91 aa) is homologous to the VirB3 protein and predicted to be a peripheral IM protein. $TraL_F$ has never been visualized in any experiment, suggesting that its expression is highly regulated, possibly due to the large amount of predicted secondary structure of the RNA sequence and subsequent cleavage by ribonucleases (Koraimann and Hogenauer, 1989). The F *lac traL311* mutant established that $TraL_F$ is needed for DNA transfer and pilus formation, but not for entry exclusion (Willetts, 1973). It is also completely resistant to f1, R17 and M13K07 phage infection suggesting its role in pilus tip formation (Anthony *et al.*, 1999). It is not plasmid-specific, being indistinguishable from *traL* of the F-like plasmids (ColV2, ColVB, R100-1 and R1-19) (Anthony *et al.*, 1996). This rarely expressed protein has been proposed to limit the number of sites for pilus assembly, since pili expression is still low under derepressed conditions (Anthony *et al.*, 1999).

$TraG_F$, a 102.5 kDa protein, is predicted to contain at least three long, hydrophobic IM transmembrane segments at its N-terminus and two larger hydrophilic C-terminal domains located in the periplasm (Firth and Skurray, 1992). This membrane topology is based on proteinase K accessibility experiments, cell fractionation data, as well as a hydropathy profile. $TraG_F$ is a unique exception to the classification system of F transfer gene products because its bifunctional nature displays a role in both F pilus assembly and mating aggregate stability. Experimental truncation and mutagenesis of $TraG_F$ established that the promoter proximal or N-terminal portion of TraG, which is located in the IM, was adequate for its role in F pilus assembly (Firth and Skurray, 1992). The N-terminal domain of $TraG_F$ is homologous with VirB6 of the Ti plasmid, which is also believed to contain multiple transmembrane segments and is necessary for pilus biosynthesis.

$TraB_F$, $TraK_F$, and $TraV_F$ are discussed last because yeast two-hybrid systems have established that these proteins form a cell envelope-spanning complex similar to that of VirB10-VirB9-VirB7 of the Ti plasmid T4SS (Harris *et al.,* 2001; Krall *et al.*, 2002).

$TraK_F$, a VirB9 homologue, is a 23.3 kDa protein (Penfold *et al.*, 1994) that has been shown to contain two separate binding domains for $TraB_F$ and $TraV_F$ (Harris *et al.,* 2001). It is uncertain whether $TraK_F$ remains in the periplasm or is localized to the outer membrane (OM) (Frost *et al.*, 1994), but its C-terminus contains a conserved secretin β-domain, exemplified by the OM protein PulD of *Klebsiella oxytoca* (Lawley *et al.*, 2003).

The β-domain, a hallmark of the secretin family, is believed to engage in multimerization in the OM. In the absence of other F transfer proteins, $TraK_F$ has been isolated from the periplasm (Firth *et al.*, 1996), suggesting that if $TraK_F$ does associate with the OM, it requires the F T4SS to do so. Because secretins serve to pass substrates through the OM, this homology offers a possible explanation for how the pilus assembles through the cell envelope. The classification of $TraK_F$ as an essential pilus tip formation protein accentuates this model.

$TraV_F$ is a 16.5 kDa OM lipoprotein that extends into the periplasm to contact $TraK_F$ (Harris *et al.*, 2001). Despite the limited similarity between the $TraV_F$ (171 aa) and VirB7 (55 aa) (Lawley *et al.*, 2003), several observations indicate that they are analogous proteins: both are lipoproteins, both interact with their respective secretin-like protein partner ($TraK_F$ or VirB9), and both contain conserved N-terminal cysteines proposed to enable multimerization (Harris *et al.*, 2001). Furthermore, $TraV_F$ is one of two core transfer proteins involved in elongating the pilus from the cell surface (Anthony *et al.*, 1999).

$TraB_F$, a 50.46 kDa IM protein, is the other core transfer protein required for pilus elongation. $TraB_F$ is predicted to be predominantly hydrophilic with a probable N-terminal membrane anchor and an extended periplasmic domain (Frost *et al.*, 1994). Limited homology has been established within the C-terminal domains of $TraB_F$ and VirB10. Bacterial two-hybrid analysis and co-immunoprecipitation experiments have

indicated that $TrhB_{R27}$ interacts with itself as well as the coupling protein $TraG_{R27}$, thereby establishing a connection between cytoplasmic DNA processing and the T4SS (Gilmour *et al.*, 2003). These interactions are attributed to an N-terminal coiled-coil domain found in all F- and H-like TraB proteins (Gilmour *et al.*, 2003).

1.4.3 F- and H-like auxiliary transfer proteins of the type IV secretion system

F-like and H-like plasmids do not contain VirB8 or VirB11 homologues, which are conserved in other conjugative systems such as the IncP plasmid RP4 (TrbF and TrbB) or the IncW plasmid R64 (TrwG and TrwD) (Table 1.1; Christie, 2001; Lawley *et al.*, 2003). However, the F plasmid has a unique group of proteins that are essential for pilus assembly and DNA transfer in F-like systems: TraF, -H, -N, -U, -W, and TrbC. The H-like plasmids have a similar group of essential auxiliary proteins (denoted Trh) with the exception that homologues to the $TraW_F$ and $TrbC_F$ gene products are found in one protein, $TrhW_H$. All of these proteins reside in the periplasm (Frost *et al.*, 1994), except the outer membrane protein $TraN_F$ (Klimke *et al.*, 2005). The transducing M13K07 phage assay demonstrated that mutations in $traF_F$, $traH_F$, and $traW_F$ affect pilus outgrowth or extension rather than tip formation (Anthony *et al.*, 1999). Previous research has established that $trbC_F$ mutants are transfer deficient and resistant to infection by F-pilus-specific phages, implying a role in pilus assembly (Maneewannakul *et al.*, 1991). $TraN_F$ and $TraU_F$ are not required for pilus production but appear to be involved in mating pair stabilization and DNA transfer (Klimke and Frost, 1998; Moore *et al.*, 1990). It should be noted that IncP plasmids, which lack the F- and H-like auxiliary

proteins, contain conjugative pili that are short, rigid, and exhibit preferential mating efficiency on solid media rather than liquid media (Bradley *et al.*, 1980). Therefore the F- and H-like auxiliary proteins may have a role in the assembly of the long, flexible F or H pilus that is capable of mediating mating in liquid media via retraction and mating pair stabilization (Lawley *et al.*, 2003).

$TraF_F$ is a predominantly hydrophilic protein that is 25.9 kDa in its mature form (Wu *et al.,* 1988). Minicell experiments suggested that $TraF_F$ associates with OM fractions (Manning *et al.*, 1981), but later cell fractionation of maxicells by osmotic shock indicated that the majority of mature $TraF_F$ localizes to the periplasmic space (Wu *et al.*, 1988). An amber mutation known as *traF13* is resistant to DNA and RNA F-specific bacteriophages (f1 and R17 respectively) and yet is fully sensitive to the M13K07 assay, which is specific to the pilus tip (Anthony *et al.*, 1999). Therefore, the supposed role for $TraF_F$ in pilus assembly is likely to be in extending the pilus from the cell surface and not in pilus tip formation.

Sequence analysis suggested that $traH_F$ encodes a 38.9 kDa precursor polypeptide that is processed to give a periplasmic protein (Ham *et al.*, 1989). In contrast, membrane fractionation studies in $traH_F$ mutants resulted in the disappearance of three inner membrane bands, implying that $TraH_F$ fractionates with the IM (Manning, 1981). Furthermore, later fractionation studies by the sucrose density gradient technique detected $TraH_F$ only in the OM fraction (Manwaring, 2001). $TraH_F$ contains three N-

terminal hydrophobic domains of approximately 20 amino acids each suggesting that it could be peripherally-associated with either membrane (Manwaring, 2001). However, the majority of $TraH_F$ is likely to be predominantly found in the periplasm because it is sensitive to guanidine-HCl and urea, which are denaturants that remove proteins residing in more hydrophilic environments (Manwaring, 2001). F- and H-like TraH proteins contain C-terminal coiled-coil domains, implying the capacity to multimerize with itself or other cell envelope transfer proteins (Lawley *et al.*, 2003).

$TraW_F$ is 210 amino acids while $TrhW_{R27}$ is 502 amino acids since its N-terminus is fused to TrbC, a separate transfer protein in the F-like system (Lawley *et al.*, 2003). The fusion of these proteins in the H-like system implies that they have related roles in F pilus assembly. Cell fractionation by maxicell analysis demonstrated that mature $TraW_F$ and $TrbC_F$ are periplasmic proteins (Maneewannakul *et al.*, 1991; Maneewannakul *et al.*, 1992). Interestingly, $TrbC_F$, whose gene is upstream adjacent to *traN*$_F$, depends upon the presence of aa residues 236 to 340 of $TraN_F$ for correct processing (Klimke, 2002).

During conjugative F DNA transfer, recipient cells form tight junctions with F^+ donor cells containing pili, and $TraN_F$ stabilizes the mating aggregate in liquid media by interacting with the major outer membrane protein OmpA in recipient cells (Klimke and Frost, 1998). $TraN_F$ is a large 66 kDa OM protein (Maneewannakul *et al.*, 1992), which is predicted to contain 20 transmembrane segments according to IS*phoA*in insertional analysis (Klimke *et al.*, 2005). Deletion analysis has shown that the amino acids 1 to 333

of $TraN_F$ may act as a specific donor cell surface receptor for recipient cell OmpA (Klimke and Frost, 1998). Although the N- and C-terminal regions (aa 1-161, and 334-602 in F) are highly conserved, a region from aa residues 162 to 333 forms a variable domain with three predicted extracellular loops that exhibits sequence divergence amongst other F-like TraN proteins (Klimke and Frost, 1998; Klimke *et al.*, 2005). This suggests that the recipient cell receptor likely varies amongst conjugative systems; after all, $TraN_{R100}$ does not require OmpA in the recipient cell for mating stabilization (Klimke and Frost, 1998). Protease K accessibility analysis displayed durable TraN digestion products of 60 kDa and 53 kDa that would indicate that most of TraN is internally localized, which might allow for associations with other transfer proteins (Maneewannakul *et al.*, 1992). In this regard, an interaction between $TraN_F$ and $TraV_F$ has been proposed since $traN_F$ mutants are often unstable in the absence of $traV_F$ (Klimke and Frost, personal communication). DNA transfer is completely abolished without a pilus, but $traN_F$ mutants still permit a minimal mating efficiency that is similar in both liquid and solid media, implying that $TraN_F$ has a greater role than just surface adherence (Kingsman and Willetts, 1978).

The resistance of *traU526* strains to F-specific phage infection led researchers to consider $TraU_F$ to be a pilus assembly protein (Miki *et al.*, 1978). However, this $traU_F$ amber mutant showed only a 50% decrease in piliation as measured by electron microscopic analysis. Later $traU_F$ null mutants were found to exhibit more pronounced DNA transfer deficiencies where piliation was still apparent at 20% wild-type frequency (Moore *et al.*,

1990). The continued presence of pili in these phage-resistant *traU*$_F$ mutants would suggest that these mutants are deficient for another aspect of phage infection and conjugation. TraU$_F$ has therefore been assigned a unique role in DNA transfer, but since there is a similar mutant phenotype among *traU*$_F$, *traN*$_F$, and *traG*$_F$, it may also aid mating pair stabilization (Lawley *et al.*, 2003).

1.5 Thioredoxin

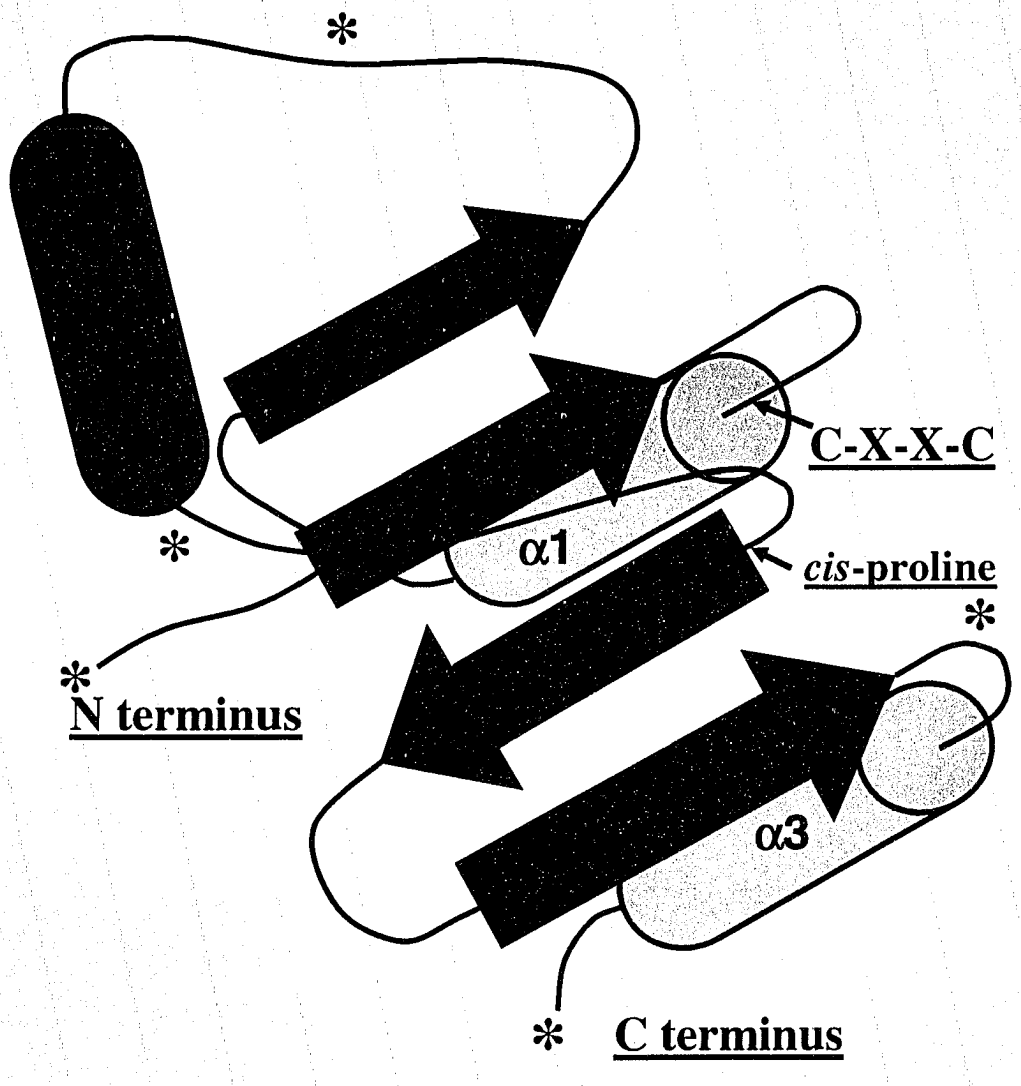
Thioredoxins form a large family of proteins that catalyze the formation and isomerization of disulfide bonds as well as other redox reactions (Raina and Missiakas, 1997). These small (12 kDa) redox proteins are ubiquitous throughout the animal, plant and bacterial kingdoms. Thioredoxins contain a well conserved active site, Cys-Gly-Pro-Cys, which is responsible for its redox potential (Arner and Holmgren, 2000). In its reduced form thioredoxin is a powerful protein disulfide oxidoreductase that ensures that cytoplasmic protein disulfides are generally reduced (Holmgren, 1984). Reduced thioredoxin also serves as a hydrogen donor for ribonucleotide reductase, an essential enzyme in DNA synthesis (Holmgren, 1989). *E. coli* thioredoxin also has two roles in bacteriophage survival: by participating as the only required host protein in filamentous phage assembly (Russel and Model, 1986; Feng *et al.*, 1999) and by increasing the processivity of T7 DNA polymerase independently of its redox potential (Tabor *et al.*, 1987). Thioredoxin is maintained in the reduced state by the transfer of electrons from NADPH via thioredoxin reductase (Holmgren, 1985). Interestingly, null mutations of

thioredoxin (*trxA*) are viable making the precise cellular physiological functions of thioredoxin unknown.

1.5.1 The thioredoxin fold

Many soluble disulfide bond forming (Dsb) proteins contain a domain with homology to the greater portion of thioredoxin, now referred to as the thioredoxin fold. The thioredoxin fold consists of at least three α-helices and a four-stranded β-sheet (Figure 1.6; Martin, 1995). The nomenclature for strands and helices of this sub-structure ($N_{trx\text{-}fold}$) may vary from that used for the structure of the entire protein, but for the purposes of this thesis the $N_{trx\text{-}fold}$ method will be used, unless specified, to avoid confusion. The thioredoxin fold entails the N-terminal β1-strand, α1-helix, and β2-strand that are joined by a loop/α2-helix to the C-terminal β3-strand, β4-strand, and α3-helix (Eklund *et al.*, 1984). The N-terminal β-strands are parallel while the C-terminal β-strands are anti-parallel. This mixed β-sheet is enclosed on one side by α-helices 1 and 3 in a parallel fashion and on the other side by α2-helix in a perpendicular orientation. The active site consists of two cysteine residues in a C-X-X-C motif and is located in a loop connecting the β1-strand and the α1-helix within the thioredoxin fold. The cysteines are reversibly reduced and oxidized during the catalytic cycle. A conserved proline residue plays an important role in both structure and function of thioredoxins by forming van der Waals interactions between the loop in which it resides and the C-X-X-C disulfide bond (Charbonnier *et al.*, 1999).

Figure 1.6. The thioredoxin fold. This figure is adapted from J. L. Martin (1995). The location of the α-helices (green cylinders) is shown with respect to the central β-sheet (red arrows). The α2-helix is illustrated in dark green because it is in front of the β-sheet whereas the α1- and α3-helices are illustrated in light green as they are behind the β-sheet. The C-X-X-C active site motif is indicated at the loop connecting the α1-helix and the β1-strand. The conserved *cis*-proline residue is indicated at the start of the β3-strand. The asterisks denote regions within this structure where additional residues have been found in other members of the thioredoxin superfamily.



1.6 Disulfide bond formation

Proteins containing disulfide bonds are predominantly found in the cell envelope (Ritz and Beckwith, 2001). However, proteins with multiple disulfide bonds are rare in *E. coli*, and not all proteins require disulfide bonds for proper folding and function (Ritz and Beckwith, 2001). With this in mind, proteins containing stable structural disulfide bonds are usually only found in cellular compartments that are highly oxidizing. Although disulfide bonds can form spontaneously in the oxidizing periplasmic environment (Anfinsen *et al.*, 1961), the disulfide bond forming potential is not merely due to oxygen but rather is facilitated *in vivo* by a number of specialized thiol-disulfide exchange enzymes, which contain C-X-X-C active site motifs within a thioredoxin fold (Ritz and Beckwith, 2001).

1.6.1 DsbA

In *E. coli*, DsbA (21.1 kDa) is a soluble, monomeric, periplasmic protein (Bardwell *et al.*, 1991) that randomly and rapidly oxidizes pairs of cysteine residues in secreted proteins through reduction of its own disulfide bond (Figure 1.7; Wunderlich and Glockshuber, 1993). There are many potential substrates for DsbA since approximately 300 *E. coli* proteins containing two or more cysteines are predicted to be localized to the periplasm (McCarthy *et al.*, 2000). Not surprisingly, *dsbA* null mutants exhibit pleiotropic phenotypes (Bardwell *et al.*, 1991) due to the accumulation of periplasmic protein thiols in the reduced form. The effects of the *dsbA* null mutation include: the rapid degradation

of cell envelope proteins such as OmpA and alkaline phosphatase (AP) (Bardwell *et al.*, 1991), a lack of motility resulting from the improper folding of the flagellar motor protein FlgI (Dailey and Berg, 1993), a hypersensitivity to metals, benzylpenicillin and dithiothreitol (DTT) (Missiakas *et al.*, 1993; Stafford *et al.*, 1999), and resistance to M13 bacteriophage infection in F^+ cells because of the absence of F pili (Bardwell *et al.*, 1991).

The active site cysteines (Cys30 and Cys33) of DsbA are found oxidized *in vivo*, which agrees with the oxidase function of DsbA (Kishigami *et al.*, 1995). The high redox potential of this Cys30-Cys33 disulfide bond (-120 mV) is further indicative of the potent oxidizing capability of DsbA (Zapun *et al.*, 1993). Although disulfide bonds typically stabilize proteins, oxidized DsbA is less stable than reduced DsbA *in vitro* (Zapun *et al.*, 1993). However, this instability would be thermodynamically favourable for the transfer of the disulfide bond from DsbA to a target protein.

The crystal structure of both oxidized and reduced DsbA has been solved (Martin *et al.*, 1993; Guddat *et al.*, 1998). It reveals that, like many other thiol-disulfide oxido-reductases, DsbA has a thioredoxin-like fold containing a C-X-X-C active site motif (Cys30-Pro31-His32-Cys33). An α-helical domain (75 aa), comprising a hydrophobic patch, is embedded within the thioredoxin fold. This domain provides a cap for the C-X-X-C active site and is thought to prevent undesirable interactions with DsbD (see below) (Haebel *et al.*, 2002). Furthermore, evidence of noncovalent interactions has suggested

that this α-helical domain could interact with unfolded protein substrates in a hydrophobic manner (Couprie *et al.*, 2000; Frech *et al.*, 1996; Guddat *et al.*, 1997). This is not surprising given that DsbA might only interact with unfolded or partially folded proteins in order to access the substrate cysteine residues prior to their internalization upon folding. The requirement for a hydrophobic interaction would also explain the very slow *in vitro* electron transfer from oxidized DsbA to reduced disulfide oxidoreductases like DsbC and DsbD (Kadokura *et al.*, 2003).

Despite great advances in the biochemical analysis of DsbA, very little is known about the regulation and control of *dsbA* expression. Activation of the Cpx pathway has been shown to induce the synthesis of DsbA 5- to 10-fold through the binding of phosphorylated CpxR to consensus elements upstream of the *dsbA* start site (Pogliano *et al.*, 1997). The Cpx two-component signal transduction system senses and responds to external stress in the *E. coli* cell envelope through the inner membrane sensor, CpxA, and the response regulator, CpxR. Thus, this upregulation of *dsbA* indicates the importance of cell envelope enzymes involved in protein folding during external stress.

1.6.2 DsbB

Genetic and biochemical evidence have established that DsbB is responsible for the reoxidation of DsbA (Figure 1.7; Bardwell *et al.*, 1993; Missiakas *et al.*, 1993). DsbA and DsbB were ascribed to the same oxidation system because a *dsbB* null mutant accumulates reduced DsbA and exhibits a phenotype similar to a *dsbA* null mutant

(Bardwell *et al.*, 1993). DsbB has also been shown to interact directly with DsbA by the isolation of a mixed disulfide between these two disulfide oxidoreductases (Guilhot *et al.*, 1995; Kishigami *et al.*, 1995).

DsbB is a 20 kDa protein believed to be anchored to the IM by four central transmembrane helices (Jander *et al.*, 1994). The N-terminus of DsbB contains a periplasmic domain with two conserved cysteines, Cys41 and Cys 44. Although this pair of cysteine residues resembles the C-X-X-C active site motif of DsbA, the region in which it is embedded is too small to form a thioredoxin fold (Nakamoto and Bardwell, 2004). Two more conserved cysteines, Cys104 and Cys130, form a disulfide bond and are located in a C-terminal periplasmic domain (Kishigami and Ito, 1996). All four of these cysteines residues are necessary for the activity of DsbB as determined by mutational analysis (Guilhot *et al.*, 1995; Jander *et al.*, 1994; Kishigami and Ito, 1996). The standard redox potentials of the Cys41–Cys44 and Cys104–Cys130 disulfide bonds of DsbB have also been determined to be −69 and −186 mV, respectively (Grauschopf *et al.*, 2003). Although the disulfide bond at Cys104-Cys130 is thought to be transferred to reduced DsbA, the redox potential of DsbA is -120 mV, which implies that this reoxidation step is thermodynamically unfavourable. However, it is predicted that the overall reaction is driven to completion by the strong oxidizing potential of the Cys41–Cys44 disulfide (Grauschopf *et al.*, 2003). DsbB would require oxidative regeneration to continue this cycle, and the fact that quinone-depleted cells accumulate reduced DsbA and DsbB, suggests that the electron transport chain provides the continuous oxidizing

Figure 1.7. Disulfide bond formation. Oxidized, monomeric DsbA rapidly oxidizes proteins secreted into the periplasm by donating its disulfide from the C-X-X-C active site to pairs of cysteines in the substrate proteins. DsbB restores the disulfide bond to reduced DsbA by using the oxidizing power of the electron transport chain.

power for protein disulfide bond formation (Kobayashi *et al.*, 1997). The *in vitro* reconstitution of this disulfide bond formation system indicates that reduced DsbB transfers two electrons to oxidized ubiquinone, which in turn is regenerated by the terminal cytochrome oxidases *bd* and *bo*, which finally transfer electrons onto oxygen (Figure 1.7; Bader *et al.*, 1998; Bader *et al.*, 1999).

1.7 Disulfide bond isomerization

There is increasing probability for incorrect disulfide bond formation in target proteins that contain more than two cysteines. Since DsbA strongly oxidizes free sulfhydryl groups quickly and nonspecifically, there is the potential for generating non-native disulfide bonds in proteins with numerous cysteines (Rietsch *et al.*, 1996). In these cases, the non-native disulfide bonds must be broken and reformed correctly. This disulfide rearrangement requires an intramolecular thiol-disulfide exchange reaction, or more specifically, a thiolate anion engages in a nucleophilic attack on the incorrect disulfide bond (Nakamoto and Bardwell, 2004). This periplasmic reaction is promoted by the disulfide bond isomerase DsbC (Figure 1.8; Missiakas *et al.*, 1994; Rietsch *et al.*, 1996) and possibly DsbG (Bessette *et al.*, 1999).

1.7.1 DsbC and DsbG

As a disulfide bond isomerase, DsbC mediates, both *in vivo* and *in vitro*, the proper folding and corrective disulfide bond rearrangements in a number of proteins with multiple cysteine residues (Maskos *et al.*, 2003). DsbC can successfully isomerize

incorrect disulfide bonds in proteins such as RNase A (Kadokura *et al.*, 2003), bovine pancreatic trypsin inhibitor (BPTI) (Zapun *et al.*, 1995), mouse urokinase (Rietsch *et al.*, 1996), and AP (Sone *et al.*, 1997) as determined *in vitro* by biochemical analysis. Modified osmotic shock combined with two-dimensional gel electrophoresis has identified RNase I and MepA as *in vivo* substrates of DsbC (Hiniker and Bardwell, 2004). DsbG, another periplasmic homologue of DsbC, may serve as a disulfide bond isomerase given that overproduced DsbG could restore BPTI processing in *dsbC* mutants (Bessette *et al.*, 1999). However, its exact cellular function remains unknown. Furthermore, DsbG, unlike DsbC, cannot efficiently catalyze insulin reduction or oxidative protein refolding of RNase, suggesting functional differences between these isomerases (Nakamoto and Bardwell, 2004).

E. coli DsbC is a 23 kDa protein consisting of an N-terminal dimer interface (residues 1-61), a hinged linker helix (residues 62-77), and a C-terminal catalytic domain (residues 78-216) containing the C-X-X-C active site motif embedded in a thioredoxin fold (McCarthy *et al*, 2000; Sun and Wang, 2000). DsbC forms a V-shaped homodimer with the C-terminal active sites from each DsbC monomer facing inwards across the cleft. This broad cleft consists mainly of uncharged or hydrophobic residues, which may allow for noncovalent binding of substrate peptides (McCarthy *et al.*, 2000; Nakamoto and Bardwell, 2004). Kinetic studies of DsbC reactions with unstructured model peptides have suggested similar noncovalent substrate binding interactions (Darby *et al.*, 1998). The hinged-linker helices direct the open and closed conformations of this cleft in

accordance with the redox state of their C-X-X-C active sites (Haebel *et al.*, 2002; McCarthy *et al.*, 2000). This flexibility gives DsbC the potential to accommodate the size and shape of the binding partner. Given that DsbG is a homodimeric protein and that it shares 49% sequence similarity with DsbC, it seems probable that it also has a structure similar to DsbC (Bessette *et al.*, 1999).

The active site cysteines of DsbC and DsbG remain reduced in the oxidizing periplasmic environment (Bessette *et al.*, 1999; Rietsch *et al.*, 1997) despite having a standard redox potential (-130 mV) that is comparable to DsbA (-120 mV) (Zapun *et al.*, 1995). Consequently, the N-terminal cysteine of the C-X-X-C active site, whose free thiol group is solvent-exposed, can attack a misformed substrate disulfide and thereby create an intermolecular disulfide bond with the target protein (McCarthy *et al.*, 2000). Conformational changes in the substrate allow an alternative thiol group from the target protein to resolve this intermolecular disulfide bond via nucleophilic attack. The result is a new disulfide bond in the target protein and an active (reduced) isomerase. Alternatively, DsbC could also exhibit a reductase activity and simply break the incorrect substrate disulfides, which would allow DsbA a second opportunity to reoxidize the target protein (Rietsch *et al.*, 1997). Regardless of the mode of action, DsbC must be maintained in the reduced state by DsbD (Figure 1.8) given the oxidizing environment of the periplasm (Stewart *et al.*, 1999). Biochemical evidence has indicated that neither DsbA nor DsbB are capable of interacting with or oxidizing DsbC *in vivo*. DsbA oxidizes DsbC very slowly *in vitro* (Zapun *et al.*, 1995), and DsbB catalyzes this reaction almost

500-fold more slowly than DsbA (Bader *et al.*, 2000). In this regard, the maintenance of disulfide bond isomerases seems to be distinct from the disulfide bond formation system.

DsbC and DsbG appear to possess molecular chaperone activities in that they promote the *in vitro* reactivation of denatured lysozyme and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Chen *et al.*, 1999), or citrate synthase and luciferase (Shao *et al.*, 2000), respectively. The ability to assist in the refolding of these denatured proteins that do not contain cysteine residues is not dependent upon the presence of the C-X-X-C active site (Liu and Wang, 2001). However, *E. coli* DsbC also contains an additional two cysteine residues (Cys141 and Cys163) that form a structural disulfide bond, which is partially solvent exposed (McCarthy *et al.*, 2000) and required for this latter activity (Liu and Wang, 2001). Interestingly, these cysteines are not highly conserved in other DsbC proteins nor are they present in *E. coli* DsbG. Furthermore, the dimeric nature of DsbC is essential for both its isomerase function and its molecular chaperone activity as the thioredoxin domain itself is inactive in both these cases (Sun and Wang, 2000; Zhao *et al.*, 2003). This dual role may permit the chaperone activity of DsbC to identify misfolded substrate proteins and the isomerase activity of DsbC to rearrange non-native disulfide bonds within the target protein.

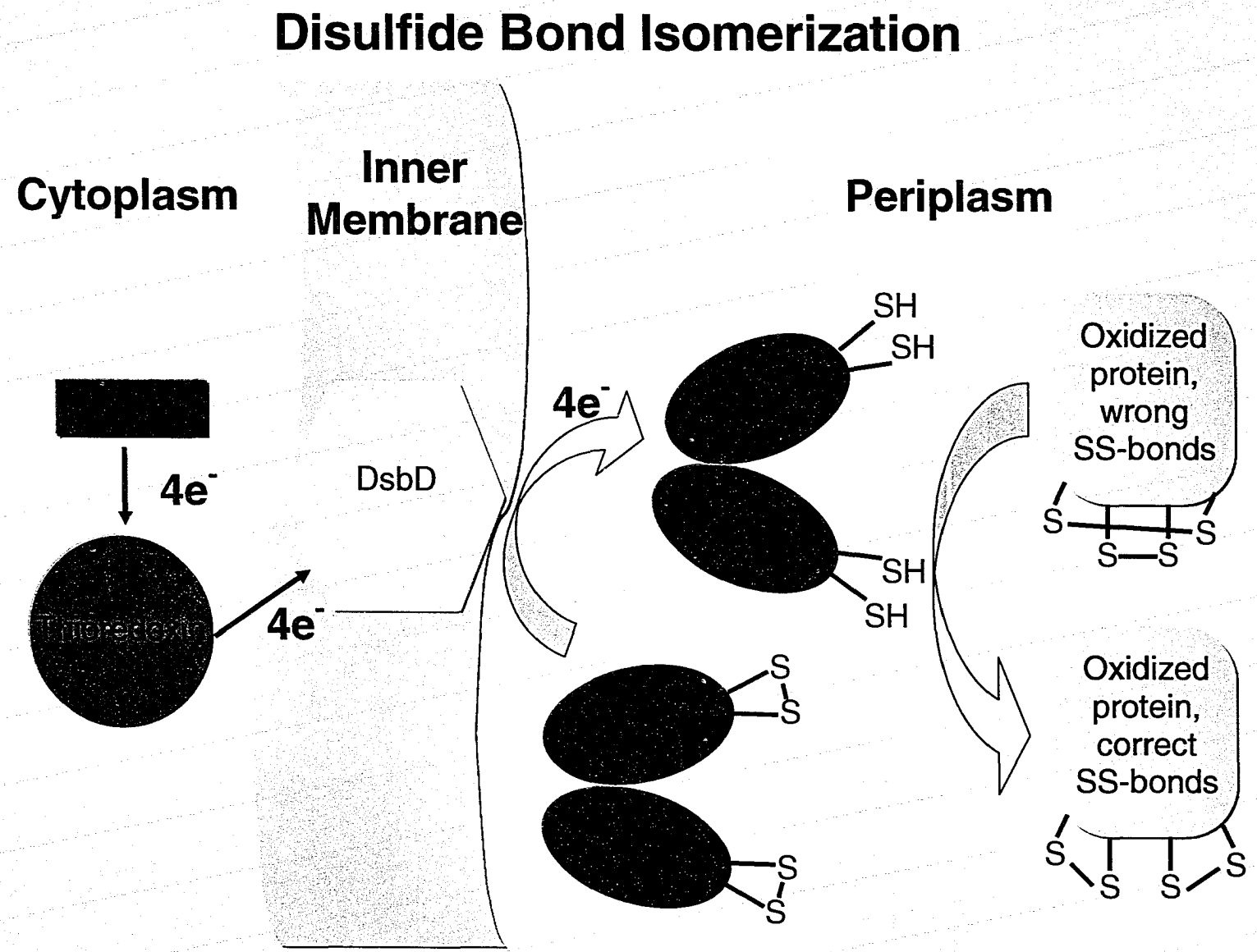
1.7.2 DsbD

DsbD, a 59 kDa IM protein, was first identified for its role in cytochrome c maturation in the *E. coli* periplasm (Crooke and Cole, 1995). DsbD transfers electrons to CcmG (also

known as DsbE) (Katzen and Beckwith, 2000), which is a periplasmic protein with an IM anchor and a thioredoxin fold domain (Fabianek *et al.*, 1998). CcmG donates electrons to the cysteines in the heme-attachment site of *c*-type apocytochromes via the C-X-X-C motif of CcmH (Fabianek *et al.*, 1999). It has also been established that the disulfide bond isomerases, DsbC (Rietsch et al., 1997) and DsbG (Bessette *et al.*, 1999), are maintained in the reduced state by DsbD, whose reducing power in turn must be regenerated by thioredoxin and thioredoxin reductase in an NADPH-dependent reaction (Rietsch et al., 1997). Consequently, in a *dsbD* null mutant, DsbC and DsbG are found in the fully oxidized state.

Topological analysis indicated that DsbD is composed of three domains: an N-terminal periplasmic domain (DsbDα) that assumes an immunoglobulin-like fold, a central IM domain made up of eight transmembrane segments (DsbDβ), and a C-terminal periplasmic domain (DsbDγ) that includes a thioredoxin-like fold (Goulding *et al.*, 2002; Stewart *et al.*, 1999). These three separate domains of DsbD can be co-expressed *in vivo* from separate plasmids to reconstitute a functional protein (Katzen and Beckwith, 2000). Mutational analysis demonstrated that pairs of cysteines (DsbDα, Cys103-Cys109; DsbDβ, Cys163-Cys185; DsbDγ, Cys461-Cys464) in each of the three domains are required for proper DsbD function, which suggests that several disulfide bond reduction steps may be required to transfer electrons across the inner membrane (Chung *et al.*, 2000; Stewart *et al.*, 1999). The isolation of mixed disulfide intermediates involving thioredoxin, DsbC, and each separate DsbD domain (Krupp *et al.*, 2001), as well as their

Figure 1.8. Disulfide bond isomerization. Reduced, dimeric DsbC resolves incorrect disulfide bonds introduced by DsbA either through isomerization to correct the bond or through reduction to give DsbA another chance to properly oxidize the protein. DsbC is kept reduced in the oxidizing periplasmic space via DsbD which utilizes the thioredoxin reductase system.



standard redox potentials (Collet *et al.*, 2002; Mossner *et al.*, 1999) indicate the following thermodynamically driven electron flow: thioredoxin (-270 mV) to DsbDβ to DsbDγ (-241 mV) to DsbDα (-229 mV) to DsbC (-130 mV). The two redox-active cysteines (Cys163 and Cys285), within the membrane portion of DsbDβ, are unusually situated for disulfide exchange reactions, limiting further characterization. Interestingly, the C-X-X-C active site embedded in the thioredoxin fold of DsbDγ does not interact with the homologous motif of DsbC but rather with the redox-active cysteines the DsbDα. It is the immunoglobulin-like fold of DsbDα that establishes binding to both DsbC subunits within the central cleft of the DsbC dimer (Haebel *et al.*, 2002). This exclusive interaction assumes a closed conformation on complex formation, which is required for disulfide exchange within the DsbC-DsbDα complex. As a result, DsbDα is unable to transfer electrons to DsbA, as determined by *in vitro* assays (Collet *et al.*, 2002), which ensures the functional separation between the disulfide bond formation and isomerization pathways.

1.8 Scope of thesis

Although the DNA transfer process of the F plasmid has been the subject of research for many years, many other aspects of conjugative apparatus remain to be investigated. In particular, the F-specific or auxiliary transfer proteins from the cell envelope are perhaps the least studied, and yet they play a vital role in pilus assembly and mating pair stabilization. The only known homologues of these F-like auxiliary transfer proteins are found in the H-like transfer regions, exemplified by IncHI1 plasmid R27. Here we report

that several of these unique F- and H-like transfer proteins bear homology to *E. coli* thioredoxin. Based on sequence analysis, two genes of the F transfer region, *traF* and *trbB*, and three genes of the R27 transfer region, *trhF*, *dsbC*, and *htdT*, are predicted to encode periplasmic proteins containing a C-terminal thioredoxin fold. The purpose of this thesis was to investigate the role of the respective gene products in F and the IncHI1 plasmid R27. Given that the limited characterization of protein-protein interactions amongst the F- and H-specific proteins, we have attempted to extrapolate the roles of the above proteins based on their ability to complement mutations in the more thoroughly studied disulfide bond formation and isomerization systems. Portions of this thesis have been submitted for publication in the Journal of Bacteriology as "F-like type IV secretion systems encode proteins with thioredoxin folds that are putative DsbC homologues" (Elton *et al.*, submitted).

Chapter 2

Materials and Methods

2.1 Bacterial strains, plasmids, and general reagents

2.1.1 Bacterial strains and growth conditions

Bacterial strains used in this study are presented in Table 2.1. Bacterial cultures were grown in Luria-Bertani Broth (LB-1% Difco Tryptone, 0.5% Difco Yeast Extract, and 1% NaCl (BDH Inc.)) at 37°C on a tube roller to mid-log phage. Growth medium was supplemented with the appropriate antibiotics at the following concentrations: ampicillin (amp) 100 μg/mL, chloramphenicol (Cm) 20 μg/mL, kanamycin (Km) 25 μg/mL, nalidixic acid (Nal) 40 μg/mL, rifampicin (Rif) 20 μg/mL, streptomycin (Sm) 200 μg/mL, and tetracycline (Tc) 10μg/mL. All antibiotic stock solutions were submitted to filter sterilization with the exception of those dissolved in ethanol. IPTG (isopropyl-β-D-thiogalactopyranoside; Sigma Chemical Co.) was added at a final concentration of 0.1 mM to 1 mM as an inducer of vectors containing IPTG-inducible promoters. In order to repress expression from an IPTG-inducible promoter, glucose was added where necessary to cultures at a final concentration of 100 mM. Arabinose was added at 0.1% to induce expression from pBAD vectors.

2.1.2 Mating assays

Mating assays have been described by Anthony *et al.* (1994) and Klimke and Frost (1998). To test transfer of pOX38-Tc, an F derivative, from RI89 (wildtype), RI90 (*dsbA*) or RI179 (*dsbC*) donor cells to XK1200 recipient cells, donor cells were grown to mid-log phase and mixed with varying concentrations of dithiothreitol (DTT; 0 mM to 7 mM) for 1 hour prior to the mating assay. 200 μL of mid-log phase donor cells and

Table 2.1 *Escherichia coli* strains used in this study

Strain	Relevant characteristics	Selective marker(s)[a]	Reference or source
CC118	*Δ(ara-leu)*7697 *araD* 139 Δ(*lac*)X74 *phoA20 galE galK thi rpsE rpoB* $argE_{am}$ *recA1*	Rif, Sp	Manoil
CC160	MC1000 *dam*	Sm	Manoil and Bailey (1997)
CC245	F^- *supF supE hsdR galK trpR metB lacY tonA dam:: kan*	Km	Manoil and Bailey (1997)
DH5α	*supE44 ΔlacU169 (Φ80 lacZΔ80M15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Nal	Hanahan (1983)
DY330	F^- IN(*rrnD-rrnE*)1 *rph*-1 *ΔlacU169 gal490 λc1857 Δ(cro-bioA)*		Yu *et al.* (2000)
MC1000	*Δ(ara-leu)*7697 *araD* 139 Δ(*lac*)X74 *galE galK thi rpsL*	Sm	
MC4100	*araD139 Δ(argF-lac)U169 rpsL150 relA1 flbB3501 deoC1 ptsF25 rbsR*	Sm	Manoil
RI89	MC1000 *phoR Δara714* leu^+	Sm	Rietsch *et al.* (1996)
RI90	RI89 *dsbA::kan1*	Km, Sm	Rietsch *et al.* (1996)
RI179	RI89 *ΔdsbC::cam*	Cm, Sm	Rietsch *et al.* (1996)
XK1200	F^- *lacΔU124 Δ(nadA aroG gal attλ bio gyrA)*	Nal	Moore *et al.* (1981)

a. Antibiotic resistances: Amp, ampicillin; Cm, chloramphenicol; Km, kanamycin; Nal, nalidixic acid; Rif, rifampicin; Sm, streptomycin; Sp, spectinomycin; Tc, tetracycline.

recipient cells were incubated at 37°C for 45 minutes. The cultures were serially diluted in saline sodium citrate (SSC) media and 10 μL droplets (between 10^{-2} and 10^{-7}) were plated out on Tc and Sm to determine the number of donor cells or Tc and Nal to determine the number of transconjugants cells. Mating efficiency was calculated as the number of transconjugant cells divided by the number of donor cells multiplied by one hundred to give transconjugants / 100 donor cells. It should be noted that if data was expressed as the number of transconjugants, then this data was normalized according to a standard control assay. If protein overproduction for vector constructs was required, then donor cells were induced with 0.1% arabinose 2 hours prior to adding the recipient cells. When testing the DTT hypersensitivity of a *dsbA* null mutant, donor cells were grown to mid-log phase and mixed with 1 mM dithiothreitol for 1 hour prior to the mating assay.

2.1.3 Qualitative phage spot tests

Phage sensitivity assays were performed as previously described (Anthony *et al.*, 1994). Briefly, bacterial cells to be tested (approximately 1 x 10^7) were mixed with 3 mL of 0.7% LB top agar, which contains tryptone (10 g), yeast extract (5 g), NaCl (5 g), agar (7 g), and milli-q (MQ) water (1 L). This mixture was used to overlay an LB agar plate containing the appropriate antibiotics. An f1 phage suspension (1 x 10^{11} pfu/mL) was serially diluted in PBS pH 7.3, which contains NaCl (8 g), KCl (0.2 g), $Na_2HPO_4 \cdot 7H_2O$ (1.15 g), KH_2PO_4 (0.2 g). 10 μL of the various serial dilutions (1 x 10^9 to 1 x 10^1 pfu) were spotted on the bacterial lawn. LB agar plates were incubate overnight at 37°C and the efficiency of phage lysis at the various dilutions was recorded accordingly: fully

sensitive for lysis at 1 x 10^2 pfu, moderately sensitive for lysis at 1 x 10^4 pfu, weakly sensitive for lysis at 1 x 10^8 pfu, and resistant for no lysis.

2.2 General DNA manipulations

2.2.1 PCR and recombinant DNA techniques

All plasmids used in this section are listed in Table 2.2. Techniques for cloning restriction fragments or PCR fragments were as described previously (Sambrook *et al.*, 1989). Small scale plasmid preparations were performed according to the method of Birnboim and Doly, 1979. DNA preparations of low-copy vectors were performed using Qiaquick spin mini-prep kits (Qiagen). Blunt-end PCR products for cloning were purified from an agarose gel using the Qiaquick gel extraction kit (Qiagen) to remove plasmid templates and were directly inserted into pCR4Blunt-TOPO (Invitrogen) according to the manufacturer's instructions. The sequences of PCR insertions into pCR4Blunt-TOPO (Invitrogen) were verified using M13 Forward (5' GTAAAACGACG GCCAG) and M13 Reverse (5' CAGGAAACAGCTATGAC) primers from Invitrogen. Restriction endonuclease digestion and ligation of DNA molecules were performed as described by Ausubel *et al.* (1989). All enzymes were supplied by Roche Applied Science except Vent polymerase (New England Biolabs) and Pfu Turbo DNA polymerase (Stratagene). Based on the method of Jonda *et al.* (1999), the plasmid pKDsbA (NheI), which contains a single NheI restriction site at the end of the DsbA signal sequence without change to the amino acid sequence, was used in the construction of plasmids for periplasmic localization of thioredoxin fold variants. Thus the portion of

Table 2.2 Plasmids and phage used in this study

Strain or Plasmid	Relevant characteristics	Selective marker(s)[a]	Reference or source
Vectors			
PCR4Blunt-TOPO	4 Kb cloning vector with covalently linked topoisomerase	Amp, Km	Invitrogen
pBAD30	Cloning vector for controlled expression from $P_{ara\text{-}BAD}$	Amp	Guzman *et al.* (1995)
pGEX-2T	Cloning vector providing an N-terminal glutathione S-transferase gene fusion under the control of P_{lac}	Amp	Amersham Pharmacia Biotech
pK184	2.4 kbp cloning vector; p15a replicon	Km	Jobling and Holmes (1990)
pMAL-p2	Cloning vector providing an N-terminal maltose-binding protein or *malE* gene fusion under the control of P_{tac}	Amp	New England Biolabs
pT7-7	2.47 kbp cloning vector; ColEl replicon; P_{T7} promoter	Amp	Tabor and Richardson (1985)
Conjugative plasmids			
pOX38-Tc	pOX38::miniTn*10*	Tc	Anthony *et al.* (1994)
pOX38-Km	pOX38 + HindIII fragment of Tn*5*	Km	Chandler and Galas (1983)
pOX38-Tc Δ*traF*::*kan*	pOX38-Tc with *kan* replacing codons 34 to 229 in *traF*	Tc, Km	This study
pOX38-Tc Δ*trbB*::*cat*	pOX38-Tc with *cat* replacing codons 14 to 160 in *trbB*	Tc, Cm	This study
F *tra* constructs			
pFTraF	F *traF* in pBAD30 (DPID)[b]	Amp	This study
pFTraF(CPYC)	F *traF* (D135C, I137Y, D138C)[c] in pBAD30 (CPYC)[b]	Amp	This study

Strain or Plasmid	Relevant characteristics	Selective marker(s)[a]	Reference or source
F *tra* constructs (continued)			
pFTrbB	F *trbB* in pBAD30 (CPYC)[b]	Amp	This study
pFTrbB(Y63H)	F *trbB* (Y63H)[c] in pBAD30 (CPHC)[b]	Amp	This study
pFTrbB(SPYS)	F *trbB* (C61S, C64S)[c] in pBAD30 (SPYS)[b]	Amp	This study
pFTraF/HTrhF	F *traF* RBS[d] + codons 1 to 30 of F *traF* + codons 54 to 243 of R27 *trhF* + codons 213 to 247 of F *traF* in pBAD30	Amp	This study
pFTraF-his6	An XbaI-HindIII fragment from pT7-TraF-his6 cloned into pBAD30	Amp	This study
pGEX-FTraF	F *traF* without the signal sequence (codons 1 to 19) in pGEX-2T	Amp	This study
pK184-FTraF	F *traF* in pK184	Km	This study
pMAL-FTraF	F *traF* without the signal sequence (codons 1 to 19) in pMAL-p2	Amp	This study
pT7-FTraF-his6	Codons 1 to 247 of F *traF* cloned into a modified pT7-7 vector containing an in frame his6 tag between the BamHI and HindIII restriction sites.	Amp	This study
R27 *trh* constructs			
pHTrhF	R27 *trhF* in pBAD30 (CQFC)[b]	Amp	This study
pHHtdT	R27 *htdT* in pBAD30 (CDGC)[b]	Amp	This study
pHDsbC	R27 *dsbC* in pBAD30 (CGFC)[b]	Amp	This study
pHDsbC(SGFS)	R27 *dsbC* (C107S, C110S)[c] in pBAD30 (SDGS)[b]	Amp	This study
E. coli dsbA constructs			
pKDsbA	*E. coli* K12 *dsbA* in pBAD30	Amp	This study

Strain or Plasmid	Relevant characteristics	Selective marker(s)[a]	Reference or source
E. coli dsbA constructs (continued)			
pKDsbA(NheI)	*E. coli* K12 *dsbA* in pBAD30 with NheI site at codons 17 and 18	Amp	This study
pFBASS	*dsbA* signal sequence with A34 to K159 of F *trbB* in pBAD30	Amp	This study
pFBASS(Y63H)	pFBASS using F *trbB* (Y63H)[c]	Amp	This study
pHFASS	*dsbA* signal sequence with Q137 to R324 of R27 *trhF* in pBAD30	Amp	This study
pHCASS	*dsbA* signal sequence with A82 to K227 of R27 *dsbC* in pBAD30	Amp	This study
Phage			
λTn*phoA*/in		Cm, Km	Manoil and Bailey (1997)
f1 filamentous phage			Anthony *et al* (1994)

a. Antibiotic resistance: Amp, ampicillin; Cm, chloramphenicol; Km, kanamycin; Nal, nalidixic acid; Rif, rifampicin; Sm, streptomycin; Tc, tetracycline.

b. Sequence of the four amino acids at the C-X-X-C motif indicated in Fig. 1 (in brackets).

c. The first letter indicates the wild-type amino acid; the second letter indicates the mutant amino acid; the number refers to the position of the amino acid within the mature protein.

d. RBS is the abbreviation used for ribosomal binding site.

trbB$_F$, *trhF*$_{R27}$ or *dsbC*$_{R27}$ containing the thioredoxin fold could be amplified by PCR and cloned into pKDsbA (NheI). Transformation of plasmids into bacterial strains was accomplished by using $CaCl_2$ competent cells or by electroporation using a Bio-Rad GenePulser.

2.2.2 DNA Sequencing

The method for automated sequencing has been described previously (Klimke and Frost, 1998). Primers used in this study are listed in Table 2.3. Primer synthesis was carried out by The Molecular Biology Services Unit (MBSU) at the University of Alberta on an Applied Biosystems DNA/RNA 392 Synthesizer or 381A DNA synthesizer (PCR-MATE). DNA sequencing was performed by MBSU using an Applied Biosystems 373 Sequencer Stretch or 3100 Capillary Genetic Analysizer. The fluorescent sequencing premix dye, DYEnamic ET (Amersham Pharmacia Biotech), was used as well.

2.2.3 Gene deletion techniques

PCR amplification of the kanamycin resistance gene (including the native promoter) from the pUC4K plasmid (Amersham Pharmacia Biotech) was obtained using primers TEL-*traF*-*kan*-For (5' GATGCAGGCTGGCAGTGGTATAACGAGAAAATAAATCC GAAAAGCCACGTTGTGTCTCAA) and TEL-*traF*-*kan*-Rev (5' TCTTCAGAAACGT TCAGGAACTGTTTTGCCAGGTCGTCCTCGCTGAGGTCTGCCTCGTGA). The resulting *kan* linear DNA cassette had overhanging arms homologous to either nucleotides 15142 to 15182 or nucleotides 15767 to 15806 of *traF*. The PCR product

Table 2.3 Primers used in this study

Construct	Primers
Gene amplification	
pFTraF For	5' GCTAAGGTACCCTCCGGTAATTATCTGG-3'
pFTraF Rev	5' GGCATAAGCTTCCTGTCATTACGCTCAG-3'
pFTrbB For	5' GGTACCGAAGGGCAGCAGGAGGGC-3'
pFTrbB Rev	5' GAAGCTTCCGGCAATGAGTAACAGCAC-3'
pHTrhF For	5' GGTACCATCATACCTCTATTCCATG-3'
pHTrhF Rev	5' CAAGCTTCAAACGGCTAACGATAAAAG-3'
pKDsbA For	5' GGTACCAGTTCTACAAGAACCCCCTTTGC-3'
pKDsbA Rev	5' GAAGCTTCAGCGGCAGGATGCATTATCAG-3'
Gene fusion amplification	
pGEX-FTraF For	5' GGATCCAAAGATGCAGGCTGGCAG-3'
pGEX-FTraF Rev	5' CAGAATTCCTCAGAAAAGAAATAACCGG-3'
pT7-7-FTraF-his6 For	5' GCATATGAATAAAGCATTACTGCC-3'
pT7-7 FTraF-his6 Rev	5' GAGGATCCAAAATTGGGTTTAAAATCTTC-3'
N-terminal FTraF Rev	5' CTCTAGATTTCTCGTTATACCACTGCC-3'
C-terminal FTraF For	5' GTCGACCCGAAACAGGGCAGTG-3'

Construct	Primers
Gene fusion amplification (continued)	
N-terminal HTrhF For	5' CTCTAGAAAAACTGAGGAAGAAATTACAG-3'
C-terminal HTrhF Rev	5' GTCGACAAGGAAAATAGTTGGAAC-3'
Truncated gene amplification	
pFBASS For	5' GCTAGCGCGGCTCCCCGCTGGTTCCG-3'
pHFASS For	5' GCTAGCGCGGCTCAGCAGTCGGTTATGAAAGATATTTTC-3'
pHCASS For	5' GCTAGCGCCGCCACTAAAACAAAATCCATCG-3'
Mutagenesis	
pFTraF(CPYC) For	5' CATGTTTTTTTACCGGGGGCAG**TG**CCCC**TACTG**CGGGCAACTGGCGCAGGTC-3'
pFTraF(CPYC)Rev	5' GACCTGCGCCAGTTGCCCG**CAGTA**GGGG**CA**CTGCCCCCGGTAAAAAAACATG-3'
pFTrbB(Y63H) For	5' CAGGGGCATTGCCCT**C**ACTGTCACCAGTTTGAC-3'
pFTrbB(Y63H) Rev	5' GTCAAACTGGTGACAGT**G**AGGGCAATGCCCCTG-3'
pFTrbB(SPYS) For	5' GTTTATGCAGGGGCATT**C**CCCTTACT**C**TCACCAGTTTGACCCGG-3'
pFTrbB(SPYS) Rev	5' CCGGGTCAAACTGGTGA**G**AGTAAGGG**G**AATGCCCCTGCATAAAC-3'
pHDsbC(SGFS) Rev	5' CTTGATGCAATTTCTGG**G**AAAAGCCG**G**ATGTAATATCCGTAAAC-3'
pKDsbA(NheI) For	5' GTTTAGTTTTAGCGTTTAGCGC**TAGC**GCGGCGCAGTATGAAGATGG-3'
pKDsbA(NheI) Rev	5' CCATCTTCATACTGCGCCGC**GCTA**GCGCTAAACGCTAAAACTAAAC-3'

Construct	Primers
Sequencing	
AKE3 for Tn*phoA*/in	5' AATATCGCCCTGAGCA-3'
5' pGEX seq. primer	5' GGGCTGGAAGCCACGTTTGGTG -3'
3' pGEX seq. primer	5' CCGGGAGCTGCATGTGTCAGAGG -3'
M13 Forward	5' GTAAAACGACGGCCAG-3'
M13 Reverse	5' CAGGAAACAGCTATGAC-3'

Numbers present in the primer names indicate amino acid mutations in the active site. Restriction sites are underlined; BamHI, KpnI, NdeI, NheI, SalI, or XbaI were used for forward primers whereas BamHI, EcoRI, HindIII, SalI, or XbaI were used for reverse primers. Nucleotide changes for mutagenic primers are in bold. The pGEX sequencing primers were obtained from Pharmacia and the M13 sequencing primers were obtained from Invitrogen.

was purified from an agarose gel using the Qiaquick gel extraction kit (Qiagen) to remove the plasmid template and electroporated into *E. coli* strain DY330/F according to the procedure outlined by Yu *et al.* (2000). The *E. coli* strain MC4100 was added as recipient cells and allowed to mate with DY330/F for 1 hour. Recombinants were selected on Kan and Sm. To verify insertion of the *kan* cassette into F, clones were sequenced with TEL1 (5' GGATCCAAAGATGCAGGCTGGCAG) and TEL2 (5' CAG AATTCCTCAGAAAAGAAATAACCGG). PCR amplification of the Tn*9* chloramphenicol acetyl transferase gene (including the native promoter) from the pBAD33 plasmid (Guzman et al., 1995) was obtained using primers TEL-*trbB-cat*-For (5' CATGTCTCTCACTAAATCACTGCTGTTCACCCTGTTGCTGCTGTGACGGAA GATCACTTC) and TEL-*trbB-cat*-Rev (5' CGTACATCTGCAAAACGGTATCCACCC GCGCCATAAAACCTTATTCAGGCGTAGCACCAG). The resulting *cat* linear DNA cassette had overhanging arms homologous to nucleotides 16855 to 16894 and nucleotides 17333 to 17372 of *trbB*. The remainder of the protocol was completed as above. To verify insertion of the *cat* cassette into F, clones were sequenced with TEL26 (5' GGTACCGAAGGGCAGCAGGAGGGC) and TEL27 (5' GAAGCTTCCGGCAAT GAGTAACACCAC).

2.2.4 Site-directed mutagenesis

All mutagenesis was completed using QuikChange (Stratagene) site-directed mutagenesis according to the manufacturer's instructions. Briefly, 25 ng of pBAD30-*trbB* was used as a template for 125 ng of mutated nucleotide primers described in Table

2.3. PCR conditions were as follows: initial denaturing at 95 °C for 2 min, followed by 18 cycles of denaturing at 95 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 7 min. After digestion with DpnI, 5 μL of PCR product was used to transform XL-1 Blue competent cells. Every mutant was sequenced to ensure that mutants were correct and in-frame.

2.3 Transposon mutagenesis

2.3.1 IS*phoA*/in transposition into $traF_F$

Insertion of IS*phoA*/in into a plasmid expressing $traF_F$ (pK184-*traF*) was accomplished according to the procedure outlined by Manoil and Bailey (1997). Infections of *E. coli* CC245 with λTn*phoA*/in phage were used to achieve a phage titre of at least 2 x 10^9 pfu. pK184-*traF* was transformed into *E. coli* CC160 and these cells were grown overnight at 37°C as standing cultures. Then in a fresh tube, 0.2 mL of this cell culture was mixed with λTn*phoA*/in at an infection multiplicity of 0.1 to 0.3 phage per cell. Final concentrations of 10 mM $MgSO_4$ and 1% maltose were also required in the infection. The infection mixture was incubated at 37°C for 10 min prior to adding 0.8 mL of LB broth and Km (25 μg/mL) to select for pK184-*traF*. The infection mixture was grown overnight at 30°C on a tube roller with aeration. 0.4 mL of undiluted culture was then plated onto LB plates containing Km (25 μg/mL) and Cm (100 μg/mL) to select for growth of cells carrying transposon insertions. After two days growth at 30°C plasmid DNA was isolated from all bacteria on the plate as previously described above. Electrocompetent *E. coli* CC118 cells were transformed with the isolated plasmid DNA

and plated onto XP^+ selection plates, which contain LB agar without NaCl, 5% sucrose, 5-bromo-4-chloro-3-indolyl phosphate (40 μg/mL)(Boehringer-Mannheim), Km (25 μg/mL) and Cm (40 μg/mL). Blue colonies were screened for correct insertions by isolating the plasmid DNA and performing a BamHI digestion. To ensure that IS*phoA*/in was inserted within $traF_F$ of pK184-*traF*, the plasmid DNA was then sequenced with primer AKE3, which sequences across the site of insertion.

2.3.2 Conversion of IS*phoA*/in insertions to in-frame 31-aa insertions

DNA isolated from IS*phoA*/in derivatives of $traF_F$ was digested with BamHI and run on an 1% agarose gel to analyze the digestion. For successful IS*phoA*/in insertions, the 3.5 kbp DNA fragment representing pK184-*traF* and phage epitope are isolated with a Qiagen Gel-extraction kit from the 4.5 kbp DNA fragment representing the left arm transposon. The 3.5 kbp DNA was ligated overnight at 16°C with T4 DNA ligase and then transformed into DH5α cells.

2.4 GST-$TraF_F$ protein interaction assay using ^{35}S-methionine-labelled cell lysate

The method for GST pull-down analysis is as described previously (Golemis, 2002). *E. coli* MC4100 cells containing either pGEX-*traF* or pGEX-2T as a control were grown overnight at 37°C on a tube roller. The overnight cultures were diluted 1/40 in 50 mL of LB broth with Amp (100 μg/mL) and then grown on a shaker at 37°C until they reached an OD_{600} of 0.4 to 0.6. The culture was induced with a final concentration of 0.5 mM IPTG and incubated on a shaker at 30°C for two hours. Uninduced and induced samples

equivalent to 0.1 OD_{600} were run on a 12% SDS-polyacrylamide gel to assess induction. Cells were pelleted at 10000 rpm for 20 min at 4°C and resuspend in 1 mL lysis buffer, which is phosphate-buffered saline (PBS) buffer containing 10 mM $MgCl_2$, 1 mM DTT, and 0.5 mg/mL lysozyme. The resuspended cells were left for 1 hour on a rocker at 4°C and then sonicated three times at 10 sec intervals. Cell debris was pelleted at 14000 rpm for 15 min and 60 μL of the supernatant containing GST-$TraF_F$ was mixed with 250 μL of glutathione sepharose 4B (Pharmacia). The protein solution was allowed to bind to the resin for 1 hour at 4°C with gentle mixing and then unbound protein was removed by extracting the supernatant from the precipitated resin.

Two mL cultures of *E. coli* MC4100 cells alone as a control or containing pOX38-Tc were grown on a roller at 37°C to an OD_{600} of 0.4. Cells were pelleted at 7000 rpm for 2 min and washed two times with 1 mL of M9 minimal media. Cells were resuspended in 1 mL of ^{35}S-methionine assay medium, which is M9 minimal media containing 2 mM $MgSO_4$, 0.2% glucose, 0.1 mM $CaCl_2$, and a 0.1% 18 amino acid mixture lacking cysteine and methionine. The cell mixture was grown for 1 hour at 30°C with shaking and then newly synthesized proteins were labeled with 25 μCi diluted ^{35}S-methionine for 1 hour at 30°C. ^{35}S-methionine-labeled proteins were extracted by sonication as above and were incubated with the glutathione sepharose 4B resin and GST-$TraF_F$ mixture overnight at 4°C. The resin was allowed to settle and the supernatant was extracted to remove unbound proteins. The resin was then washed 4 times with 0.5 mL of binding buffer, which is 50 mM Tris-HCl (pH 8) containing 100 mM NaCl, 0.1 mM DTT, 10 mM $MgCl_2$, 10% glycerol, and 0.1% Nonidet P-40. GST-$TraF_F$ and any interacting

proteins were eluted from the glutathione sepharose 4B resin using 50 μL of glutathione elution buffer, which is 50 mM Tris-HCl (pH 8) containing 10 mM reduced glutathione. Protein samples were run on a 12% SDS-polyacrylamide gel, and the gel was sealed in a film cassette for 2 weeks at -80°C before developing the film.

2.5 Protein computer analysis

2.5.1 Multiple sequence alignment F- and H-like protein sequences

Sequences similar to F TraN and TraG were found by using The PSIBLAST algorithm was used to search the Microbial Finished and Unfinished Genomes Database (http://www.ncbi.nlm.nih.gov/Microb_blast/unfinishedgenome.html) to find sequences similar to $TraF_F$, $TrbB_F$, $TrhF_{R27}$, $DsbC_{R27}$, and $HtdT_{R27}$. Only sequences from complete genome sequencing projects were used in this study. Multiple alignments were obtained for all sequences using ClustalW (http://clustalw.genome.ad.jp/) with a gap open penalty of 5 and a gap extension penalty of 0.01 with the GONNET weight matrix. The alignment was then configured with Genedoc (www.psc.edu/biomed/genedoc).

2.5.2 Signal sequence, conserved domain, and secondary structure prediction

F- and H-like proteins that had not been reported to be secreted to the cell envelope were analyzed using SignalP predictions (http://www.cbs.dtu.dk/services/SignalP) to detect leader peptides. A search for conserved domains within $TraF_F$, $TrbB_F$, $TrhF_{R27}$, $DsbC_{R27}$, $HtdT_{R27}$ and their homologues was performed using the program CD-Search (Marchler-Bauer *et al.*, 2004). JPRED, a consensus method for protein secondary structure

prediction (http://www.compbio.dundee.ac.uk/~www-jpred/), was then used to compare the predicted structures of these F- and H-like proteins to the known structures of *E. coli* thioredoxin (Jeng *et al.*, 1994) and DsbC (McCarthy *et al.*, 2000).

Chapter 3

Results

3.1 The thioredoxin folds of F- and H-like transfer proteins

3.1.1 Alignment of F- and H-like transfer regions

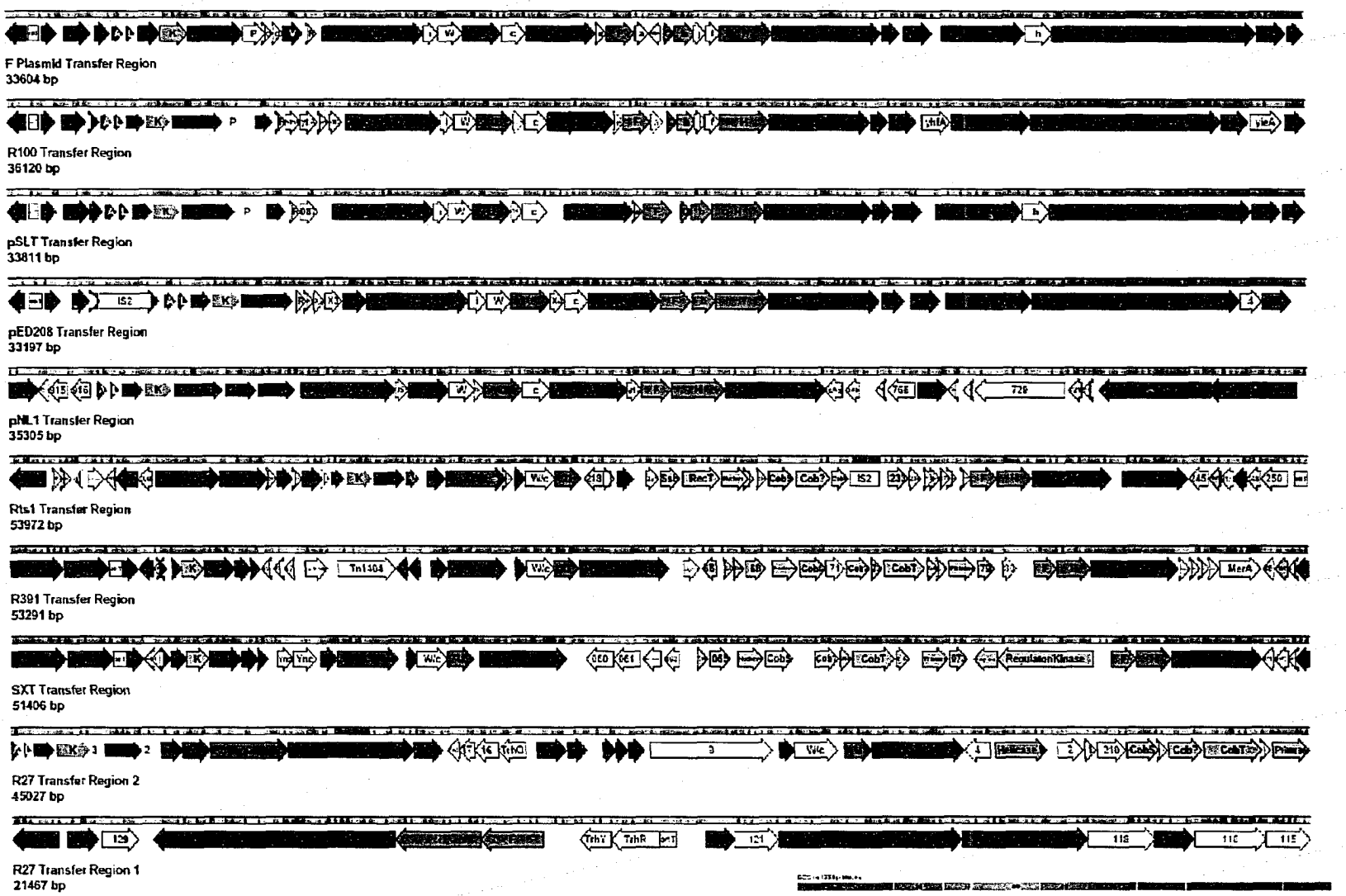
Recent advances in gene and protein sequence analysis have allowed researchers to search for homologous sequences in numerous genome databases. However, the significance of data obtained from such programs as PSIBLAST (Altschul *et al.*, 1997) must be left to the discretion of the researcher. In this study, we were interested in obtaining multiple alignments to compare the conservation and deviation amongst relevant protein homologues of the F *tra* gene products. This search was confined by ensuring that the following parameters were met: the F protein homologue must be encoded by a putative transfer operon, the putative transfer operon must contain the majority of T4SS components including the F-specific or auxiliary proteins, and the coding region from which the homologue was identified must be a complete GenBank submission. Consequently, an alignment of all transfer regions similar to plasmid F was required prior to assessing the sequence variation within individual gene products (Figure 3.1). Extensive analysis of PSIBLAST search results for all F T4SS components revealed 13 plasmid transfer regions bearing similarity to the F plasmid.

Irrespective of plasmid incompatibility grouping or DNA regulation systems, two relatively distinct types of transfer regions were identified in regards to their T4SS gene organization and sequence conservation. First, the F-like transfer regions exemplified by the IncFI plasmid F of *Escherichia coli* (Frost *et al.*, 1994) includes the following plasmids: R100 of *Salmonella enterica* serovar typhimurium and *Shigella flexneri*

(Anthony *et al.*, 1999), pSLT of *S. typhimurium* (McClelland *et al.*, 2001), pED208 of *Salmonella enterica* serovar typhi (Lu *et al.*, 2002), and pYJ016 of *Vibrio vulnificus* (Chen *et al.*, 2003). Second, the H-like transfer regions exemplified by the IncHI1 plasmid R27 of *S. typhi* (Sherburne *et al.*, 2000) includes the following plasmids: R478 of *Serratia marcescens* (Gilmour *et al.*, 2004), pHCM1 of *S. typhi* (Parkhill *et al.*, 2004), pCAR1 of *Pseudomonas resinovorans* (Maeda *et al.*, 2003), Rts1 of *Proteus vulgaris* (Murata *et al.*, 2002), R391 of *Providencia rettgeri* (Böltner *et al.*, 2002), SXT of *V. cholerae* (Beaber *et al.*, 2002), and pNL1 of *Novosphingomonas aromaticivorans* (Romine *et al.*, 1999). With the exception of *N. aromaticivorans*, the organisms from which all these plasmids were isolated are members of the γ-proteobacteria; these organisms are specifically from the Enterobacteriaceae, Pseudomonadaceae, and Vibrionaceae families. *N. aromaticivorans*, the host organism of pNL1, belongs to the α-proteobacteria.

The F-like transfer regions identified maintain the same genetic organization of these T4SS components: $traA_F$, $-L_F$, $-E_F$, $-K_F$, $-B_F$, $-V_F$, $-C_F$, $-W_F$, $-U_F$, $trbC_F$, $traN_F$, $traF_F$; $trbB_F$, $traH_F$, and $traG_F$ (Figure 3.1). However, individual F-like transfer regions may contain additional genes that interrupt this pattern. Some of these additional genes, such as the pilin processing genes, are conserved in other F-like members. The pilin chaperone, encoded by $traQ_F$, is only conserved in plasmids F, R100, and pSLT; the pilin acetylase, encoded by $traX_F$, is conserved in plasmids F, R100, pSLT, and pED208. Regardless of pilin processing, the $traA_F$ genes exhibit similarity but not with $trhA_H$

Figure 3.1. Comparison of the F- and H-like transfer regions. The F-like transfer regions included in this figure are derived from the following plasmids: F of *Escherichia coli* (Frost *et al.*, 1994), R100 of *Salmonella enterica* serovar typhimurium and *Shigella flexneri* (Anthony *et al.*, 1999), pSLT of *S. typhimurium* (McClelland *et al.*, 2001), and pED208 of *Salmonella enterica* serovar typhi (Lu *et al.*, 2002). The H-like transfer regions included in this figure are derived from the following plasmids: R27 of *S. typhi* (Sherburne *et al.*, 2000), Rts1 of *Proteus vulgaris* (Murata *et al.*, 2002), R391 of *Providencia rettgeri* (Böltner *et al.*, 2002), SXT of *Vibrio cholerae* (Beaber *et al.*, 2002), and pNL1 of *Novosphingomonas aromaticivorans* (Romine *et al.*, 1999). Transfer genes that are homologous are represented with the same colour. Gene sizes are proportional within each plasmid transfer region but not between plasmids. The line running above each transfer region indicates the percent GC content according to the legend located in the bottom right-hand side.



genes. Pilin genes of F-like plasmids are very similar and differ only significantly at the amino-terminus of the mature pilin subunits (Frost *et al.*, 1985). With the exception of pYJ016, $trbI_F$ is conserved in the F-like transfer region.

The H-like transfer regions are more diverse for the following reasons: they may be found in plasmids as well as ICEs, their T4SS component genes may be found in multiple transfer operons, and certain genes such as $trhA_H$ or $trhN_H$ may often vary in their genetic location. However, certain genes are always found organized together such as $trhL_H$, -E_H, -K_H, and –B_H or $trhF_H$, -G_H, and –H_H (Figure 3.1). In addition to the *trh* T4SS genes, H-like transfer regions also contain a pilin peptidase gene, $traF_P$, as well as putative disulfide bond isomerase genes, $htdT_H$ or $dsbC_H$. Interestingly, the $trhW_H$ genes encode proteins that resemble a fusion between the $TrbC_F$ and $TraW_F$ proteins. Plasmid pNL1 has an unusual transfer region in that it contains $dsbC_{pNL1}$, two distinct $traW_{pNL1}$ and $trbC_{pNL1}$ genes, as well as $orf851_{pNL1}$, which appears to be a fusion of $trbI_F$ and the peptidase $traF_P$ gene. Given that the transfer region of pNL1 is a mosaic of F- and H-like T4SS genes, it is difficult to designate a classification for this system. For the purposes of this thesis, pNL1 will be referred to as an H-like plasmid.

3.1.2 F- and H-like transfer regions encode putative thiol-disulfide oxidoreductases

The non-redundant protein sequence database was searched with the program PSIBLAST (Altschul *et al.*, 1997) to find homologues of $TraF_F$ in the F plasmid (Frost *et al*, 1994;

Gubbins *et al.*, 2005). PSIBLAST detected several TraF homologues in the transfer regions of other F-like (E-values = $4e^{-21}$ to e^{-136}) and H-like (E-values = $6e^{-4}$ to $3e^{-18}$) plasmids (Bart Hazes, personal communication) (Table 3.1). Using the position-sensitive scoring matrix (PSSM) based on this set of TraF homologues, a second cycle of PSIBLAST found a group of proteins that displayed homology to approximately 100 amino acids of the C-terminal region of $TraF_F$ (E-values = $2e^{-6}$ to $4e^{-8}$) (Table 3.1). This group of proteins included $TrbB_F$ and its F-like homologues in R100 (Anthony *et al.*, 1999), pSLT (McClelland *et al.*, 2001), pED208 (Lu *et al.*, 2002), and pYJ016 (Chen *et al.*, 2003), as well as a few hypothetical proteins (not shown in Table 3.1) annotated as thiol-disulfide isomerases. Subsequent cycles of PSIBLAST using a PSSM inclusion threshold of 0.001 revealed extensive homology to the thioredoxin/thiol-disulfide isomerase family exemplified by *E. coli* thioredoxin (E-values = $<8e^{-10}$) (Table 3.1). A search for conserved domains within homologues of $TraF_F$ using the program CD-Search (Marchler-Bauer *et al.*, 2003) revealed thioredoxin-like regions (COG0526, TrxA) within TraF of R391 (Böltner *et al.*, 2002) and SXT (Beaber *et al.*, 2002). A consensus method for protein secondary structure prediction was performed on all $TraF_F$ and $TrbB_F$ homologues using the program JPred (Cuff *et al.*, 1998). The predicted secondary structures of the C-terminal regions of these homologues showed considerable similarity to the thioredoxin fold as determined by comparison to the known structure of *E. coli* thioredoxin (Jeng *et al.*, 1994) (Figure 3.2). Although $TraF_F$ lacks the C-X-X-C motif that forms the catalytic site of thioredoxins, closely related TraF orthologues from the H-like T4SS as well as all TrbB proteins do contain this active site motif (Fig. 3.2).

Table 3.1 Homologies amongst TraF, TrhF, TrbB, DsbC, and HtdT proteins from F-like, H-like and I-like Plasmids

Plasmid	Inc Group	Organism	Plasmid References	BLAST Query: F TraF		BLAST Query: R27 DsbC	
				protein	E value	protein	E value
F-like							
F	FI	*Escherichia coli*	Frost *et al.*, 1994; Gubbins *et al.*, 2005	TraF	e^{-136} (1)[a]		
				TrbB	$4e^{-07}$ (2)		
R100	FII	*Salmonella enterica serovar Typhimurium*	Anthony *et al.*, 1999	TraF	e^{-133} (1)		
				TrbB	$8e^{-08}$ (2)		
pSLT		*Salmonella enterica serovar Typhimurium*	McClelland *et al.*, 2001	TraF	e^{-107} (1)		
				TrbB	$2e^{-06}$ (2)		
pED208	FV	*Salmonella enterica serovar Typhi*	Lu *et al.*, 2002	TraF	$1e^{-51}$ (1)		
				TrbB	$4e^{-08}$ (2)		
pYJ016		*Vibrio vulnificus*	Chen *et al.*, 2003	TraF	$4e^{-21}$ (1)		
				TrbB	$1e^{-07}$ (2)		

Plasmid	Inc Group	Organism	Plasmid References	BLAST Query: F TraF		BLAST Query: R27 DsbC	
				protein	E value	protein	E value
H-like							
pNL1		*Novosphingomonas aromaticivorans*	Romine *et al.*, 1999	TraF	$3e^{-18}$ (1)	Orf883	$2e^{-21}$ (2)
Rts1	T	*Proteus vulgaris*	Murata *et al.*, 2002	Orf240	$8e^{-09}$ (1)	Orf212	$2e^{-16}$ (2)
R391	J	*Providencia rettgeri*	Böltner *et al.*, 2002	TrhF	$5e^{-13}$ (1)	DsbC	$3e^{-23}$ (2)
SXT		*Vibrio cholerae*	Beaber *et al.*, 2002	TrhF	$4e^{-13}$ (1)	S054	$3e^{-23}$ (2)
pCAR1		*Pseudomonas resinovorans*	Maeda *et al.*, 2003	TrhF	$1e^{-09}$ (1)	DsbC	$2e^{-14}$ (2)
pHCM1	HI1	*Salmonella enterica serovar Typhi*	Parkhill *et al.*, 2001	TrhF	$7e^{-05}$ (1)	DsbC	e^{-140} (1)
						HtdT	$4e^{-04}$ (2)
R27	HI1	*Salmonella enterica serovar Typhi*	Lawley *et al.*, 2002; Lawley *et al.*, 2003; Sherburne *et al.*, 2000	TrhF	$7e^{-05}$ (1)	DsbC	e^{-140} (1)
						HtdT	$3e^{-04}$ (2)
R478	H2	*Serratia marcescens*	Gilmour *et al.*, 2004	TrhF	$6e^{-04}$ (1)	DsbC	e^{-120} (1)
						HtdT	$1e^{-04}$ (2)

Plasmid	Inc Group	Organism	Plasmid References	BLAST Query: F TraF		BLAST Query: R27 DsbC	
				protein	E value	protein	E value
I-like							
R64	I1	*Salmonella enterica serovar Typhimurium*	Furuya *et al.*, 1996			TrbB	$5e^{-15}$ (3)
ColIb-P9	I1	*Shigella sonnei*	Rees *et al.*, 1987			TrbB	$5e^{-15}$ (3)
pEL60		*Erwinia amylovora*	Foster *et al.*, 2004			TrbB	$3e^{-07}$ (2)
pCTX-M3		*Citrobacter freundii*	Accession # NC_004464			TrbB	$4e^{-06}$ (3)
none	None	*Escherichia coli*		TrxA	$8e^{-10}$ (3)		
none	None	*Escherichia coli*				DsbC	$5e^{-32}$ (1)
none	None	*Escherichia coli*				DsbG	$5e^{-16}$ (2)

a. The numbers in parenthesis indicate the number of cycles of PSI-BLAST.

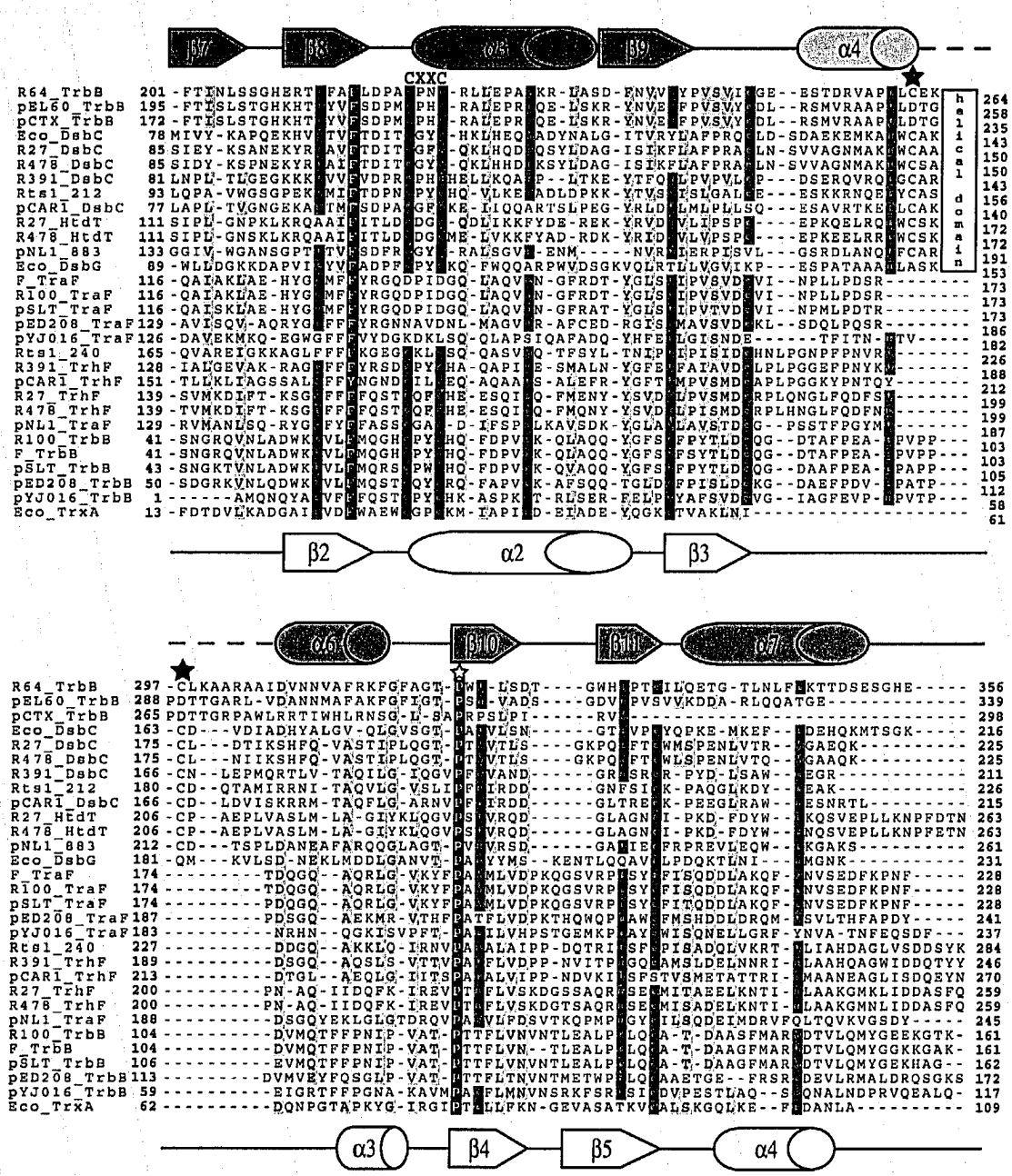
Interestingly, TraF orthologues that lack the C-X-X-C motif are only found on F-like plasmids that carry TrbB. In contrast, TraF homologues that contain a C-X-X-C motif are only found on H-like plasmids that lack TrbB. This suggests that TrbB may compensate for the lost redox function of TraF.

Although the H-like plasmids encode TraF homologues with C-X-X-C motifs, these plasmids also encode DsbC proteins, which contain the same motif. The IncHI1 R27 transfer protein $DsbC_{R27}$ is annotated as a putative disulfide-interchange protein (Accession number = AAF69969) (Lawley *et al.*, 2002; Lawley *et al.*, 2003; Sherburne *et al.*, 2000). A PSIBLAST search with this protein sequence revealed $DsbC_H$ homologues in the transfer regions of H-like plasmids pHCM1 (Parkhill *et al.*, 2001) and R478 (Gilmour *et al.*, 2004) (E-values = e^{-140} and e^{-120} respectively) as well as the well characterized *E. coli* chromosomally encoded DsbC (E-value $5e^{-32}$) (Table 3.1). The second cycle of PSIBLAST identified another group of $DsbC_H$ homologues from other H-like plasmids (E-values = $2e^{-14}$ to $3e^{-23}$), establishing homology to H-like HtdT proteins (E-value = $4e^{-4}$) and the chromosomally encoded *E. coli* DsbG (E-value = $5e^{-16}$) (Table 3.1). Subsequent cycles of PSIBLAST using a PSSM inclusion threshold of 0.001 revealed homology to the TrbB proteins of the IncI plasmids R64 (Furuya *et al.*, 1996), ColIb-P9 (Rees *et al.*, 1987), pEL60 (Foster *et al.*, 2004) and pCTX-M3 (Accession number = NC_004464) (E-values = $4e^{-6}$ to $5e^{-15}$), which are not directly related to the F-like T4SS TrbB proteins. Furthermore, these I-like T4SS do not contain homologues to any of the F- and H-like specific or auxiliary transfer proteins. CD-Searches also

revealed that all $DsbC_H$ and $HtdT_H$ homologues, with the exception of $HtdT_{R478}$, share homology with COG1651 (DsbG) showing E-values between $7e^{-16}$ and 0.004. All these proteins conserve the C-X-X-C active site motif of thioredoxins (Fig. 3.2). Secondary structure predictions by JPred were compared to the known structure of *E. coli* DsbC, and the results indicated that these proteins exhibit a thioredoxin domain reminiscent of *E. coli* DsbC. The C-terminal domain (residues 78-216) of *E. coli* DsbC is divided in two regions: the thioredoxin sub-domain (residues 78-122, 167-216) and the α-helical sub-domain (residues 123-166), which is inserted between the β2-strand and the α2-helix of the thioredoxin fold (McCarthy *et al.*, 2000). Cys^{141} and Cys^{163} within this sub-domain form an intramolecular disulfide bond that is important for the stability of the molecule (Liu and Wang, 2001). Despite variations in length, all $DsbC_H$ and $HtdT_H$ proteins contain this extra domain, which includes the two conserved cysteine residues (Fig 3.2).

Disulfide isomerization activity requires hydrophobic or aromatic amino acids in the third position of the C-X-X-C active site (Bessette *et al.*, 2001). All $DsbC_H$ and $TrbB_F$ proteins contain aromatic amino acids in this position. Most $TraF_F$ or $TrhF_H$ proteins with an active site have either aromatic or hydrophobic amino acids in this position, with the exception of $TraF_{pNL1}$, which has an alanine, and the $HtdT_H$ proteins, which have glycine. A proline from the β3-strand is found in close structural proximity to the active site of α1-helix, and this residue is required for maintaining an active conformation in *E. coli* thiol-disulfide oxidoreductases (Kadokura *et al.*, 2004). This β3-proline is conserved

Figure 3.2. Multiple sequence alignment of TraF, TrhF, TrbB, HtdT, and DsbC from the F-like, H-like, and I-like transfer regions with the TrxA, DsbC and DsbG proteins from *E. coli*. Each protein in the alignment is preceded by the plasmid name or a three letter abbreviation for the organism if the gene is encoded on the chromosome (Eco = *Escherichia coli*). The information regarding these proteins is arranged below as plasmid name (if necessary), protein name, Genbank accession number, and host organism: R64 TrbB **BAB91646** *S. typhimurium*; pEL60 TrbB **AAQ97939** *E. amylovora*; pCTX-M3 TrbB **AAN87722** *C. freundii*; DsbC **AAC75931** *E. coli*; R27 DsbC **AAF69969** *S. typhi*; R478 DsbC **CAE51723** *S. marcescens*; R391 DsbC **AAM08034** *P. rettgeri*; Rts1 Orf212 **BAB93774** *P. vulgaris*; pCAR1 DsbC **BAC41659** *P. resinovorans*; R27 HtdT **AAF69865** *S. typhi*; R478 HtdT **CAE51541** *S. marcescens*; pNL1 Orf883 **AAD03960** *N. aromaticivorans*; DsbG **AAC73705** *E. coli*; F TraF **BAA97961** *E. coli*; R100 TraF **BAA78873** *E. coli*; pSLT TraF **AAL23500** *S. typhimurium*; pED208 TraF **AAM90720** *S. typhi*; pYJ016 TraF **BAC97739** *V. vulnificus*; Rts1 *orf240* **BAB93802** *P. vulgaris*; R391 TraF **AAM08018** *P. rettgeri*; pCAR1 TraF **BAC41668** *P. resinovorans*; R27 TrhF **AAF69964** *S. typhi*; R478 TrhF **CAE51730** *S. marcescens*; pNL1 TraF **AAD03949** *N. aromaticivorans*; R100 TrbB **BAA78876** *E. coli*; F TrbB **BAA97965** *E. coli*; pSLT TrbB **AAL23502** *S. typhimurium*; pED208 TrbB **AAM90721** *S. typhi*; pJY016 TrbB **BAC97741** *V. vulnificus*; TrxA **AAA24694** *E. coli*. Proteins from other plasmid systems with identical sequences to a protein listed above were not included in Fig. 1, but there accession numbers are included below: ColIb-P9 TrbB **BAA75142** *S. sonnei*; pHCM1 DsbC **CAD09838** *S. typhi*; SXT S054 **AAL59715** *V. cholerae*; pHCM1 HtdT **CAD09683** *S. typhi*; SXT TraF **AAL59678** *V. cholerae*; pHCM1 TrhF **CAD09843** *S. typhi*. The secondary structure of DsbC (top) and thioredoxin (bottom) are as determined by X-ray structure and NMR analysis (Jeng *et al.*, 1994; McCarthy *et al.*, 2000). The active sites are labeled as CXXC. The black stars indicate cysteines known or proposed to form a disulfide bond. The white star indicates a conserved proline residue that is found in close proximity to the active site of DsbA, DsbC and thioredoxin from *E. coli*. The box marked "helical domain" indicates the presence of an extra helical domain in some DsbC-like proteins. Residues in black indicate 100% conservation, residues in dark gray indicate more than 80% conservation, and residues in light gray indicate at least 50% conservation.



in *E. coli* DsbA, -C, -G, and thioredoxin as well as in all $TraF_F$, $TrhF_H$, $TrbB_F$, $HtdT_H$, and $DsbC_H$ proteins discussed here (Fig. 3.2).

3.1.3 $TraF_F$, $TrhF_H$, $TrbB_F$, $DsbC_H$, and $HtdT_H$ are predicted to be soluble periplasmic proteins

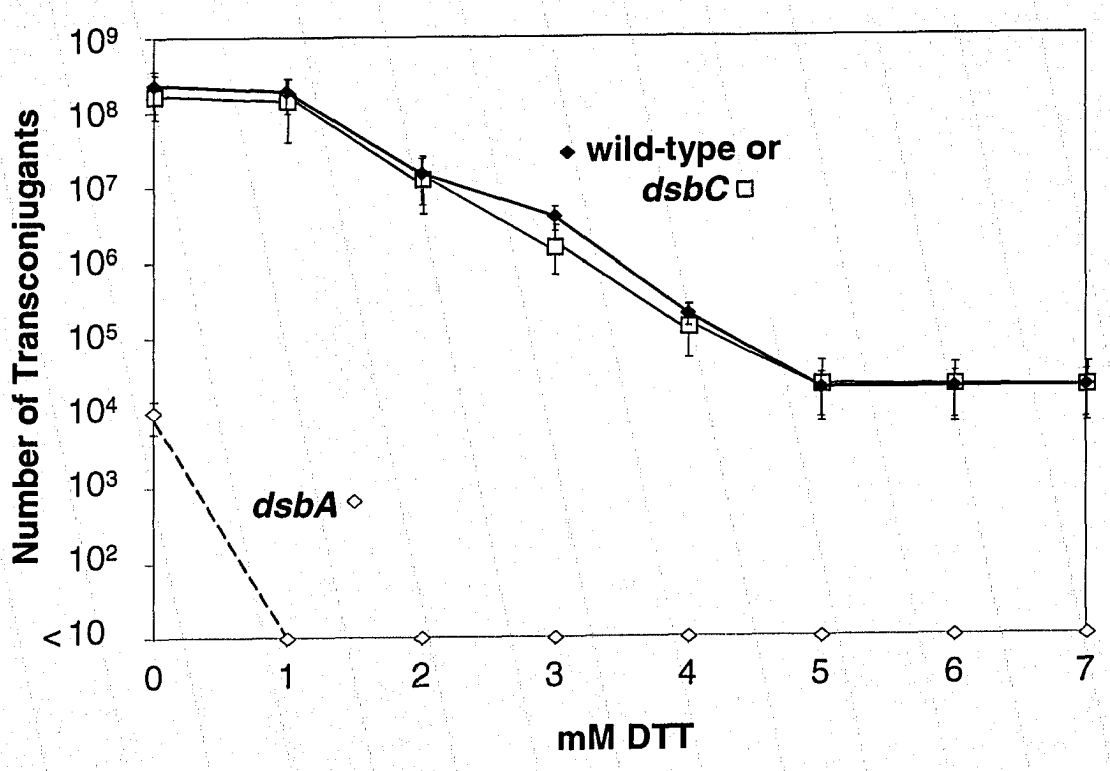
$TraF_F$ and $TrbB_F$ are known to contain leader peptides (Wu *et al.*, 1988; Wu and Ippen-Ihler, 1989), and SignalP predictions (Nielsen *et al.*, 1997) detected leader peptides in all but one of the $TrhF_H$, $HtdT_H$ and $DsbC_H$ proteins. The exception is $HtdT_{R27}$ (Accession number = AAF69865), for which a leader sequence could not be found; however, when compared to the sequence of the nearly identical $htdT_{pHCM1}$ (Accession number = CAD09683) it became apparent that $htdT_{R27}$ should begin 35 codons upstream from its current predicted start, at the rare TTG start codon. Analysis of this longer sequence revealed a 22 residue leader peptide in this protein. No other putative transmembrane regions were predicted in the mature proteins. Full length $TraF_F$ and $TrbB_F$ can be expressed as soluble proteins (Audette *et al.*, 2004; L. S. Frost, unpublished data) suggesting $TrhF_{R27}$, $DsbC_{R27}$, and $HtdT_{R27}$ are also soluble periplasmic proteins. In contrast, the I-like TrbB proteins identified from the PSIBLAST search using $DsbC_{R27}$ were not predicted to contain leader peptides according to SignalP predictions. Furthermore, subcellular protein localization predictions using the program PSLpred (Bhasin *et al.*, 2005) placed all $TrbB_I$ proteins in the cytoplasm. This suggests that the I-like TrbB proteins serve a different role than the F-like TrbB proteins in their respective transfer systems.

3.1.4 Transfer efficiency of pOX38-Tc is affected by DTT or *dsbA* null mutations

Null mutations in *dsbA* have previously been reported to affect assembly of the flagellar motor and the conjugative F pilus, as measured by electron microscopy (Bardwell *et al.*, 1991; Dailey and Berg, 1993). Furthermore, these *dsbA* null mutants also exhibit a hypersensitivity to DTT (Missiakas *et al.*, 1993). This phenotype was established by a genetic library screen of *E. coli* λTn*10* insertional mutants intended to isolate chromosomal mutations that render *E. coli* sensitive to otherwise non-lethal concentrations (7 mM) of dithiothreitol (DTT) (Missiakas *et al.*, 1993). DTT is a small reducing agent that can diffuse into the periplasm and maintain sulfhydryl groups in the reduced state *in vivo*. Without DsbA, the primary periplasmic oxidant, DTT exhibits a more pronounced effect upon these mutant cells because it is anticipated that cell envelope proteins such as OmpA and β-lactamase are prevented from forming their required disulfide bonds.

In order to further characterize the effect of *dsb* mutations on F conjugation, we assayed the mating efficiencies of wild-type, *dsbA* and *dsbC* mutant donor cells containing the F derivative pOX38-Tc. The mating assays were subsequently repeated following incubation of the donor cells with varying concentrations of DTT for one hour. Since wild-type bacteria have been shown to tolerate DTT concentrations up to 7 mM (Missiakas *et al.*, 1993), we sought to determine: i) if F conjugation was dependent upon cell envelope disulfide bond formation, and ii) if the DTT hypersensitivity of certain *dsb*

Figure 3.3. Mating efficiency of pOX38-Tc is greatly decreased by the reducing agent DTT. The normalized mating efficiencies of *E. coli dsbA* and *dsbC* mutants containing pOX38-Tc were plotted against increasing concentrations of DTT (mM).



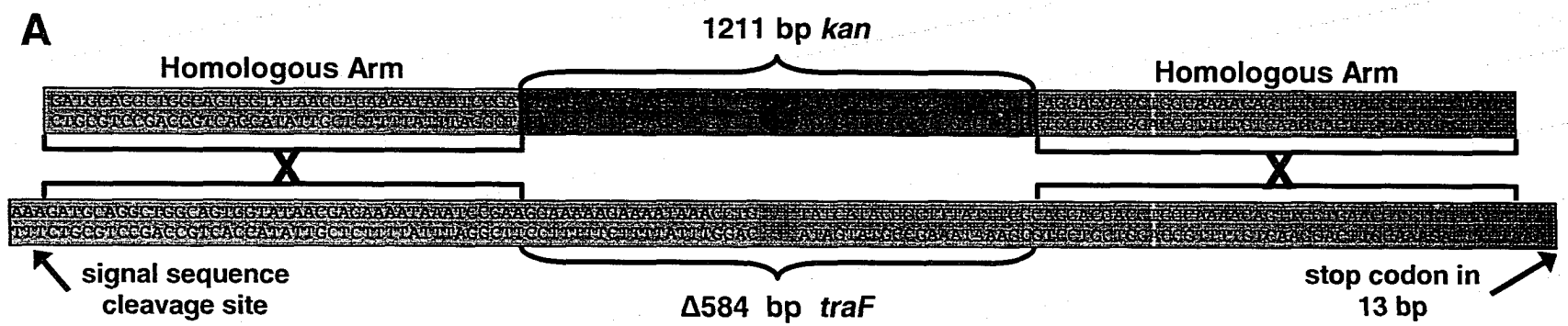
mutants could also be detected as measured by conjugative mating assays using non-lethal DTT concentrations. The mating efficiency of wild-type and *dsbC* donor cells was not affected by 1 mM DTT, but higher concentrations reduced mating efficiency up to four orders of magnitude in a dose-dependent manner before reaching a plateau at 5 mM DTT (Fig. 3.3). Donor cells with a chromosomal *dsbA* null mutation had transfer efficiencies four logs lower than that of wild-type cells; addition of 1 mM DTT to these cells completely abolished DNA transfer, indicating a DTT-hypersensitive phenotype for this strain (Fig. 3.3).

3.1.5 Construction of $traF_F$ and $trbB_F$ null mutants of pOX38-Tc

The essential role of $TraF_F$ in pilus formation was initially determined using a $traF_F$ insertion mutant that could produce a C-terminally truncated $TraF_F$ protein (Wu *et al.*, 1988). To rule out any effect of the N-terminal region of $TraF_F$, we used the λ Red recombination system to replace codons 34 to 229 with a *kan* cassette, thus creating pOX38-Tc Δ*traF*::*kan* (Fig. 3.4, Table 2.2). This mutation completely abolished DNA transfer and phage f1 infection, an indication that no F pili were assembled. The effects could be fully complemented by $traF_F$ supplied *in trans* on pFTraF (Fig. 3.4).

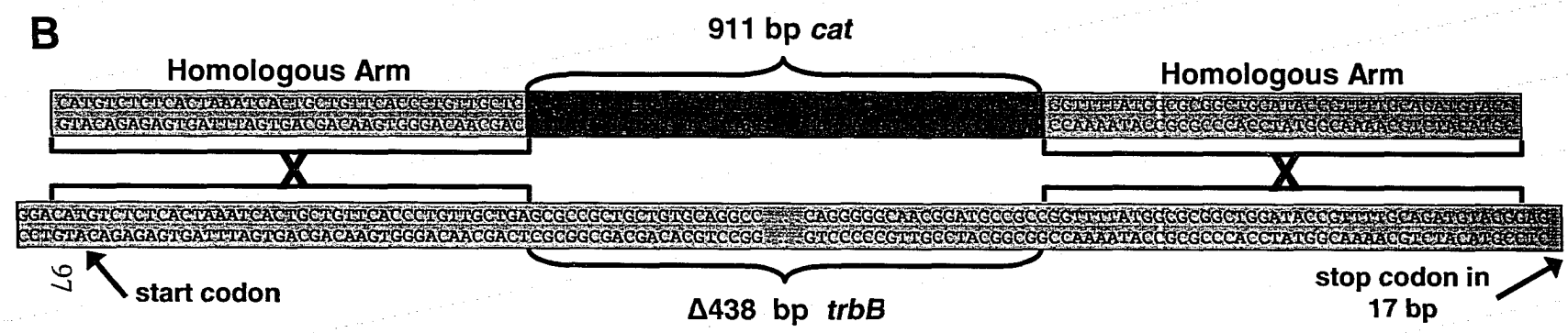
Similarly, the previous insertion of a *kan* cassette between codons 66 and 67 of $trbB_F$ might express a truncated version of $TrbB_F$ with a functional thioredoxin motif (Kathir and Ippen-Ihler, 1991). To ensure against this, codons 14 to 160 of $TrbB_F$ were replaced with the chloramphenicol acetyltransferase (*cat*) gene from Tn*9* to give pOX38-Tc

Figure 3.4. Gene disruptions of F *traF* (A) and F *trbB* (B). Adjoining this page are schematic diagrams of a *kan* and *cat* cassette insertion into F *traF* and F *trbB* respectively through homologous recombination. The native *kan* and *cat* promoters are present in both gene disruptions. Between the diagrams are the mating efficiencies and f1 phage sensitivities of the *traF* and *trbB* null mutants.



Construct	Mating efficiency (no. of transconjugants)	f1 phage sensitivity
pOX38-Tc RI89 (wildtype)	1.2×10^8	S
pOX38-TcΔ*traF*::*kan* RI89	< 10	R
+ pFTraF uninduced	8.1×10^7	S
+ pFTraF induced	1.1×10^8	S

Construct	Mating efficiency (no. of transconjugants)	f1 phage sensitivity
pOX38-Tc RI89 (wildtype)	1.2×10^8	S
pOX38-TcΔ*trbB*::*cat* RI89	1.2×10^8	S



Δ*trbB*::*cat* (Fig. 3.4, Table 2.2). This null mutation neither affected plasmid transfer nor f1 phage infection (Fig. 3.4).

3.1.6 Mating efficiency is reduced in an *E. coli dsbC-trbB*$_F$ double null mutant

The *trbB*$_F$ null mutant has no obvious phenotype, similar to several *E. coli* periplasmic thiol-redox proteins (Kadokura *et al.*, 2003). Thus, significant functional redundancy could exist among this group of disulfide isomerase proteins (Missiakas *et al.*, 1994; Ritz and Beckwith, 2001). To investigate whether the absence of both *E.coli* DsbC and the putative disulfide bond isomerase, TrbB$_F$, could affect F conjugation, a *trbB-dsbC* double mutation was created by introducing pOX38-Tc Δ*trbB::Cm* into RI179 (*dsbC*) cells. As a control, individual mutants were generated by introducing pOX38-Tc Δ*trbB::Cm* into RI189 (wild type) or pOX38-Tc into RI179 (*dsbC*) cells. Mating assays revealed that transfer efficiency decreased 10-fold in the *dsbC* strain (RI179) containing pOX38-Tc Δ*trbB::Cm* as compared to donor/plasmid combinations that contained either *trbB* or *dsbC* single mutations or completely wild-type donor cells (Table 3.2). Furthermore, complementation of RI179/pOX38-Tc Δ*trbB::Cm* with TrbB$_F$ supplied *in trans* from pFTrbB resulted in the restoration of wild type mating efficiency (Table 3.2).

3.1.7 Overproduced TrbB$_F$, DsbC$_{R27}$ or HtdT$_{R27}$ complement the DTT hypersensitivity of a *dsbA* null mutant

The *E. coli dsbC* gene was first detected in a multicopy *E. coli* chromosomal DNA library as a clone that could simultaneously suppress the sensitivity to DTT (10 mM) and

Table 3.2 Effect of *dsbC* and *trbB* null mutations on pOX38-Tc transfer

Construct	Number of transconjugants	Mating efficiency (+/- 10) (transconjugants/100 donor cells)
pOX38-Tc/RI89 (wild type)	1.2×10^8	80
pOX38-Tc/RI179 (*dsbC*)	1.1×10^8	73
pOX38-TcΔ*trbB*::*cat*/RI89 (wild type)	1.0×10^8	67
pOX38-TcΔ*trbB*::*cat*/RI179 (*dsbC*)	1.1×10^7	7
pOX38-TcΔ*trbB*::*cat*/RI179 (*dsbC*) + pFTrbB	1.0×10^8	67
pOX38-TcΔ*trbB*::*cat*/RI179 (*dsbC*) + pBAD30	1.0×10^7	7

benzylpenicillin (30 μg/mL) exhibited by a *dsbA* null mutant (Missiakas *et al.*, 1994). The *Erwinia chrysanthemi dsbC* gene was identified concurrently by an independent study, which transformed *E. coli dsbA* mutant strains with a multicopy *E. chrysanthemi* gene library and looked for motile transformants that displayed alkaline phosphatase activity (Shevchik *et al.*, 1994). In both cases over-expressed *dsbC* could partially compensate for *dsbA* null mutants.

We endeavored to ascertain whether the over-production of F- or H-like transfer proteins, which contain putative thioredoxin folds, could alleviate the general hypersensitivity to DTT exhibited by *dsbA* null mutants as measured by conjugative mating assays. For this experiment we relied on the arabinose-inducible pBAD30 expression system to over-produce selected transfer proteins, as well as on the presence of 1 mM DTT, a concentration we previously showed to abolish conjugative DNA transfer in a *dsbA* null mutant. Although $TrbB_F$, expressed by pFTrbB (Table 2.2), could not directly complement the effect of the *dsbA* mutation on the mating ability of RI190 (*dsbA*)/pOX38-Tc cells (data not shown), it could overcome the hypersensitivity to DTT (Table 3.3). This complementation required that pFTrbB be first induced with 0.1% arabinose, as uninduced samples showed no effect (data not shown). Similarly, over-expression of $dsbC_{R27}$ or $htdT_{R27}$ (pHDsbC or pHHtdT, respectively, Table 2.2) yielded comparable results (Table 3.3).

Table 3.3 Effect of overproduction of F- and H-like transfer proteins in a *dsbA* mutant

E. coli strains	Plasmid Constructs			Number of transconjugants
pOX38-Tc/RI89 (wild type)				1.2×10^8
pOX38-Tc/RI90 (*dsbA*)				1.1×10^4
pOX38-Tc/RI90 (*dsbA*)	+ 1mM DTT			<10
pOX38-Tc/RI90 (*dsbA*)	+ 1mM DTT	+ pBAD30		<10
pOX38-Tc/RI90 (*dsbA*)	+ 1mM DTT	+ pFTraF		<10
pOX38-Tc/RI90 (*dsbA*)	+ 1mM DTT	+ pFTraF(CPYC)		<10
pOX38-Tc/RI90 (*dsbA*)	+ 1mM DTT	+ pFTrbB	**F-like**	9.0×10^3
pOX38-Tc/RI90 (*dsbA*)	+ 1mM DTT	+ pFTrbB(Y63H)		3.0×10^3
pOX38-Tc/RI90 (*dsbA*)	+ 1mM DTT	+ pFTrbB(SPYS)		<10
pOX38-Tc/RI90 (*dsbA*)	+ 1mM DTT	+ pHTrhF		<10
pOX38-Tc/RI90 (*dsbA*)	+ 1mM DTT	+ pHHtdT	**H-like**	8.0×10^3
pOX38-Tc/RI90 (*dsbA*)	+ 1mM DTT	+ pHDsbC		1.0×10^4
pOX38-Tc/RI90 (*dsbA*)	+ 1mM DTT	+ pHDsbC(SGFS)		<10

All vector constructs were induced with the addition of 0.1% arabinose 2 hours prior to adding the recipient cells for the mating assay.

3.1.8 The C-X-X-C motif of $TrbB_F$ and $DsbC_{R27}$ is required to complement DTT-hypersensitive *dsbA* mutants

The replacement of at least the N-terminal cysteine residue of the C-X-X-C active site of DsbA, DsbC, and DsbG completely inactivates Dsb protein function (Andersen *et al.*, 1997; Grauschopf *et al.*, 1995; Missiakas *et al.*, 1994). Furthermore, DsbA is able to catalyze disulfide bond formation only when it contains the active Cys30-Cys33 disulfide bond, whereas reduced DsbC is able to catalyze disulfide bond isomerization only when its N-terminal active cysteine residue (Cys98) can form a mixed disulfide bond with substrate proteins (Nakamoto and Bardwell, 2004).

To determine whether the C-X-X-C motif of $TrbB_F$ and $DsbC_{R27}$ were important for the rescue of *dsbA* donor cells in the presence of DTT, site-directed mutagenesis (QuikChange, Stratagene) was used to convert the putative active site cysteines to serines to give pFTrbB(SPYS) and pHDsbC(SGFS), respectively. RI190 (*dsbA*)/pOX38-Tc donor cells containing either pFTrbB(SPYS) or pHDsbC(SGFS) showed a negligible mating efficiency, even after induction with 0.1% arabinose (Table 3.3). Thus, the C-X-X-C motif appears to be essential for the function of plasmid-encoded $TrbB_F$ and $DsbC_{R27}$, and it seems to act as an active redox site in these proteins. Because $TraF_F$ lacks the putative active site cysteines, site-directed mutagenesis was used to restore a C-P-Y-C motif at amino acids 135 to 138 of the mature protein. This mutant, pFTraF(CPYC), when supplied *in trans,* complemented the transfer deficiency of RI89/pOX38-Tc Δ*traF*::*kan* donor cells (Table 3.6) without induction with arabinose, suggesting that

basal expression of $TraF_F$ was sufficient for mating to proceed. However, mating was undetectable when either the wildtype (pFTraF) or mutated (pFTraF(CPYC)) $TraF_F$ were provided *in trans* in RI90 (*dsbA*) donor cells with 1 mM DTT (Table 3.3). Wildtype $TrhF_{R27}$ (pHTrhF) was also unable to complement the DTT hypersensitivity when provided *in trans* even though $TrhF_{R27}$ contains a C-X-X-C motif (Table 3.3).

3.1.9 Periplasmic secretion of the C-terminal thioredoxin folds of $TrbB_F$ and $DsbC_{R27}$, but not $TrhF_{R27}$, partially complements a *dsbA* deficiency.

E. coli thioredoxin, a disulfide oxidoreductase, can be secreted to the periplasm via the DsbA signal sequence. Although periplasmic wild-type thioredoxin cannot replace DsbA, thioredoxin variants containing the Grx-type (C-P-Y-C) or DsbA-type (C-P-H-C) active site sequences could complement a DsbA deficiency by approximately 40 to 60% as measured by motility on a swarm plate (Jonda *et al.*, 1999). Furthermore, a DsbC variant with a 76 amino acid deletion in its dimerization domain is able to complement the effect of a *dsbA* null mutation by 44% as measured by alkaline phosphatase activity (Bader *et al.*, 2001).

To determine whether the C-terminal thioredoxin folds of $TrbB_F$, $DsbC_{R27}$ or $TrhF_{R27}$ could complement a DsbA deficiency during conjugation, we constructed fusions between the DsbA signal sequence and our truncated proteins using the method of Jonda *et al.* (1999) (Table 2.2). Mating was assayed using RI190 (*dsbA*)/pOX38-Tc donor cells in the absence of DTT. As a control, the expression of *E. coli dsbA* from uninduced

pKDsbA was shown to nearly fully complement a *dsbA* mutation as measured by mating efficiency (Table 3.4). The expression of the C-terminal putative thioredoxin domains of $trbB_F$ and $dsbC_{R27}$ (pFBASS and pHCASS, respectively) were also shown to increase mating efficiency by 11- to 20-fold, in the *dsbA* mutant (Table 3.4). To test whether having a DsbA-type (C-P-H-C) active site rather than the wildtype (C-P-Y-C) active site of truncated $TrbB_F$ would be more effective in complementing the *dsbA* mutation, we constructed pFBASS(Y63H) (Table 2.2). pFBASS(Y63H) was similar in functional assays to pFBASS and pHCASS (Table 3.4), implying that DsbA has attributes other than the thioredoxin domain that contribute to its activity, which are lacking in $TrbB_F$. In contrast, the mating efficiency of pOX38-Tc in RI90 (*dsbA*) did not increase when the putative thioredoxin domain of $trhF_{R27}$ was expressed from pHFASS despite the presence of a C-X-X-C motif (Table 3.4).

Interestingly, induction of these constructs (pKDsbA, pFBASS, and pHCASS) with 1% arabinose gave a decrease in mating efficiency. Perhaps this overexpression caused an excessive increase of oxidizing potential in the periplasm that interferes with the disulfide bond isomerization. Overproduction of DsbB has been noted to result in a defect in periplasmic disulfide bond formation supporting the idea that isomerization has an optimal upper limit (Jander *et al.*, 1994).

Table 3.4 Effect of periplasmic secretion of C-terminal putative thioredoxin folds of F- and H-like transfer proteins in a *dsbA* mutant

E. coli strains	Plasmid Constructs	Number of transconjugants
pOX38-Tc/RI89 (wild type)		1.2×10^8
pOX38-Tc/RI90 (*dsbA*)		1.1×10^4
pOX38-Tc/RI90 (*dsbA*)	+ pBAD30	1.0×10^4
pOX38-Tc/RI90 (*dsbA*)	+ pKDsbA uninduced	8.0×10^7
pOX38-Tc/RI90 (*dsbA*)	+ pKDsbA induced	5.5×10^4
pOX38-Tc/RI90 (*dsbA*)	+ pFBASS uninduced	1.2×10^5
pOX38-Tc/RI90 (*dsbA*)	+ pFBASS induced	1.3×10^3
pOX38-Tc/RI90 (*dsbA*)	+ pFBASS(Y63H) uninduced	1.6×10^5
pOX38-Tc/RI90 (*dsbA*)	+ pFBASS(Y63H) induced	1.1×10^3
pOX38-Tc/RI90 (*dsbA*)	+ pHFASS uninduced	3.0×10^4
pOX38-Tc/RI90 (*dsbA*)	+ pHFASS induced	2.1×10^4
pOX38-Tc/RI90 (*dsbA*)	+ pHCASS uninduced	2.0×10^5
pOX38-Tc/RI90 (*dsbA*)	+ pHCASS induced	1.1×10^3

Induced refers to the addition of 1% arabinose 2 hours prior to adding the recipient cells for the mating assay; uninduced refers to the continued growth for 2 hours without the addition of arabinose.

3.2 Analysis of the role of $TraF_F$

3.2.1 Analysis of IS*phoA*/in insertions in *traF*$_F$ by mating assays

The Tn*phoA*/in system (Manoil and Bailey, 1997) was used to produce transposon mutants of *traF*$_F$. This system provides an in-frame PhoA fusion to the target gene so that if the target gene product (or portion there of) is secreted to the periplasm, the fused active alkaline phosphatase (AP) enzyme should be active and can be selected on plates containing 5-bromo-4-chloro-3-indolyl-phosphate (BCIP). Furthermore, a BamHI restriction endonuclease digestion of Tn*phoA*/in insertions of interest, followed by religation of the plasmid, results in the deletion of the Tn*phoA*/in insertion leaving an in-frame 31-aa epitope in the target protein.

The plasmid pK184-FTraF was used as a target for transposon mutagenesis because it is a low copy plasmid, which is capable of complementing a *traF*$_F$ null mutation at close to wild-type levels in a conjugative mating assay (Table 3.5). Seven independent Tn*phoA*/in inserts were obtained, of which only Tn*phoA*1 could not be converted to the corresponding 31-aa derivative (Figure 3.5). The locations of the six remaining 31-aa insertion mutants were determined by sequencing, using a primer specific to the 31-aa epitope (AKE3) (Table 2.3). The positions of each 31-aa insertions within *traF*$_F$ were then compared to the secondary structure of $TraF_F$ predicted by JPred. Sequencing of Tn*phoA*2/31-aa revealed its position at the N-terminus of $TraF_F$, whereas Tn*phoA*3 through -7/31-aa were located within the predicted thioredoxin fold domain (Figure 3.5). The 31-aa insertion mutants of $TraF_F$ were tested for complementation ability of pOX38-

Figure 3.5. Is*phoA*/in insertions into F *traF*. The location of the 7 Is*phoA*/in insertions and subsequent 31-aa epitopes is indicated within the entire protein sequence of F TraF. The asterisk denotes the signal sequence cleavage site of F TraF. The secondary structure predictions by JPred are illustrated as cylinders for α-helices and arrows for β-strands. The 4 β-strands and 3 α-helices predicted to form a thioredoxin fold are indicated in red and green respectively, whereas α-helices in the N-terminal domain are indicated in blue. Secondary structures that carry on to the next line are designated by the dotted lines. A double-headed arrow shows known insertions that modify the basic thioredoxin fold in other members of the thioredoxin superfamily.

Table 3.5 Analysis of IS*phoA*/in insertions in $traF_F$ in pK184-FTraF by conjugative assays

E. coli strains	Plasmid Constructs	Number of transconjugants from uninduced samples	Number of transconjugants from induced samples
pOX38-Tc MC4100		9.0×10^7	9.0×10^7
pOX38-TcΔ*traF*::*kan* MC4100		< 10	< 10
pOX38-TcΔ*traF*::*kan* MC4100	+ pK184-FTraF	< 0	2.0×10^7
pOX38-TcΔ*traF*::*kan* MC4100	+ pK184-FTraF /Tn*phoA*1*	< 0	< 0
pOX38-TcΔ*traF*::*kan* MC4100	+ pK184-FTraF /Tn*phoA*2 31-aa	< 0	< 0
pOX38-TcΔ*traF*::*kan* MC4100	+ pK184-FTraF /Tn*phoA*3 31-aa	< 0	< 0
pOX38-TcΔ*traF*::*kan* MC4100	+ pK184-FTraF /Tn*phoA*4 31-aa	< 0	1.7×10^6
pOX38-TcΔ*traF*::*kan* MC4100	+ pK184-FTraF /Tn*phoA*5 31-aa	< 0	2.4×10^6
pOX38-TcΔ*traF*::*kan* MC4100	+ pK184-FTraF /Tn*phoA*6 31-aa	< 0	< 0
pOX38-TcΔ*traF*::*kan* MC4100	+ pK184-FTraF /Tn*phoA*7 31-aa	< 0	< 0

Induced refers to the addition of 1 mM IPTG 2 hours prior to adding the recipient cells for the mating assay; uninduced refers to the continued growth for 2 hours without the addition of IPTG.

* This transposon mutant could not be converted to the corresponding 31-aa derivative

TcΔ*traF*::*kan* /MC4100 by mating into XK1200; only Tn*phoA*4/31-aa and -5/31-aa were effective in complementation (Table 3.5). It is intriguing to note that only the 31-aa derivatives of Tn*phoA*4 and -5, which were capable of complementing a $traF_F$ null mutation, did not interrupt the predicted α-helices and β-strands of this thioredoxin fold domain.

3.2.2 Analysis of $traF_F$ gene fusion constructs by mating assays

Gene fusion systems, such as the glutathione-S-transferase (GST) (Amersham) or the maltose binding protein (MBP) (New England Biolabs) systems, provide for simple expression and purification of fusion proteins using affinity chromatography. These fusion proteins can be detected by immunological assays using antibodies developed against the GST or MBP moieties. In addition, gene fusions can also be used to produce chimeric proteins consisting of combined segments of homologous proteins. In this regard, researchers can assess whether certain segments of one protein prevent its ability to complement its counterpart in a homologous system.

The pMAL-FTraF construct was created to express a protein fusion between MBP and the mature $TraF_F$ gene product, which would be secreted to the periplasm. When induced with 1mM IPTG, this construct was capable of complementing pOX38-TcΔ*traF*::*kan* /MC4100 as assayed by mating into XK1200 with 70% efficiency (Table 3.6). This suggests the N-terminal fusion of MalE to $TraF_F$ does not interfere to any great extent with the role of $TraF_F$ during F conjugation. However, the pFTraF-his6 construct, which

should produce a periplasmic $TraF_F$ gene product with a C-terminal his6-epitope tag, was unable to complement the same mating assay even with induction by 1% arabinose (Table 3.6). This result might suggest that the integration of additional residues at the C-terminus of $TraF_F$ prevents its function in pilus assembly in the periplasm. However, other researchers have shown that only a small proportion of C-terminal his6-tagged $TraF_F$ produced is actually processed and exported to the periplasm (Anthony *et al.*, 1998).

We have previously determined that $TraF_F$ and $TrhF_{R27}$ are homologous proteins. However, $TrhF_{R27}$, when expressed from pHTrhF, is unable to counteract the absence of $TraF_F$ in a mating assay between pOX38-TcΔ*traF*::*kan* /MC4100 and XK1200. The multiple sequence alignment by ClustalW combined with the secondary structure predictions by JPred for $TraF_F$ and $TrhF_{R27}$ reveal that most of both proteins produce a sequence alignment with few gaps and with an overlapping predicted secondary structure. However, $TrhF_{R27}$ contains an additional 17 residues at its N-terminus with no predicted secondary structure and an additional 70 residues at its C-terminus, which are predicted to include one α-helix and at least one β-strand. In order to establish whether the additional residues of $TrhF_{R27}$ affect its ability to complement a $traF_F$ null mutation, a pFTraF/HTrhF plasmid was constructed to produce a chimeric protein. The N-terminus of this chimera consists of residues Lys1 to Lys11 of $TraF_F$ fused with Arg30 to Val219 of $TrhF_{R27}$. There is also the substitution of a serine residue for Thr29 in $TrhF_{R27}$ as a consequence of the XbaI site. The $TrhF_{R27}$ segment is then fused precisely at its C-

Table 3.6 Analysis of *traF*$_F$ gene fusion constructs by conjugative assays

E. coli strains	Plasmid Constructs	Number of transconjugants from uninduced samples	Number of transconjugants from induced samples
pOX38-Tc MC4100		1.4×10^8	1.4×10^8
pOX38-TcΔ*traF*::*kan* MC4100		< 10	< 10
pOX38-TcΔ*traF*::*kan* MC4100	+ pFTraF	7.7×10^7	1.2×10^8
pOX38-TcΔ*traF*::*kan* MC4100	+ pFTraF(CPYC)	7.2×10^6	1.9×10^7
pOX38-TcΔ*traF*::*kan* MC4100	+ pHTrhF	< 0	< 0
pOX38-TcΔ*traF*::*kan* MC4100	+ pFTraF/HTrhF	3.0×10^3	1.0×10^4
pOX38-TcΔ*traF*::*kan* MC4100	+ pFTraF-his6	< 0	< 0
pOX38-TcΔ*traF*::*kan* MC4100	+ pMAL-FTraF	4.3×10^5	9.9×10^7

Induced refers to the addition of 1% arabinose or 1 mM IPTG 2 hours prior to adding the recipient cells for the mating assay; uninduced refers to the continued growth for 2 hours without the addition of arabinose or IPTG.

terminus to Asp194 to Phe228 of $TraF_F$. This chimeric protein consists of approximately 80% of $TrhF_{R27}$ amino acids and 20% of $TraF_F$ amino acids. With induction by 1% arabinose, expression from pFTraF/HTrhF/MC4100 resulted in 1.0×10^4 XK1200 transconjugants using the above conjugative mating assay. Although the mating efficiency is about 4 orders of magnitude lower than the wild-type assay involving pOX38-Tc, the ability of this chimera to partially complement a *traF*$_F$ null mutation suggests that both $TraF_F$ and $TrhF_{R27}$ have relatively similar roles in conjugation.

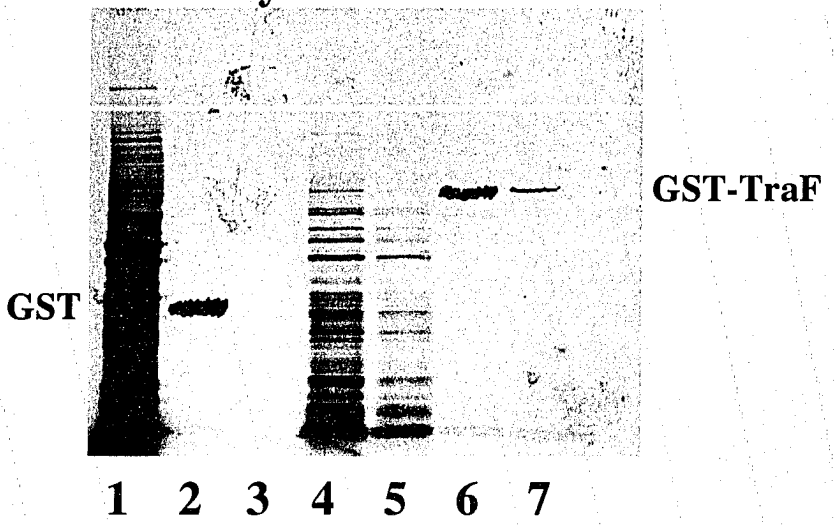
3.2.3 Analysis of GST-$TraF_F$ protein-protein interactions

The pGEX-FTraF plasmid was used to overexpress a cytoplasmic $TraF_F$ with an N-terminal fusion to GST. Given that the previous N-terminal fusion of $TraF_F$ with MalE did not affect $TraF_F$ function in a mating assay, it was anticipated that a GST-$TraF_F$ fusion would not disrupt potential protein-protein interactions in a GST pull-down assay as long as the structure of $TraF_F$ remained intact. A GST-$TraF_F$ pull-down assay was performed using ^{35}S-methionine labeled lysate from MC4100 and pOX38-Km /MC4100 to detect possible F transfer protein interactions. After the eluted samples were run on a 12% SDS polyacrylamide gel and left to expose an X-ray film, the results revealed a protein band of approximately 45 kDa that was present only in the pOX38-Km /MC4100 lysate. The only F transfer proteins predicted to have a similar molecular weight are the IM protein TraB (50.46 kDa), which is known to interact with TraK (Harris *et al.*, 2001), and the periplasmic protein TraH (47.8 kDa). Using yeast two-hybrid analysis, other researchers have indicated an interaction between TraF and TraH (Harris *et al.*, 2004).

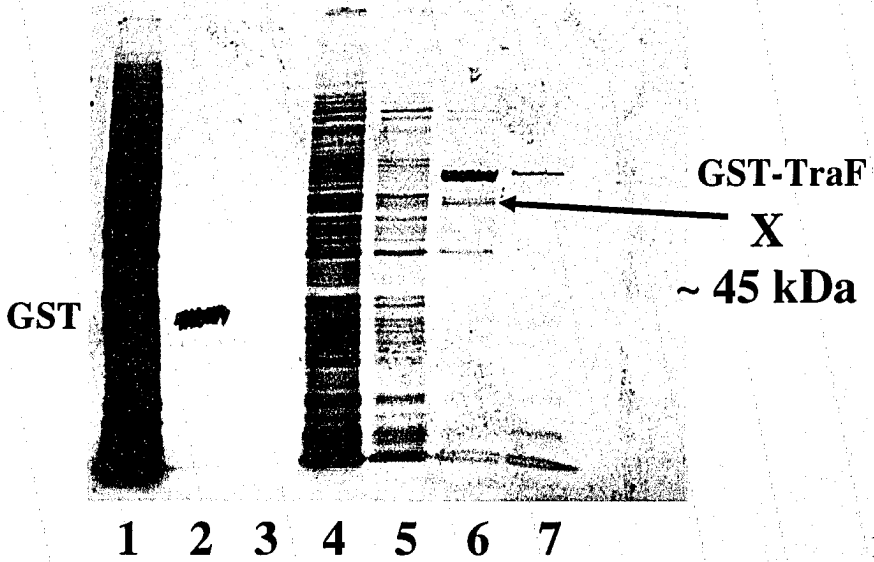
Figure 3.6. GST-TraF pulldown analysis. The lane markings are as follows: 1) ^{35}S-methionine labeled lysate, 2) eluent from the GST control, 3) boiled glutathione sepharose 4B beads from the GST control, 4) removal of supernatant containing proteins unbound to GST-TraF, 5) First wash of GST-TraF sample, 6) eluent from GST-TraF sample, 7) boiled glutathione sepharose 4B beads from the GST-TraF sample. For both images, the location of GST (lane 2) is indicated on the left hand side, whereas the location of GST-TraF (lane 6 and 7) is indicated on the right hand side. In the bottom image, an arrow indicates the location of the ~ 45 kDa protein from the lysate containing pOX38-Km that eluted with GST-TraF. The F transfer region proteins of this size include the IM protein TraB (50.46 kDa) and the periplasmic protein TraH (47.8 kDa).

Identification of Protein-Protein Interactions using GST-TraF

GST-TraF Pulldown using ^{35}S-methionine labeled lysate from MC4100



GST-TraF Pulldown using ^{35}S-methionine labeled lysate from pOX38-Km/MC4100



Chapter 4

Discussion

4.1 Discussion

The purpose of this thesis was to analyze the putative thioredoxin folds in proteins from plasmid F and R27. Based on sequence analysis, two genes of the F transfer region, *traF* and *trbB*, and three genes of the R27 transfer region, *trhF, dsbC* and *htdT,* are predicted to encode periplasmic proteins containing a C-terminal thioredoxin fold. The C-X-X-C active site motif of thioredoxins is present in all of these proteins except $TraF_F$. *E. coli* proteins containing thioredoxin folds are often noted for their role in disulfide bond formation and isomerization (Ritz and Beckwith, 2001). We therefore further characterized the effect of *dsb* mutations on F conjugation by showing that *E. coli* carrying a *dsbA* mutation, which is deficient in disulfide bond formation, is also greatly impaired in plasmid transfer and exhibits hypersensitivity to DTT. Furthermore, conjugation is sensitive to the cellular redox state since the reducing agent dithiothreitol (DTT) lowers mating efficiency. We then proposed that $TrbB_F$, $DsbC_{R27}$ and $HtdT_{R27}$ are putative disulfide bond isomerases for their respective transfer systems based on mutational analysis combined with complementation studies, which revealed similar results to those found for the *E. coli* disulfide bond isomerase DsbC. Although $TraF_F$ is shown to be essential for conjugation, the loss of the putative catalytic cysteines in the thioredoxin fold suggests that it has a function other than disulfide bond formation. Using GST pulldown analysis we suggested a possible protein-protein interaction between $TraF_F$ and $TraH_F$. Furthermore, we established a functional similarity between $TraF_F$ and $TrhF_{R27}$ as determined by complementation studies using a chimeric protein construct.

4.1.1 Putative disulfide bond isomerases of F- and H-like transfer systems

Sequence homology searches detected a putative thioredoxin fold within the C-terminal region of $TraF_F$ and $TrbB_F$ in the F-like transfer regions and $TrhF_H$, $DsbC_H$, and $HtdT_H$ within or near to the H-like transfer regions. Despite the lack of sequence identity between the above F- or H-like proteins and known members of the thioredoxin family, secondary structure predictions gave results for each protein that overlap the known structures of DsbC and thioredoxin. With the exception of $TraF_F$, the predicted thioredoxin fold within these transfer proteins also contains the C-X-X-C active site motif, as well as the essential β3-strand proline residue that is adjacent to the active site loop. Biophysical studies on *E. coli* thioredoxin have indicated that this proline is important both for the stability and the folding of this cytoplasmic disulfide reductant (Georgescu *et al.*, 1998; Kelley and Richards, 1987). The NMR structure of human thioredoxin complexed with a protein target, Ref-1, has revealed that the *cis* conformation of this proline residue places it in close proximity to the mixed disulfide formed with the substrate protein; consequently two hydrogen bonds can form between amino acids adjacent to this proline and the target peptide chain (Qin *et al.*, 1996). For the major periplasmic disulfide oxidant, DsbA, mutations of this conserved *cis* proline to alanine result in the loss of stabilizing van der Waals interactions with the active site disulfide (Charbonnier *et al.*, 1999), whereas mutations to threonine cause mixed disulfides between DsbA and its substrates to accumulate (Kadokura *et al.*, 2005). Although the C-X-X-C motif and β3-strand proline are conserved in the thioredoxin superfamily, the central dipeptide (-X-X-) sequence of the C-X-X-C motif greatly affects

the inherent redox properties of the active-site disulfide of thioredoxin family members (Chivers *et al.*, 1997). For example, mutational and biophysical studies of the sequence motif Cys30-Pro31-His32-Cys33 of DsbA have indicated that DsbA is very highly oxidizing because its reduced form is favoured through the stabilization of the thiolate form of Cys30 by His32 (Grauschopf *et al.*, 1995). As a result, the alteration of the DsbA sequence motif can reduce its oxidation potential by more than 1000-fold (Grauschopf *et al.*, 1995). In contrast, a screen of *E. coli* DsbC mutants for increased disulfide bond isomerization of mouse urokinase (12 disulfide bonds) revealed that selected mutants contain hydrophobic or more commonly aromatic residues at the third position of the C-X-X-C motif whereas the second position is subject to variation (Bessette *et al.*, 2001). Since the $TrbB_F$ and $DsbC_H$ proteins all contain aromatic residues at the third position, their active site sequence is consistent with a possible role in disulfide bond isomerization. Furthermore, the variation at the second position of F- and H-like proteins containing thioredoxin folds may suggest substrate specificity in each system since mutations at the second position of the *E. coli* thioredoxin sequence motif were noted to interfere with its known substrate interactions without affecting its redox potential (Lin and Chen, 2004). Two H-like T4SS proteins, $DsbC_H$ and $HtdT_H$, closely resemble the host DsbC protein. In particular, these two H-like proteins conserve the extra helical domain that is present in *E. coli* DsbC, including the disulfide bond between residues Cys^{141}-Cys^{163}. This disulfide bond is required for the chaperone activity of *E. coli* DsbC (Liu and Wang, 2001) further suggesting that $DsbC_H$ and $HtdT_H$ are functional homologues of *E. coli* DsbC.

Using $DsbC_{R27}$ as a query, PSIBLAST searches also revealed homology to the TrbB protein of the IncI plasmids, a protein required for the efficient transfer of plasmid R64 (Furuya and Komano, 1996). However, I-like plasmids do not contain any of the F- or H-specific transfer proteins, and the $TrbB_I$ proteins are predicted to be cytoplasmic, thereby precluding their involvement in disulfide bond isomerization. If the C-X-X-C motifs of these proteins are involved in redox reactions, the putative thioredoxin folds of cytoplasmic $TrbB_I$ proteins are far more likely to act as disulfide reductants similar to thioredoxin. Another possibility is that these $TrbB_I$ proteins could be involved in cell signaling via thiol redox control, given the critical role in oxidative stress response of the *E. coli* thioredoxin/glutaredoxin systems through the enzymatic reduction of the transcriptional regulator OxyR (Kumar *et al.*, 2004).

Proteins that promote disulfide bond formation have previously been shown to be essential for the proper assembly of a number of periplasmic protein complexes, including the *E. coli* flagellar apparatus, the enteropathogenic *E. coli* bundle-forming pilus, the *E. coli* pap pili, the *Klebsiella oxytoca* type II secreton, and the *Shigella flexneri* type III secretion system (Bouwman *et al.*, 2003; Pugsley *et al.*, 2001; Yu *et al.*, 2000). Furthermore, pertussis toxin, a multisubunit protein containing 11 disulfide bonds, relies upon DsbA for its assembly but upon DsbC for its secretion by the Ptl T4SS (Stenson and Weiss, 2002), indicating that disulfide bond isomerization can be an important consideration for T4SS. In addition, the F-like plasmid pSLT has been shown to encode SrgA, a DsbA homologue, which is essential for the production of plasmid-

encoded fimbriae (Bouwman *et al.*, 2003). SrgA, but not DsbA, is efficient at forming a disulfide bond necessary for the stability of PefA, the major fimbriae subunit (Bouwman *et al.*, 2003). This is the first known example of a substrate-specific disulfide oxidoreductase being expressed by a plasmid, and it leads to the possibility that other plasmid-encoded systems may benefit from encoding their own substrate-specific Dsb proteins.

In addition to previous studies on the effect of a *dsbA* mutation on F piliation as measured by filamentous phage sensitivity and electron microscopy (Bardwell *et al.*, 1991), we observed that the mating efficiency of the F plasmid is also affected by the presence of a *dsbA* mutation in the donor cell and that the addition of DTT further decreases mating ability to undetectable levels. Interestingly, the F-like T4SS-specific proteins TraN, -U, -H, -W and TrbC have 22, 11, 6, 1 and 2 conserved cysteines, respectively. The F-like universal T4SS envelope proteins TraB, -V and -G have 2, 3 and 2 cysteines, respectively, suggesting there are many candidates for disulfide bond formation and isomerization. Nonreducing gel electrophoresis has demonstrated that wild-type TraV and a $TraV_{C18S}$ mutant can form mixed disulfides with many cell envelope proteins (Harris and Silverman, 2002). Similar experiments based on protein mobility under nonreducing conditions have determined the presence of intra- and intermolecular disulfide bonds in TraN (Klimke *et al.*, 2005). In addition, the T4SS of the Ti plasmid, which has no apparent thioredoxin-like homologues, relies on disulfide bond formation for the stability of its transfer protein complex. In this case, VirB7

stabilizes VirB9 by a disulfide crosslink and the B7-B9 dimer in turn stabilizes other VirB proteins (Cascales and Christie, 2004). Since the *dsbA* mutation affects F piliation, TraB, -G, -H, -V, -W and TrbC, which are involved in pilus assembly, are the best candidates. The effect of a *dsbA* mutation on other proteins that are specific to F-like T4SS, which are involved in mating pair stabilization and possibly pilus retraction, cannot be readily assessed in the absence of the pilus.

Since the $DsbC_H$, $HtdT_H$, and $TrbB_F$ proteins are all believed to localize to the periplasm, it follows that the C-X-X-C active sites of their putative thioredoxin folds could be utilized for disulfide bond formation or isomerization. $DsbC_{R27}$, $HtdT_{R27}$, as well as $TrbB_F$ are more likely to act as disulfide bond isomerases, rather than disulfide bond-forming proteins, for several reasons. First, unlike the DsbA homologue SrgA, which can partially complement a *dsbA* null mutation as measured by AP activity and motility assays (Bouwman *et al.*, 2003), $TrbB_F$ cannot complement a host *dsbA* mutant. Second, $TrbB_F$, $DsbC_{R27}$ and $HtdT_{R27}$ can alleviate the DTT hypersensitivity of a *dsbA* null mutant when overexpressed, similar to the *E. coli* DsbC disulfide bond isomerase (Missiakas *et al.*, 1993; Shevchik *et al.*, 1995). Furthermore, the C-X-X-C motif of $TrbB_F$ and $DsbC_{R27}$ is required for this activity, suggesting that these transfer proteins are capable of redox reactions. Third, vector constructs, which express a DsbA signal sequence fused to the putative thioredoxin folds of truncated $TrbB_F$ and $DsbC_{R27}$ proteins, can partially complement a *dsbA* null mutation without induction, according to mating assays. A similar effect has been observed in AP activity assays using a DsbC

variant with a 76 aa deletion in its dimerization domain (Bader *et al.*, 2001). This could suggest that N-terminal domains of $TrbB_F$ and $DsbC_{R27}$ restrict the activities of the thioredoxin fold by dimerization just as the dimerization domain of *E. coli* DsbC protects its active site from oxidation by DsbB (Bader *et al.*, 2001). On this note, $DsbC_{R27}$ exhibits a similar conservation of residues found in the dimerization interface of *E. coli* DsbC. Fourth, $DsbC_{R27}$ and $HtdT_{R27}$ both conserve the extra helical domain of *E. coli* DsbC, including the important disulfide bond between Cys^{141}-Cys^{163} that determines specificity for chaperone activity. Lastly, single $trbB_F$ and *E. coli dsbC* null mutants have no phenotype whereas a *trbB/dsbC* double mutant has a 10-fold decrease in mating efficiency, suggesting that both proteins may have a related function.

There are more proteins with disulfide bonds than there are disulfide bond isomerases, suggesting there may be overlapping specificities that can explain the lack of a phenotype in $trbB_F$ and *E. coli dsbC* null mutants (Kadokura *et al.*, 2003). Interestingly, in many large, F- and H-like conjugative plasmids there is at least one Dsb homologue containing the C-X-X-C motif. H-like transfer regions such as those of pNL1 (Romine *et al.*, 1999), SXT (Beaber *et al.*, 2002), R391 (Böltner *et al.*, 2002), Rts1 (Murata *et al.*, 2002) or pCAR1 (Maeda *et al.*, 2003) contain either $DsbC_H$ or $HtdT_H$ but not both, whereas R27 (Lawley *et al.*, 2002; Lawley *et al.*, 2003; Sherburne *et al.*, 2000), R478 (Gilmour *et al.*, 2004) and pHCM1 (Parkhill *et al.*, 2001) have two transfer regions, each encoding either $HtdT_H$ or $DsbC_H$. Both F- and H-like transfer regions encode TraF or TrhF, however TrbB is only found in plasmids encoding TraF lacking the C-X-X-C

motif. The fact that there are TraF orthologues with and without the C-X-X-C motif suggests that the motif has been lost after gene duplication of $traF_F$ resulting in $trbB_F$. The strict conservation of at least one functional Dsb homologue in each plasmid suggests a need for assisted disulfide bond formation. This conserved presence of plasmid-derived Dsb homologues, in spite of the chromosomally expressed DsbC, might be due to the relatively low isomerase activity of *E. coli* DsbC compared to eukaryotic protein disulfide isomerase (PDI) (Chen *et al.*, 1999). Since very few periplasmic and outer membrane proteins require more than two disulfide bonds per subunit, a high isomerase activity may not be necessary (Chen *et al.*, 1999), whereas a resident plasmid producing several cell envelope proteins containing multiple disulfide bonds might need auxiliary isomerase activities. Furthermore, disulfide bond isomerases may not be expressed as highly as the disulfide oxidant DsbA. RNA half-life studies have indicated that the *dsbC* transcript persists for only 0.8 min in wild-type cells. The short half-life of DsbC mRNA is attributed to the mRNA processing of RNase E, the major endonuclease involved in the RNA decay pathway (Zhan et al., 2004). Even though the expression of DsbC is limited due to its mRNA instability, DsbG is only expressed at approximately 25% the level of DsbC (Nakamoto and Bardwell, 2004). Therefore, the inclusion of auxiliary isomerase genes on F- and H-like plasmids may enhance the host disulfide bond isomerization system to prevent the misfolding of transfer proteins containing multiple disulfide bonds, which might otherwise elicit a stress response.

Since this project was completed, new genome sequencing projects have revealed an F-like transfer region in the 65 kbp pathogenicity island of *Legionella pneumophila*, the causative agent of Legionnaires' disease (Brassinga *et al.*, 2003; Chien *et al.*, 2004). Although the gene arrangement strongly resembles the F plasmid, these researchers could only account for *trbC* of the *trb* genes. However, two contiguous genes are referred to as *traF*; *traF*1 encodes a 260 aa polypeptide without a C-X-X-C motif whereas *traF2* encodes a 140 bp polypeptide with a C-X-X-C motif, which bears homology to $trbB_F$. New F-like transfer regions have also been detected in plasmid pG8786 from *Yersinia pestis* (Golubov *et al.*, 2004), the causative agent of plague, as well as in plasmid pPBPR1 from *Photobacterium profundum* (Accession number = NC_005871), a deep-sea piezophile. A new H-like transfer region has been identified in the gonococcal genetic island (GGI) of *Neisseria gonorrhoeae*, but unlike other H-like systems, this novel T4SS is able to secrete chromosomal DNA into the medium (Hamilton *et al.*, 2005). The distinction between F- and H-like transfer regions was based not only on the organization of the transfer genes, but also on sequence divergence within the pilin gene ($traA_F$ or $trhA_H$) as well as the type of pilin-processing genes present ($traQ_F$ and $traX_F$ or $traF_H$). Furthermore, all F-like plasmids encode *trbB* whereas all H-like plasmids encode *dsbC* or *htdT*, yet all three genes are believed to encode putative disulfide bond isomerases. Although homologues of *E. coli* DsbA and DsbB are found in many gram-negative bacteria, DsbC and DsbD are mostly restricted to the β- and γ-subdivisions of eubacteria (Katzen et al., 2002). Therefore, it is intriguing that the known F- and H-like plasmids or conjugative elements were all isolated from members of the β- or γ-

proteobacteria with the exception of pNL1. The plasmid pNL1 was isolated from the α-proteobacteria *Novosphingomonas aromaticivorans*, whose genome has not been completely sequenced. It is conceivable then, that during their evolution, plasmids encoding F- or H-like T4SS have preferentially selected host organisms containing a complete disulfide bond isomerization system as their niche. The presence of plasmid-encoded disulfide bond isomerases may possibly have arisen to offer substrate specificity to transfer proteins or to alleviate stress on the host disulfide bond isomerization system.

4.1.2 Role of $TraF_F$ and $TrhF_{R27}$ in conjugation

$TraF_F$ is essential for pilus assembly even though it does not contain a C-X-X-C active site motif nor any other cysteines. When a C-P-Y-C motif was introduced by mutagenesis, $TraF_F$ remained able to complement a *traF*$_F$ mutation *in trans* but could not alleviate the DTT hypersensitivity of a *dsbA* donor cell. Similarly, $TrhF_{R27}$, which has the conserved C-X-X-C motif, is unable to counteract the hypersensitivity of a *dsbA* mutant to DTT. Furthermore, the cloned C-terminal domain of $TrhF_{R27}$, containing the thioredoxin fold element, was unable to complement the *dsbA* mutation. $TrhF_{R27}$ might have substrate specificity or redox potential requirements that do not allow it to affect general periplasmic disulfide bond formation. Gene disruption analysis has indicated that $TraF_F$ is essential for conjugation, but neither $TraF_F$ nor $TrhF_{R27}$ appear to have a function involved in disulfide bond isomerization. However, we cannot discount the possibility that these proteins do in fact contain a thioredoxin fold. Analysis of IS*phoA*/in insertions in *traF*$_F$ revealed that only two of the 31-aa insertion mutants (Tn*phoA*4 and -

5) could complement a null mutation of *traF*$_F$ as measured by conjugative mating assays. Interestingly, only these two mutants contained epitopes that did not interrupt the predicted α-helices and β-strands of the putative thioredoxin fold of TraF$_F$, and Tn*phoA*5 was located in a region known to accept insertions in the thioredoxin fold (Martin, 1995). Furthermore, Liu and Wang (2001) have shown that the active site cysteine residues of *E. coli* DsbC are necessary for enzyme activity but are not required for substrate binding and chaperone function. Therefore, TraF$_F$ and TrhF$_{R27}$ may act as chaperones during pilus assembly. Analysis of GST-TraF$_F$ protein-protein interactions revealed a ~45 kDa protein derived from pOX38-Km that could possibly be TraB (50.46 kDa) or TraH (47.8 kDa), of which both are required for F pilus elongation. Yeast two-hybrid studies have indicated that TraF$_F$ interacts with TraH$_F$ (6 cysteines) (Harris and Silverman, 2004), which has a C-terminal coiled-coil domain (Lawley *et al.*, 2003), making it an excellent candidate for chaperone-assisted assembly in the periplasm. Given that *E. coli* DsbC can assist in that folding of proteins lacking in disulfide bonds (Chen *et al.*, 1999) and that this chaperone activity does not require a C-X-X-C active site (Liu and Wang, 2001), it would seem more likely that it is the C-terminal thioredoxin fold domain of TraF$_F$, rather than the N-terminal domain, that is able to engage in protein-protein interactions with TraH$_F$. TrhF$_{R27}$ may likely have a similar role since a TrhF$_{R27}$-TraF$_F$ chimeric protein, which removed the additional residues from TrhF$_{R27}$ not found in TraF$_F$, was found to partially complement the absence of TraF$_F$ during F conjugation. However, it should be noted that a gene disruption of *traH*$_F$ greatly diminishes mating efficiency, but does not abolish conjugation (Frost, unpublished data), whereas a gene disruption of *traF*$_F$ has

already been demonstrated to completely abolish mating efficiency. Therefore, it is likely that $TraF_F$ has an additional function that does not necessarily rely on its interaction with $TraH_F$.

4.1.3 Future Directions

Although our initial genetic analysis has indicated a possible role for $TrbB_F$, $DsbC_{R27}$, and $HtdT_{R27}$ as disulfide bond isomerases for their respective T4SS, we cannot confirm this role without subsequent biochemical analysis. If $TrbB_F$ is to be purified, it would be beneficial to subject this protein to size exclusion chromatography to assess whether the protein is dimeric like all other known disulfide bond isomerases. Furthermore, using a protein-protein interaction assay similar to what was done with GST-$TraF_F$, it may be possible to detect substrates of $TrbB_F$. However, this interaction assay would likely require modification given that disulfide bond exchange reactions that occur through the C-X-X-C motif of Dsb proteins are relatively transient. In this regard, a mutation of the previously discussed β3-strand proline to threonine has been shown to trap mixed disulfide intermediates between DsbA and its substrate target proteins (Kadokura *et al.*, 2005), whereas removal of the C-terminal cysteine residue of the C-X-X-C motif in thioredoxin has been used to trap disulfide intermediates with its target proteins (Bunik *et al.*, 1999). However, $TrbB_F$ is not believed to be an oxidant like DsbA, so mutations of the above conserved residues may not necessarily trap disulfide intermediates of $TrbB_F$. Furthermore, thioredoxin acts as a reductant and thus relies on its C-terminal cysteine residue of the C-X-X-C motif in order to resolve mixed disulfides formed with substrate

proteins. In contrast, $TrbB_F$ is believed to be an isomerase, which could utilize free sulfhydryl groups in the substrate protein in order to resolve mixed disulfides formed with its target. Therefore, addition of chemicals like iodoacetamide, which blocks free thiol groups, may be useful to prevent free sulfhydryl groups from resolving mixed disulfides that could possibly form between $TrbB_F$ and other transfer proteins that contain multiple cysteines.

Unfortunately, we cannot determine definitively at this time whether these plasmid-encoded proteins are disulfide bond isomerases for their respective DNA transfer systems since there are no assays for their activity beyond pilus formation and conjugation. An alternative approach might be to examine which of the many candidate proteins in the F- and H-like transfer regions beside TraN (Klimke et al., 2005) require DsbA for pilus assembly and transfer. Numerous vector constructs involving *traH*$_F$, *traW*$_F$, *trbC*$_F$, and *traU*$_F$ have been created during my program, which may be useful for the further characterization of the F-specific transfer proteins and/or possible substrates of $TraF_F$ and $TrbB_F$.

One of the assumptions of this work is that if $TrbB_F$, $HtdT_{R27}$, and $DsbC_{R27}$ are disulfide bond isomerases, then they likely act to ensure the correct confirmation of structural disulfide bonds in transfer proteins that contain multiple cysteine residues. However, it should be questioned why proteins like $TraN_F$ (22 cysteines), $TraU_F$ (11 cysteines), and $TraH_F$ (6 cysteines) contain so many conserved cysteines? Disulfide bond formation has

previously been shown to be important for the T4SS of the Ti plasmid of *Agrobacterium tumefaciens*. In this system, the formation of a disulfide bond between VirB7 ($TraV_F$ homologue) and VirB9 ($TraK_F$ homologue) is important for the stabilization of other VirB proteins (Cascales and Christie, 2004). Interestingly, $TraK_F$ is incapable of forming such a disulfide bond because it lacks cysteine residues in its mature form. However, it was previously mentioned that $TraV_F$ is capable of forming mixed disulfides with several unidentified cell envelope proteins (Harris and Silverman, 2002). It is therefore possible that the above mentioned F-like transfer proteins form disulfide bonds that help to maintain portions of these transfer proteins as a stable framework. Harris *et al.* (2004) have proposed from yeast two-hybrid analysis that $TraH_F$, $TraF_F$, $TraW_F$, $TraU_F$, $TrbI_F$, and $TrbB_F$ form a periplasmic interaction group that could control F-pilus extension and retraction. Very little is known about the mechanism of F pilus retraction. Retraction does not require the synthesis of ATP because pili retract in the presence of energy poisons (Sowa *et al.*, 1983); therefore, something other than ATP is responsible for the shift from elongation to retraction. With this in mind, it is conceivable that thiol-disulfide exchange reactions may occur between the numerous cysteines from the proteins of the above interaction complex to act as a signaling switch between pilus extension and retraction. Furthermore, oxidation of a critical SH group will generally lead to a changed biological function (Arner and Holmgren, 2000), and consequently the above transfer proteins may have dual roles in pilus elongation/retraction depending on the redox state of their cysteines. Although many possibilities exist, the indication of novel roles for

$TrbB_F$, $DsbC_{R27}$, and $HtdT_{R27}$ has introduced the potential importance of disulfide bond isomerization for these T4SS.

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