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Analysis of the Mating Pair Stabilization System of the F plasmid

by

William Albert Klimke

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Analysis of the Mating Pair Stabilization System of the F Plasmid** submitted by William Albert Klimke in partial fulfillment of the requirements for the degree of **Doctor of Philosophy, in Molecular Biology and Genetics.**

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Abstract

F plasmid-mediated conjugation proceeds through an ordered series of steps from production of an extracellular filament termed the F pilus, to contact with a recipient cell, retraction of the pilus, stabilization of the mating pairs and DNA transfer. Two proteins in the donor cell envelope, TraN and TraG, stabilize the two cells by an unknown mechanism. Two components in the recipient cell envelope, the outer membrane protein OmpA and lipopolysaccharide, are required for the formation of stable mating pairs with the F^+ donor cell, but not with cells carrying the F-like plasmid R100. Furthermore, two plasmid-encoded products, TraT in the outer membrane and TraS in the inner membrane, block conjugation when they are present in the recipient cell, serving to reduce redundant donor-to-donor DNA transport.

This study utilized the unique plasmid-specificity exhibited by a mutation in *ompA* in the recipient cell to discover that the plasmid encoded protein, F TraN is specific for OmpA. R100 TraN appears to require a different outer membrane protein for a receptor. Analysis of the F and R100 TraN protein sequences indicates that a variable region in the middle of F TraN might be responsible for OmpA-specificity. The surface exclusion protein, TraT, does not appear to be specific for TraN in the recipient cell. The entry exclusion protein, TraS, appears to be specific for TraG.

F TraN contains a large number of conserved cysteine residues, several of which form intramolecular disulfide bonds, that are required for protein stability. F TraN also interacts with other transfer-encoded proteins through intermolecular disulfide bonds. The requirement for the outer membrane lipoprotein, TraV, for TraN stability prompted an investigation into the nature of TraN multimerization based on a comparison with other transport systems that encode stabilizing outer membrane lipoproteins.

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I would especially like to thank my family, for without which, this work would not exist. My mother, Wanda Klimke, my father Harold Klimke, and especially my brother and sister, David and Evangeline Klimke. In memory of my grandfather, James Edwards.

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List of Abbreviations

α	alpha
β	beta
γ	gamma
Δ	delta or deletion
ϕ	phi
aa	amino acid
ABC	ATP-binding cassette
Amp	ampicillin
AP	alkaline phosphatase
ATP	adenosine triphosphate
BCIG	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
bp	base pair
BSA	bovine serum albumin
cam	CAT cassette
CAT	chloramphenicol acetyltransferase
ccd	coupled cell division
Cm	chloramphenicol
Con^-	conjugation deficient
$ConF^-$	conjugation deficient with F^+ donor
$ConR100^-$	conjugation deficient with $R100^+$ donor
CT	cholera toxin
Da	daltons
DNA	deoxyribonucleic acid
ds	double-stranded
Dsb	disulfide bond formation
EDTA	ethylenediaminetetraacetic acid
Eex	entry exclusion
EPEC	enteropathogenic *E. coli*
F Factor	fertility factor, also F plasmid
FHA	filamentous hemagluttinin
Flm	F leading region maintenace locus
GFP	green fluorescent protein
Hfr	high frequency of recombination
hrs	hours
HRP	horse radish peroxidase
IgG	immunoglobulin G
IHF	integration host factor
IM	inner membrane
Inc	incompatibility
IPTG	isopropyl-β-D-thiogalactopyranoside
IS	insertion sequence
kan	kanamycin resistance cassette
kDa	kilodaltons
Km	kanamycin

LB	Luria-Bertani medium
LPS	lipopolysaccharide
MCS	multiple cloning site
min	minutes
MFP	membrane fusion protein
Mpf	mating pair formation
MPS	mating pair stabilization
mq	milli-q (double destilled and deionized)
MW	molecular weight
NTP	nucleoside triphosphate
OM	outer membrane
OMP	outer membrane protein
PG	peptidoglycan
PhoA	alkaline phosphatase
PPEA	pyrophosphorylethanolamine
psi	pounds per square inch
Psi	prevention of SOS induction
Ptl	*Bordetella pertussis* toxin secretion system
sec	seconds
Sec	secretion system responsible for insertion into, or crossing of the inner membrane by proteins
Sfx	surface exclusion
Sop	stability of plasmid
ss	single-stranded
SSB	single-stranded DNA binding protein
TCA	trichloro acetic acid
Ti	tumor inducing
Tn	transposon
Tra	transfer
TTSS	type III secretion system
Vir	virulence

Chapter 1

Introduction

Horizontal gene transfer in bacteria results in the acquisition of genes including antibiotic resistance genes, heavy metal resistance genes, as well as new metabolic enzymes that enable the bacterial cell to grow in inhospitable environments. Transfer of entire operons from bacteria to bacteria as well as inter-kingdom DNA transfer has been observed. The consequence of the spread of antibiotic resistance genes via conjugation, is a number of pathogenic organisms that are now untreatable by typical antibiotic regimens. Two highly studied DNA transfer systems are the T-DNA transport system (Vir) of the Ti plasmid of *Agrobacterium tumefaciens*, and the conjugative F plasmid of *Escherichia coli.*

The mechanism of conjugation is not well understood. The reactions that take place in the cytoplasm of the donor cell, the DNA processing events that lead to DNA transfer, are more thoroughly understood than the events that result in the transfer of DNA through the donor cell and recipient cell envelopes, and into the recipient cell cytoplasm. The DNA processing machinery consists of a few DNA binding proteins and enzymes, whereas the transenvelope complex is encoded by at least 13 proteins in the F plasmid system. The exact functions of only a few are understood at all. Some protein components appear to be structurally involved in spanning the bacterial cell envelope, although the exact stoichiometry, as well the composition of the complete structure is not known. Discerning the functions of the individual proteins during organization of this structure, as well as their specific role during DNA transport has been difficult. The *virB* genes and the F plasmid *tra* genes that encode proteins comprising the transenvelope complex share some homologies between their inner membrane components whereas the outer membrane components are different. This may reflect the differences in transferring DNA from a bacterial to a plant cell in the case of the VirB system, and inter-bacterial transfer in the case of the F system. This also suggests that the inner membrane components function in a similar manner.

Recent research on the F system has shed light on some of the protein components in the cell envelope, although the exact role of every protein as well as their collective functions during pilus synthesis and during DNA transport remains largely unknown. Since the focus of this thesis is on two elements that are components of the F plasmid transenvelope complex, the nature of the bacterial cell envelope, as well as the secretion systems that have evolved to bypass this structure, will be discussed below. Much more is known about the type I, II, III, and flagellar transport systems, while relatively less is known about the DNA transport systems. The two most thoroughly studied DNA transport systems, Vir and F will be discussed as well.

1.1 The Gram-Negative Bacterial Cell Envelope. Gram-negative bacteria differ from gram-positive bacteria in that they have a complex cell envelope composed of an inner membrane (IM), a periplasmic space, a thin peptidoglycan (PG) layer, and an outer membrane (OM), whereas gram-positive bacteria have an IM and multi-layered PG (Koebnik *et al.*, 2000). The peptidoglycan layer in gram-negative organisms is linked to the outer membrane through outer membrane proteins (OMPs) that interact either covalently or non-covalently with the PG layer. The lipid bilayer of the inner membrane is symmetrical and composed of the phospholipids phosphatidylethanolamine, phosphatidylglycerol and cardiolipin. The outer membrane is asymmetric, with the inner leaflet composed of phospholipids, and the outer leaflet composed of lipopolysaccharide (LPS).

The complete envelope, especially the outer membrane, provides a formidable barrier to the movement of large molecules (Nikaido and Vaara, 1985). For example, antibiotics and PG-lytic enzymes that can normally penetrate the gram-positive cell envelope are unable to enter gram-negative organisms (Schleifer and Kandler, 1972). Gram-positive organisms have evolved various types of peptidoglycan to avoid this type of attack, while gram-negative organisms have nearly identical PG types. Bacteria control the influx of compounds, such as metabolites, through the outer membrane by the synthesis of diffusion channels and transport systems that are specific for their target compounds. They have also evolved a number of specific secretion

systems for the movement of large molecules from within the bacterium to the extracellular space.

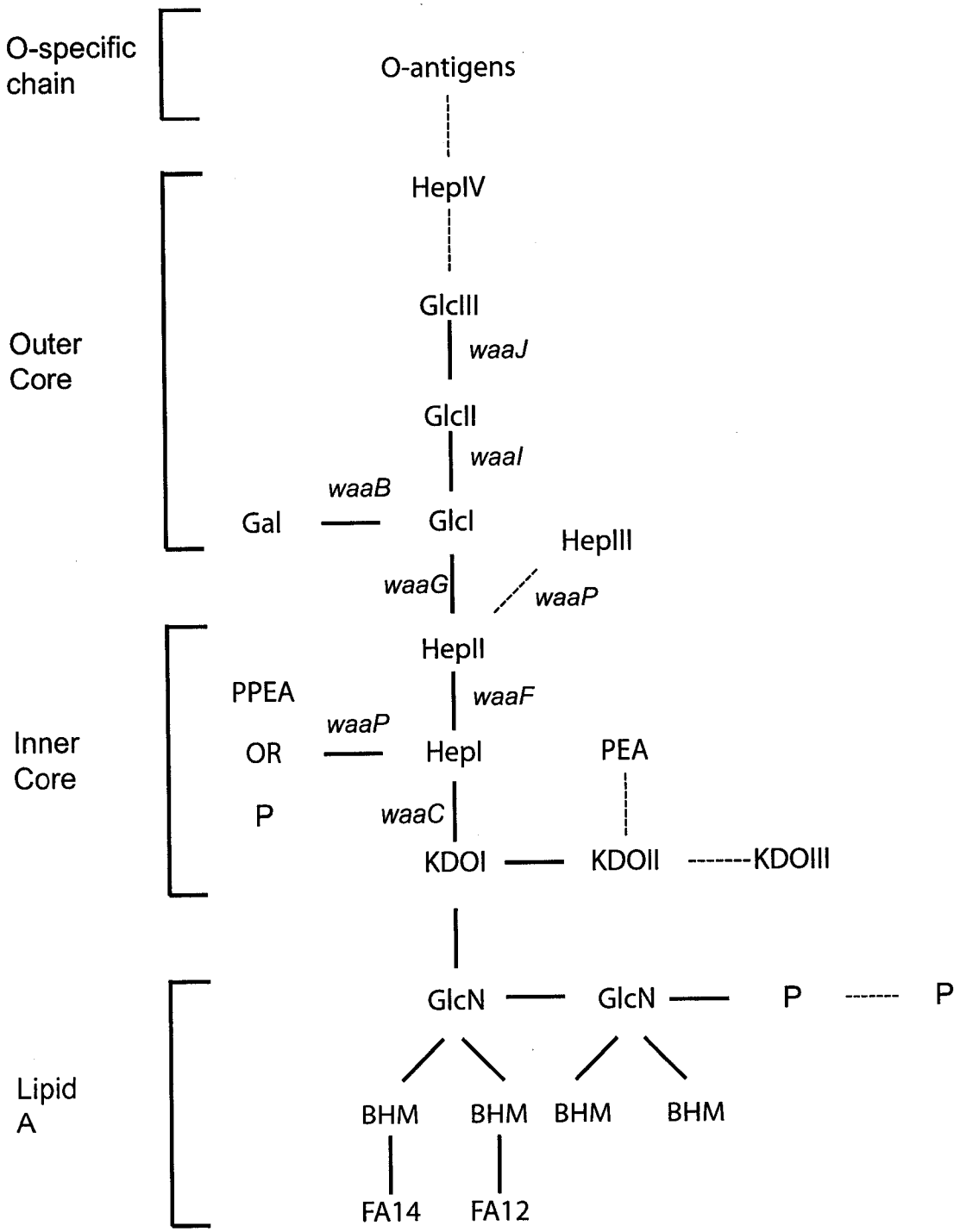
1.1.1 Lipopolysaccharide. LPS is a complex structure consisting of an acylated disaccharide core that is modified by the addition of numerous carbohydrate and phosphorylated derivatives (Figure 1-1). The disaccharide unit consists of two N-acetylglucosamine (GlcN) molecules, linked to 6-7 acyl chains (Schnaitman and Klena, 1993; Heinrichs *et al.*, 1998). One of the GlcN molecules is linked to a 2-keto-3-deoxyoctonic acid (KDO) carbohydrate, which forms the first branch point in the structure. The inner core is modified with phosphate, phosphoethanolamine (PEA) and pyrophosphorylethanolamine (PPEA) additions, and these negatively charged phosphate moieties of the LPS have high affinity for Ca^{2+} and Mg^{2+} ions, leading to the formation of a salt-bridged network, which prevents the penetration of the outer membrane by lipophilic compounds.

Gram-negative bacteria produce three classes of outer membrane proteins that allow influx through the OM: 1) non-specific channels, 2) specific channels, 3) high affinity energy-dependent transporters (Figure 1.2; Koebnik *et al.*, 2000).

1.1.2 Porins. The outer membrane does not allow compounds larger than 600 Daltons to penetrate the lipid bilayer, and molecules of this size or smaller pass through non-specific diffusion channels (porins) that are embedded in the outer membrane (Nikaido, 1994). *E. coli* contains 3 trimeric porins, OmpF, OmpC, and PhoE. The porins are each approximately 30-50 kDa in size, and each monomer contains a pore.

Crystal structures of all three porins have been obtained and they constitute a unique class of structures called the β-barrel (Weiss *et al.*, 1991; Cowan *et al.*, 1992). Each porin monomer consists of a β-barrel structure made up of amphipathic antiparallel β-strands that compose the transmembrane segments, with 16 transmembrane segments per monomer. The transmembrane

Figure 1.1. Lipopolysaccharide structure. The typical structure of an *E. coli* K-12 LPS molecule is shown (Schnaitman and Klena, 1993). The LipidA, Inner Core, Outer Core, as well as the link to O-specific side chains are shown. *E. coli* K-12 does not produce O-antigen. Partial substituents are indicated with a dashed line. FA14, myristate; FA12, laurate; BHM, β-hydroxymyristate; GlcN, N-acetylglucosamine; KDO, 2-keto-3-deoxyoctonic acid; Hep, heptose; Glc, glucose; Gal, galactose. The number after the substituent refers to the order of addition. P, phosphate; PEA, phosphorylethanolamine; PPEA, pyrophosphorylethanolamine. The genes (italicized) responsible for some of the additions are listed.



segments are connected by short periplasmic loops, and long extracellular segments, with the third and longest extracellular loop (L3) from each monomer folding back into the pore of an adjacent monomer. This interdigitation results in a constriction zone in the pore structure, resulting in a small cross-sectional area for solutes to pass through.

OmpF and OmpC differ only in the size of their restriction zones: OmpF has a 1.2 nm pore, while the pore of OmpC is 1.1 nm in diameter. Diffusion rates through the OmpF pore are, therefore, significantly higher than those through OmpC and the synthesis of both OmpF and OmpC are osmoregulated (Pratt *et al.*, 1996). OmpC predominates when bacteria are exposed to the intestinal environment, while in a nutrient poor environment such as fresh water, OmpF is the predominant porin. PhoE shows anion preference, while OmpF and OmpC prefer neutral ions and cations.

1.1.3 Specific Diffusion Channels. The *E. coli* outer membrane also contains a number of specific diffusion channels that mediate the movement of substrates too large to enter the porins, or that cross the porin channel too slowly. LamB, Tsx, and ScrY are examples of specific diffusion channels for maltodextrins, nucleosides, and sucrose, respectively. Crystal structures of LamB and ScrY have been obtained, and are very similar to the porins and to each other. Each monomer of the trimeric channel forms a beta-barrel (Schirmer *et al.*, 1995; Forst *et al.*, 1998). The specific channels contain two important differences from the porins: they contain 18 antiparallel beta-strands, and there are amino acid (aa) residues at the constriction zone that facilitate uptake of the specific substrate. In the case of the LamB pore, there are a number of aromatic residues ("greasy slide") in the pore that aid maltose binding, surrounded by a number of polar residues ("ionic track"), resulting in a stepwise movement of maltose through the pore (Dutzler *et al.*, 1995; Meyer *et al.*, 1997).

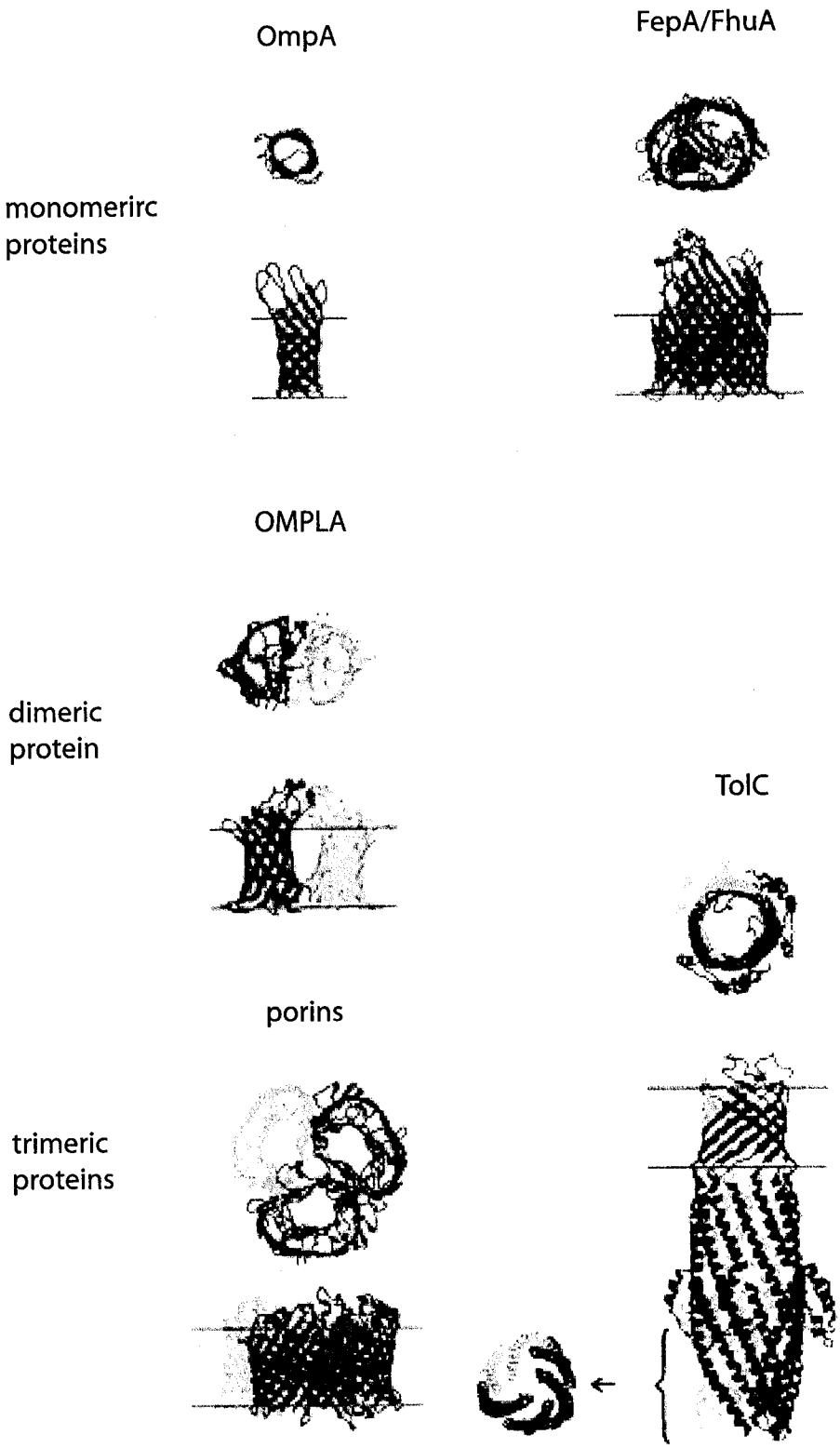
1.1.4 High Affinity Receptors. A number of high affinity receptors (FhuA and FepA) are produced by *E. coli*. These mediate the uptake of compounds such as vitamin B12 and iron-

(ferrichrome and enterobactin) complexes. Uptake of these compounds is energy dependent, and translocation across the outer membrane requires an inner membrane complex, TonB-ExbBD, that transmits electrochemical energy from the proton motive force to the OM receptor. The crystal structures of FhuA and FepA show that they contain C-terminal transmembrane β-barrels formed by 22 transmembrane β-strands, and an N-terminal plug domain that fits inside of the barrels (Figure 1.2; Ferguson *et al.*, 1998; Killman *et al.*, 1998; Buchanan *et al.*, 1999). In the case of FhuA, ferrichrome binds on top of the plug domain, and presumably TonB-dependent conformational changes would result in internalization of the substrate.

1.1.5 Other Outer Membrane Proteins. *E. coli* produces a number of OMPs that do not form pores and some structures have been determined by X-ray crystallography. These include enzymes such as the monomeric protease OmpT, the dimeric enzyme phospholipase A, the virulence-associated monomeric adhesin OmpX, and the monomeric OmpA. In all cases the transmembrane domain always consists of antiparallel β-strands (Vogt and Schulz, 1999; Snijder *et al.*, 1999; Vandeputte-Rutten, *et al.*, 2001). The structure of the periplasmic trimeric lipoprotein, that binds the PG monolayer, as well as the extracellular domain of the intimin receptor of enteropathogenic *E. coli* (EPEC) have also been obtained (Luo *et al.*, 2000; Shu *et al.*, 2000)

OmpA is an example of a β-barrel OMP that does not form a pore. Based on the crystal structure of the transmembrane domain, the pore structure contains an intricate hydrogen-bonded network of salt bridges and water filled cavities, and contains no room for solute transit, a common element of these non-pore forming OMPs (Pautsch and Schultz, 1998). OmpA consists of an N-terminal β-barrel domain, with 8 amphipathic, antiparallel β-strands, and a C-terminal domain that binds peptidoglycan. OmpA contributes to outer membrane integrity, is involved in conjugation (discussed below), is a receptor for phage and colicin binding, and is important for virulence and pathogenicity (Foulds and Barrett, 1973; Sonntag *et al.*, 1978; Morona *et al.*, 1985; Ried and Henning, 1987; Weiser and Gotschlich, 1991; Prasadarao *et al.*, 1996). There are approximately 100 000 copies of OmpA per cell.

Figure 1.2. Representative structures of some outer membrane proteins (from Andersen *et al.*, 2000). Monomeric OmpA and FhuA, dimeric phospholipaseA, and trimeric porin and TolC are shown. Top and side views of the proteins embedded in the OM are shown. The bottom view of the TolC multimer at the outer face of the inner membrane is also shown. The beta-barrel structure of each protein is shown.



1.1.6 Outer Membrane Structure and β-Barrels. Even though the OMPs perform a variety of functions, from passive and specific channels to high-affinity receptors and enzymes, their structures have a number of common elements (Koebnik *et al.*, 2000). The most obvious shared motif is the β-barrel structure. It is believed that gram-negative bacteria have evolved the amphipathic β-barrel structure in order to bypass the IM. Since IM proteins typically have hydrophobic α-helical transmembrane segments, the β-barrel structure results in export to the periplasm. The amphipathic nature of the beta-strands, with a hydrophobic external residue facing the lipid bilayer, and an internal hydrophilic or hydrophobic residue, allows insertion into the outer membrane. The minimal length of the beta strand is 6 aa, and the maximal length is 25 aa, with an average length of 12.5 residues found among all OM proteins (excluding TolC and OmpT; Koebnik *et al.*, 2000). The tilt of the β-strand with respect to the plane of the membrane allows for a wide range in β-strand length. Most of the OMPs have short periplasmic loops, and long extracellular loops, except for TolC. In almost all cases studied, the terminal transmembrane segment contains an aromatic residue, which is important for membrane assembly of some proteins, such as PhoE, but not others (Struyve *et al.*, 1991; Koebnik, 1999). OMPs also contain a girdle of aromatic residues in the transmembrane segments that would position these aromatics just below the polar headgroups of the phospholipids and LPS moieties. This aromatic girdle is found in all outer membrane proteins and is thought to anchor the protein in the membrane.

Prediction of the topology and structure of the outer membrane proteins have led to a few accurate and a few inaccurate models. Based on the crystal structure of OmpF, the predicted structure of PhoE was very close to the final structure (Cowan *et al.*, 1992; van der Ley *et al.*, 1986). LamB and FhuA, however, were not correctly predicted. It was presumed that LamB would contains 16 transmembrane segments as was found with the OmpF, but the resolved structure showed 18 transmembrane segments (Schirmer and Cowan, 1993; Schirmer *et al.*, 1995). The FhuA structure prediction completely missed the presence of the N-terminal plug that is responsible for gating the pore channel, and it was originally assumed that an extracellular loop

would fold back into the pore based on the OmpF structure to provide the gating found in this protein (Koebnik and Braun, 1993; Ferguson *et al.*, 1998). Topological methods have included mutational studies for binding sites such as phage receptors, insertional mutagenesis with epitopes in both the extracellular and periplasmic turns, mutagenesis that allows labeling with residue-specific compounds, and predictive methods for determining amphipathic beta sheets (Stathopoulos, 1999). However, X-ray crystallography has been the only reliable method of structure determination.

A number of supposed constraints on outer membrane structure have fallen by the wayside in recent years (Koebnik *et al.*, 2000). The crystal structure of TolC provides ample evidence of the existence of very long periplasmic extensions, as well as the existence for a single pore structure within a trimeric protein (Koronakis *et al.*, 2000). The structure of the intimin receptor shows clearly that the N- and C-termini of OMPs do not necessarily have to sit in the periplasm, as the C-terminus of intimin is clearly outside the cell (Luo *et al.*, 2000). Topological analysis of the FhaC outer membrane transporter for *B. pertussis* filamentous hemagglutinin revealed an odd number of beta-strands, and insertion of a c-Myc epitope two residues from the N-terminus indicated cell surface exposure (Guedin *et al.*, 2000).

1.1.7 Peptidoglycan. The PG layer of gram-negative bacteria provides a highly cross-linked structure that is resistant to mechanical stress, allowing the bacteria to maintain a high internal osmotic pressure, while also limiting the passage of large macromolecules (Pink *et al.*, 2000). The PG layer of gram-negative organisms typically is 1-3 layers high, and is bound, either covalently or electrostatically, by lipoproteins in the outer membrane. The PG of *E. coli* consists of long chains of repeating disaccharide units, N-acetylglucosamine (GlcN) and N-acetylmuramic acid (MurN), crosslinked via septa-, octa- or nonapeptide stems that branch off and connect MurN moieties in adjacent glycan strands.

The PG layer of *E. coli* has been found to allow molecules up to 2 nm in size to pass through (Demchik and Koch, 1996). Computer modeling of a peptidoglycan mesh under normal stress levels also predicts holes of the same size, 6 nm long slits that are 2 nm wide (Pink *et al.*, 2000). The bacterium must control these holes in the PG layer, since the internal pressure of the bacterium would rupture this thin mesh, resulting in lysis of the bacteria. Gram-negative bacteria have evolved a number of lytic hydrolases, not only to degrade the PG layer and add new PG strands during growth, but also to open holes in the PG at specific sites for the export of substrates (Shockman and Holtje, 1994; Dijkstra and Keck, 1996).

1.2 Inner Membrane Insertion and Translocation. Proteins that are targeted to the inner membrane are typically recognized by the signal recognition particle (SRP), while those targeted to spaces beyond the inner membrane are typically recognized by the SecB tetramer upon translation, and delivered to the SecA dimer at the inner surface of the inner membrane (de Gier and Luirink, 2001). SecA, bound to the SecYEGDFYajC inner membrane complex, binds SecB, and then recognizes the leader and mature domains of the targeted protein. Once the preprotein associates with SecA, ATP is bound by SecA, driving insertion of the N- and C-terminal domains of SecA into the membrane, via the SecYEGDFYajC complex, resulting in insertion of the preprotein. ATP hydrolysis results in release of the preprotein by SecA, while subsequent ATP binding and hydrolysis results in deinsertion of the SecA dimer. New rounds of preprotein binding can begin again resulting in either complete insertion of IM proteins, or crossing of the IM barrier by periplasmic proteins and OMPs. Hydrophilic domains are targeted to the periplasm, while hydrophobic domains remain in the inner membrane lipid bilayer.

A second system for membrane translocation has been discovered that is Sec-independent. The twin-arginine translocation (Tat) system that is so named due to the presence of a twin-arginine motif in the N-terminus of the secreted substrates (Weiner *et al.*, 1998; Robinson and Bolhuis, 2001). The Tat system in bacteria is homologous to a ΔpH-dependent transport system found in thylakoid membranes of chloroplasts. The Tat system is capable of targeting fully folded proteins,

including those bound to a cofactor, across the IM. The Tat system translocates two phospholipases prior to their being secreted by the type II secretion system in Pseudomonas (Voulhoux *et al.*, 2001). The Tat system is capable of transporting the green fluorescent protein (GFP) into the periplasm, while the Sec system cannot (Santini *et al.*, 2001; Thomas *et al.*, 2001). Conversely, the Sec system can transport the periplasmic enzyme PhoA, while the Tat system cannot (Stanley *et al.*, 2002).

1.3 Periplasmic Transit. Preproteins targeted to the periplasm and beyond contain signal peptides at their N-terminus. The signal sequence is either cleaved by leader peptidase, releasing the protein into the periplasm, or the signal sequence is cleaved by signal peptidase II, and a lipid moiety is attached resulting in the formation of a lipoprotein.

The periplasm of *E. coli* contains a set of enzymes involved in the catalysis of disulfide bond formation. The Dsb enzymes (disulfide bond formation) are required for formation and isomerization of disulfide bonds in the periplasm, and are members of the thioredoxin superfamily (Bardwell, 1994; Ritz and Beckwith, 2001). The periplasmic enzyme DsbA is the main disulfide oxidoreductase (Bardwell *et al.*, 1991). Correct disulfide bond formation is a requirement for the correct folding, targeting, and function of a variety of proteins and enzymes. DsbA donates its disulfide bond (DsbA-S2) to reduced protein substrates, thereby oxidizing them, and forming DsbA-SH2. DsbA is reoxidized by the inner membrane protein DsbB. DsbC is the disulfide isomerase, and rearranges incorrectly formed disulfide bonds, either those that form spontaneously or are incorrectly made by DsbA (Schevchik *et al.*, 1994; Missiakis *et al.*, 1994; Missiakis and Raina, 1997). DsbD in turn recycles the DsbC enzyme back to a functional form. DsbB is kept oxidized by the electron transport chain, while DsbD, also an inner membrane protein, is oxidized by cytoplasmic thioredoxin.

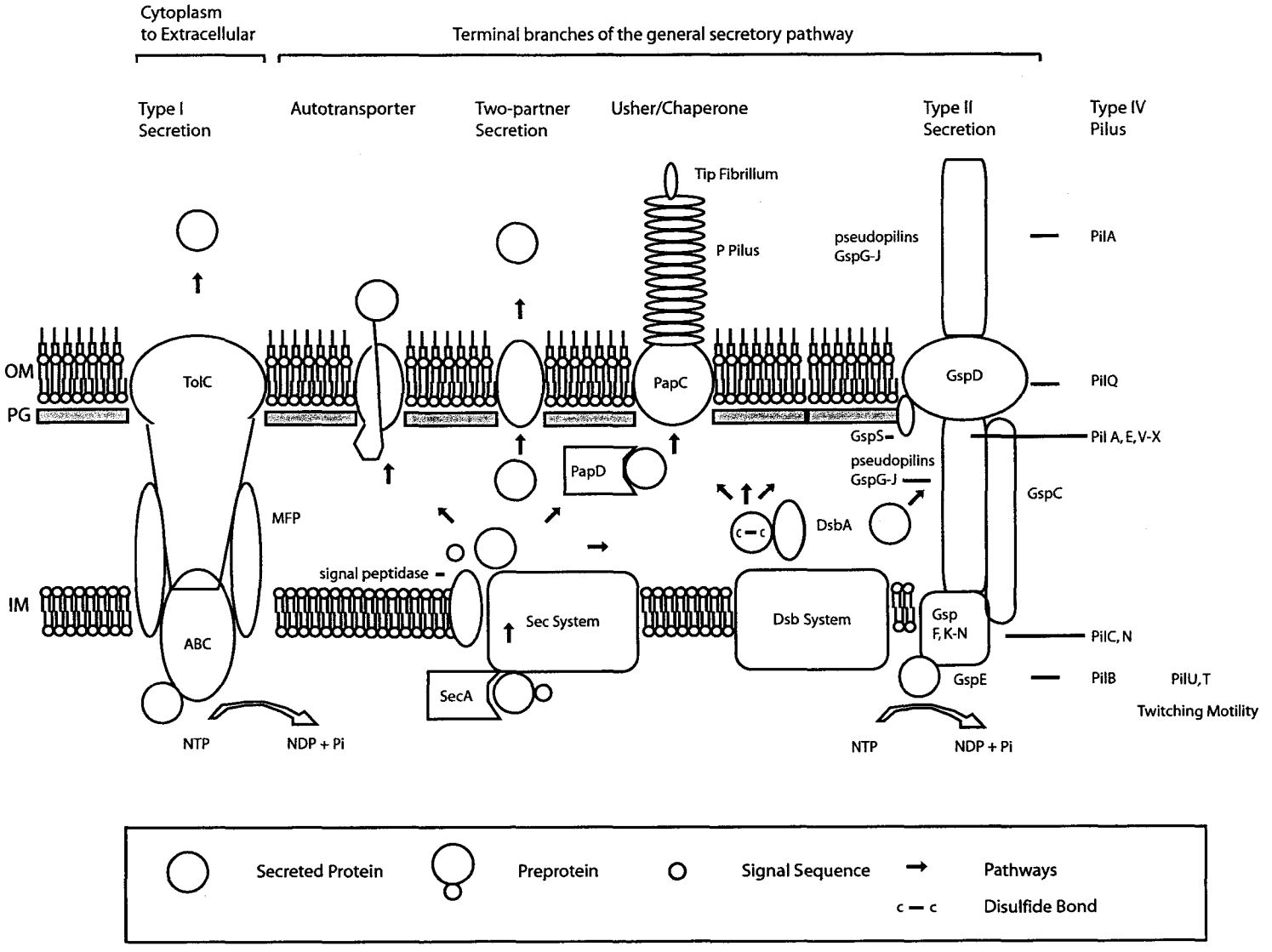
A number of chaperones facilitate outer membrane insertion, or protein secretion, and some proteins have secondary chaperone functions in addition to their normal roles. For example, the

peptidyl-prolyl isomerase (PPIase), SurA, appears to facilitate OmpF and LamB insertion into the outer membrane as these two proteins accumulate as folded monomers in a *surA* null mutant (Lazar and Kolter, 1996). DegP is a periplasmic protease that also functions as a chaperone for the substrates MalS and citrate synthase (Spiess *et al.*, 1999; Kim *et al.*, 1999). Another enzyme, DsbA, also functions as a chaperone in the secretion of pullulanase, an activity that is independent of its role in disulfide bond formation (Pugsley, 1992; Sauvonnet and Pugsley, 1998). The Skp protein is a chaperone that ensures the correct membrane insertion of OmpA, OmpC, OmpF, and LamB (Chen and Henning, 1996; Rizzitello *et al.*, 2001). LPS may also function in outer membrane insertion, as a number of mutations that affect LPS biosynthesis also affect insertion of outer membrane proteins (de Cock *et al.* 1996). Specific chaperones, such as those found in the biogenesis of P pili, are discussed in their respective sections.

1.4 Specific Secretion and Transport Systems. In order to bypass the normal routes of protein targeting to the inner membrane, periplasm, or outer membrane, bacteria have evolved a number of specific systems that facilitate the movement of substrates extracellularly. These systems provide important mechanisms that mediate interactions with the environment, especially during bacterial infection of host cells.

1.4.1 Autotransporters. Autotransporters compose the simplest secretion pathway possible (Figure 1.3). A single protein contains a secreted, or passenger domain, and a carboxy-terminal β-barrel domain (Hendersen *et al.*, 1998). Once the autotransporter has crossed the inner membrane via the Sec pathway, the C-terminal domain inserts into the outer membrane, forming a pore. The passenger domain is then threaded through the pore, to the extracellular space. The first known example of this pathway was the immunoglobulin A1 protease (IgA1) of *Neisseria gonorrhoeae* (Pohlner *et al.*, 1987). Once the passenger domain of IgA1 becomes extracellular, it autocatalytically cleaves itself, and is released into the media. Substrates are thought to be transported through the periplasm in an unfolded state, as an autotransporter that contained a heterologous passenger domain, with cysteine residues, resulted in disulfide bond formation, and

Figure 1.3. Secretion systems. The type I, as well as the terminal branches of the general secretory pathway are schematically illustrated. Type I is composed of 3 components and secretes exoproteins from the cytoplasm. The other systems take proteins that have crossed the IM boundary via the Sec system and exports them. The Sec system binds preproteins and by using energy derived from ATP hydrolysis, forces them across the IM. Signal peptidase I cleaves off the signal peptides. The exoproteins are now free to interact with the disulfide bond formation system, if required, or to interact with their respective secretion systems. The autotransporter is a single protein with an exodomain and pore domain. Two partner secretion systems encode similar functions in two separate proteins. The usher/chaperone pathway takes periplasmic intermediates and assembles them into a complete pilus structure. The type II system takes periplasmic intermediates, and through the actions of a set of 12 proteins, secretes them extracellularly. The secretin (GspD) is the only OM component of the type II system, and is homologous to secretins involved in filamentous phage extrusions and type III secretion. The type II system encodes a number of pseudopilin proteins, that are processed in the same manner as the type IV pilus subunit. Recently it has been shown that the GspG subunit can be assembled into a pilus-like structure on the surface of the *E. coli* cell when the type II system is overexpressed. It is assumed that the pseudopilins are also involved in crossing the periplasmic space, although evidence for such a structure is lacking. Related proteins involved in type IV pilus biogenesis, twitching motility (and competence) are shown on the far right. The GspE ATPase is part of a family of ATPases in type II secretion, type IV pilus biogenesis, twitching motility, and some type IV secretion systems. Only some protein components are shown. Adapted from Thanassi and Hultgren (2000).



the protein was not secreted unless in a *dsbA* background (Jose *et al.*, 1996). Other autotransporters remain bound to the outer membrane domain, either by remaining covalently linked, or, after cleavage has occurred, remaining noncovalently associated. Secreted proteins include proteases, toxins, adhesins, and invasins.

1.4.2 Two partner secretion. The two partner secretion (TPS) system is similar to the autotransporter system, except that in this case, two proteins make up the pore and the exoprotein (Figure 1.3; Jacob-Dubuisson *et al.*, 2001). Examples include the FHA adhesin (filamentous hemagglutinin) of *Bordetella pertussis*, and the ShlA haemolysin of *Serratia marcescens*. The exoprotein crosses the inner membrane via the Sec system, and contains a secretion domain near its N-terminus (110 aa) that is apparently recognized by the outer membrane pore. The TPS systems may require other accessory proteins for functionality.

1.4.3 Chaperone/Usher Pathway. The chaperone/usher pathway also requires two proteins, in this case an outer membrane pore (usher), and a periplasmic chaperone (Figure 1.3). This pathway is responsible for the formation of adhesive virulence structures such as the P and type I pili of uropathogenic *E. coli*. The pili are composed of multiple subunits, and the chaperone recognizes and binds each subunit to prevent premature association. The chaperone delivers the subunits in an ordered fashion to the OM usher. The P pilus usher has been shown by electron microscopy to form a 2-3 nm oligomeric pore structure (Thanassi *et al.*, 1998).

Structural determination of the chaperone bound to a subunit revealed that the chaperone contributes a hydrophobic strand to the subunit, thus completing an immunoglobulin fold, and preventing premature interaction with other subunits, a process which has been termed donor strand complementation (Choudhury *et al.*, 1999; Sauer *et al.*, 1999). In the complete pilus structure, each subunit donates its own strand to an adjacent subunit, thereby forming an interlocked polymer.

1.4.4 Type I Secretion. Type I Secretion is one of the simplest and most widespread secretion systems, composed of three components: the ATP-binding cassette inner membrane protein (ABC), the inner membrane fusion protein (MFP), and the outer membrane pore (Figure 1.3; Binet *et al.*, 1997). This system uses ATP hydrolysis to direct the secretion of a number of substrates, including toxins, proteases, and lipases, across the bacterial cell envelope in one step, bypassing the periplasm. The transport of α-hemolysin by *E. coli* is an example of this pathway, with the α-hemolysin gene (*hlyA*), and the ABC (*hlyB*) and MFP (*hlyD*) genes arranged together on the chromosome (Mackman *et al.*, 1986). The gene for the outer membrane pore, *tolC*, is not linked to genes for the ABC and MFP proteins, however, and the TolC protein is not specific for this system, as it can function in a number of export pathways including the expulsion of noxious compounds (Fath and Kolter, 1991). Closely related multidrug (MDR) efflux pumps use a similar set of proteins, but in this case use energy derived from the proton motive force to direct secretion (Zgurskaya and Nikaido, 2000).

Secreted protein substrates do not contain a typical N-terminal signal sequence, and are not secreted via the Sec system. Instead, a signal found in the C-terminal 60 aa serves as a type I secretion signal, consisting of a glycine-rich set of repeats and a signal patch at the end of the protein (Mackman *et al.*, 1986). Different signals are found within each class of exoproteins, as the secreted proteases contain different signals than the secreted toxins. Once a substrate has bound the inner membrane complex of HlyD and HlyB via the secretion signal, ATP binding results in complete association with TolC (Letoffe *et al.*, 1996; Thanabalu *et al.*, 1998). Release of the substrate is predicted to occur once ATP hydrolysis takes place, resulting in secretion of the compound. The complete structure is dynamic, and TolC can associate with different inner membrane complexes to secrete discrete substrates (Andersen *et al.*, 2000).

The TolC structure has been resolved at 2.1 angstroms (Andersen *et al.*, 2000; Koronakis *et al.*, 2000). The pore structure is composed of three TolC monomers with each monomer contributing 4 transmembrane segments to the complete beta-barrel structure. The cross-sectional area of the

pore is quite large, 960 angstroms, fifteen times larger than the OmpF pore by comparison, and has a diameter of 35 angstroms. The unusual feature of the TolC protein is that a long alpha-helical segment that extends into the periplasm precedes each outer membrane-spanning segment. The end result is a long, tapered cylinder, 140 angstroms long, 100 angstroms comprising the alpha-helical domain, and 40 angstroms forming the β-barrel domain. The periplasmic domain is thought to be closed where the alpha-helical domains come together, and are presumably opened during transport of substrates.

1.4.5 Type II secretion. Type II secretion is also called the main terminal branch of the general secretory pathway, although it is one of at least 4 secretion systems that transport proteins to the extracellular milieu that have crossed the inner membrane via the Sec system (Figure 1.3). Type II secretion systems are capable of transporting a variety of proteins, including toxins, proteases, cellulases, pectinases, and lipases (Sandkvist, 2001a; Sandkvist, 2001b). Analysis of the *Vibrio cholera* toxin (CT) has indicated that secretion takes place once the CT subunits have crossed the inner membrane, been processed, and folded into the complete structure. CT toxin is composed of a pentameric ring of B subunits, and a single copy of the A subunit. The archetype of type II secretion is the pullulanase secretion system of *Klebsiella oxytoca* that is responsible for transport of the starch-hydrolyzing lipoprotein, pullulanase (Pugsley *et al.*, 1997). A common secretion motif has not been found in any of the secreted substrates of the type II secretion systems. It has been suggested that a common patch, present in the completely folded structure of these substrates, may signal transport across the outer membrane.

The Type II secretion system is relatively complex compared to the previous systems. At least 12-15 proteins are required for secretion via this system and the gene products have been assigned a standard nomenclature of *gspA-gspO*, assigned based on their gene order within the type II secretion operon. Only one protein, GspD, is localized to the outer membrane, and the majority of Gsp proteins are associated with the inner membrane. GspD proteins (secretins) and their homologues are found in a number of secretion systems and have been discovered to form large

multimeric pore structures that allow substrate passage through the outer membrane (discussed below).

Of particular interest are the pseudopilins GspG through GspK, which are homologous to the type IV pilin subunits, and are actually processed in a similar manner (Nunn and Lory, 1991; Nunn and Lory, 1992; Strom *et al.*, 1993; Nunn, 1999). These pseudopilin preproteins are N-terminally processed and methylated by GspO, the prepilin peptidase. In addition, the GspD, -E, and –F proteins are homologous to proteins required for type IV pilus biogenesis. These two observations suggest that the type II secretion apparatus is structurally similar to the type IV pilus. A pilus-like structure was found when the type II secretion genes from *K. oxytoca* (Pul genes) were overexpressed in *E. coli* (Sauvonnet *et al.*, 2000). The extracellular filament was composed of the pseudopilin PulG, as determined by immunogold labeling. No similar structure was observed when the *pul* genes were expressed from a chromosomal location.

It is likely that a pilus-like structure is assembled by the type II secretion system, and forms a complex that spans the cell envelope. It is not known how the pilus filament contributes to protein secretion. The diameter of the pilus is approximately 6-7 nm, while the pore of the GspD complex is within the range 7.6 to 9.5 nm, limiting the possibility of completely folded substrates from co-transferring with the pilus through the GspD complex (Parge *et al.*, 1995; Linderoth *et al.*, 1997; Bitter *et al.*, 1998; Nouwen *et al.*, 1999).

1.4.6 Type IV Pilus and Twitching Motility. It has been suggested that the pilus may act like a piston, continuously retracting, then pushing substrates out through the cell envelope (Alm and Mattick, 1995; Shevchik *et al.*, 1997; Filloux *et al.*, 1998). The type IV pilus of *Neisseria gonorrhoeae*, which is involved in twitching motility, has been found to retract with a significant force of 80 pN (pico Newtons) (Merz *et al.*, 2000). Two ATPases are required for twitching motility *in N. gonorrhoeae*, PilT and PilF, both of which are homologous to the GspE ATPase, and may function in a similar manner (Figure 1.3; Merz *et al.*, 2000; Wolfgang *et al.*, 2000). The PilT

ATPase appears to be involved in pilus retraction, while the PilE ATPase appears to be required for pilus extension. The pilus may function in a similar manner during protein secretion via the type II system. Type IV pili are approximately 6 nm in diameter, up to 4 μm in length, are typically found at a polar location on the cell surface and function in adhesion, cell motility, natural competence, and phage infection.

1.4.6.1 Natural Competence Mediated by Type IV Pili. Natural competence is the efficient uptake of macromolecular DNA from the environment and is found in both gram-negative and gram-positive bacteria (Dubnau, 1999). Competence is distinct from artificial transformation such as electroporation, requires a set of genes that are similar to those required for type IV pili biogenesis, and is found in both the gram-negative and gram-positive divisions (although see below). The genes required for competence include, in the case of *N. gonorrhoeae*, PilT, PilG, PilE, PilD, and PilQ. The function of PilT in twitching motility has already been discussed. The PilG protein is an inner membrane protein with three predicted transmembrane segments. PilE is the pilin subunit that is processed by PilD, and in some cases N-methylated. PilQ is the large OM secretin. One other protein, PilC, is required for *N. gonorrhoeae* competence, but is not required for pilus formation or twitching motility. It is suggested to function at the cell surface, as addition of purified PilC to the media rescued a *pilC* mutant (Rudel *et al.*, 1995). PilC has been found to promote the formation of a DNase-resistant state during uptake (Link *et al.*, 1998). Two other proteins, ComL and Tpc, both involved in PG hydrolysis, are also required for competence, and have no function in pilus biogenesis (Fussenegger *et al.*, 1996a; Fussenegger *et al.*, 1996b)

1.4.7 Filamentous phage extrusion. Bacteriophages have two options for release by an infected cell: lysis, or extrusion. Lysis results in the release of a number of particles, but is limiting in that no more phage can be released upon cell death. Extrusion is beneficial, as a continuous number of phage particles can be produced continuously, however, the phage has to have a secretion system in place in order to benefit from this. The system for filamentous phage extrusion has an

outer membrane protein, pIV in the f1 system, which is homologous to the secretins (Russel *et al.*, 1997).

Filamentous phage are 900 nm long, 6.5 nm wide, and contain a single ss (single-stranded) DNA molecule wrapped in a protein sheath (Russel *et al.*, 1997). The phage genome produces 11 proteins, five that make up the phage particle, 3 that are required for DNA synthesis and processing, and three that function in secretion of the complete phage particle. Phage assembly and secretion occur at the same time. All five structural proteins are anchored in the inner membrane as are the secretion proteins, pI, pXI, while pIV is an outer membrane protein. The three secretion proteins, along with host cell thioredoxin, serve to secrete the phage DNA as it is being packaged by the structural proteins.

Phage infection, replication, and assembly occur in a series of ordered steps. Newly injected ss DNA is converted to super-coiled ds (double-stranded) DNA via the host cell enzymes (Model and Russel, 1988). Replication is mediated by pII, a site-specific relaxase, that results in rolling circle replication, producing ss DNA molecules that are converted to new ds DNA forms. Once sufficient expression of pV is reached, it begins to bind the ss DNA and sequesters it from the replication machinery. pV forms a dimer and binding to phage DNA results in the formation of a flexible nucleoprotein complex with the packaging signal exposed at one end, forming the substrate for phage assembly (Bulsink *et al.*, 1988; Bauer and Smith, 1988).

The pI protein is thought to recognize the packaging signal in the substrate DNA, thioredoxin, and to interact with the major phage coat protein, thus initiating the packaging and extrusion of the phage. pXI is an internal translation initiation within pI, and therefore shares the C-terminal one-third of pI. pI and pXI might interact with the outer membrane protein pIV to complete the transenvelope complex needed for phage extrusion. pIV is the filamentous phage secretin, and shares homology with type II and type III secretins. Examination of phage extrusion demonstrated

that although the pIV complex was open to allow f1 phage, it and could not at the same time allow the influx of maltooligosaccharides (Marciano *et al.*, 2001).

1.4.8 Type III Secretion. The Type III secretion system (TTSS) is even more complex than the Type II system (Hueck, 1998; Galan and Collmer, 1999; Cheng and Schneewind, 2000; Cornelis and Gijsegem, 2000; Plano *et al.*, 2001). At least 20 secretion components are required to secrete effector proteins extracellularly, as well as into the host cell membrane and cytoplasm (Figure 1.4). TTSSs are found in *Salmonella typhimurium, Yersinia* spp., *E. coli, Shigella flexneri* as well as many others, and mediate virulence in all of these organisms.

The TTSS is ancestrally related to the flagellar machinery. Ten of the eleven conserved proteins found in TTSSs in all organisms are also homologous to components of the flagellar apparatus (Table 1.1; Figure 1.4). The YscN family of proteins are ATPases, and are related to the catalytic subunit of F_0F_1 ATPases (Woestyn *et al.*, 1994). The YscQ, R, S, T, U, and V proteins are all IM proteins, while YscN, YscL, and YscQ are cytoplasmic or peripheral membrane proteins. Interestingly, the flagellar machinery does not contain a secretin homologue (discussed below). Most of the homologues are proteins that associate with the C and MS rings in flagella (Figure 1.4).

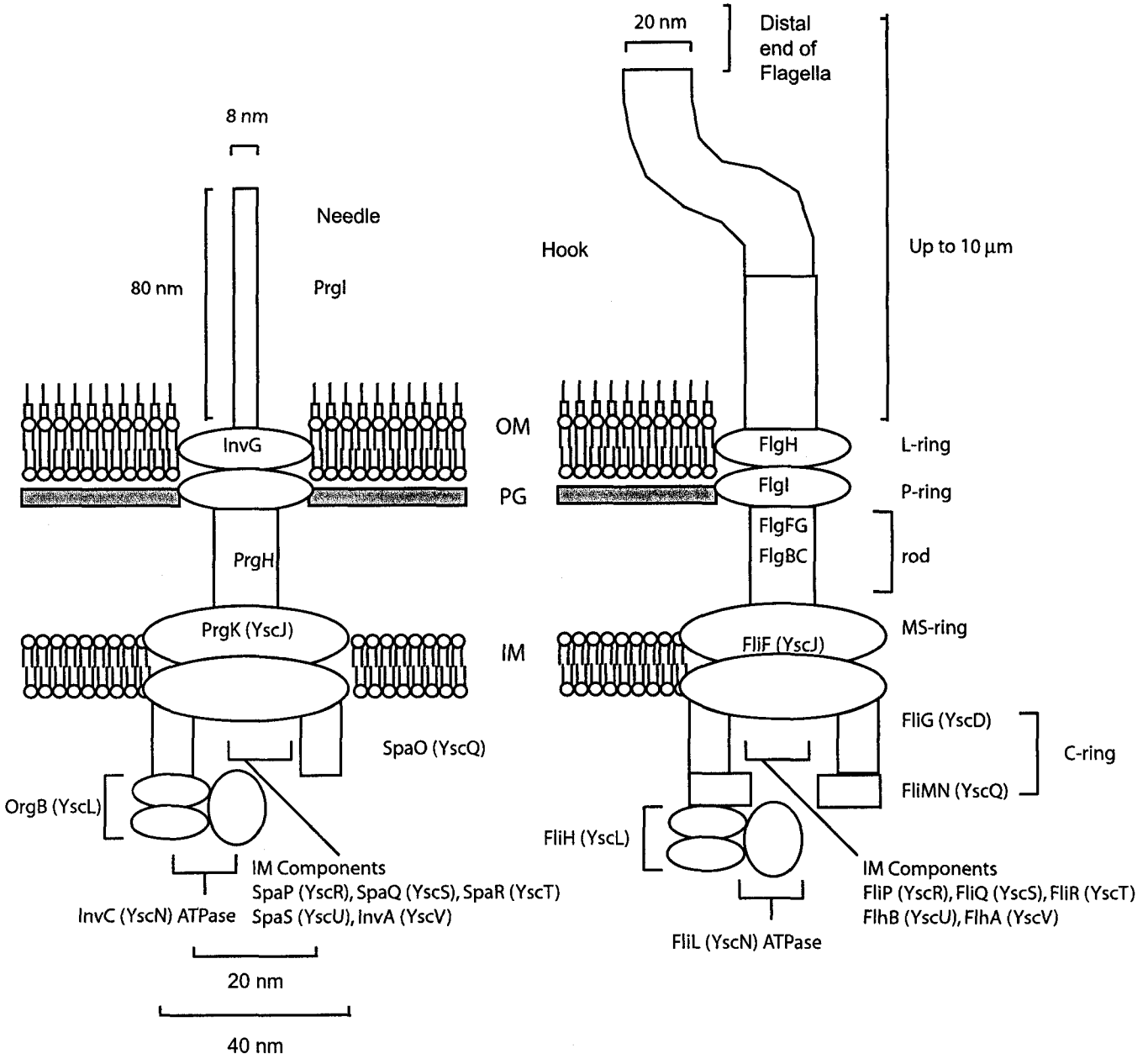
The conservation of components of the TTSS and the flagellar apparatus prompted an ultrastructural examination of the secretion system in the same manner as had been done with the flagellar machinery (Kubori *et al.*, 1998). The TTSS forms a needle complex that spans the bacterial cell envelope, with a short protrusion extending 80 nm away from the bacterial surface, and a basal body consisting of two rings in the inner and outer membrane connected by a rod (Figure 1.4). The needle structure is conserved and similar in all organisms (Blocker *et al.*, 2001; Sekiya *et al.*, 2001). The *Yersinia enterocolitica* needle is capable of penetrating host cell membranes (Hoiczyk and Blobel, 2001). Interestingly, only one of the three components of the

Table 1.1 Conserved Flagellar and Type III Secretion Components[a]

Salmonella typhimurium	*Yesinia pestis*	Flagellar Biosynthesis
-	YscD	FliG
PrgK	YscJ	FliF
OrgB	YscL	FliH
InvC	YscN	FliL
SpaO	YscQ	FliN
SpaQ	YscR	FliP
SpaR	YscT	FliR
SpaS	YscU	FlhB
InvA	YscV	FlhA

a. Adapted from Plano *et al.*, 2001. Only proteins that are homologous are shown.

Figure 1.4. Model of type III secretion system needle complex (NC) and flagellar apparatus. The *Salmonella typhimurium* NC is shown on the left, and the flagella on the right. Conserved proteins are listed with the homologue found in *Yersinia* needle complexes (in parentheses, see Table 1.1). Most of the homologous proteins found between the NC and flagella are found in the IM components. The two structures are not shown to scale. The TTSS secretin, InvG, is not homologous to the flagellar L-ring, and it is thought that proteins pass through the hollow tube made up of the rod and hook. *Salmonella typhimurium* does not have a YscD homologue. The proteins responsible for energizing transport are just shown for clarity, and do not represent their actual position in the structure. Not shown are the Mot protein complexes that surround the MS-ring in the flagellar system, and drive rotation of the flagella via the proton motive force. Adapted from Plano *et al.* (2001)



Type III Secretion Apparatus

Flagellar Machinery

TTSS basal body is homologous to the flagellar export system. YscJ (TTSS) shares limited identity with FliF (flagella).

1.4.9 Flagellar Machinery. The flagellum is a long protein filament embedded in the bacterial cell envelope, with approximately 5 to 10 peritrichous filaments (randomly) found in the typical *E. coli* or *Salmonella typhimurium* cell (Macnab, 1999). A motor at the base of the structure drives the flagellar filaments either clockwise or counterclockwise based on input signals from the chemotaxis system.

The flagellar structure (Fig 1.4) is composed of a number of rings (basal body) embedded in the cell envelope: the C ring, which contains the motor/switch proteins, the MS ring in the inner membrane, the proximal rod that connects the MS ring to the external basal body components, the P ring, in the peptidoglycan layer, the L ring in the outer membrane, and the distal rod, extending through the L ring (Plano *et al.*, 2001). The external components of the filament include the hook, connected to the distal rod, the hook-filament junction, the filament, composed of flagellin (FliC), and the filament cap. Flagella are approximately 20 nm in diameter, and can be up to 10 μM long.

The flagellar structure itself can also function as a secretion apparatus (Macnab, 1999). The distal elements of the structure, including all extracellular components (rod, hook, hook cap, hook-filament junction, filament, and filament cap), are secreted through the flagellar structure in the cell envelope. Three other non-structural proteins are also secreted, including an anti-sigma factor (FlgM) that is no longer required at that stage of flagellar assembly, a flagellum-specific muramidase, presumably to allow penetration of the peptidoglycan layer, and a protein that functions in hook length control (Minamino and Macnab, 1999). Interestingly, it has been shown that the flagellum in *Yersinia enterocolitica* is capable of secreting a virulence associated phospholipase (YplA), and that the *Pseudomonas aeruginosa* FlhA flagellar-associated protein, is important for internalization of the bacterium during pathogenesis (Young *et al.*, 1999; Fleiszig *et*

al., 2001). Protein secretion via the flagella requires ATP hydrolysis, while the proton motive force drives flagellar rotation.

The flagellum does not contain a secretin homologue, and it is thought that the flagellar rod and hook structures, including the L-ring lipoprotein, FlgH, function in an analogous manner. In fact, the only homologue found in both the flagellar and TTSS structures is the inner membrane component, FliF, which is found associated with the C and MS rings. The rod and hook structure potentially forms a conduit through which proteins are secreted via the flagellar export pathway.

1.5.1 Secretin Structure. A number of systems that have been studied contain large protein complexes that form multimeric ring-shaped structures in the outer membrane. These include the PapC usher of the usher/chaperone family, WzaK30 for translocation of capsular polysaccharide, the TolC protein involved in Type I secretion, the PulD, pIV and related proteins from Type II secretion, and the InvG, YscC family of TTSS secretins (see specific sections for references; Drummelsmith and Whitfield, 2001). A pore complex participates in *Streptococcus pyogenes* (gram-positive) secretion of cytolysin (Madden *et al.*, 2001; Gauthier and Finlay, 2001). Type II, type III and filamentous phage secretion all occur through OM secretins.

Secretins are highly resistant to denaturation in SDS, and multimeric PulD must be boiled in SDS and phenol in order to dissociate it (Nouwen *et al.*, 1999). When pIV and PulD were purified as multimeric complexes, by taking advantage of their SDS-resistant structures, they displayed ring-shaped structures when examined under transmission electron microscopy (Linderoth *et al.*, 1997; Nouwen *et al.*, 1999). The secretins have central cavities with diameters ranging from 5.3 nm for PilQ (type IV pilus) to 9.5 nm for XcpQ (type II secretion; Bitter *et al.*, 1998). As the secretins in type II systems are the only known OM protein, this suggested that they formed the OM pore complex.

Further work using purified secretins reconstituted into artificial bilayers supported the idea that both pIV and PulD form large gated channels (Nouwen *et al.*, 1999; Marciano *et al.*, 1999). When a single mutation was obtained in pIV, S324G, it made the bacterial outer membrane porous to large molecular weight compounds such as the antibiotic vancomycin (MW 1334) and large MW sugars such as maltohexaose when tested in a *lamB* strain (Marciano *et al.*, 1999). The BfpB secretin, involved in bundle-forming pili biogenesis, is not gated, however, this secretin and the operon in which it resides, is only induced under specific conditions (Schmidt *et al.*, 2001).

High-resolution microscopy of both the PulD-PulS multimer and the PilQ secretin revealed a dodecameric arrangement (Nouwen *et al.*, 1999; Nouwen *et al.*, 2000; Collins *et al.*, 2001). A side view of PulD and pIV revealed a stacked ring structure, long enough to be capable of penetrating the OM (Linderoth *et al.*, 1997; Nouwen *et al.*, 2000).

1.5.2 Secretin Function. Secretins serve to pass substrates through the outer membrane. Besides their stability in SDS and ring-shaped structure, secretins have a number of other shared characteristics. They typically contain a conserved C-terminal domain, that is homologous to secretins in both Type II and Type III systems, while the N-terminal domain is divergent and genus-specific. It is thought that the C-terminal domain is actually inserted into the outer membrane, while the N-terminal domain is in the periplasm. The N-terminus is thought to interact with substrates and to provide substrate specificity to the secretion mechanism. Deletion of aa 66 to 116 of *Erwinia chrysanthemi* OutD abolished exoprotein secretion and binding, but other deletions only abolished exoprotein secretion with no affect on binding (Shevchik *et al.*, 1997). The N-terminal 149 aa of pIV^{f1}, when fused to the C-terminal domain of pIV^{IKe}, supported f1 filamentous phage extrusion, but not IKe phage extrusion (Daefler *et al.*, 1997b). However, when the N-terminal domain of OutD was fused to the C-terminal domain of PulD, pullulanase secretion occurred normally (Guilvout *et al.*, 1999).

Most secretins seem to require an outer membrane pilot protein, either for insertion into the outer membrane, or for multimerization. This pilot protein is typically a lipoprotein. PulS is required for insertion of PulD in the outer membrane, and protects PulD from proteolysis (Hardie *et al.*, 1996). Further work demonstrated that the pilot protein binds to the extreme C-terminal 65 aa of PulD (Daefler *et al.*, 1997a). This region is highly divergent in all secretins. Interestingly, the pIV secretin does not contain this C-terminal tail. However, if the binding site for PulS at the C-terminal end of PulD is fused onto pIV, then pIV required PulS for stability. PulS co-purifies with PulD, and the pIV-C-terminal PulD chimera (Daefler *et al.*, 1997a; Nouwen *et al.*, 1999).

1.5.3 The Transport Process Through Needle Structures and Pili. An examination of the TTSS of a plant pathogen, *Pseudomonas syringae* pv tomato, revealed it also had a needle complex (Brown *et al.*, 2001). Immunogold labeling using antibodies against a secreted effector protein, HrpZ, showed gold labeling in a track along the needle, suggesting that secreted proteins are transported along the outside of the needle itself like a conveyor belt. Further examination demonstrated binding of HrpN and DspA/E along the length of the needle, but also demonstrated these substrates apparently being extruded through the tip of the needle, suggesting that secretion occurred through the hollow needle itself (Jin *et al.*, 2001a; Jin *et al.*, 2001b).

Secreted proteins have not been examined for extracellular localization to the pilus in the type II system, however, studies on the EpsD protein (type II secretin) in *Vibrio cholerae* have shown that EpsD is required for secretion of both cholera toxin and CTXϕ phage (Davis *et al.*, 2000). As the CTXϕ phage does not require any of the other Eps proteins, this suggests that the secretin component can become uncoupled from the secretion apparatus and recouple with a different secretion apparatus (Sandkvist, 2001a; Sandkvist, 2001b). This is similar to what is seen with the *TolC protein of the Type I secretion apparatus.*

Secreted effectors are taken directly from the periplasm in the case of the chaperone/usher pathway, the autotransporter and TPS pathway, and the Type II secretion system. The Type I, Type III, and the majority of the Type IV substrates are taken directly from the cytoplasm.

1.6 Type IV Secretion. Type IV appears to be the most complex secretion system known since it appears to be capable of transporting the widest variety of substrates, and requires up to 20 proteins for complete functionality (Christie, 2001). Some type IV systems transport proteins from the cytoplasm, periplasm, or outer membrane, while others transport nucleoprotein complexes or ss (single-stranded) DNA molecules. Substrates are targeted to either the extracellular space, or into another bacterial or host cell. Type IV systems also serve as entry points for phage infection, and at least some type IV proteins are involved in competence.

Genes encoding type IV systems are commonly found within virulence islands on the chromosomes of pathogens, or on conjugative plasmids. Pathogenic organisms that encode type IV secretion systems on their chromosomes include, but are not limited to, *Helicobacter pylori*, *Legionella pneumophila*, *Neisseria gonorrhoeae*, and *Salmonella enterica*. Conjugative plasmids are found in both gram-negative and gram-positive species, although only gram-negative plasmids related to type IV secretion systems are discussed here. These plasmids are categorized based on their incompatibilities, which is the inability of two plasmids to coexist in the same cell (Novick, 1987). Some Inc groups along with their representative plasmids include IncF (F), IncH (R27), IncI (R64), IncN (pKM101), IncP (RP4), and IncW (R388). The IncF group is subdivided further into 7 groups including IncFI (F), IncFII (R100) and IncFV (pED208) as well as others (Ippen-Ihler and Skurray, 1993). Two extensively studied type IV systems are the Vir system of the Ti plasmid found in *Agrobacterium tumefaciens*, and the conjugative F plasmid of *E. coli*. Homologues between them, as well as other conjugation and type IV secretion systems has been noted (Table 1.2; Christie, 2001).

Table 1.2. Similarities of Transenvelope Type IV Secretion Proteins [a]

Substrate[b]	System[c]	Proteins[d]											
			Pilin		ATPase		Transenvelope Complex					ATPase	ATPase
T-DNA Vir		B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	D4
RP4 (IncP)	Trb	N	C	D	E	F					I	B	TraG
F (IncF)	Tra	orf169	A	L	C	E	**K**[e]	**V**			B		D
B. pertussis Toxin	Ptl		A	B	C		D	I	E	F	G	H	
H. pylori Cag	Cag				E			T		528	527	525	524
L. pneumophila	Lvh	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	D4
H. pylori competence	Com				B4			B7	B8	B9	B10		
Type II Secretion												GspE	

a. Adapted from Christie, 2001.
b. Substrates are listed if known.
c. Representative systems. Not all genes have the same prefix, for example orf169 is not Traorf169.
d. Only proteins based on homology to the Vir system are listed.
e. TraK and TraV are considered analogues of VirB6 and VirB7, respectively.

1.6.1 Type IV Protein Secretion. The *Bordetella pertussis* toxin (Ptl) transport system is an example of a type IV protein secretion system. The toxin is comprised of six subunits that are synthesized as preproteins and are thought to cross the inner membrane via the Sec system (Locht and Keith, 1986; Nicosia *et al.*, 1986). The holotoxin must completely assemble prior to being exported (Farizo *et al.*, 2000). The S1 subunit has been found in the outer membrane, suggesting it nucleates assembly of the entire holotoxin before transport begins (Farizo *et al.*, 2002). The Ptl system is homologous to the Vir system of *Agrobacterium tumefaciens* that mediates secretion of nucleoprotein complexes into the host cell nucleus (Weiss *et al.*, 1993).

Helicobacter pylori contains two type IV secretion systems. The first is encoded within the *cag* pathogenicity island, and is responsible for transport of the CagA protein into the host cell (Tummuru *et al.*, 1995; Censini *et al.*, 1996). CagA becomes tyrosine phosphorylated in the host cell and induces changes in the host cell cytoskeleton by affecting signal transduction pathways, an important step for *H. pylori* virulence (Odenbreit *et al.*, 2000; Stein *et al.*, 2000). This is similar to what is observed with the translocated intimin receptor (Tir) in the TTSS of EPEC, which is tyrosine phosphorylated as well, and induces cytoskeletal changes in the host cell leading to pedestal formation (Kenny *et al.*, 1997). The *cag* pathogenicity island encodes proteins that are homologous to the Vir proteins of the Ti plasmid. The second system in *H. pylori* mediates natural competence and is composed of at least five proteins (ComB4, ComB7-ComB10) that are also homologous to the Vir system (VirB4 and VirB7-VirB10; Hofreuter *et al.*, 2001). Mutations in any of these genes either abolish, or significantly decrease competence efficiency.

1.6.2 Vir System. The Ti plasmid of *Agrobacterium tumefaciens* encodes two type IV secretion systems. One system promotes plasmid transfer from one bacterium to another, while the other is involved in selectively transporting a ss DNA molecule called the T-strand, which is carried on the Ti plasmid, into host plant cells (Stachel and Zambryski, 1986). This T-strand is directed to the nucleus of the host cell, where it integrates into the plant cell chromosome and directs the synthesis of opines which the bacterial cell uses as a nutrient source, and plant cell hormones

that cause proliferation of the affected cells. T-strand export is mediated by the Vir system, whose genes are encoded within several operons on the Ti plasmid (*virA*, *B*, *C*, *D*, *E* and *G*). Expression of these operons is controlled by a two-component signal transduction pathway that is sensitive to the presence of plant phenolic compounds, monosaccharides, low pH and low phosphate levels present at a wound site on the plant (Winans, 1992).

1.6.2.1 Vir Transport. The non-self-mobilizable plasmid RSF1010 (IncQ) is capable of utilizing the Vir system for transfer into a plant cell (Buchanan-Wollaston *et al.*, 1987). VirB proteins, along with some of the proteins encoded by other *vir* operons, are homologous to proteins involved in mating pair formation (Mpf) in the IncP, -N, and –W groups, and their genetic organization is very similar (Winans, 1992). The VirB proteins are involved in T-DNA transfer, RSF101 transfer, are homologous to conjugative proteins, and are predicted to be, or have been found in the bacterial membranes. All of these results suggested that the VirB proteins, along with VirD4, form the transenvelope complex that transports the T-DNA into plant cells (Fullner, 1988; Thompson *et al.*, 1988; Beijersbergen *et al.*, 1994; Shirasu *et al.*, 1994; Christie, 1997). Once the VirB proteins are assembled into a membrane transporter competent for transfer, T-strand transfer can initiate. The VirD2 endonuclease cleaves a sequence found at the right border of the T-DNA in a site- and strand-specific manner, which leads to covalent attachment of the enzyme to the 5' end of the DNA (Yanofsky *et al.*, 1986; Young and Nester, 1988; Pansegrau *et al.*, 1993). A ss DNA binding protein, VirE2 coats the DNA, and the entire nucleoprotein complex is then transported through the cell envelope of the bacteria and into the host plant cell cytoplasm (Christie *et al.*, 1988; Citovsky *et al.*, 1988; Das, 1988). Nuclear localization signals in both VirD2 and VirE2 target the complex into the host cell nucleus, where the T-strand is incorporated into the host cell chromosome (Citovsky *et al.*, 1992; Howard *et al.*, 1992; Tinland *et al.*, 1992; Winans, 1992; Rossi *et al.*, 1993; Citovsky *et al.*, 1994). A number of studies have indicated that VirE2 and the T-strand can transfer independently of one another, and that VirE2 can form channels in artificial membranes that are capable of transporting ss DNA, suggesting that VirE2 forms a pore in the plant membrane, and actually coats the transported DNA once it is in the plant cell (Otten, 1984;

Citovsky *et al.*, 1988; Binns *et al.*, 1995; Chen, 2000; Dumas, 2001). VirE1 is apparently a chaperone that facilitates VirE2 transport, while VirD1 assists the enzymatic activity of VirD2 (Scheiffele, *et al.*, 1995; Sundberg *et al.*, 1996).

The products of the *virB2-virB11* genes, as well as the *virD4* gene, are required for T pilus formation, T-DNA transfer, VirE2 secretion, and RSF1010 mobilization (Berger and Christie, 1994; Lin and Kado, 1993). The VirB1 protein does not appear to be essential, as mutations in *virB1* decrease virulence by only 100-fold (Berger and Christie, 1994). VirB1 is homologous to a number of lytic transglycosylases, and may be involved in locally opening the peptidoglycan layer surrounding the transport complex, although this process does not appear to be absolutely required. VirB2 is the T-pilus subunit, and is processed in an unusual manner (Eisenbrandt *et al.*, 1999). Once VirB2 is inserted in the membrane via the Sec system, a long N-terminal leader peptide is cleaved off, leaving 74 aa. A cyclization reaction occurs that is catalyzed by host proteins in *A. tumefaciens,* but not in *E. coli,* resulting in a cyclic VirB2, a process that is important for pilus elongation and T-DNA transport. The T pilus is assembled from VirB2 subunits, a process that depends on the other VirB proteins, and possibly contains VirB5 as a minor subunit, and potentially also VirB7 (Schmidt-Eisenlohr *et al.*, 1999; Lai *et al.*, 2000; Sagulenko *et al.*, 2001).

Extensive protein interactions have been discovered amongst the remaining VirB proteins that comprise the transenvelope complex. The outer membrane lipoprotein, VirB7, forms homodimers as well as heterodimers with VirB9, a process that is mediated by intermolecular disulfide bonds (Anderson *et al.*, 1996; Fernandez *et al.*, 1996; Spudich *et al.*, 1996). The VirB7-VirB9 heterodimer appears to be involved in nucleating the rest of the complex. VirB9-VirB8, VirB9-VirB9, VirB9-VirB10, VirB8-VirB10, and VirB10-VirB10 interactions and complexes have been found (Ward *et al.*, 1990; Beaupre *et al.*, 1997; Das and Xie, 2000). VirB8 appears to be important for positioning of the complex as a lack of VirB8 results in VirB9 and VirB10 moving from typically tightly clustered complexes, to a more random profile across the cell envelope

(Kumar *et al.*, 2000). The polytopic inner membrane protein VirB6 appears to be important for the stabilization of two other Vir proteins, VirB3 and VirB5, as well as other proteins in the complex (Hapfelmeier *et al.*, 2000). VirB3 localization to the outer membrane is dependent on VirB4, and is required for DNA transfer (Jones *et al.*, 1994).

1.6.2.2 Vir ATPases. Three ATPases are required for T-DNA transport, VirB4, VirB11, and VirD4. Homologues of VirB4 and VirD4 are found in all known conjugative plasmids. VirB4 is an inner membrane ATPase that appears to be involved in production of the pilus (Berger and Christie, 1993; Fullner *et al.*, 1994; Shirasu *et al.*, 1994; Dang *et al.*, 1997). VirB4 oligomerizes through its N-terminal domain, a process that stimulates the uptake of the RSF1010 plasmid when VirB4, along with a subset of other VirB proteins, is present in the recipient cell (Bohne *et al.*, 1998; Dang *et al.*, 1999). This suggested that oligomerization of VirB4 leads to the formation of proper VirB protein-protein interactions.

VirB11 is homologous to a large family of ATPases involved in many secretion systems, including the GspE protein of type II secretion, the PilT ATPase involved in twitching motility, ComG1, which is involved in competence in gram-positive bacteria, and TrbB of RP4. (Planet *et al.*, 2001). VirB11 hydrolyzes ATP *in vitro*, oligomerizes via domains in both the N- and C-terminal halves, and is associated with the inner face of the cytoplasmic membrane (Christie *et al.*, 1989; Zhou and Christie, 1997; Rashkova *et al.*, 2000). Homologues of VirB11 have been purified and shown to exist as hexameric ring structures when examined by electron microscopy (Krause *et al.*, 2000a; Krause *et al.*, 2000b). The crystal structure of the VirB11 homologue, HP0525 from *Helicobacter pylori*, has been solved, and the protein forms a hexameric pore complex that is closed at one end of the tube (Yeo *et al.*, 2000). VirB11 homologues contain 4 conserved motifs, Walker A and B boxes involved in ATP-binding and hydrolysis, and Aspartate and Histidine boxes. Mutation or insertion in these regions led to the conclusion that pilus production and substrate transport can be uncoupled (Sagulenko *et al.*, 2001). Mutations in the Walker A box resulted in a lack of pilus synthesis or substrate export, although the other VirB proteins

accumulated to wild-type levels, suggesting that the transenvelope complex was properly assembled. A number of other mutations were obtained that selectively blocked either T pilus production or substrate translocation, but not both together. For example, an I256T mutation in the conserved His box completely abolished T pilus production, as measured by the presence of extracellular VirB2 protein, with no effect whatsoever on substrate transport. Since introduction of the plasmid RSF1010 *in A. tumefaciens* has a Pil^{+} Tra^{-} phenotype, in that the cells are pilus proficient, but T-DNA transfer is strongly inhibited, it was suggested that VirB11 functions as a chaperone ATPase that serves to facilitate the movement of DNA and other substrates, such as the pilin subunit, across the cell envelope by controlling access to the transport machinery (Ward *et al.*, 1991; Sagulenko *et al.*, 2001).

VirD4 belongs to a large family of ATPases called the coupling proteins. Mutations in VirD4 have no effect on pilus production, but do affect T-DNA transfer and VirE2 export (Christie, 1997; Lai *et al.*, 2000; Lin and Kado, 1993; Vergunst *et al.*, 2000). VirD4 is important for substrate selection and mobilization efficiency of mobilizable plasmids (Cabezon *et al.*, 1997; Hamilton *et al.*, 2000). VirD4 is an integral inner membrane protein (Okamato *et al.*, 1991). The crystal structure of the R388 TrwB protein, the homologue of VirD4, revealed a hexameric ring structure with a 2 nm central cavity, indicating that it forms a pore in the inner membrane (Gomis-Ruth *et al.*, 2001).

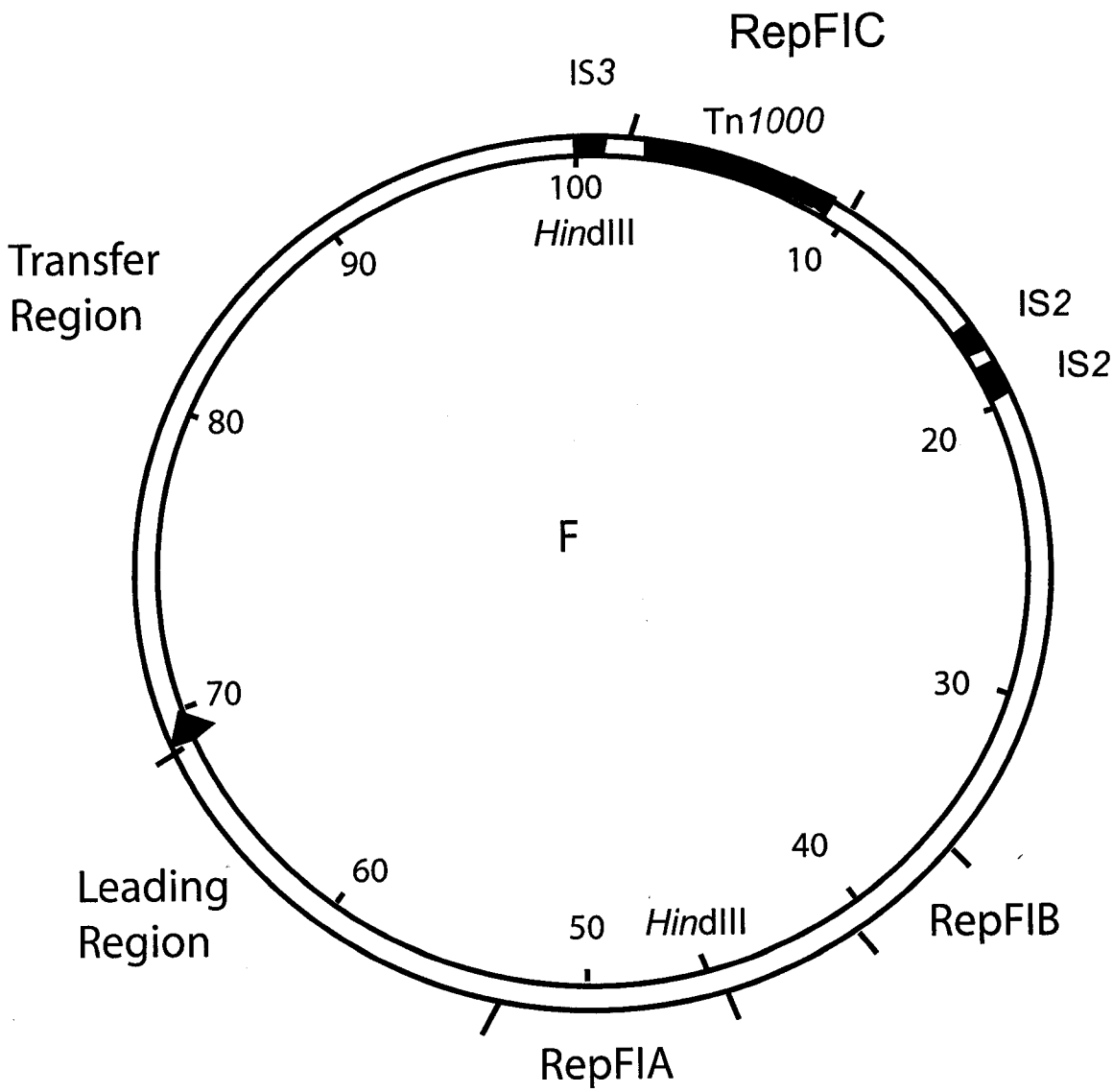
1.6.2.3 Vir Complex Assembly and Transport. The assembly processes have been suggested to occur in the following fashion (Sagulenko *et al.*, 2001). Once the *vir* operons are induced, expression of *virB* results in construction of the transenvelope complex. VirB7 is processed to a lipoprotein and forms a disulfide-linked heterodimer with VirB9, which is then inserted into the outer membrane. The VirB7-VirB9 complex associates with the inner membrane proteins VirB8 and VirB10 and aids in oligomerization of VirB10. Association with VirB4 and VirB6 is expected to occur. This initial complex, when present in the recipient cell, promotes uptake of RSF1010 from donor cells, and since these core proteins, minus VirB6, are homologous to the Com proteins involved in competence in *H. pylori*, it is presumed that this complex forms the core membrane-

spanning structure through which substrates can transit. VirB11 associates with the complex and oligomerizes, at which point it can direct the assembly of the pilus from the inner membrane pool of VirB2 subunits, along with the VirB5 protein. This assembly process is not absolutely required, and VirB11, along with VirD4, can translocate various DNA substrates, such as the VirD2-T-DNA complex or the RSF1010 plasmid, independently of pilus elongation. Transport of VirE2 results in a pore forming in the plant cell membrane, through which the VirD2-T-DNA complex is moved. The ss DNA is coated by VirE2 and the entire complex is localized to the plant nucleus by signals in VirE2 and VirD2.

1.7 F Plasmid. Lederberg and Tatum originally discovered bacterial conjugation in 1946. The F, or Fertility factor, was used extensively in mapping studies of the *E. coli* chromosome by use of Hfr, or high frequency of recombination, strains where F had integrated into the chromosome (Cavalli *et al.*, 1953; Hayes, 1953). Since it is the earliest known conjugative plasmid, it has been extensively studied over the years, and a great deal is now known about the actual mechanism of F plasmid conjugation, although many functions remain unclear.

1.7.1 Organization of the F plasmid. The entire sequence of the 99 159 bp (base pairs) of the F plasmid is now known (accession number AP001918). The major features of the F plasmid include the 33.3 Kb transfer operon, the leading region, which is the first DNA to enter the recipient cell during mating, the replication origins, RepFIA and RepFIB, a large region that encodes genes not necessary for conjugation or plasmid maintenance, as well as a number of insertion elements that have important consequences for the F plasmid (Figure 1.5; Firth *et al.*, 1996). An insertion of an IS*3* element into the *finO* regulatory gene results in constitutive expression of the transfer genes (Cheah and Skurray, 1986). All 4 insertion elements mediate integration of the F plasmid into the *E. coli* chromosome. During early studies on the F plasmid, a number of derivatives were constructed, including pOX38 (Guyer *et al.*, 1980). This 55 Kb *Hin*dIII fragment from the end of RepFIA to the *Hin*dIII site in the IS*3* element in *finO* eliminates

Figure 1.5. Physical map of the F plasmid. Coordinates are marked on the interior in Kb. Actual size of the F plasmid is 99 159 bp. The site of the *Hin*dIII sites that result in the 55 Kb pOX38 derivative are marked as well, the top site is at the left hand side of the IS*3* element in *finO*. Major regions are marked, including all three RepF replicons, the leading region, and the transfer region. The site of the origin of transfer between the left end of the transfer operon, and the leading region, as well as the direction of transfer, is marked with an arrow.



everything but the transfer operon, the leading region, and RepFIA. pOX38 functions indistinguishably from that of the F plasmid.

The leading region is the first piece of DNA transferred to the recipient cell during conjugation and encodes a number of genes required for F plasmid stability and maintenance in the recipient cell (Ray and Skurray, 1983). One of the more interesting functions encoded in the leading region, by *orf169*, which is related to lytic transglycosylases (Loh *et al.*, 1989; Bayer *et al.*, 1995). A number of homologues exist on other conjugative plasmids, including VirB1 from the Ti plasmid and P19 from plasmid R1. *orf169* is not necessary for F plasmid transfer, while mutations in *gene19* decrease transfer efficiency of R1 by 10-fold, similar to what is observed with *virB1* (Loh *et al.*, 1989; Berger and Christie, 1994; Bayer *et al.*, 1995). It is thought that these enzymes might be responsible for locally opening up the PG layer around the transenvelope complex, and consistent with that, overexpression of P19 induces localized breakage of the peptidoglycan layer, resulting in extrusion of the cell contents at spatially localized regions in the cell envelope (Bayer *et al.*, 2001).

Other genes in the leading region, the first DNA to enter the cell, include *psiB* (plasmid SOS inhibition), a single-stranded DNA binding protein (*ssb*), *flmA, B, C* (F leading region maintenance locus), as well as genes with an unknown function, *psiA*, *orf273*, *orf95*, and two single-stranded initiation sites, *ssiD* and *ssiE*. PsiB prevents SOS induction when the F plasmid enters the recipient cell as a single strand (Bailone *et al.*, 1988; Dutreix *et al.*, 1988; Golub *et al.*, 1988). SSB is similar to the *E. coli* SSB, which has roles in DNA replication, recombination, and repair (Chase *et al.*, 1983; Kolodkin *et al.*, 1983). The Flm (F leading maintenance) proteins ensure the killing of plasmid-free cells, thereby maintaining the F plasmid in a population of cells (Loh *et al.*, 1988; Gerdes *et al.*, 1997. The *flm* locus is similar to the *hok/sok* locus of plasmid R1 and is found in a number of other plasmids and on the *E. coli* chromosome (Pedersen and Gerdes, 1999). The considerable conservation of this region between distantly related plasmids suggests that it encodes important functions for plasmid survival (Manwaring *et al.*, 1999).

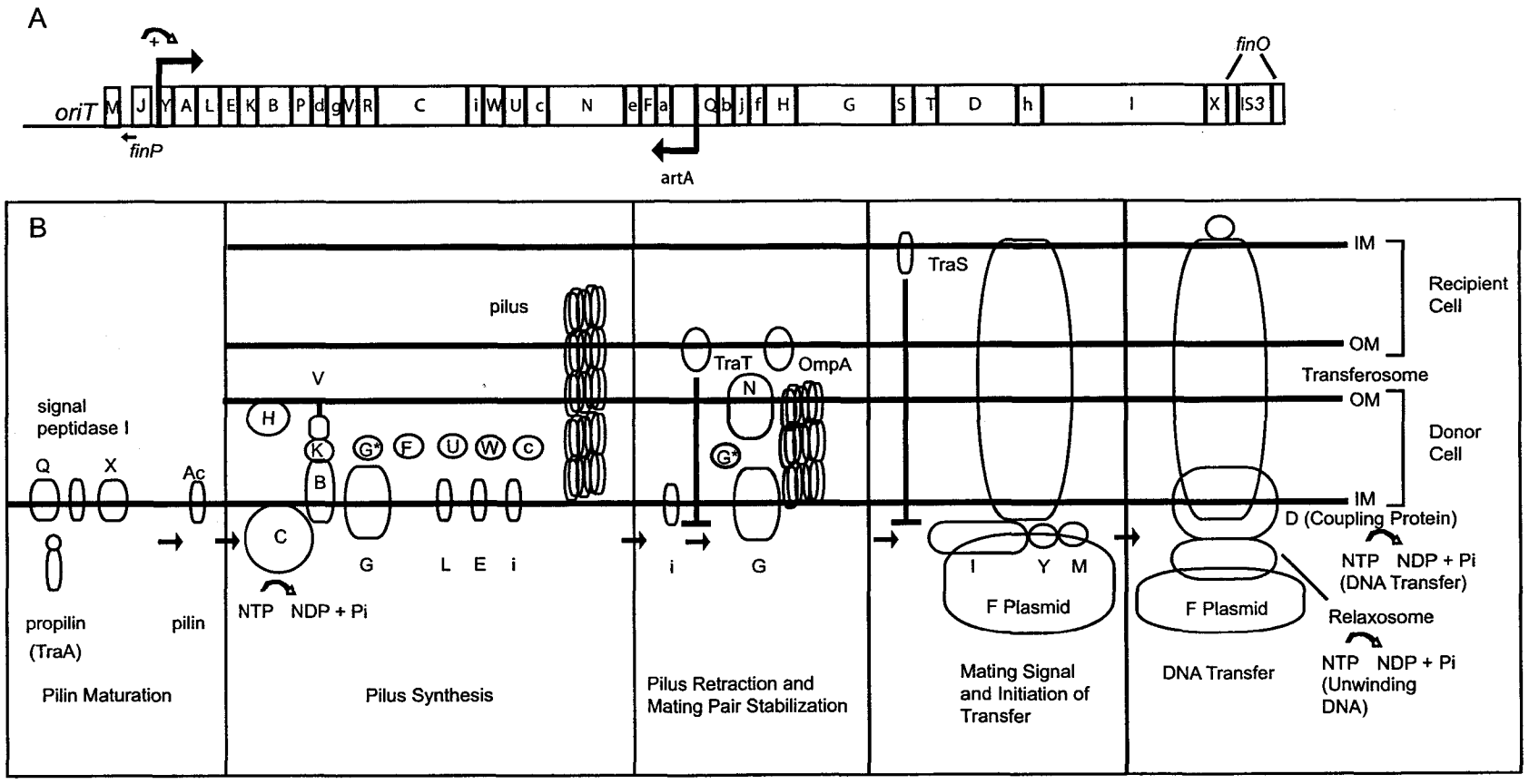
The F plasmid contains 3 different replicons, RepFIA, RepFIB, and RepFIC. RepFIA is the major replicon and contains uni- (*oriS*) and bi-directional (*oriV*) replication initiation sites, as well as a partitioning region, *sopABC* (Eichenlaub *et al.*, 1977; Lane, 1981). *oriS* and the replication protein RepE are absolutely required for replication. An AT rich region, two DnaA boxes, and the *incB* and *incC* loci, which mediate plasmid incompatiblity, are also found in this region. *oriS*, *repE* and *incC* are required for proper regulation of F plasmid replication (Uga *et al.*, 1999; Uga *et al.*, 2000). RepFIB can function in the absence of RepFIA, while RepFIC is interrupted by Tn*1000* (Lane and Gardner, 1979; Lane, 1981; Saadi *et al.*, 1987). The F plasmid is in part maintained in a population of cells by the *ccd* locus (coupled cell division). CcdA, which is unstable, is an inhibitor of CcdB, which prevents cell division and persists in a stable form (Karoui *et al.*, 1982; Ogura and Hiraga, 1983; Miki *et al.*, 1984). Loss of the F plasmid results in concomitant loss of CcdA, with the result that CcdB kills the cell. CcdB is a poison for DNA gyrase, leading to blockage of DNA replication machinery, and double-strand breaks in the DNA (Bernard and Courtier, 1992; Bahassi *et al.*, 1999). CcdA and CcdB coordinately regulate their own expression (Afif *et al.*, 2001; Dao-Thi *et al.*, 2002). The partitioning function of SopA and SopB (stability of plasmid), which act at *sopC*, a centromere-like sequence, ensures that plasmids are delivered to daughter cells prior to cell division (Hayakawa *et al.*, 1985; Mori *et al.*, 1989). SopB can function at a single binding site to form a nucleoprotein complex (Biek *et al.*, 1994). SopB is found at a polar location within the cell, suggesting that localization drives partitioning of the F plasmid (Kim and Wang, 1998). SopA and SopB negatively regulate their own expression in conjunction with *sopC* (Yates *et al.*, 1999). Visualization of the F plasmid by use of a LacI-GFP fusion (green fluorescent protein) and a multicopy insertion of the *lacO* into the F plasmid as a marker indicated that the F plasmid was at the mid-point of a newly divided cell, and that after replication, daughter plasmids moved to the ¼ and ¾ positions of the cell (Gordon *et al.*, 1997; Niki *et al.*, 1997). Replication and partitioning functions ensure that the F plasmid is maintained at 1-2 copies per cell (Frame and Bishop, 1971; Collins and Pritchard, 1973).

1.7.2 The Transfer Operon. The 33.3 Kb transfer operon encodes all of the functions necessary for F plasmid transfer (Figure 1.6; Frost *et al.*, 1994). A total of 37 genes are encoded within this operon. At the far left hand of the transfer operon is the origin of transfer (*oriT*; Willetts, 1972) All *tra* genes, starting with *traM* and extending to *finO*, are to the right of *oriT*. The direction of DNA transfer during conjugation results in the leading region, which is to the left of *oriT*, entering the recipient cell first, and the transfer operon entering last. Most of the transfer genes are encoded within a polycistronic operon starting at the P_{traY} promoter at the left end of the operon, and extending to the right end, although there are promoters for P_{traD}, P_{trat} P_{traS}, and P_{trbF} downstream of P_{traY} (Ham *et al.*, 1989). A number of genes that have their own promoters are upstream of P_{traY}, including *traM*, *finP* and *traJ*. The promoters for *finP* and *artA* are in the opposite orientation of the other genes. Genes encoded within the polycistronic operon typically have overlapping start and stop codons. The transfer genes are categorized into 5 functional groups. 1) pilus synthesis, 2) mating pair stabilization (MPS), 3) surface and entry exclusion, 4) DNA processing, 5) regulation. A number of genes have unknown phenotypes as mutations in these genes have no affect on DNA transfer.

1.7.2.1 Regulation. The major P_{traY} promoter is positively activated by the TraJ protein (Willetts, 1977). Expression of TraJ is controlled negatively by the FinP antisense RNA transcript, which binds to part of the untranslated leader sequence of the *traJ* mRNA, and is expected to occlude the ribosome binding site (Finnegan and Willetts, 1971; Mullineaux and Willetts, 1985). The FinO protein is an RNA binding protein that binds both the FinP and *traJ* RNA molecules, and facilitates duplex formation, while also preventing FinP degradation (Lee *et al.*, 1992; van Biesen and Frost, 1994; Jerome *et al.*, 1999; Ghetu *et al.*, 1999). In cells expressing FinO, the transfer operon is in a repressed state, such that in a population of cells, only about 1% become derepressed and capable of transfer. The IS*3* insertion element in the F plasmid *finO* gene results in a constitutively derepressed state in vegetatively growing cells, so that all cells carrying the F plasmid are capable of transfer (Cheah and Skurray, 1986; Yoshioka *et al.*, 1987).

Figure 1.6. A. Organization of the transfer operon (adapted from Frost *et al.*, 1994; Firth *et al.*, 1996). All genes encoded within the 33.3 Kb transfer operon are listed. *tra* genes are in capital, *trb* genes are in lowercase. The approximate size of each gene product is indicated by the size of the boxed region. The P_{traY} and P_{artA} promoters are shown with arrows. The antisense RNA *finP* is indicated below the TraJ transcriptional activator. The origin of transfer is at the left end of the transfer operon, and transfer in the 5' to 3' direction results in the leading region (not shown) entering the recipient cell first. The insertion of the IS*3* element in *finO* is shown, rendering the F plasmid derepressed.

B. Subcellular location and step in which each protein is required is indicated. Size of the protein is approximately indicated. The major steps related to the mating cycle (see Figure 1.7). TraT and TraS function in the recipient cell envelope to block redundant donor to donor cell transfer. The transferosome is expected to be capable of crossing both cell envelopes in order to facilitate transfer, although evidence of such a structure is lacking.

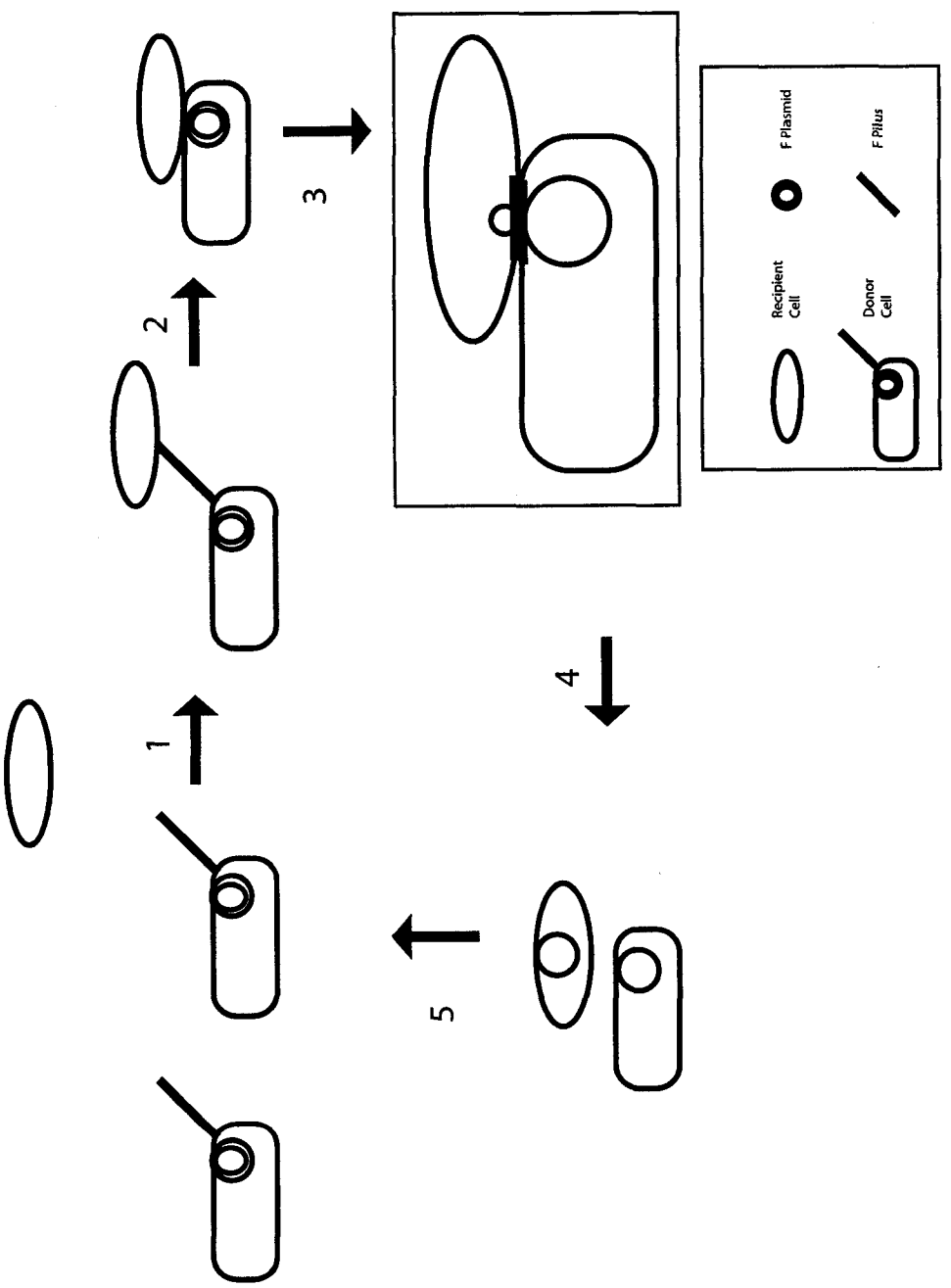


Regulation of the transfer operon is complex, and other factors appear to be involved in regulation of P_{traY}. The TraY protein binds to its own promoter, and mutations in *traY* result in a decrease in transcription from P_{traY}, suggesting that the protein autoregulates itself (Nelson *et al.*, 1993; Maneewannakul *et al.*, 1996; Silverman and Scholl, 1996). IHF also affects transcription from P_{traY}, and mutations in *ihf* decrease P_{traY} promoter activity (Silverman *et al.*, 1991). The host encoded ArcA protein, which is part of a two-component signal transduction system involved in sensing changes in the redox state of the bacterial cell, is important for promoter activity of P_{traY} (Silverman *et al.*, 1991; Lynch and Lin, 1996). TraJ expression is affected by the Cpx signal transduction pathway, a system that is involved in measuring environmental stress in the periplasm, as efficient TraJ expression requires CpxA (Silverman *et al.*, 1993; Danese and Silhavy, 1998). The CRP (cAMP receptor protein) has inhibitory effects, and a potential binding site for CRP was discovered to overlap the *traJ* transcription start site (Harwood and Meynell, 1975; Paranchych *et al.*, 1986).

1.8 Mechanism of Conjugation. The remainder of the genes in the transfer operon are involved in the actual DNA transport event itself. Analysis of mutations in these genes led to the model of the mating cycle, which is composed of 5 steps (Figure 1.7; Achtman and Skurray, 1977; Manning *et al.*, 1981). 1) Pilus contact with a recipient cell and retraction, 2) leading to close wall to wall contacts, 3) stabilization of these contacts and triggering of DNA processing events at *oriT*, 4) DNA transfer, and finally 5) disaggregation and second strand synthesis in both donor and recipient cells leading to the formation of two cells capable of transferring the F plasmid. Proteins involved in these steps will be discussed below.

1.8.1 F Pilin Processing. The propilin precursor, TraA, is an unusual protein of 121 aa that contains a very long leader sequence of 51 aa (Frost *et al.*, 1984). Insertion of propilin into the inner membrane requires the inner membrane chaperone TraQ, and is independent of the SecA pathway (Moore *et al.*, 1982; Majdalani and Ippen-Ihler, 1996; Majdalani *et al.*, 1996). F pilin binding to TraQ has been demonstrated by yeast two-hybrid analysis (Harris *et al.*, 1999). Propilin

Figure 1.7. The Mating Cycle. The major steps of F plasmid mating are indicated by numbers: 1) pilus binding to the recipient cell, 2) pilus retraction and initial wall to wall contact, 3) mating pair stabilization and initiation of DNA transfer (occurs concomitantly), 4) disaggregation of the mating pairs; 5, strand synthesis in both donor and recipient cell to generate ds DNA. Donor cell is rectangular, recipient cell is oval, the pilus is a black line at 45°, double circles represent ds F plasmid DNA, single circles represent ss F plasmid DNA.



is then cleaved by signal peptidase I, leaving the N- and C-termini exposed in the periplasm, with two transmembrane segments in the inner membrane separated by a short cytoplasmic loop. The pilin subunit is further processed by the product of the *traX* gene, resulting in N-acetylation, a process that is not absolutely required for pilus synthesis (Moore *et al.*, 1993; Maneewannakul *et al.*, 1995). The *traX* gene is conserved in other F-like plasmids, and acetylation of pED208 and ColB2 pilin has been observed (Frost *et al.*, 1983; Finlay *et al.*, 1984; Yoshioka *et al.*, 1990; Cram *et al.*, 1991). Antibody binding characteristics of pili products from *traX* cells are altered (Grossman *et al.*, 1990). Pili expressed from *traX* cells also tend to aggregate and show different adsorption characteristics for R17 phage, although the phage can infect (Grossman and Silverman, 1989; Grossman *et al.*, 1990). These results suggest that lack of acetylation alters pilus structure but not pilus function. Major antigenic and phenotypic differences are attributed to the allele of pilin expressed by the cell. There are five groups based on the pilin sequences from F, ColB2, pED208, R1-19 and R100, with most differences occurring at the N- and C-terminal regions.

1.8.2 Pilus Synthesis. The majority of proteins expressed from the transfer operon are involved in pilus synthesis. These include TraL, TraE, TraK, TraB, TraV, TraC, TrbI, TraW, TraU, TrbC, TraF, TraH, and the N-terminal domain of TraG. The homology between some of these proteins and the Vir proteins has been noted (Table 1.2; Firth *et al.*, 1996; Cao and Saier., 2001). Once mature pilin accumulates in an inner membrane pool, these proteins act to erect the pilus (Moore *et al.*, 1981; Moore *et al.*, 1993). Mutations in most of these genes completely block pilus synthesis and DNA transfer (Achtman *et al.*, 1971). Mutations in the distal end of *traG* affect MPS, while proximal mutations affect pilus synthesis (Achtman *et al.*, 1971; Manning *et al.*, 1981; see below). Insertion of a kanamycin resistance cassette in *trbI* results in cells producing extremely long pili, suggesting that TrbI functions in control of pilus retraction or elongation (Maneewannakul, S. *et al.*, 1992b). Mutations in *traU* do not greatly reduce the number of pili on the cell, but do significantly alter transfer efficiency (Moore *et al.*, 1990). The *traC1044* mutation results in lack of an elongated pilus, but cells expressing this allele are still capable of being

infected by filamentous phage (Schandel *et al.*, 1987). Insertion of a *kan* cassette into *trbC* results in a similar phenotype of pilus$^-$ cells, but phage infection can still occur (Maneewannakul *et al.*, 1991).

The majority of these proteins fractionate with the cell envelope, suggesting that they form a transenvelope complex for pilus assembly. TraV is an outer membrane lipoprotein (Doran *et al.*, 1994). Interactions between TraV-TraK and TraK-TraB have been discovered by yeast two-hybrid analysis (Harris *et al.*, 2001). TraB and TraK fractionate with the outer membrane, which is probably mediated through their protein-protein interactions with TraV (Harris *et al.*, 2001). TraH has been demonstrated to be an outer membrane protein, although it does not appear to be cell-surface exposed (Manwaring, Ph.D.). TrbC, TraF, TraU, and TraW have all been found in the periplasm (Wu *et al.*, 1988; Maneewannakul *et al.*, 1991; Maneewannakul *et al.*, 1992a; Moore *et al.*, 1990). TrbI, and TraG are inner membrane proteins (Moore *et al.*, 1981; Maneewannakul *et al.*, 1991). A cleavage product of TraG, TraG*, has been discovered in the periplasm (see below; Firth and Skurray, 1992). TraE and TraL are predicted to be inner membrane proteins (Frost *et al.*, 1984). TraC is unusual in that, by itself, it is found to be cytoplasmic, but in the presence of the other transfer proteins, is found to be associated with the IM (Schandel *et al.*, 1990; Schandel *et al.*, 1992). TraC is homologous to VirB4, contains an ATP-binding site, and is thought to provide the energy for pilus elongation. Complementation analysis of F*lac* and pOX38 transfer mutants with F and R100 clones suggested that TraC, TraE, TraK, TraL, and the N-terminal region of TraG, along with the pilin subunit, are involved in formation of the pilus tip at the cell surface (Anthony *et al.*, 1999). It is interesting that homology exists between inner membrane components of the F plasmid that are involved in pilus tip formation and the VirB inner membrane components (Table 1.2; see above).

1.8.3 Pilus Structure and Function. The F pilus was originally discovered when it was found that donor-specific RNA phages bound to the length of a pilus on donor but not recipient cells (Crawford and Gesteland, 1964; Brinton *et al.*, 1964). The F pilus was considered a virus-like

structure that could transfer DNA since it was observed under the light microscope that donor and recipient cells moved together as if they were separated by an invisible strand (Brinton, 1971). Shearing the pilus off of cells by blending them resulted in an inability of cells to transfer DNA (Brinton, 1965; Novotny *et al.*, 1969). A pilus-specific DNA phage was discovered that bound the pilus tip (Caro and Schnos, 1966). The pilus is a long, flexible filament, 1 to 2 μm in length, and 8 nm in diameter (Frost, 1993). The pilin subunits are arranged helically along the length of the pilus; 25 subunits are arranged in two turns, with a pitch of 16 nm, a unit rise of 1.28 nm, and the crystallographic repeat is 32 nm. Structural analysis of the pilus indicates that the center is hollow, with a diameter of 2 nm (Marvin and Folkhard, 1986). DNA transfer has been reported when donor and recipient cells were kept apart in a micromanipulation experiment, as well as when the donor and recipient cells are separated by a thick (6 μm) and porous (0.01 to 0.1 μm diameter) membrane (Ou and Anderson, 1970; Harrington and Rogerson, 1990). These results suggested that the DNA is transferred through the lumen of an extended pilus.

An alternative hypothesis has also emerged, based in part on observations of pilus retraction (Curtiss, 1969; Marvin and Hohn, 1969). In the micromanipulation experiment described above, close contact between donor and recipient was observed more frequently than contact at a distance, and DNA transfer occurred at greater efficiency when this occurred (Ou and Anderson, 1970). Conjugal pairing of cells undergoing DNA transfer was noticed by Lederberg in 1956. DNA transfer cannot occur when the donor and recipient cell are separated by a fritted glass disc (Davis, 1950). The pilus appears to be capable of retracting or depolymerizing in response to external agents (Novotny and Fives-Taylor, 1974). Competition between a filamentous phage that binds the pilus tip, and conjugation, suggested that the pilus was required for DNA transport (Jacobson, 1972; Ippen *et al.*, 1967; Novotny *et al.*, 1968). Addition of SDS to pre-formed mating pairs cells, at concentrations that depolymerize the extended pilus, have little effect on mating efficiency, suggesting that DNA transfer does not occur through an extended pilus (Achtman *et al.*, 1978a). The pilus may continually polymerize and depolymerize, and transfer may only initiate when the pilus contacts a recipient cell (Sowa *et al.*, 1983). It has been suggested that the pilus

may merely serve to attach the recipient cell, and retract, a process that is required for transfer, but that the pilus does not serve as the conduit for DNA transfer. However, the recent evidence that secreted proteins are observed coming out of the tip of the needle in type III secretion lends support to the idea that the pilus can function as a conduit for transfer (see above; Jin *et al.*, 2001).

1.8.4 Donor Conjugal DNA Synthesis. It had been suggested, based on the replicon model of chromosome replication, that replication of the F plasmid allows DNA transfer (Jacob and Brenner, 1963). While replication does occur during DNA transfer, use of a double mutant strain (*thy dnaB*ts), which completely blocks donor conjugal DNA synthesis, had no effect on DNA transfer (Jacob *et al.*, 1963; Sarathy and Siddiqi, 1973). Donor conjugal DNA synthesis had been a useful tool in distinguishing the events that lead to initiation of DNA transfer from the actual transfer event itself (see below).

1.8.5 Mating Pair Stabilization. Mating pairs are stabilized when the mating occurs on a solid surface (Matney and Achenbach, 1962; Wood, 1968). Conjugative plasmids exhibit one of two mating types: 1) universal or liquid mating ability, and 2) surface-dependent mating (Bradley *et al.*, 1980). The IncP plasmid RP4, which produces a thick rigid pilus that is unlike the long flexible pilus of the F plasmid, requires a solid surface for efficient conjugation. The IncI plasmid R64 however, does not require a solid surface, and mates efficiently in liquid culture. R64 produces two types of pili, a conjugative pilus like that of RP4, and a number of thin flexible pili that are homologous to type IV pili (Bradley, 1983; Bradley, 1984; Kim and Komano, 1997) . The type IV pili allow R64-containing cells to bind to cells in liquid culture by contacting and stabilizing the cells during mating while the thick pilus is involved in conjugation (Komano *et al.*, 1990; Komano *et al.*, 1995). Recently it has been shown that the tip of the R64 type IV pilus specifically uses LPS in the recipient cell as a receptor (Ishiwa and Komano, 2000).

Mating aggregates of up to 20 cells were noticed by electron microscopy (Achtman, 1975). Two types of aggregates were noticed by Coulter counter analyses, which places the cells under high shear during cell counting procedures: 1) shear-sensitive and 2) shear-resistant. Shear-sensitive aggregates occur when the F pilus contacts a recipient cell and retracts, but the cells have not yet stabilized their interactions. Mutations in *traN* and the distal part of *traG* affected the formation of stable mating pairs, but did not affect donor conjugal DNA synthesis (Kingsman and Willetts, 1978; Manning *et al.*, 1981). These mutations did not alter F pilus binding to the recipient cell, nor the formation of unstable wall-to-wall contacts (Manning *et al.*, 1981). Although the mutations in *traN* and *traG* allowed initiation of DNA synthesis during mating, the DNA was not transported to the recipient cell, suggesting a defect in DNA transport (Kingsman and Willetts, 1978). This suggested that the mating signal is not dependent on mating pair stabilization, and that TraN and TraG mediate interactions with the recipient cell to bring about the formation of stable mating pairs.

1.8.5.1 TraN. The product of the *traN* gene was found to be a 602 aa protein that was processed to 584 aa forming a 64 kDa protein (Maneewannakul *et al.*, 1992a). The presence of a putative ATP binding site was also noted, which did not correspond with its location in the outer membrane, as determined by sucrose gradient fractionation and protease accessibility assays. Incubation of maxicells expressing TraN with 250 μg/mL of proteinase K for 18 hours resulted in the disappearance of full-length TraN and the appearance of two truncated products of 60 kDa and 53 kDa (Maneewannakul *et al.*, 1992a). This suggested that only small regions of TraN at the N- or C-terminal regions were extracellularly exposed. TraN overproduction due to multicopy effects was found to interfere with TrbC processing when expressed from the same plasmid (Maneewannakul *et al.*, 1992a). C-terminal deletions were used to find if any region of TraN was responsible for this interference, and it was shown that aa residues 236 to 340 may play a part in TrbC processing.

1.8.5.2 TraG. TraG was found to be a 100 kDa inner membrane-associated protein (Manning *et al.*, 1981; Moore *et al.*, 1981). Analysis of the amino acid sequence suggested that TraG contains three putative transmembrane segments, resulting in a periplasmic loop in the N-terminal region (aa 52-371), and the entire C-terminal region after aa 448 in the periplasm (Firth and Skurray, 1992). When antibodies directed against the C-terminal region of TraG were used, a TraG cleavage product was found in the periplasm (TraG*), which was thought to be due to cleavage of the periplasmic C-terminal domain. Examination of the protein sequence in this region led to the suggestion of a signal peptidase I cleavage site at aa 451 (von Heijne, 1984; Firth and Skurray, 1992). Deletion analysis confirmed that the N-terminal region is responsible for pilus synthesis as was found with previous frameshift mutations (see above).

1.8.5.3 Recipient Cell Requirements. An examination of recipient cells for mutations that specifically inhibited mating (conjugation deficient, Con$^-$) revealed two different classes of mutants; mutations that affect the LPS structure, and mutations in the gene for the OmpA protein. Initially, strains defective in bacteriophage infection were isolated, and then tested for their ability to function as recipient cells (Monner *et al.*, 1971; Reiner, 1974; Skurray *et al.*, 1974). R100-1 however, was not affected by these mutations. R100-1 is similar enough to F that complementation of F *tra* mutants with the corresponding gene in R100-1 result in efficient transfer in most cases, although key differences do exist (Skurray *et al.*, 1974; Anthony *et al.*, 1999). Specific ConF$^-$ mutations were found in a mutational screen, with the result that only defects in LPS and OmpA were recovered (Havekes and Hoekstra, 1976a; Havekes *et al.*, 1976b).

The ConF$^-$ mutations in *ompA* also affected bacteriophage infection by K3 and TuII*, while the LPS mutations were found to produce a heptose-less LPS (Henning *et al.*, 1976; Schweizer and Henning, 1977; van Alphen *et al.*, 1977). Mutations that affected R100-1 mating were also found, but most of these were also ConF$^-$, with the exception of two specific ConR100-1$^-$ mutations, which were not further characterized (Havekes *et al.*, 1977). Since the *ompA* mutations were also

pleiotropic, it was not clear if they affected mating directly. Addition of purified OmpA protein as well as purified LPS, showed specific inhibition in F plasmid mating, indicating OmpA and LPS were important components of the recipient cell (Schweizer and Henning, 1977). The actions of these mutations were further refined to the stage of mating pair stabilization, as ConF$^-$ mutations allowed F pilus specific contacts, close cell-to-cell contacts, and produced more efficient mating on solid surfaces, but did not form stable mating aggregates (Achtman, 1975; Havekes and Hoekstra, 1976; Achtman *et al.*, 1978b). Mutations in *ompA* and LPS biogenesis were not additive, suggesting that they functioned together (Manoil and Rosenbusch, 1982). A single mutation was isolated in *ompA*, G154D, which blocked mating, but did not affect bacteriophage K3 infection (Ried and Henning, 1987).

E. coli strains carrying mutations in LPS synthesis genes (*waa*), showed that the mutations in *waaP* specifically affect mating (Anthony *et al.*, 1994). The product of the *waaP* gene is responsible for two additions to the LPS inner core, addition of PPEA to heptose I, and extension of heptose II by heptose III (Figure 1.1; Klena and Schnaitman, 1993). Addition of purified PEA to mating cells led to a decrease in transfer efficiency, suggesting that it is the PPEA addition step that specifically affects transfer (Anthony *et al.*, 1994). Complementation of an F *traA* knockout with R100-1 *traA* revealed that the ConF$^-$ phenotypes associated with the recipient cell were not due to the specific pilin subunit present in the donor cell. This suggested that either a specific component at the pilus tip or some other F plasmid-encoded factor was responsible for recognizing OmpA and LPS in the recipient cell.

1.8.5.4 Conjugational Junctions. Thin section analysis of conjugating cells revealed an electron dense structure at the interface between the two cell walls of the donor and recipient bacterium (Durrenberger *et al.*, 1991). This structure was not observed in cultures of pure donors, or in cultures of pure recipients, suggesting it was formed during conjugation. The observed size of these conjugational junctions in cross-section indicated that they were not simply the close apposition of the donor and recipient outer membranes. This suggested that a plasmid-encoded

component was involved. No bridge between the inner and outer membranes of the donor was observed, however, those bridges may be too small to be observed. It was suggested that the mating pair stabilization proteins would constitute some of this electron dense material. However, cells containing the plasmid RP4 also exhibit these conjugational junctions, even though they do not appear to encode homologues of the MPS genes, *traN* and *traG* (Samuels *et al.*, 2000). The low levels of TraN protein found in F^+ cells also suggested that TraN would not be a major component of these junctions (Silverman, 1997).

1.8.6 Surface and Entry Exclusion. Cells that contain an F plasmid make poor recipients. This is due in part to both plasmid incompatibility, which is based on the replication system, and surface and (Sfx) entry exclusion (Eex), which are not based on replication. Only recipient cells containing a related plasmid inhibit DNA transfer, while those of closely related systems do not (Willetts and Maule, 1986). This process, termed surface exclusion, was found to be dependent on two genes, *traS* and *traT* (Achtman *et al.*, 1977; Achtman *et al.*, 1980). The combined action of TraT and TraS reduces transfer by several hundred-fold. Recipient cells expressing TraT were found to be very deficient in forming stable mating aggregates, and DNA transfer was inhibited by approximately 10- to 20-fold (Achtman *et al.*, 1977). In the same study, recipient cells expressing TraS alone formed normal mating aggregates, but DNA transfer was severely inhibited, up to 200-fold. TraT was found to be an outer membrane protein of approximately 26 kDa, and was present at up to 90 000 copies per cell (Achtman *et al.*, 1977; Minkley and Ippen-Ihler, 1977). TraS was found to be an inner membrane protein of 18 kDa (Achtman *et al.*, 1977). Analysis of donor to donor matings using donor conjugal DNA synthesis as a marker indicated that the presence of the F factor in the recipient cell inhibited DNA labeling, suggesting that the events leading up to DNA transfer were inhibited (Kingsman and Willetts, 1978). Surface exclusion and entry exclusion, therefore, inhibit two different reactions in the donor cell, stabilization of mating cells, and initiation of DNA transfer.

TraT is a lipoprotein exposed at the cell surface, and fusions to the C-terminus result in extracellular exposure of an epitope (Manning *et al.*, 1980; Perumal and Minkley, 1984; Chang *et al.*, 1999). TraT has been found in multimeric form, and when purified, exhibits exclusion functions when added to cells prior to mating (Minkley and Willetts, 1984). The plasmid-specificity exhibited by TraT is due to a single aa change, whereby R100 TraT has an alanine in place of the glycine found in F TraT at position 141 (Harrison *et al.*, 1992). TraT apparently blocks mating aggregate formation, suggesting it interferes with pilus tip binding to the recipient cell, however, specificity was not due to the plasmid-specific pilin subunit (Achtman *et al.*, 1977; Anthony *et al.*, 1994). Based on the observation that TraT inactivates some OmpA-specific phages, it was suggested that TraT interfered with access to the OmpA protein in the recipient cell (Riede and Eschbach, 1986).

1.8.7 DNA Processing. DNA processing occurs at a *nic* site within the *oriT* region at the left end of the transfer operon. The *oriT* region includes binding sites for TraM, TraY, IHF (integration host factor) and TraI. TraI is the relaxase responsible for enzymatic cleavage of *nic* in a site- and strand-specific manner (Matson and Morton, 1991; Reygers *et al.*, 1991; Sherman and Matson, 1994). TraI is a bifunctional protein that contains an N-terminal relaxase domain that is functionally analogous to VirD2, and a C-terminal helicase domain (Abdel-Monem *et al.*, 1983; Lahue *et al.*, 1988; Traxler and Minkley, 1988; Dash *et al.*, 1992). Both processes are required for DNA transfer, as mutations in either the nickase or helicase domains completely block transfer (Matson *et al.*, 2001). The transesterification reaction promoted by the TraI relaxase domain results in covalent attachment of the DNA to the protein (Matson *et al.*, 1993). Based on homology to the R388 relaxase, TrwC, it is predicted that the enzymatic reaction would require two tyrosine residues in the N-terminal relaxase domain of F TraI (Grandoso *et al.*, 2000; Zechner *et al.*, 2000). Cleavage and religation continuously occur in equilibrium (Sherman and Matson, 1994). Once the mating signal is presented to the relaxosome, the helicase activity unwinds the DNA, resulting in DNA transfer. At this point, donor conjugal DNA synthesis can proceed. The relaxase activity of F TraI requires two other proteins, IHF and TraY (Howard *et al.*, 1995; Nelson

et al., 1995). IHF and TraY are also required for R100 TraI nicking (Inamato *et al.*, 1991; Inamato *et al.*, 1994). TraY and IHF both bend DNA, and this process may affect the nicking reaction (Thompson and Landy, 1988; Luo *et al.*, 1994). The relaxosome refers to the complex of proteins bound at the *oriT* region (Zechner *et al.*, 2000).

TraM binds to the *oriT* region but is not required for the relaxation reaction at the *nic* site (Everett and Willetts, 1980; Di Laurenzio *et al.*, 1992). Since mutations in *traM* block donor conjugal DNA synthesis, but allow stable mating pairs to form, it was suggested that TraM functions at some point after nicking occurs, but prior to DNA transfer (Kingsman and Willetts, 1978; Manning *et al.*, 1981). TraM binds to three sites in the *oriT* region, two of which appear to be involved in autoregulatory functions (*sbmAB*), since they overlap the two *traM* promoters, and one of which is important for transfer (*sbmC*) and is closest to the *nic* site (Di Laurenzio *et al.*, 1992; Penfold *et al.*, 1996). Analysis of TraM from the related R1 plasmid suggested that it formed tetramers in solution and on the DNA, and had a tendency to form larger aggregates (Verdino *et al.*, 1999). TraM cooperatively bound to all three binding sites since binding to the high affinity *sbmA* and *sbmB* sites facilitated binding to the low affinity *sbmC* site (Fekete and Frost, 2002). At high concentration TraM may form a nucleoprotein complex with the DNA wrapped around the oligomerized protein. Since TraM appears to bind the coupling protein, TraD, in the inner membrane, it was suggested that TraM delivers the relaxosome machinery to the transferosome (Disque-Kochem and Dreiseikelmann, 1997).

1.8.8 DNA Transfer. Once the TraI helicase unwinds the DNA, it is transferred to the recipient cell. The TraD protein mediates coupling of the relaxosome functions, possibly through TraM, to the transferosome, through the pilus machinery. TraD is homologous to VirD4 (described above; Lessl *et al.*, 1992). Mutations in *traD* result in a transfer-deficient phenotype, but donor-conjugative DNA synthesis, nicking at *oriT*, and piliation are apparently normal, suggesting that DNA transfer initiates and the DNA is unwound, but transfer does not proceed (Kingsman and Willetts, 1978; Everett and Willetts, 1980; Panicker and Minkley, 1985). When a temperature-

sensitive allele of *traD* was examined at the non-permissive temperature during conjugation, it was observed that the cells were in stable mating pairs, suggesting that TraD functions after both mating pair stabilization and DNA processing reactions have taken place (Panicker and Minkley, 1985). Some *traD* mutations result in overpiliated cells, suggesting a link to the pilus machinery (Armstrong *et al.*, 1980). Topological analysis indicated that the N- and C-termini of the protein were in the cytoplasm, with a short periplasmic loop, and a long cytoplasmic C-terminal tail (Lee *et al.*, 1999). This predicts that the two ATP-binding sites, as well as the region for plasmid-specificity, are cytoplasmic (Sastre *et al.*, 1998). TraD has DNA-dependent ATPase activity, and is also required for penetration of the F pilus-specific RNA phages R17 and MS2, suggesting it forms a pore in the IM to allow the passage of nucleic acids (Paranchych, 1975; Schoulaker-Schwarz and Engelberg-Kulka, 1983; Panicker and Minkley, 1992).

1.8.9 Completion of Transfer. DNA transfer occurs rapidly, and rates of 0.15 transconjugants per donor per minute have been measured (Andrup and Andersen, 1999). Once the full-length DNA molecule is transferred to the recipient cell, TraI terminates the event by religating the previously cleaved ss DNA. The ss DNA molecules left in both the donor and the newly transferred strand in the recipient undergo replication, resulting in two F^+ transfer-proficient cells. Since Hfr matings persist in stable mating pairs, while F^+ matings do not, it has been suggested that disaggregation is an active process that occurs at completion of DNA transfer (Achtman *et al.*, 1978a). The gradient of decay in transfer efficiency of chromosomal markers in Hfr matings can be attributed to either instability of components of the transferosome or relaxosome, or inefficiency in DNA recombination in the recipient cell (Achtman *et al.*, 1978a).

1.9 Scope of Thesis. The F plasmid system is one of the most well characterized transfer systems known. The key question in understanding conjugation is how is the DNA transported from the cytoplasm of a donor cell to the cytoplasm of a recipient cell? Recent studies have shown that a remarkable number of transport systems function in an analogous manners, including the production of extracellular appendages, the formation of outer membrane pore

structures through which secreted substrates are passed, and a transenvelope complex that is needed for transport.

The mechanism of DNA transport, however, is not as clearly understood. All of these systems function in two pathways, the first is the production of a morphogenetic structure that spans the bacterial cell envelope while the second is involved in changes that result upon contact with the recipient cell and DNA transport. Some detail about the transenvelope complexes in both the VirB and F systems are beginning to emerge, but a fundamental understanding of DNA transport will only come about when both the core components, as well as system specific functions are both known.

The F plasmid MPS system consisting of inner membrane TraG and outer membrane TraN appears to be uniquely situated at the crossover point between these two pathways, from production of the pilus structure to active DNA transport as well as interactions with the recipient cell. The MPS system appears to be unique to F-like plasmids, with other conjugative systems using type IV pili for MPS, and other systems not encoding any MPS functions at all, yet the F-like MPS system appears to be a core component of DNA transport. By studying the functions and interactions of TraN and TraG during pilus synthesis, MPS, and DNA transport, greater insight into the formation of the transenvelope complex as well as the interactions that take place during DNA transfer will be achieved.

In Chapter 3, the allelic differences between the related F and R100 plasmids will be exploited to understand the functions of TraN, OmpA, and TraT during the events that take place during mating pair stabilization. Sequence analysis of the F and R100 TraN proteins suggested that receptor specificity might be encoded in a divergent region in the middle of both proteins. Sequence analysis also indicated that TraN contains conserved cysteine residues. A topological analysis of F TraN in Chapter 4 revealed that the region of sequence divergence appears to be mostly periplasmic, and not extracellular, which was expected. The role of the cysteine residues

in TraN, as well as possible protein-protein interactions of F TraN with other transfer proteins was examined in Chapter 5 using antibodies directed against an epitope internally localized in F TraN. It was found that disulfide bond formation, as well as the outer membrane lipoprotein TraV, contribute to the stability of F TraN. These two disparate elements may function together, through an intermolecular disulfide bond between TraV and TraN. Chapter 6 is an examination of the topology of TraG as well as the cellular localization of a GFP fusion to TraG. The allelic differences between F and R100 were used again to clarify the function of TraG and TraS in entry exclusion.

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. Cells were grown in Luria Bertani Broth (LB-1% Difco Tryptone, 0.5% Difco Yeast Extract, and 1% NaCl (BDH Inc.)) at 37°C with appropriate antibiotics on a tube roller or shaker to mid-log phase, or left standing overnight, and subsequently diluted 1:100 in fresh medium. Glucose was added to cultures to a final concentration of 100 mM to repress expression from an IPTG inducible promoter, or as a general growth enhancer. Antibiotics (Sigma Chemical Co.) were added at the following concentrations: ampicillin (Amp) 50 μg/mL; chloramphenicol (Cm) 20 μg/mL; kanamycin (Km) 25 μg/mL; spectinomycin (Sp) 100 μg/mL; streptomycin (Sm) 200 μg/mL; tetracycline (Tc) 10 μg/mL; rifampicin (Rif) 50 μg/mL; naladixic acid (Nal) 16 μg/mL). All antibiotic stock solutions were filter sterilized unless dissolved in ethanol. IPTG (isopropyl-β-D-thiogalactopyranoside; Sigma Chemical Co.) was added as an inducer and BCIG (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; Sigma Chemical Co.) was added as an indicator where needed. Arabinose was added at 0.05% to induce expression from pBAD vectors.

2.1.2. Mating Assays. Mating assays were performed as previously described (Anthony *et al.*, 1994). Briefly, 2 mL cultures were grown to mid- to late-log phase in LB with appropriate antibiotics. Glucose was added to some donor cultures to a final concentration of 100 mM. Cells were pelleted and washed once with 4°C LB to remove antibiotics then resuspended in an equal volume of LB. One hundred microlitres of donors and recipients were mixed with 800 μL of 4°C LB, vortexed, and allowed to mate for 30 min at 37°C. Matings were then vortexed vigorously and put on ice to prevent further mating. After a 10 min incubation on ice, matings were serially diluted 10-fold in 4°C LB, and 10 μL was plated separately on selective plates for donors and for transconjugants. Colonies were counted after overnight incubation, and the number of transconjugants per 100 donor cells calculated. Typically 1 X 10^7 to 1 X 10^8 donor cells/mL were used in each mating assay. Multiple cultures were assayed in each experiment and each culture was started from a single independent colony. Surface exclusion indices were calculated

Table 2.1. Bacterial strains used in this study.

Strain	Relevant characteristic	Source
ED24	Spc^R, F^- Lac^-	Achtman *et al.* (1971)
ED2149	F^- *lacΔU124* Δ(*nadA aroG gal att* I *bio*)	Laboratory Collection
XK100	Spc^R derivative of BL21(DE3) carrying T7 RNA polymerase under *lacUV5* control In the chromosome	Studier and Moffat (1986)
DH5α	*supE44 ΔlacU169 (Φ80 lacZΔ80M15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Hanahan (1983); Laboratory Collection
MC4100	Sm^R, *araD139 Δ(argF-lac)U169 rpsL150 relA1 flbB3501 deoC1 ptsF25 rbsR*	Manoil; Laboratory Collection
JC3272	Sm^R, F^- *lacΔX74 gal his trp lys rpsL tsx*	Achtman *et al.* (1971); Laboratory Collection
DY330	W3110 *ΔlacU169 gal490 λcI857 Δ(cro-bioA)*	Yu *et al.*, (2000)
$DY330Rif^R$	Rif^R derivative of DY330	Lawley and Taylor
CC118	*araD139 Δ(ara-leu)7697 ΔlacX74 phoA20 galE galK thi rpsE rpoB* $argE_{am}$ *recA1*	Manoil
CC160	*araD139 Δ(ara-leu)7697 ΔlacX74 phoA20 galE galK thi rpsL dam*	Manoil and Bailey (1997)
CC245	*supF supE hsdR galK trpR metB lacY tonA dam::kan*	Manoil and Bailey (1997)
RI89	MC4100 *phoR-Δara714 leu+*	Rietsch *et al.* (1996)
RI90	RI89 *dsbA::kan*	Rietsch *et al.* (1996)
RI179	RI89 *ΔdsbC::cam*	Rietsch *et al.* (1996)
JMR201	*degP* derivative of MC4100	T. Raivio
XK1200	Nal^R, F^- *lacΔU124 Δ(nadA aroG gal attl bio gyrA)*	Moore *et al.* (1981); K. Ippen-Ihler
MV1193	*Δ(lac-proAB) rpsL thi endA sbcB15 hsdR4 Δ(srl-recA) 306::*Tn*10(*tet^R*) F'[traD36 proAB*$^+$ *lacI*^{q} *lacZΔM15]*	Zoller and Smith, 1987. Common lab stock
JE2571-1	Sm^R, Nal^R, *leu thr* Fla^- pil^- λ^+	David Bradley
RD17	$\Delta(pro\text{-}lac)_{XIII}$ λ^- *recA56 rel-1 supE44 thi-1*	Tsai *et al.* (1987)
HB101	NalR, *supE44 hsdS20 recA13 ara-14 proA1 lacY1 galK2 rpsL20 xyl-5 mtl-1*	Boyer and Roulland-Dussoix, 1969; spontaneous NalR in Lessl *et al.* (1993)

Strain	Relevant characteristic	Source
Omp and LPS mutants		
CC102	*galE28* of JC3272	C. Manoil
CS180-2	*thr leuB6 proA argE his thi galK lacY trpE mtl xyl ara-14 rpsL* Su^+	CS180 re-isolated; Austin *et al.* (1990); C. Schnaitman
CC277	Km^R, *ompA902*::Tn*5* of CC102	Manoil and Rosenbusch (1982); C. Manoil
CC650	Sm^R, *ompA886* in MC4100	Manoil (1983); C. Manoil
CC651	Sm^R, *ompA889* of MC4100	C. Manoil
CS1834	*recB21 recC22 sbcB15 sbcC201 argE3 his-4 leuB6 proA2 thr-1 ara-14 galK2 mtl-1 xyl-5 thi-1 rpsL32 supE44 Bx-33* $\Delta(argF\text{-}lac)U169\ \Delta(trpEA)_2$	Pradel and Schnaitman (1991); C. Schnaitman
CS1999	Δ*lac* of CS180 Mu^R	Klena *et al.* (1992); C. Schnaitman
CS2198	Km^R, *rfaJ19*::Tn*lacZ* of CS1999	Pradel *et al.*, (1992); C. Schnaitman
CS2187	Km^R, *rfaG13*::Tn*lacZ* of CS1834	C. Schnaitman
CS2193	Km^R, *rfaP23*::Tn*lacZ* of CS1834	C. Schnaitman
C600	*supE44 hsdR thi-1 thr-1 leuB6 lacY1 tonA21*	Hanahan (1983), J. Tommassen
pop725	*lamB*⁻ derivative of C600	
CE1107	F^- *thr leu thi pyrF thy ilvA his lacY argG tonA tsx rpsL cod dra rtr glpR ompB471*	Lugtenberg *et al.* (1978)
CE1217	*thr*$^+$ *leu*$^+$ *phoR18* derivative of CE1107($PhoE^+$)	Koretland *et al.* (1982); J. Tommassen
CE1238	TC45 resistant *phoE, pro* derivative of CE1237 (no porin)	
CE1239	SDS resistant derivative of CE1238 ($OmpF^+$ $OmpC^+$)	
CE1240	Me1 resistant derivative of CE1239 ($OmpF^+$)	
CE1241	Tula resistant derivative of CE1239 ($OmpC^+$)	

previously described (Anthony *et al.*, 1994). Cold (4°C) minimal saline solution appeared to inhibit the growth of the donor cells after plating, therefore cold LB was used instead. As well, the growth of donor cultures prior to mating required the addition of glucose for reasonably fast growth. Since pKI375 and related plasmids were not stably maintained in the cells, only fresh transformants were used for all mating assays, and glycerol stocks were not used except for *E. coli* ED24 containing pOX38-Tc, pOX38-Km, or F*lacN548*-Tc, and *E. coli* JE2571-1 or XK1200 containing R100-1, and all recipient strains.

2.1.3. Antibiotic Sensitivity. *E. coli* strains carrying various *traN* derivatives were assayed for antibiotic (vancomycin and bacitracin; Sigma Chemical Co.), detergent (SDS (GibcoBRL) and deoxycholate (Sigma Chemical Co.)) sensitivity using an inhibition disc assay (Russel, 1994). For plate assays, 25 mL of LB containing appropriate antibiotics for selection of TraN-expressing plasmids was inoculated with 250 μL of an overnight culture of MC4100 cells containing pOX38*N1*::CAT and TraN-expressing plasmids or vectors. Cells were grown to mid-log phase, mixed with 150 ML of LB agar at 52°C, and poured into a 20 cm X 20 cm dish. The agar was allowed to cool and small Millipore disks (1 cm) were placed in a grid array on the plate (4 X 4). A small volume of antibiotics or detergents was placed on each disc. Final amounts were: 3, 30, and 300 μg of vancomycin and bacitracin, and 0.02, 0.2, and 2 mg of SDS and deoxycholate. Plates were incubated inverted at 37°C for 18 hrs and the diameter of the zone of inhibition was measured for each disc.

2.2 General DNA Manipulations

2.2.1 Cloning. All plasmids used in this study are listed in Table 2.2. General techniques were performed as described in Ausubel *et al.* (1987) unless otherwise indicated. All DNA restriction and modification enzymes were from Roche. Vent polymerase (proofreading$^+$) was from New England Biolabs. Large scale plasmid preparations were done according to Qiagen except that, after resuspension of the DNA pellet in TE buffer, the DNA was subjected to two phenol extractions, and the aqueous phase was collected and ethanol precipitated as normal. Small

Table 2.2. Plasmids and phage strains used in this study.

Plasmid/Phage	Relevant characteristic	Source
Vectors		
pBS KS+/SK+	Amp^R, cloning vector, 3.0 Kb	Stratagene
pK184	Km^R, 2.4 Kb cloning vector, p15a replicon	Jobling and Holmes (1990)
pK194	Km^R, 2.4 Kb cloning vector, p15a replicon	Jobling and Holmes (1990)
pCR4Blunt-TOPO	Amp^R, Km^R, 4 Kb cloning vector with covalently linked topoisomerase at MCS for cloning of PCR products directly	Invitrogen
pDSW209	GFP fusion vector	Weiss *et al.* (1999)
pBAD24	Amp^R, cloning vector for controlled expression from P_{ara}	Guzman *et al.* (1995)
pT7.3	Amp^R, ColE1 replicon	Stratagene
F *traN*		
pKI375	Amp^R, 3.0 Kb *Asp*7001 fragment of F plasmid containing *trbC*, *traN*, *trbE*	Maneewannakul *et al.* (1992a)
pKI375*N1*::CAT	Amp^R, replacement of *Bbr*PI/*Eco*RV fragment of *traN* in pKI375 with CAT cassette	This study
pKI375B$^-$	pKI375, removal of *Bam*HI site for Tn*phoA* mutagenesis	This study
pBK184N	3.0 Kb fragment (*Eco*RI/*Hin*dIII)of pKI375 containing *trbC*, *traN*, and *trbE* in pK184	This study
pS55, pS53, pS84, pS63 pS62, pS83, pS52, pS82	Amp^R, 3' deletions of *traN* in pKI375 see Figure 1	Maneewannakul *et al.* (1992a) K. Ippen-Ihler
pBK2K90T	Amp^R, K90T mutation in putative ATP-binding motif of *traN* in pKI375, *trbC*$^+$, *traN*$^+$, *trbE*$^+$	This study
R100-1 *traN*		
pBK7	Amp^R, 6.2 Kb *Sac*I/*Sal*I fragment of R100-1 in pBS/SK+ containing *trbI, traW, traU, orfF. trbC, traN, trbE,* and *traF*	This study
pBK8	Amp^R, 3.5 Kb *Nsi*I fragment of pBK7 in pBS/SK+, *traN*$^+$, *trbE*$^+$, *traF*	This study
pBK8-2818	Amp^R, 2.8 Kb, 3' deletion of pBK8, *traN*$^+$, *trbE*$^+$	This study

Plasmid/Phage	Relevant characteristic	Source
F *traG*		
pRS1670	Amp^R, 3.0 Kb fragment of F plasmid containing *traG*	Firth and Skurray (1992)
pBK20	Amp^R, 3.0 Kb fragment of pRS1670 in pBS SK+	This study
pBADTraG	Amp^R, 3.0 Kb fragment of F TraG in pBAD24	Manchak
R100 *traG*		
pKAR6	Amp^R, 3.0 Kb fragment of R100 traG in pT7.3	Anthony *et al.* (1999)
F *traT*		
pK194T	Km^R, 1.1 Kb PCR amplified *traT*$^+$ fragment in pK194	Anthony *et al.* (1994)
R100 *traS/traT*		
pBK16	Amp^R, PCR amplified fragment of R100 *traS/traT* in pT7.3	This study
pED208 *traS/traT*		
pBF106	Amp^R, containing pED208 *traS/traT* in pBR322	Finlay and Paranchych (1986)
Miscellaneous		
pUC4C	Amp^R, Cm^R, 3.6 Kb, end-filled *Asu*II fragment of chloramphenicol cassette (pBR327) cloned into *Pst*I digested pUC4K (to remove kanamycin resistance cassette)	Maneewannakul *et al.* (1992a)
Conjugative plasmids		
pOX38	IncFI, Tra$^+$, RepFIA$^+$, f1 *Hin*dIII fragment of F	Guyer *et al.* (1980)
pOX38-Km	Km^R, pOX38 + *Hin*dIII fragment of Tn*5*	Chandler and Galas (1983)
pOX38-Tc	Tc^R, pOX38::miniTn*10*	Anthony *et al.* (1994)
pOX38N1::CAT	Cm^R, *traN1*::CAT of pOX38	This study
pOX38N1::CAT-Km	Cm^R, *traN1*::CAT of pOX38::Km	This study
pOX38N1::CAT-Tc	CmR, *traN1*::CAT of pOX38::Tc	This study
pOX38N317-Km or -Tc	TraNG317 epitope replacement of *N1*::CAT in pOX38-Km or -Tc	This study
pOX38N465-Km or -Tc	TraNK465 epitope replacement of *N1*::CAT in pOX38-Km or -Tc	This study

Plasmid/Phage	Relevant characteristic	Source
Conjugative plasmids		
pOX38-*tra715*	Major P_Y promoter for *tra* genes replace with T7 RNAP promoter	Maneewannakul, K. *et al.* (1992)
F*lacN548*	*traN* amber mutant of F*lac*	Miki *et al.* (1978)
F*N548*::Tc	Tc^R, miniTn10 in *lac*	Anthony *et al.* (1994)
R100-1	IncFII, Tra^+, Cm^R Fa^R Sm^R Sp^R Su^R Tc^R	Lawn *et al.* (1967); Willetts and Maule (1986)
pRS29	Tc^R, 15 Kb partial *Eco*RI fragment of F containing *traN*, cloned into pSC101	Achtman *et al.* (1978c)
pRS29*N548*	*traN* amber mutant of pRS29	K. Ippen-Ihler
F*lac* and pOX38 *tra* mutants		
F*lactraB2*	amber mutation in *traB*	Willetts and Achtman (1972)
F*lactraE18*	amber mutation in *traE*	Willetts and Achtman (1972)
F*lactraK105*	frameshift mutation in *traK*	Willetts and Achtman (1972)
F*lactraF13*	amber mutation in *traF*	Willetts and Achtman (1972)
F*lactraH80*	amber mutation in *traH*	Willetts (1973)
F*lactraV569*	amber mutation in *traV*	Doran *et al.* (1994)
F*lactraU526*	amber mutation in *traU*	Miki *et al.* (1978)
F*lactraW546*	amber mutation in *traW*	Miki *et al.* (1978)
FlactraG101	frameshift mutation in distal end of *traG*	Achtman *et al.* (1972)
F*lactraG106*	frameshift mutation in proximal end of *traG*	Achtman *et al.* (1972)
pOX38*trbI463*	*kan* insertion in *trbI*	Maneewannakul *et al.* (1992b)
pOX38*trbI472*	*kan* insertion in *trbI*	Maneewannakul *et al.* (1992b)
pOX38*trbC460*	*kan* insertion in *trbI*	Maneewannakul (1991)
Phage		
λTn*phoA*/in	Cm^R Km^R	Manoil and Bailey (1997)

scale plasmid preparations were performed as described (Birnboim and Doly, 1979). Qiagen kits were used for DNA preparations of low-copy vectors. Transformation was done by electroporation on a Bio-Rad GenePulser or by formation of $CaCl_2$ competent cells.

2.2.2. DNA Sequencing. Primers used in this study are listed in Table 2.3. Primer synthesis and DNA sequencing were carried out by The Molecular Biology Services Unit at the University of Alberta on an Applied Biosystems DNA/RNA 392 Synthesizer and an Applied Biosystems 373 Sequencer Stretch (or Applied Biosystems 391 or 381A DNA synthesizer (PCR-MATE), and Applied Biosystems 373 DNA Sequencer, STRETCH). The Sequenase sequencing kit was used as well (United States Biochemical).

2.2.3 Site-Directed Mutagenesis. Single-stranded DNA was produced using the method of Vieira and Messing (1987). Site-directed mutagenesis was performed according to Zoller *et al.* (1985) using the two-primer method and primers LFR53 and SRNF. Heteroduplex DNA was then transformed into *E. coli* DH5α, and the colonies were screened by colony hybridization. Colony lifts were done according to Sambrook *et al.* (1989) on nitrocellulose membranes. The DNA was cross-linked to the membrane using a Bio-Rad GS Gene Linker UV Chamber, and $\gamma[^{32}P]$-labeled LFR53 was hybridized in 5X Denhardt's Solution (2.5 X SSC; 0.5% SDS; 90 mM Tris, pH 7.5; 0.9 M NaCl; 6 mM EDTA; 100 μg/mL heat-denatured calf thymus DNA). Membranes were washed in 50 mL of 6X SSC (0.9 M NaCl; 0.09 M sodium citrate; pH 7.0), 0.1% SDS for 15 minute intervals starting at room temperature and by successively raising the temperature by 5^0C each time until potentially positive colonies could be distinguished above the background. The membranes were autoradiographed with Kodak X-Omat AR film at -70^0C using an intensifying screen. DNA was extracted from potentially positive colonies, and the presence of the mutation was checked by automated sequencing.

2.2.4 Construction of pOX38*N1*::CAT. A null mutant of *traN* was constructed by inserting the chloramphenicol resistance cassette (chloramphenicol acetyltransferase; CAT) from pUC4C into

Table 2.3. Primers

Primer	Sequence 5'-3'[a]	Use
F *traN*		
LFR53	ACCGGAACAACCATC	mutagenic primer, K90T mutation in putative ATP site in TraN
LFR57	TTCGAATTCCTTACTGTCCTGCCTG PCR	amplify TraN (*Eco*RI)
LFR58	GCAGAATTCGACAGCAACAGTGATTACC PCR	amplify TraN without signal seq (*Eco*RI)
SRNF	GATCCGCAGGTGGCTCATGATTTAC	amplify entire F *traN* gene
SRNR	GAATTCTTACCGTATCCCACGACC	amplify entire F *traN* gene
R100 *traN*		
LFR55	CACAGGAGAGTATCC	sequencing primer
LFR56	TTCATCAGGTCTTCG	sequencing primer
LFR59	ACTCCGACACTGACGGTCAG	sequencing primer
LFR60	AGTACTGCACACGGACTGCC	sequencing primer
LFR61	CAGACAGGTTCCCTCAGCGG	sequencing primer
BIK28	GCACGTGTGAACGGGTTGAAACTGGTGTTC	PCR of R100 TraN variable region (*Bbr*PI)
BIK29	GATATCCAGACCCCAGCCACCATCCTTAC	PCR of R100 TraN variable region (*Eco*RV)
pED208 *traN*		
LFR165	CGGAGCTCCACACCATTGAG	sequencing primer pBF106
LFR166	GAACGGGCAGCTTTCAGACC	sequencing primer
LFR167	GTGCGTGTGCAGTACTGCTC	sequencing primer
LFR 81	GGAGCTGACTGGGTTGAAGG	from *Sal*I site in pBR322
LFR102	GGATGTTTTTGACTCACAGG	sequencing primer for ColB2 TraN from 3' end across variable region

Primer	Sequence 5'-3'	Use
traG		
LFR140	GCCCAAGCTTTCCAACTCACTACTGATAATTTGTT	pcr primer (*Hin*dIII)
LFR141	CGCGGATCCGCGCTGCTGGTTGTCGATCGTC	PCR primer (*Bam*HI)
BIK1	GCCTCTTCATCTATCAGTCC	sequencing primer
BIK2	TCAGCCTGCTGGTTAATGTC	sequencing primer
BIK3	CCGTCAGGATAGTCTGGACC	sequencing primer
BIK4	CCCTACCCAACATGTTATG	sequencing primer
BIK9	GCGGCACAACAGGAAATGG	sequencing primer
BIK10	GAAATCATCAATCTGTTCCAG	sequencing primer
BIK11	CCAGTGACAGAGTCACTGC	sequencing primer
G22218	TGCTGTTCTGAGTATCTCCG	sequencing primer
G20360	GTAACTATGCGAAACCTGCC	sequencing primer
G19374	AACAACTATCACTTCGGAGG	sequencing primer
BIK25	AGGCCATCAGGTCTTCCAGC	sequencing primer
BIK15	GGACCTGTTATTCAGACAA	sequencing primer
BIK16	TAGGCATAAGTGTATTGGG	
BIK33	GGCCGGATCCGTGAATGAAGTTTATGTGATTG	GFPTraG fusion primer
BIK34	GCGCAAGCTTTTATTCTTTATGCTGGTAACTC	GFPTraG fusion primer
Miscellaneous primers		
AKE3	AATATCGCCCTGAGCA	sequencing primer for Tn*phoA*in inserts

a. Underlined residues indicate restriction enzyme cleavage sites

traN. The cassette was removed by *Bam*HI digestion of pUC4C and run on a 1.5% agarose gel. The fragment was purified by the GlassMAX DNA Isolation Matrix System(Gibco BRL). The ends were filled in with Klenow to blunt the fragment, and the entire cassette (978 bp) was ligated to pKI375 digested with *Eco*RV and *Bbr*PI (which removes 859 bp and creates blunt ends) to generate pKI375*N1*::CAT. In order to generate pOX38*N1*::CAT, a triparental mating was conducted (Moore *et al.*, 1987). Donor cells containing pOX38 (RD17/pOX38) and recipient cells containing pKI375*N1*::CAT (XK100/pKI375*N1*::CAT) were grown to mid-log phase and mixed at equal volumes and incubated for one hour at 37^{0}C to allow plasmid transfer and recombination. Recipient HB101 cells at mid-log phase were added at a 4-fold excess volume and incubated for a further hour at 37^{0}C to allow the newly generated pOX38*N1*::CAT to transfer. Correct recombinants were Nal^R/Cm^R and Amp^S.

2.2.5. Recombination in strain DY330Rif^R. pOX38*traN* derivatives were generated using PCR amplification and recombination of PCR products in strain DY330Rif^R (Yu *et al.*, 2000; Lawley and Taylor, personal communication). Two types of derivatives were made: pOX38*N1*::CAT-Tc or pOX38N1::CAT-Km were initially generated, and then replacements of the CAT cassette in *traN* with DNA amplified from pAKN317 or pAKN465 to form single-copy 31-codon insertion mutants of *traN*. Typically matings were performed at 30°C with pOX38-Tc or pOX38-Km in MC4100 as the donors, and DY330Rif^R as the recipient. Transconjugants were selected on plates containing appropriate antibiotics (Km or Tc) and Rif. PCR products were transformed at concentrations up to 1 mg DNA per transformation. pOX38*N1*::CAT cassette derivatives were selected on plates containing Cm and Rif, and correct insertion was checked by PCR amplification and subsequent restriction enzyme analysis (an *Eco*RI site is present in the CAT cassette). The pOX38*N1*::CAT derivatives were mated out of the DY330Rif^R strain to MC4100 or ED24 and *in trans* complementation with pKI375, demonstrated correct insertion of the CAT cassette into *traN*. Once correct insertion had been established, the pOX38*N1*::CAT derivatives of pOX38-Km and pOX38-Tc were conjugated to DY330Rif^R and selected for on plates containing Cm and Rif. TraN 31-aa insertion mutants (pAKN317 and pAKN465) were amplified by PCR and single-copy

recombinants were generated as above, except that after recombination had taken place, replacing the CAT cassette, the recombinants were mated into MC4100 cells for 1 hour at 30°C. Transconjugants were selected on plates containing Km and Sm or Tc and Sm at 37°C. The transconjugants were checked for mating efficiency, and cells were examined for the presence of the epitope-tagged TraN using anti-31-aa antibodies.

2.2.6. Generation of 3' deletions of pBK8. Deletion of the 3' end of pBK8 was done according to the Erase-a-Base system (Promega Corporation, 1991) using approximately 10 μg of plasmid DNA (pBK8) digested with *Bam*HI and *Sac*I to generate 5' and 3' overhangs suitable for uni-directional nuclease digestion. The first three time points were screened for deletions of the appropriate size by restriction digests using *Eco*RI of small-scale plasmid preparations. The exact endpoint of the deletion was determined by sequencing using Sequenase and the Reverse sequencing primer of plasmid pBS/SK$^+$.

2.3 Transposon Mutagenesis

2.3.1. IS*phoA*/in transposition into TraN. Insertion of IS*phoA*/in into TraN expressing plasmids was done according to Manoil and Bailey (1997). Either pKI375B$^-$ or pBK184N were transformed into *E. coli* CC160. Cells were grown as standing overnight cultures at 37°C. The OD_{600} was determined and 0.2 mL of cell culture was aliquoted into a fresh tube. λTn*phoA*/in was added at a multiplicity of infection of 0.1 to 0.3 phage/cell. $MgSO_4$ was added to a final concentration of 10 mM and maltose was added to a 1% final concentration. The tubes were left standing for 10 minutes at 37°C and then 0.8 mL of LB was added. Tubes were incubated overnight at 30°C with aeration. The entire culture was then plated onto LB plates containing either Amp (100 μg/mL) and Cm (100 μg/mL) for cells containing pKI375B$^-$ or Km (25 μg/mL) and Cm (100 μg/mL) for cells containing pBK184N. After two days growth at 30°C, bacteria from the entire plate were collected and plasmid DNA was isolated as above. CC118 cells were transformed by electroporation with the isolated DNA and plated onto LB agar lacking NaCl but containing 5% sucrose, 5-bromo-4-chloro-3-indolyl phosphate (Boehringer-Mannheim) and either Amp (100

μg/mL) and Cm (40 μg/mL) for pKI375B$^-$ derivatives or Km (25 μg/mL) and Cm (40 μg/mL) for pBK184N derivatives. Blue colonies were screened for correct insertions by either testing for kanamycin sensitivity (pKI375B$^-$) or plasmid isolation and subsequent *Bam*HI digestion (pBK184N). DNA from potential clones was then sequenced with primer AKE3, which sequences across the site of insertion.

2.3.2. IS*phoA*/in insertion into F *traG*. A 3.0 Kb fragment containing F *traG* was subcloned from pRS1670 into pBS SK+ eliminating the *Bam*HI site in the MCS in the process. This construct (pBK20) was used to generate permissive PhoA fusions in TraG using IS*phoA*/in transposition. Experimental procedures were done as above.

2.3.3. Conversion of IS*phoA*/in insertions to in-frame 31-aa insertions. DNA isolated from IS*phoA*/in derivatives of *traN* was treated with *Bam*HI, and the correct DNA fragment (6 Kb for pKI375, 5.3 Kb for pBK184N) was isolated, after electrophoresis, from a 1% agarose gel using the Qiagen Gel-extraction kit. The DNA was ligated with T4 DNA ligase and transformed into DH5α cells. DNA isolated from these clones was screened for the production of a single, linear DNA fragment of the correct size upon *Bam*HI digestion. Insertions in *traG* were treated in a similar manner.

2.4 Protein Analysis

2.4.1. SDS-PAGE and Immunodetection of proteins. Proteins were separated by SDS-PAGE (Moore *et al.*, 1982). For immunoblotting, the gels were transferred to Immobilon-P (Millipore) and blocked in 10% skim milk in 1X TBST. Anti-31-aa antibodies were used at 1/2000 to 1/7500 dilution in 10% skim milk. Anti-OmpA antibodies were used at $1/10^6$ dilution in 5% BSA (Boehringer-Mannheim) to avoid cross-reaction with skim milk. Anti-rabbit IgG HRP conjugate was used at 1/5000 to 1/10 000 dilution for detection of anti-31-codon antibodies, or 1/30 000 dilution for detection of anti-OmpA antibodies. The membranes were washed three times as above, and then chemiluminescent reagent (Western Lighting Chemiluminescence Reagent Plus;

NEN Life Sciences) was added for 2 minutes. Membranes were exposed to X-ray film for one minute (Kodak). The procedure for stripping and reprobing membranes with a different primary antibody was as described (Ausubel *et al.*, 1989). Briefly membranes to be reprobed were washed sequentially in mq H_2O, 0.5 M NaOH, and mq H_2O before being reblocked. All washes were for 10 min.

2.4.2. Trypsin accessibility experiments. Trypsin digestion of osmotically sensitized or whole cells is a modification of the procedures in Matos *et al.* (1996) and Merck *et al.* (1997). Briefly, *E. coli* ED24 cells containing pOX38*N1*::CAT and expressing various 31-aa insertion mutants of TraN were grown to an approximate OD_{600} of 0.5. Cells were pelleted at 6000 X g for 2 min at 4°C and resuspended at an OD_{600} of 0.5 in 0.5 mL: either 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$ (whole cells) or 50 mM Tris-HCl, pH 8.0, 0.5 M sucrose (osmotically-sensitized cells). Cells were incubated on ice for 20 minutes whereupon half the reaction was removed and trypsin (Roche) was added to a final concentration of 25 μg/mL. Cells were incubated on ice for 20 minutes, and then 6 μL of a solution of Complete Protease Inhibitor was added (Roche; 1 pellet/1.5 mL mqH_2O (double-distilled and deionized H_2O)) and the cell suspension was mixed by inversion. Cells were pelleted as above and the supernatant was removed. Cell pellets were resuspended in 20 μL of 1X sample buffer, and 8 μL (equivalent to an OD_{600} of 0.1) was loaded onto a 10% SDS-PAGE.

2.4.3. Antibodies. Antibodies to the C-terminal region of OmpA were a generous gift from Dr. Glen Armstrong. Antibodies to the C-terminal region of TraG were a gift from Dr. Neville Firth (Firth and Skurray, 1992). Initial experiments with 31-aa insertion mutants of TraN were performed with anti-31-aa antibodies from Dr. Colin Manoil. The peptide CS3317 BbNle-GGTEPFPFSIQGDPRSDQET-$CONH_2$ was synthesized by the Alberta Peptide Institute and used to generate anti-31-aa epitope antiserum. This peptide is almost identical to the one described by Manoil and Bailey (1997) except for the glycine linker at the N-terminus, and the blocked C-terminus. Polyclonal antiserum was derived from a rabbit immunized with KLH-CS3317 conjugate.

2.4.4. *In vivo* crosslinking. Crosslinking was performed on pOX38-Km, pOX38*N1*::CAT/ED24 or F*lactra* mutant cells with or without various 31-aa insertion mutants of TraN using the crosslinker BS3 (Bis(Sulfosuccinimidyl)suberate; Sigma). Cells were grown to mid-log phase and immediately chilled on ice. After 10 minutes, a volume of cells corresponding to an OD_{600} of 1.0 was pelleted at 6000 X g for 2 min in a refrigerated microfuge. The cells were washed 2X with 1 mL of ice cold 50 mM HEPES (BDH Inc.) buffer, pH 8.0; 10 mM $MgCl_2$. One hundred µL was then added to 1.5 mL Eppendorf tubes containing various concentrations of BS3 (stock 25 mM in 50 mM HEPES pH 8.0) and the cells incubated at room temperature for 30 min. The reactions were quenched with 6 µL of 1 M Tris-HCl, pH 7.5, for 15 min at room temperature and the cells were pelleted twice at 6000 X g, at 4°C. The supernatant was removed, and the cell pellets were resuspended in 1X sample buffer. Proteins were separated on a 3.5%/7.5% stacking/separating SDS-PAGE, and transferred to Immobilon-P (Millipore) (Kazmierczak *et al.*, 1994). Initial crosslinking experiments were done by incubating the entire membrane after protein transfer in standard dilutions of antibodies (1/5000 dilution for anti-31-aa antibodies, 1/10 000 dilution for anti-rabbit HRP conjugate). In later experiments the membrane was split at the 79 kDa marker, and the top half was probed with anti-31-aa antibodies (1/2000) and anti-rabbit HRP conjugate (1/5000), and the bottom half was probed with anti-31-aa antibodies (1/5000) and anti-rabbit HRP conjugate (1/10 000). The membrane was reassembled prior to exposure to X-ray film.

2.5 Cell Fractionation

2.5.1. Isolation of cell membranes. Cells were grown to an approximate OD_{600} of 0.5 in 200 mL of LB plus appropriate antibiotics. Cells were pelleted at 1465 X g for 10 min at 4°C in a Sorvall GSA rotor. Cells were resuspended in 10 mM Tris-HCl, pH 7.5, 50 µg/mL RNAse A, 100 U DNase I (Roche), and 100 µL of Complete Protease Inhibitor solution. Cells were disrupted by 3 passages through an Aminco French press at 13 000 psi. The unbroken cells were removed by centrifugation at 1475 X g for 15 min in a Sorvall SS34 rotor, and the total membranes were pelleted at 27 000 X g for 45 min.

2.5.2. Density flotation of membranes. Density flotation was done according to Grahn *et al.* (2000). All sucrose solutions were made up (w/v) in 10 mM Tris-HCl, pH 7.5, 5 mM EDTA. Cell membranes isolated as above were resuspended in 2 mL of 55% sucrose. Gradients were prepared in a stepwise fashion with layers of 0.5 mL 60%, 2 mL sample, 3 mL 50%, 3 mL 45%, 2 mL 40%, 1 mL 35%, 0.5 mL 30% sucrose. Gradients were centrifuged in a Beckman SW41 rotor at 210 000 X g for 72 hrs. Fractions of 750 μL were collected from the top. The refractive index, protein content, and the NADH oxidase activity levels of all odd-numbered fractions were measured (Osborn *et al.*, 1972).

2.5.3. Detergent solubilization of TraN. To assess solubility characteristics of TraN, crude membranes were isolated from cells expressing TraNG317 from the plasmid pBKN317. Membranes were resuspended in 100 mM NaCl, 5 mM EDTA, 10mM Tris-HCl, pH 7.5 (resuspension buffer). The resuspension was mixed with an equal volume of detergent in 20% glycerol; 20 mM Tris-HCl, pH 7.5 (detergent buffer). The mixture was incubated at 37°C on a rocking platform for 30 min. Insoluble components were pelleted at 98 600 X g in a TLA-55 rotor centrifuged in a Beckman OptimaMAX ultracentrifuge. To assess solubility, the insoluble components were resuspended in an equal volume of resuspension buffer. Equal volumes from both the pellet and soluble components were analyzed by SDS-PAGE and immunoblotting. Once a detergent was decided upon, the procedure was scaled up for use with a 200 mL cell culture. Membranes were resuspended in 5 mL resuspension buffer, and mixed with 5 mL of the detergent buffer.

2.5.4. Size exclusion chromatography. Membranes were solubilized as above in 1% SDS or 1% n-lauroyl sarcosine. Once the insoluble components had been pelleted, the supernatant was dialyzed against 100 mM NaCl; 10 mM Tris-HCl; 5 mM EDTA; and 0.1% of the detergent (SDS or n-lauroyl sarcosine) overnight. Dialysis was done with a 100-fold volume of dialysis solution as compared to the sample volume, and 4 complete changes of the dialysis solution were made

during the course of dialysis. The dialyzed solution was then concentrated with a Millipore concentration column (Millipore Ultrafree Biomax; 30 000 molecular weight cutoff) to 1.5 ml total volume. The insoluble components were pelleted at 21 000 X g in a Micromax tabletop refrigerated centrifuge. The concentrated solution was loaded onto a Pharmacia HiPrep Sephacryl S200 or S300 column equilibrated in the same buffer. A constant flow rate was achieved with a Pharmacia peristaltic P-1 pump at speed 1. Fractions of approximately 4.5 ml (12 minutes each) were collected for 4 hrs total, and the presence of protein was analyzed by A_{280}. Fractions were analyzed for the presence of TraNG317 after precipitation of total proteins by TCA (500 mL). Precipitated proteins were then resuspended in 1X SDS sample buffer, electrophoresed on a 10% SDS-PAGE, and the presence of TraNG317 was examined by immunoblot. Samples from the Q sepharose column that were to be loaded onto an S300 column were pooled and concentrated using a Millipore concentration column, and the sample was loaded immediately on an S300 column equilibrated in 0.1% SB-12; 100 mM NaCl; 10 mM Tris-HCl, pH 7.5; 5 mM EDTA.

2.5.5. Anion exchange chromatography. To separate TraNG317 on an anion exchange column, membranes were solubilized as above in n-lauroyl sarcosine, but were dialyzed against a buffer containing 0.2% SB-12 instead of 0.1% n-lauroyl sarcosine. The dialyzed solution was not concentrated, and the entire 10 ml was loaded onto a Pharmacia HiLoad Q sepharose 16/10 column. TraNG317 was separated by fast performance liquid chromatography (FPLC). Flow control was achieved with Unicorn 3.2.1 software. The FPLC machine was an AKTA Explorer 100A, and fractions of 1 mL were collected with a FRAC-900 fraction collector. After washing the column with 0.5 volumes of loading buffer, TraNG317 was eluted with a salt gradient from 0.1-2 M NaCl, pH 7.5, over 10 column volumes and 120 fractions of 1 mL were collected. Protein peaks were analyzed by A_{280}, and the presence of TraNG317 examined by immunoblot after TCA precipitation of the total proteins in the fractions.

2.5.6. Velocity sedimentation. Velocity sedimentation of TraNV584 was done according to Kazmierczak *et al.*, (1994). Cells containing pBKN584 were grown to mid-log phase, washed in ice-cold 50 mM HEPES buffer, pH 8.0, and resuspended at 2 X 10^9 cells/mL (1 mL total volume) in ice cold extraction buffer (50 mM HEPES, pH 8.0; 5 mM EDTA; 2% (v/v) Triton X-100; 100 mL of Complete Protease Inhibitor stock solution). The cell mixture was incubated on ice overnight at 4°C, and insoluble material was pelleted at 21 000 X g in a Micromax refrigerated tabletop centrifuge at 4°C. The supernatant (whole cell extraction) was removed and 0.5 mL was loaded onto a 5-20% continuous sucrose gradient. Sucrose solutions were made in 50 mM HEPES, pH 8.0; 100 mM NaCl; 5 mM EDTA; 1% Complete Protease Inhibitor Solution. A 50% sucrose cushion was poured into the bottom of the gradient, and the gradient was formed by using a gradient mixing apparatus. The sample was loaded on top of the gradient and centrifuged at 110 000 X g for 43 hours at 4°C in a Beckman ultracentrifuge (SW41 rotor). Fractions were collected from the bottom of the gradient.

2.6. DNA and Protein Computer Analysis

2.6.1. Multiple sequence alignment of TraN and TraG sequences. Sequences similar to F TraN and TraG were found by using the BLAST algorithm to search the Microbial Finished and Unfinished Genomes Database (http://www.ncbi.nlm.nih.gov/Microb_blast/unfinishedgenome.html). Six TraN homologues come from finished genomes and plasmids: F TraN, P24082; R100 TraN, AAB61941; *Salmonella typhimurium*, NP_490579; R27, NP_058219; *Salmonella enterica*, AAK02035; *Novosphingobium aromaticivorans*, NP_049155; pED208 TraN was sequenced in this lab, AY046069 (Lu *et al.*, unpublished data), while the *Neisseria gonorrhoeae* TraN sequence was a generous gift from Dillard and Seifert (personal communication). Four TraN homologues were found in the incomplete genomes databases of the following organisms: *Acidothiobacillus ferrooxidans* (from TIGR: www.tigr.org), *Klebsiella pneumoniae* from The Genome Sequencing Centre (http://genome.wustl.edu/gsc/Projects/K.pneumoniae/), *Legionella pneumophila* (http://genome3.cpmc.columbia.edu/~legion/index.html), and *Rhodobacter sphaeroides* (Institute

http://www.jgi.doe.gov/JGI_microbial/html/index.html). TraG homologues were found in similar organisms and plasmids and TraG homologues (TraG and Sac4) from *Neisseria gonorrhoeae* are from Dillard and Seifert (2001). All TraN sequences underwent multiple alignment with 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 highlighted using Genedoc (www.psc.edu/biomed/genedoc).

2.6.2. Topological modeling of TraN. Topological modeling was performed by combining two predictive methods: 1) an algorithm for prediction of outer membrane transmembrane segments (Gromiha *et al.*, 1997; see Appendix) and 2) a computer algorithm for prediction of beta-structure (PHD; http://www.embl-heidelberg.de/predictprotein/predictprotein.html). See Chapter 4 and Chapter 8.

2.7. Fluorescence Microscopy. *E. coli* F*lactraG106*/ED24 was transformed with pJY1, pBK20, and pBS SK+, and grown overnight at 37°C on LB agar plates containing Amp (100 mg/mL). Colonies were inoculated into 3 mL of LB broth, and grown standing overnight at 37°C, at which point the cultures were diluted 1/100 into fresh LB and grown at 37°C with aeration until the cells had reached mid-log phase. Expression of GFP-TraG fusion was achieved with 50 μM IPTG for 1 hr. at 37°C with aeration. Cells were pelleted in a microfuge, and washed 1X with 1 mL of MOPS minimal media supplemented with 0.5% glucose and 20 aa mixture (Gordon *et al.*, 1997). Cells were pelleted as above and resuspended in 20 mL of the same media. One ml of the resuspended cells was added to a slab of MOPS + 0.5% glucose/1.5% agarose on a microscopic slide. A coverslip was placed on top of the slide and sealed. GFP was visualized using an UV light source from a Leica DMRE microscope and equipped with an HQF filter and Cooke SensiCam (Gilmour *et al.*, 2001). Images were collected (from 200 ms to 2.5 s) and processed using SENSICONTROL 4.0. Images were cropped in Photoshop 6.0 (Adobe).

Chapter 3

Genetic analysis of the role of TraN in mating pair stabilization during F and R100 mediated conjugation

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3.1. Introduction

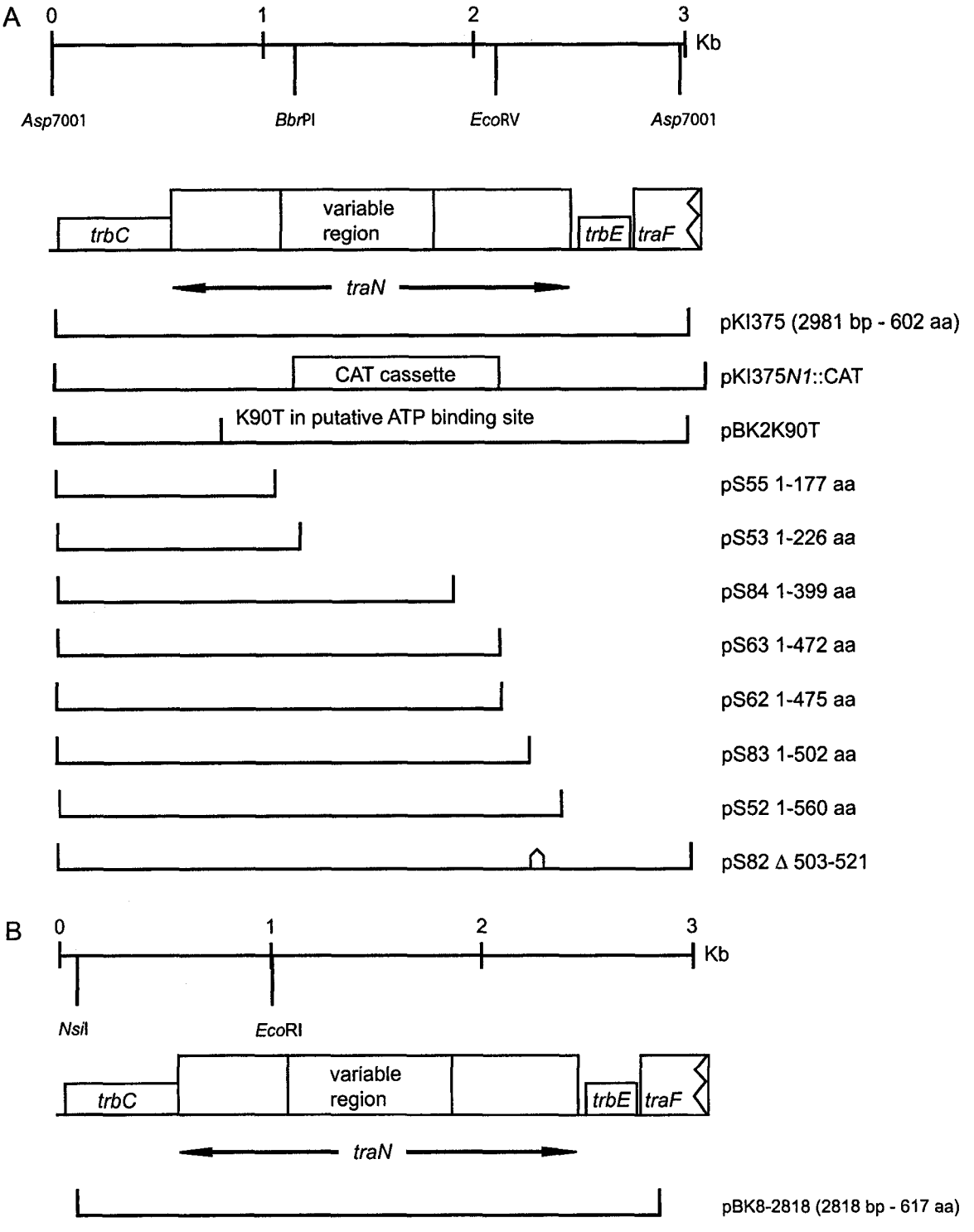
Once a cell containing the F plasmid has expressed a pilus, it is competent for mating. Pilus contact with a recipient cell triggers pilus retraction, bringing the cells into close contact, at which point MPS serves to lock the two cells together to ensure efficient plasmid transfer (Manning *et al.*, 1981). Two proteins mediate MPS, TraN in the outer membrane, and TraG in the inner membrane (Firth and Skurray, 1992; Maneewannakul *et al.*, 1992a). TraN was sensitive to digestion by exogenous proteinase K, and therefore, contains some cell surface exposed regions, potentially regions that could interact with the recipient cell to enable MPS (Maneewannakul *et al.*, 1992a). Mutations in *ompA* in the recipient cell inhibit F plasmid transfer, but do not block R100 plasmid entry (Skurray *et al.*, 1974; Havekes and Hoekstra, 1976). It was originally presumed that these ConF- mutations specifically blocked pilus binding, although purified F pili bound to ConF- cells (Achtman *et al.*, 1978b). The specificity of OmpA interactions is not due to the allele of pilin subunit present in the donor cells, as both F and R100 *traA* could complement pOX38*traA*::CAT at wild-type levels to *ompA::kan* recipient cells (Anthony *et al.*, 1994). Based on these observations, it was suggested that other transfer proteins, such as F TraN, were responsible for interactions with OmpA and LPS in the recipient cell.

3.2. Results

3.2.1. Genetic Analysis of F *traN*. The plasmid F*lactraN548* carrying the amber allele *traN548* was used to originally identifiy the *traN* gene (*Miki et al.*, 1978). When using this plasmid for complementation analysis, a high level of reversion of the amber mutation occurred, resulting in loss of the mutation and affecting complementation levels. A complete knockout of the gene was obtained in this study by inserting a chloramphenicol acetyltransferase (CAT) cassette into the F *traN* gene on pKI375, replacing some of the native sequence (Figure 3.1).

Initially a CAT cassette without a promoter was inserted into *traN* in order to test expression levels of TraN, however, no chloramphenicol resistant colonies were obtained in the tri-parental mating. It was possible that insufficient expression of the *traN* gene resulted in a lack of CAT

Figure 3.1. Physical map of *traN* constructs. A) F plasmid derivatives. B) R100-1 plasmid derivatives. The region corresponding to the F or R100-1 plasmids from which each construct is generated is shown; sizes are marked at the top, restriction enzyme sites are shown underneath. *tra* and *trb* genes are boxed, large and small respectively. The variable region in both F and R100 TraN are shown as well (aa 162-333 of F; aa 162-348 of R100). Each construct is shown approximately to scale, and the expected length of each TraN derivative is shown. The F plasmid deletions and mutants are all derivatives of pKI375, which is a 3 Kb *Asp*7001 fragment of the F plasmid cloned into pBS KS+/*Eco*RV (Maneewannakul *et al.*, 1992a). The R100-1 *traN* clone is a 3' deletion derivative of a 3.5 Kb *Nsi*I fragment of R100-1 cloned into pBS SK+/*Pst*I. pS82 is an in-frame deletion of aa 503-521. The region replaced by the CAT cassette in pKI375 is boxed (859 bp of *traN* replaced with 978 bp of CAT cassette). Transcription proceeds left to right for all derivatives, and left to right for the CAT cassette. The K90T is a lysine to threonine mutation at residue 90 in TraN that is part of a putative ATP-binding site.



expression. A CAT cassette containing a promoter upstream of the CAT gene was obtained from plasmid pUC4C, and inserted into pKI375 digested with *Bbr*PI and *Eco*RV, replacing approximately 850 base pairs (bp) of the *traN* gene with the 980 bp CAT fragment and generating pKI375*N1*::CAT. Restriction enzyme analysis indicated the correct replacement had occurred by using a convenient *Eco*RI site found in the CAT cassette.

A triparental mating was employed to recombine the *N1*::CAT insert into the pOX38 plasmid. pOX38 was mated from RD17 cells to XK100 cells containing pKI375*N1*::CAT. After recombination had occurred, the resultant pOX38*N1*::CAT derivative was mated into HB101 cells and correct transconjugants were selected for on $Cm^R Nal^R$ plates. Complementation with pRS29 (*traN*$^+$) at 11 transconjugants per 100 donor cells indicated that wild-type levels could be restored with a *traN* expressing plasmid, while complementation with pRS29*N548* (*traN* amber mutant *N548*) at 0.018 transconjugants per 100 donor cells indicated that correct disruption of only *traN* had occurred. f1 bacteriophage sensitivity demonstrated normal pilus production from pOX38*N1*::CAT, and that there were no polar effects on downstream genes that are required for pilus synthesis.

3.2.1.1.Cloning and sequencing of R100 *traN*. R100 *traN* was initially cloned as a 6.2 kb *Sac*I/*Sal*I fragment of the R100 plasmid into pBS SK+ to give pBK7. A 3.5 kb *Nsi*I fragment of pBK7 containing *traN*, *trbE* and *traF* was subcloned into pBS SK+ to give pBK8. As pKI375 contains F *trbC*, *traN*, and *trbE*, exonuclease III was used to perform 3' deletions to remove the R100 *traF* gene from pBK8 to construct the plasmid pBK8-2818. R100 *traN* was sequenced after PCR amplification using priers SRNF and SRNR. After PCR amplification, primers were used to sequence in both directions the entire R100 gene.

3.2.1.2. Comparison of F and R100 TraN. A comparison of F and R100 TraN revealed that they are 73.5% identical and 82.3% similar (Figure 3.1). The non-homologous residues are not scattered randomly throughout the protein sequences. F TraN is 602 aa long, while R100 TraN is

617 aa. Schematically, the F and R100 TraN proteins share conserved N- and C-terminal regions, while the middle region is divergent (Figure 3.1). The conserved N-terminal region spans aa 1-163, and the conserved C-terminal region spans aa 333-602 of F. The middle region is highly divergent, and R100 TraN appears to contain an extra 15 aa in this region, from aa 164-332 of F TraN and aa 162-348 of R100 TraN.

3.2.2. Mating to *ompA*⁻ recipient cells. The plasmid pOX38*N1*::CAT was complemented with both F and R100 *traN*, and the mating efficiency to both *ompA*⁺ (MC4100) and *ompA*⁻ (CC277) cells was determined (Table 3.1). The wild-type controls, pOX38-Tc (F) and R100-1 (R100) showed that, as expected, mating efficiency of the pOX38-Tc plasmid decreased when conjugated to a recipient cell containing a kanamycin resistance cassette inserted in *ompA*, while R100-1 was not affected. When complementing pOX38*N1*::CAT with F *traN* (pKI375), a significant decrease in mating occurred with the *ompA*⁻ recipient as with the wild-type recipient. Contrasted with this, complementation of pOX38*N1*::CAT with R100 *traN* (pBK8-2818) showed no decrease in mating efficiency when the recipient cell was *ompA::kan*, similar to the results obtained with R100-1. This result suggests that F TraN in the donor cell is specific for OmpA in the recipient cell during conjugation. R100 TraN is not specific for OmpA, and mates with wild-type efficiency to an *ompA*⁻ recipient cell. Expression from both F and R100 *traN* expressing plasmids was driven by native expression from the vector, and no induction took place.

A number of specific point mutations in *ompA* have been found to inhibit F plasmid entry. The strain CC650 carries one of these point mutants, *ompA886* (Manoil and Rosenbusch, 1982; Manoil, 1983). Complementation of pOX38*N1*::CAT with F (pKI375) and R100 (pBK8-2818) *traN* revealed that the missense mutation in *ompA* inhibits mating with pKI375 but not with pBK8-2818, although the decrease is not to the same level as observed with the *ompA::kan* recipients, the missense mutation in *ompA* inhibits mating efficiency approximately 40-fold (Table 3.2). These data confirm that the specific inhibition exhibited by *ompA::kan* on F plasmid entry is due to the presence of F TraN in the donor cell.

Table 3.1. Complementation of pOX38*N1*::CAT with F and R100 *traN*.

Conjugating plasmid	Relevant genotype	Complementing plasmid	Relevant genotype	Mating efficiency *ompA*[a]	Mating efficiency *ompA*[+b]
pOX38*N1*::CAT	*traN1*::CAT	pBSKS+	vector	0.00038	0.00043
pOX38*N1*::CAT	*traN1*::CAT	pKI375	F *traN*	0.04	160
pOX38-Tc	wild-type	-	-	0.12	67
pOX38*N1*::CAT	*traN1*::CAT	pBK8-2818	R100 *traN*	56	99
R100-1	R100 wild type	-	-	233.3	200[c]

a. The *ompA*⁻ mutant is a null mutant, strain CC277, carrying a kanamycin resistance cassette in the ompA gene and is a derivative of JC3272.
b. The wild-type strain is MC4100. Similar results were obtained when JC3272 was used as a wild-type control.
c. The wild-type strain is CS2198, since R100-1 is Sm^R, MC4100 (Sm^R) cannot be used as a wild-type control. The results of the R100-1 mating assay are from a separate experiment from the other mating assays presented in this table.

Table 3.2. Effect of a missense mutation in *ompA* on F and R100 TraN.

Conjugating plasmid	Relevant genotype	Complementing plasmid	Relevant genotype	Mating efficiency CC650[a]	Mating efficiency MC4100[b]
pOX38*N1*::CAT	*traN1*::CAT	pBSKS+	vector	0.00058	0.00076
pOX38*N1*::CAT	*traN1*::CAT	pKI375[c]	F *traN*	1.9	73.2
pOX38-Tc[d]	F wild-type	-	-	1.1	38.5
pOX38*N1*::CAT	*traN1*::CAT	pBK8-2818[c]	R100 *traN*	66.2	32.2
R100-1[d]	R100 wild type	-	-	102.5	77.7

a. A ConF$^-$ strains carrying missense mutation *ompA886*
b. The wild-type strain is MC4100
c. Results for pKI375 and pBK8-2818 are the average of 3 independent experiments
d. Results for pOX38-Tc and R100-1 are the average of 2 independent experiments

3.2.3. Mutations of putative ATP binding site in TraN. Although TraN has been shown to be an outer membrane protein, it also contains a putative ATP binding site near the N-terminus (84-ATGETGKT-91) which resembles a Walker box (Walker *et al.*, 1982; Maneewannakul *et al.*, 1992b). The sequence of R100 TraN also contains the same motif. These ATP binding sites contain an important lysine residue for catalytic activity, K90 in the F TraN sequence (Story and Steitz, 1992; Logan and Knight, 1993). Using site-directed mutagenesis, the lysine at position 90 was converted to a threonine. When this mutant (pBK2K90T) was tested in a complementation analysis, it exhibited wild-type activity (Table 3.3). Therefore, F TraN does not appear to contain an ATP binding site.

3.2.4. Complementation with F *traN* 3' deletions. The plasmid pKI375 carries a 3.0 kb fragment containing *trbC*, *traN*, and *trbE* in the high copy vector pBS KS+. Maneewannakul *et al.*, (1992b) had found that expression of TraN from this plasmid interfered with signal sequence processing of the periplasmic protein TrbC, while expression from a low copy vector carrying the same insertion resulted in normally processed TrbC. A series of 3' deletions in pKI375 were employed to attempt to map the region of TraN responsible for interference of TrbC signal peptide processing (Figure 3.1; Maneewannakul *et al.*, 1992a). C-terminal deletions that resulted in a TraN of only 340 aa still inhibited TrbC processing, while a deletion that resulted in a TraN of only 236 aa allowed complete TrbC processing, suggesting that residues 236 to 340 of TraN affected TrbC processing.

These 3' deletions of *traN* were used to examine their functionality in complementation of the pOX38*N1*::CAT mutant to both wild-type and *ompA::kan* cells in an attempt to determine the region of TraN specific for OmpA interactions (Table 3.3). The plasmid carrying the smallest deletion, pS52, which removes only the last 40 C-terminal amino acids of TraN resulted in inviable cells when transformed into pOX38*N1*::CAT/ED24 cells and could not be tested. The other 7 deletions complemented at varying levels. Five deletions, pS55, pS53, pS63, pS83, and

Table 3.3. Complementation analysis of F *traN* mutants[a]

Plasmid type	TraN expressed[b]	Mating efficiency *ompA*$^{+c}$	% of wild-type mating efficiency (deletion/pKI375)	Mating efficiency *ompA*$^{-c}$
pKI375	wild-type	160	100	0.04
pS55	aa 1-177	0.0011	0.00052	ND
pS53	aa 1-226	0.0019	0.00089	ND
pS84	aa 1-399	0.83	1.46	0.023
pS63	aa 1-472	<0.0078[d]	<0.012	<0.0052[d]
pS62	aa 1-475	0.16	0.15	<0.00023[d]
pS83	aa 1-502	0.011	0.013	0.008
pS82	Δ 503-521	0.006	0.015	<0.0023[d]
pBK2K90T	K90T	162	90	ND

a. Complementation of pOX38*N1*::CAT
b. See Figure 3.
c. OmpA$^+$ and OmpA$^-$ are as defined in Table 3.1.
d. Represents the fact that no transconjugants were found at the lowest dilution, and the number presented represents the theoretical limit if one colony had been present.

pS82 complemented at 1 log above the vector control (0.0011, 0.0019, <0.0078, 0.011, and 0.006 transconjugants per 100 donor cells respectively). However, the deletions pS84 and pS62 complemented at a much higher level, 0.83 and 0.16 transconjugants per 100 donor cells respectively. When these deletions were tested to see if complementation decreased when mating pOX38*N1*::CAT to a recipient cell containing the *ompA::kan* mutation, all of the C-terminal deletions up to pS84 were affected. pS84 is a deletion resulting in only the first 399 aa of TraN being expressed and mating to *ompA::kan* decreased mating efficiency by approximately 36 fold, suggesting that at least some part of the first 399 aa of TraN is responsible for OmpA specificity.

3.2.5. Receptor for R100 conjugation. ConR100$^-$ recipient cells had been obtained, but the exact nature of the mutations was never determined (Havekes *et al.*, 1976). In an attempt to characterize the exact R100 receptor a number of strains deficient in other *omp* genes were used as recipients (Table 3.4). A specific decrease in R100 mating efficiency would be expected if a recipient strain carried a mutation in an outer membrane receptor for R100 TraN. None of the mutant strains tested showed a specific decrease in R100 mating. pOX38-Tc and R100-1 mated with equal efficiency into all strains, although many of the strains appeared not to be capable of supporting plasmid transfer, since no transconjugants were recovered (strains CE1239, CE1240, CE1241). The exact nature of the R100 TraN receptor is unknown.

3.2.6. Mating to LPS-deficient cells. Mutations in the LPS inner core in recipient cells also have an affect on F plasmid mating efficiency (Anthony *et al.*, 1994). To determine if this was due to the specificity of TraN or not, complementation of pOX38*N1*::CAT with both F and R100 *traN* was used (Table 3.5). As was the case with the *ompA::kan* recipients, LPS-defective recipients serve as poor recipients for F plasmid mating. Complementation of pOX38*N1*::CAT with F and R100 *traN* revealed that this specificity is probably due to F TraN since a 28-fold reduction in mating efficiency was found when complementing pOX38*N1*::CAT with pKI375 when mating to recipient cells containing an *rfaP* mutation (CS2198). This reduction was abolished when either R100 traN

Table 3.4. Conjugation to other *omp* deficient strains[a]

Conjugating plasmid	Mating efficiency pop725[b]	Mating efficiency C600[c]	Mating efficiency CE1217[d]	Mating efficiency CE1238[e]	Mating efficiency CE1239[f]	Mating efficiency CE1240[g]	Mating efficiency CE1241[h]
pOX38-Tc[i]	10.88	19.81	0.6	0.13	-[j]	-	-
R100-1[i]	33.93	10.3	0.13	0.13	-	-	-

a. See Table 2.1.
b. pop725 is a *lamB*⁻ strains
c. C600 is the wild-type version of pop725
d. CE1217 is PhoE⁺, *ompF*⁻, *ompC*⁻
e. CE1238 is *omp*⁻
f. CE1239 is *phoE*⁻, OmpF⁺ OmpC⁺
g. CE1240 is *phoE*⁻, OmpF⁺, *ompC*⁻
h. CE1241 is *phoE*⁻, *ompF*⁻, OmpC⁺
i. Mating efficiency is average of two independent experiments
j. Represents the fact that there were no transconjugants

Table 3.5. Effect of LPS deficient recipients on complementation ability of F and R100 *traN*.

Conjugating plasmid	Relevant genotype	Complementing plasmid	Relevant genotype	Mating efficiency *rfaP*[a]	Mating efficiency *rfaP*[+b]
pOX38*N1*::CAT	*traN1*::CAT	pBSKS+	vector	0.0024	0.00023
pOX38*N1*::CAT	*traN1*::CAT	pKI375	F *traN*	4.68	130.2
pOX38-Tc	F wild-type	-	-	0.8	27.5
pOX38*N1*::CAT	*traN1*::CAT	pBK8-2818	R100 *traN*	44.8	72.8
R100-1	R100 wild type	-	-	188.3	200

a. The *rfaP* mutant is strain CS2193.

b. The wild-type strain is CS2198, which is *rfaJ19*::Tn*lacZ* and was chosen since it is Km^R, but does not affect mating efficiency.

was used in the donor cell, or the recipient cells were wild-type (CS2193). This suggests that LPS may be part of the receptor for F TraN during conjugation

3.2.7. TraT exclusion functions in the recipient cell. TraT is a plasmid-specific entry exclusion protein (Achtman *et al.*, 1977; Harrison *et al.*, 1992). Complementation of pOX38*N1*::CAT was used to determine if this specificity was due to the presence of the *traN* allele present in the donor cell (Table 3.6). Recipient cells expressing either F TraT from the plasmid pK194T or the vector alone (pK194), were used. The wild-type plasmid pOX38-Tc was excluded 77 fold from entering the recipient cells expressing F TraT, while R100-1 was excluded only 1.5 fold. Complementation of pOX38*N1*::CAT with both *traN* alleles revealed that there was no difference in mating efficiency when the recipient cells were expressing F TraT, and both F *traN* and R100 *traN* expressing donor cells were excluded at very similar levels (245 fold for F *traN*, 284 fold for R100 *traN*). This suggests that the specificity exhibited by TraT dependent exclusion is not due to TraN and the nature of TraT dependent allele-specific entry exclusion is not known.

3.2.8.1.Sequencing of pED208 *traN*. pED208 is an F-like plasmid belonging to the IncFV group. Sequence analysis of pED208 TraN revealed that it is similar to both F and R100 TraN with the same basic features.

3.2.8.2.Comparison of other TraN proteins. F-like *tra* genes have been discovered in a number of organisms in the Proteobacter group of gram-negative bacteria. Twelve different traN homologues have been found on both conjugative plasmids, and in pathogenicity islands of a number of virulent organisms. TraN is found on the conjugative plasmids F, R100, pED208, on pSLT from *Salmonella typhimurium* LT2, on pNL1 from *Novosphingobium aromativoricans*, and on R27. TraN homologues are in the chromosomes contained within pathogenicity islands of *Klebsiella pneumoniae*, *Legionella pneumophila*, *Rhodobacter sphaeroides*, *Salmonella enterica*, *Neisseria gonorrhoeae*, and *Acidothiobacillus ferrooxidans*. Four of the sequences are from unfinished genome projects and may not be accurate, including *Klebsiella pneumoniae*,

Table 3.6. Surface exclusion indices of different alleles of *traN*.

Conjugating plasmid	Relevant genotype	Complementing plasmid	Relevant genotype	Mating efficiency *traT*$^{+a}$	Mating efficiency *traT*$^{-b}$	Sfx index^{c}
pOX38*N1*::CAT	*traN1*::CAT	pBSKS+	vector	<0.00025^{d}	0.0024	>9.5
pOX38*N1*::CAT	*traN1*::CAT	pKI375	F *traN*	0.28	61.1	245.1
pOX38-Tc	F wild-type	-	-	0.13	10	76.9
pOX38*N1*::CAT	*traN1*::CAT	pBK8-2818	R100 *traN*	0.037	10.3	284
R100-1	R100 wild type	-	-	60	90.9	1.52

a. Recipient cells were pK194T/JC3272 expressing TraT from the P_{traT} promoter.
b. Recipient cells were pK194/JC3272.
c. Surface exclusion index is mating efficiency to the vector control (pK194) divided by mating efficiency of TraT expressing cells (pK194T).
d. There were no colonies present, therefore 0.00025 is the theoretical upper limit if only one colony existed at the lowest dilution.

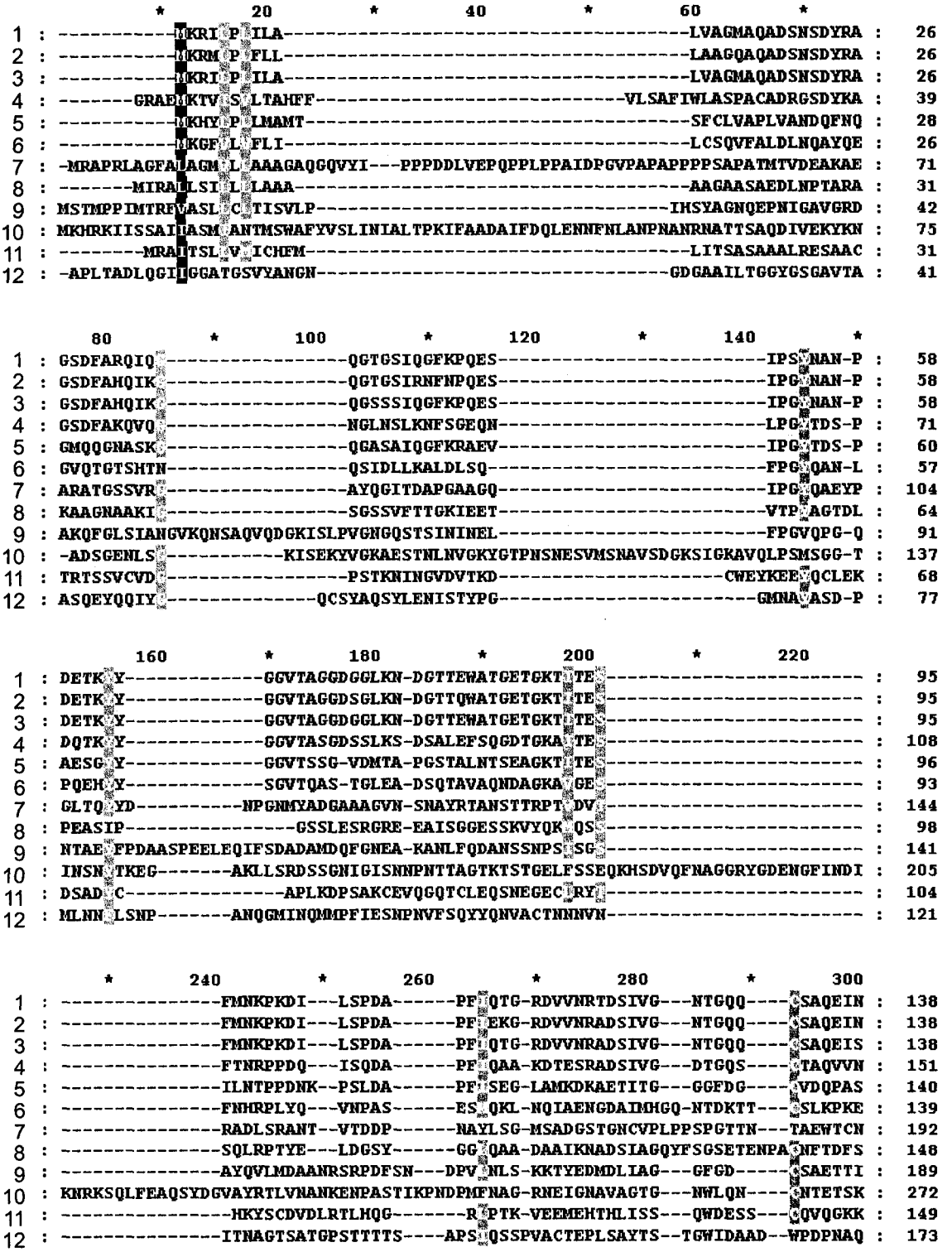
Legionella pneumophila, *Rhodobacter sphaeroides*, and *Acidothiobacillus ferooxidans*. Other TraN homologues may exist, but those identified by BLAST analysis are fragmentary at best. A comparison of the 12 TraN homologues reported here revealed the same basic features of TraN, conserved N- and C-terminal regions and a divergent middle region (Figure 3.2). The extreme C-terminal region of all TraN homologues becomes divergent again (aa 584-602 of F TraN). The TraN proteins vary in size from 559 aa for *Neisseria gonorrhoeae* TraN, to 1058 aa for R27 TrhN. The longer sequences found in some of these proteins appears to map in between more conserved regions found in the TraN homologues, suggesting they are probably in periplasmic or extracellular loops. Topology will be examined in Chapter 4.

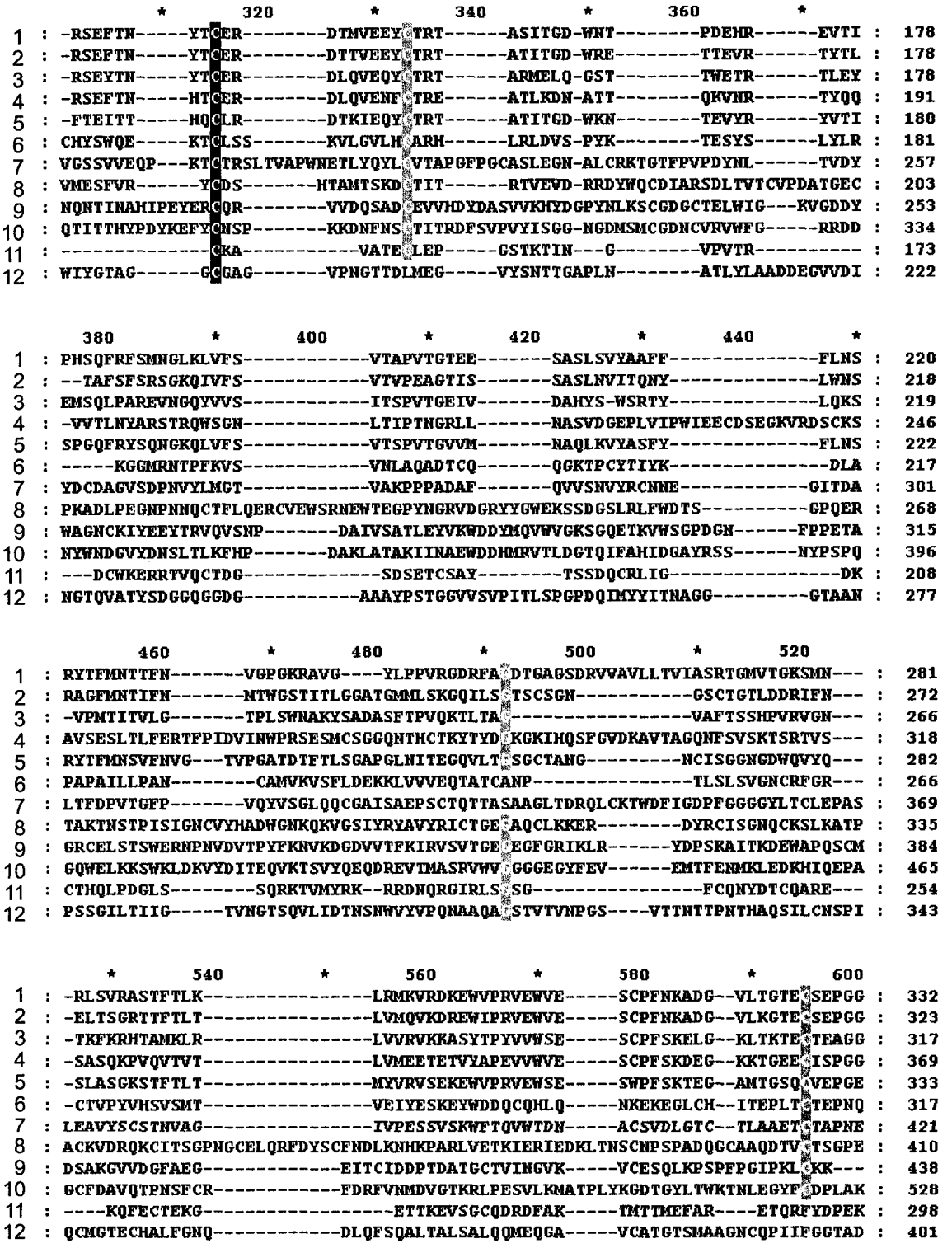
One of the most interesting features of TraN is the presence of a large number of conserved cysteine residues. F TraN contains 22 cysteines, and at least 20 of them appear in greater than 50% of the other TraN homologues. As the periplasm of *E. coli* contains a set of enzymes involved in disulfide bond formation and maintenance (Dsb proteins), TraN likely contains a number of disulfide bridges. This issue will be explored in Chapter 5.

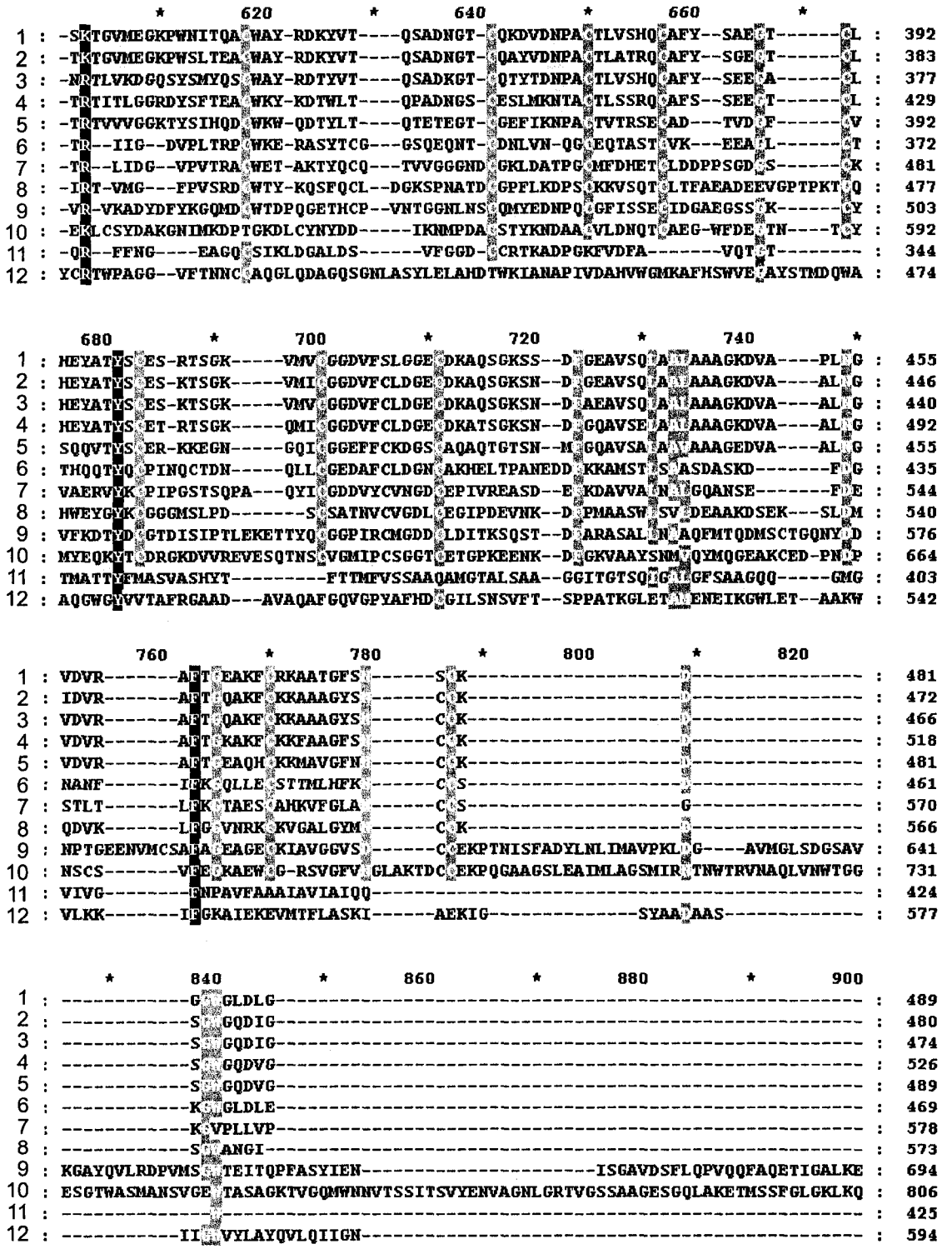
3.3. Discussion

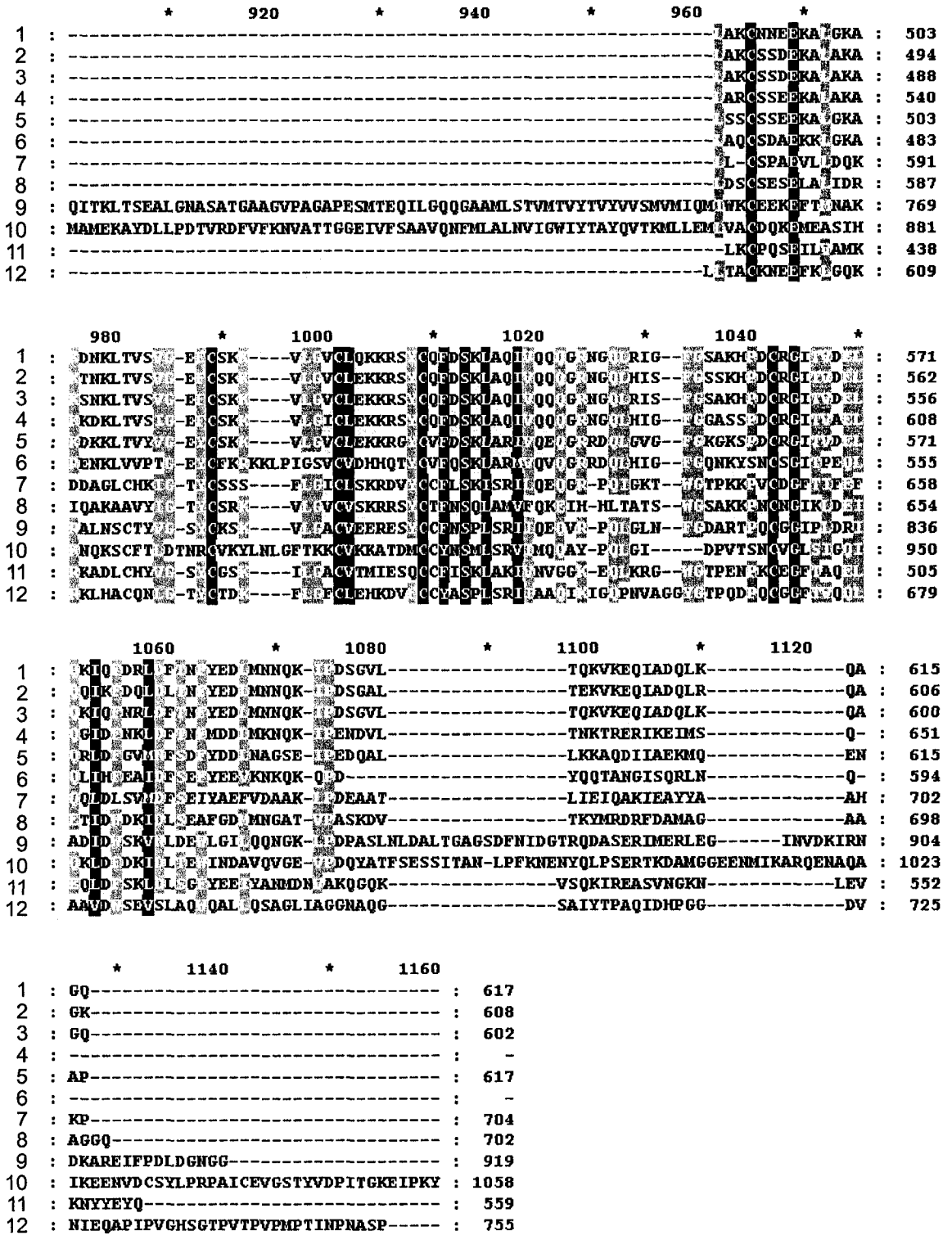
The mating pair stabilization protein TraN, resides in the outer membrane where it interacts with the recipient cell during conjugation to ensure efficient plasmid delivery. The genetic analysis presented here shows that F TraN probably interacts with OmpA in the recipient cell to enable MPS. Both a complete knockout, and a missense mutation in *ompA*, inhibited plasmid entry when donor cells contained F *traN*, but did not inhibit donor cells that contained R100 *traN*. TraN probably interacts directly with OmpA during mating, although this has not been shown. The extracellular segments of TraN are potentially responsible for this interaction. An examination of a series of C-terminal deletions indicated that at least some portion of the first 399 aa of F TraN is responsible for this interaction as cells expressing this TraN derivative (pS84) were still inhibited in promoting plasmid transfer to an *ompA::kan* recipient. Interestingly, aa 162-332 of F TraN is a highly divergent region, that may be responsible for specific receptor interactions.

Figure 3.2. Multiple sequence analysis of all TraN homologues. 1) R100 plasmid TraN, 2) *Salmonella typhimurium* LT2 pSLT plasmid TraN, 3) F plasmid TraN, 4) *Klebsiella pneumoniae* TraN, 5) pED208 plasmid TraN, 6) *Legionella pneumophila* TraN, 7) *Novosphingobium aromativoricans* pNL1 plasmid TraN, 8) *Rhodobacter sphaeroides* TraN, 9) *Salmonella enterica* TraN, 10) R27 plasmid TrhN, 11) *Neisseria gonorrhoeae* TraN, 12) *Acidothiobacillus ferrooxidans* TraN. 4, 6, 8 and 12 are from the Unfinished Microbial Genomes Database. 12 is a gift from Dillard and Seifert. 4, 6, 8, 11, and 12 are found in the chromosome (and not on a conjugative or other plasmid). Residues conserved at 100% are shaded black, 80% shaded dark grey, and 60% shaded light grey (allowing for conservative substitutions).









An attempt to find the specific R100 TraN receptor was not successful. Strains carrying mutations in other major outer membrane proteins did not show a specific decrease in mating efficiency of donors carrying R100 *traN*. Mating efficiency was decreased for both F and R100 plasmids, suggesting that these strains serve as poor recipient regardless of what plasmid is being transferred. The possibilities are that none of the major outer membrane proteins are the specific R100 TraN receptor, or that R100 TraN is capable of interacting with multiple receptor proteins, or that the effect of a ConR100$^-$ phenotype was masked due to the poor receptive nature of these strains.

A decrease in mating efficiency was also observed when donor cells carrying F TraN were mated to recipient cells containing a mutation in the LPS biosynthetic gene, *waaP*. This gene is responsible for two alterations in the LPS inner core, addition of PPEA to HepI, and branching of HepII by HepIII (Figure 1.1). Anthony *et al.*, (1994) had shown a decrease in F-mediated conjugation when PEA was added to the cells prior to mating, suggesting that it is the PPEA addition which specifically affects F plasmid transfer. The OmpA protein was co-crystallized with an intact LPS molecule, which was imaged at atomic resolution (Pautsch and Schultz, 1998). This suggests that the mobility of the LPS molecule is sufficiently hindered in order to be resolved at these resolutions. Mutations that specifically inhibit F plasmid entry map to the 4th extracellular loop of OmpA, a loop that is closest to the bound LPS moiety in the crystal structure (Ried and Henning, 1987). This suggests that the OmpA-LPS interface might serve as the receptor for TraN-mediated MPS. The other possibility is that mutations in LPS affect the folding of OmpA, rendering it slightly defective as a TraN receptor.

The plasmid pKI375 contains the F *trbC*, *traN*, and *trbE* genes, whereas the plasmid pBK8-2818 contains R100 *traN* and *trbE*. TrbE is not likely to be the cause of OmpA specificity as an insertion of a *kan* cassette into *trbE* had no effect on mating efficiency, and TrbE is predicted to be an inner membrane protein. TrbC is not expressed from pBK8-2818.

The knockout of *traN* in plasmid pOX38*N1*::CAT revealed that *traN* is more important for conjugation than previously assumed. The amber mutant F*lactraN548* mates with approximately 100-fold lower efficiency than wild-type, while the pOX38*N1*::CAT mutant mates at 6 logs below wild-type levels. It appears that the amber mutant in F*lactraN548* might be leaky, allowing a small amount of expression to occur, and rescue of the mutant. The MPS functions of both TraN and TraG increase transfer efficiency significantly, suggesting that MPS is a required function for F plasmid transfer, and does not merely serve to increase mating in liquid culture. Mutations in *ompA* can be rescued when mating on a solid surface, but mutations in *traN*, or the distal end of *traG*, are not completely rescued on solid surfaces (Manning *et al.*, 1981).

Two proteins mediate entry and surface exclusion, TraT in the outer membrane, and TraS in the inner membrane. Both are plasmid-specific; F TraT appears to block mating pair stabilization, and this specificity is due to a single amino acid change, while TraS blocks DNA entry via an unknown mechanism. TraT has been suggested to inhibit mating pair stabilization, but this was not due to the allele of pilin present in a donor cell carrying a pOX38*traA*::CAT plasmid (Anthony *et al.*, 1994). Specificity was not due to TraN, as recipient cells expressing F TraT inhibited donor cells regardless of the *traN* allele present. Therefore, TraT specifically interacts with some other transfer protein to inhibit mating. TraT was found to inhibit infection by some OmpA-specific phages, a result that seems to disagree with the results presented here (Riede *et al.*, 1986). As TraT has also been found to mediate serum resistance, possible by inhibiting complement attack complexes from penetrating or assembling correctly at the bacterial outer membrane, it may be that TraT simply serves to inhibit membrane penetration, by either phages, or the pilus (Moll *et al.*, 1980; Ogata *et al.*, 1982; Montenegro *et al.*, 1985).

Chapter 4

Topological analysis of F TraN

4.1. Introduction

Based on the complementation analysis with F and R100 *traN*, F TraN interacts with OmpA during conjugation to form stable mating pairs. F TraN probably interacts with OmpA through extracellular segments exposed at the cell surface, and a highly divergent region in F TraN between aa 162-332 might contribute to some of these extracellular loops. TraN was found to contain at least some extracellular segments as demonstrated by sensitivity to exogenously added proteinase K (Maneewannakul *et al.*, 1992a).

A number of methods have been developed to study the topology of membrane proteins (Stathopoulos, 1999). If the protein is a known receptor for a bacteriophage, then mutational analysis can elucidate phage binding sites in the extracellular loops, a method that was used extensively to map OmpA (Morona *et al.*, 1984; Morona *et al.*, 1985). Monoclonal antibodies have been used to map extracellular loops, and were used to map the cell surface loops of FepA, PhoE, and OmpF (van der Ley *et al.*, 1986; Klebba *et al.*, 1990; Murphy *et al.*, 1990). Short peptides can be inserted into the protein, which either contain antibody binding sites, protease cleavage sites, or chemically sensitive residues. Insertion of the C3 epitope from poliovirus into FhuA was used to determine the topology of that protein, while another study used protease sensitive sites in the same protein (Koebnik and Braun, 1993; Moeck *et al.*, 1994).

F TraN has not been found to be a receptor for any bacteriophage. Random insertions can result in inactivation of the protein. Random C-terminal deletions of F *traN* resulted in 5 out of 7 derivatives effectively inactivated (Table 3.3), so a more selective procedure was used.

Alkaline phosphatase (AP) is a periplasmic enzyme that is often used as a marker for periplasmic sites (Manoil, 1991). In-frame fusions of AP in membrane proteins identify periplasmically localized regions, and several systems use transposon delivery vehicles to randomly insert the AP gene into a sequence of interest. Fusions that generate active AP enzyme are selected on

plates containing 5-bromo-4-chloro-3-indolyl-phosphate. Active AP suggests that the fusion protein is not being misfolded or degraded.

The system employed here is the Tn*phoA*/in system developed by Manoil and Bailey (1997). This system has the added benefit of not only supplying an in-frame PhoA fusion to a gene of interest, but removal of the transposon and subsequent religation leads to a residual in-frame 31-aa epitope remaining in the protein. Antibodies directed against the 31-aa epitope allow analysis of the resultant epitope-tagged derivatives. In addition, the 31 aa epitope has been found to be highly sensitive to trypsin digestion and F TraN had been found not to contain trypsin-sensitive extracellular segments (Maneewannakul *et al.*, 1992a; Lee *et al.*, 1999). Protease accessibility can be used to map potential insertion sites.

4.2. Results

4.2.1. Transposon mutagenesis of pKI375B$^-$. Initially, the plasmid pKI375B$^-$ containing *trbC*, *traN*, and *trbE* was used as a target for transposon mutagenesis (Figure 3.1). Seven independent Tn*phoA*/in inserts were obtained using this plasmid, but only 3 could be converted to their corresponding 31-aa derivatives. Upon *Bam*HI digestion, religation, and transformation, the other 4 inserts apparently resulted in deletions. This suggested that the high-copy nature of the parent vector of pKI375B$^-$, pBluescript KS+, was deleterious to cells when it carried Tn*phoA*/in inserts in *traN*. An additional problem was the number of false positives obtained by Tn*phoA*/in transposition into the β-lactamase gene on the vector.

The 3.0 Kb insert containing *trbC*, *traN*, and *trbE* was subcloned into the low copy vector pK184 which contains a kanamycin resistance gene instead of an ampicillin resistance gene. The resulting derivative pBK184N was used to generate 65 new Tn*phoA*/in inserts (Table 4.1). Forty-five of these were in *traN*, 11 were in *trbC*, 1 was in *trbE*, and 7 used the partial coding sequence that contains the 5' end of *traF* present at the distal end of the 3 Kb insert for expression of stable

Table 4.1. Properties of IS*phoA*/in and 31-aa insertions of *traN*.

Plasmid/ Insertion	Number Of insertions[a]	Converted[b]	Position/ Mutation[c]	Plasmid[d]	Complementation Efficiency[e]	% wild-type[f]
traN derivatives						
pOX38*N1*::CAT	N/A	N/A	Knockout	pOX38*N1*::CAT	0.004	0.04
pOX38N317-Km	N/A	N/A	G317	pOX38N317-Km	5	-
pOX38N456-Km	N/A	N/A	K465	pOX38N465-Km	6	-
pBK184N	N/A	N/A	Wild-type	pBK184N	69.2	100
pKI375B$^-$	N/A	N/A	Wild-type	pKI375B$^-$	10	100
pBAD24N317 +Arabinose	N/A	N/A	G317	pBAD24N317	25	-
pBAD24N317 +Glucose	N/A	N/A	G317	pBAD24N317	60	-
pKI375 Derivatives						
1	1	No	K62	N/A	-	-
2	1	No	A255	N/A	-	-
3	1	Yes	G317	pAKN317	15.3	153
4	1	Yes	R319	pAKN319	22.2	222
5	1	No	Y331	N/A	-	-
6	1	Yes	K465	pAKN465	5	50
7	1	No	A543	N/A	-	-
8	1	No	K544	N/A	-	-
pBK184N Derivatives						
9	1	Yes	I42	pBKN42	35.7	51.6
10	6	No	G66	N/A	-	-
11	1	Yes	T197	pBKN197	5	7.2
12	2	Yes	A255	pBKN255	27.5	39.7
13	3	Yes	Y288	pBKN288	69.2	100
14	1	Yes	Y337	pBKN337	39.1	56.5
15	24	Yes	T361	pBKN361	23.0	33.2
16	1	Yes	G397	pBKN397	15.8	22.8
17	1	Yes	G398	pBKN398	10	14.5

Table 4.1. *cont.*

Plasmid/ Insertion	Number Of insertions[a]	Converted[b]	Position/ Mutation[c]	Plasmid[d]	Complementation Efficiency[e]	% wild-type[f]
18	1	Yes	D442	pBKN442	10	14.5
19	1	Yes	G498	pBKN498	0.05	0.007
20	3	Yes	V584	pBKN584	27.2	39.3
trbC derivatives						
21	4	No	K35	N/A	-	-
22	4	No	T145	N/A	-	-
23	2	No	T153	N/A	-	-
24	1	No	Q172	N/A	-	-
trbE derivatives						
25	1	No	I51	N/A	-	-
traF derivatives						
26	2	No	E48	N/A	-	-
27	5	No	N/A	N/A	-	-

a. Number of independently isolated and sequenced inserts. N/A, not applicable.
b. Only some of the inserts in pKI375 were convertible to 31-aa epitopes, the rest resulted in deletions (upon *Bam*HI digestion, religation and transformation). Only one insert in pBK184N resulted in a deletion. Yes, converted; No, unable to be converted; N/A, not applicable.
c. Position is the amino acid directly preceding the 31-aa epitope.
d. Only those IS*phoA*/in inserts that could be converted to 31-aa derivatives are indicated (along with wild-type counterparts). N/A, not applicable.
e. Plasmids containing 31-aa insertion mutants of *traN* were tranformed into pOX38*N1*::CAT/ED24 and the mating efficiency to MC4100 was determined. Efficiency is defined as transconjugants/100 donor cells. Only wild-type and 31-aa mutants were tested. Inserts in other genes were not converted nor tested.
f. Percent wild-type related derivatives of pBK184N to pBK184N, and derivatives of pKI375B$^-$ to pKI375B$^-$.

PhoA fusions. Many of the fusions were redundant, with the result that only 12 were unique inserts in *traN*, 4 were unique in *trbC*.

When the Tn*phoA*/in inserts in pBK184N were converted to their corresponding 31-aa in-frame epitopes, 11 of the 12 in *traN* could be converted. In contrast, number 10 resulted in a deletion, and a stable 31-aa epitope at that site was never recovered, similar to that observed with the 4 recalcitrant inserts in pKI375. Two inserts were identical, number 2 and number 12. Number 2 is an insert in pKI375B^{-}, and the corresponding 31-aa insert was never recovered. Number 12 is an insert at the identical position in pBK184N, and was easily converted to the corresponding 31-aa epitope. The Tn*phoA*/in inserts in *trbC* and *trbE* were not characterized further.

4.2.2. Generation of single-copy and miscellaneous versions of 31-aa derivatives of TraN. Single-copy versions of the 31-aa derivatives were used to assess expression levels from the pOX38 plasmid. Recombination and replacement of a *traN1*::CAT derivative was used as a selectable system to assess proper generation of the 31-aa derivative. pOX38*N1*::CAT-Km and pOX38*N1*::CAT-Tc were created by using PCR to amplify *traN1*::CAT from pKI375*N1*::CAT. The PCR product was subsequently recombined into pOX38-Km and -Tc plasmids in the highly recombinogenic strain DY330Rif^{R} (Yu *et al.*, 1994; Lawley and Taylor, personal communication). Recombinants were mated out of DY330Rif^{R} into MC4100 and tested for correct insertion of the CAT cassette as was done previously with pOX38*N1*::CAT.

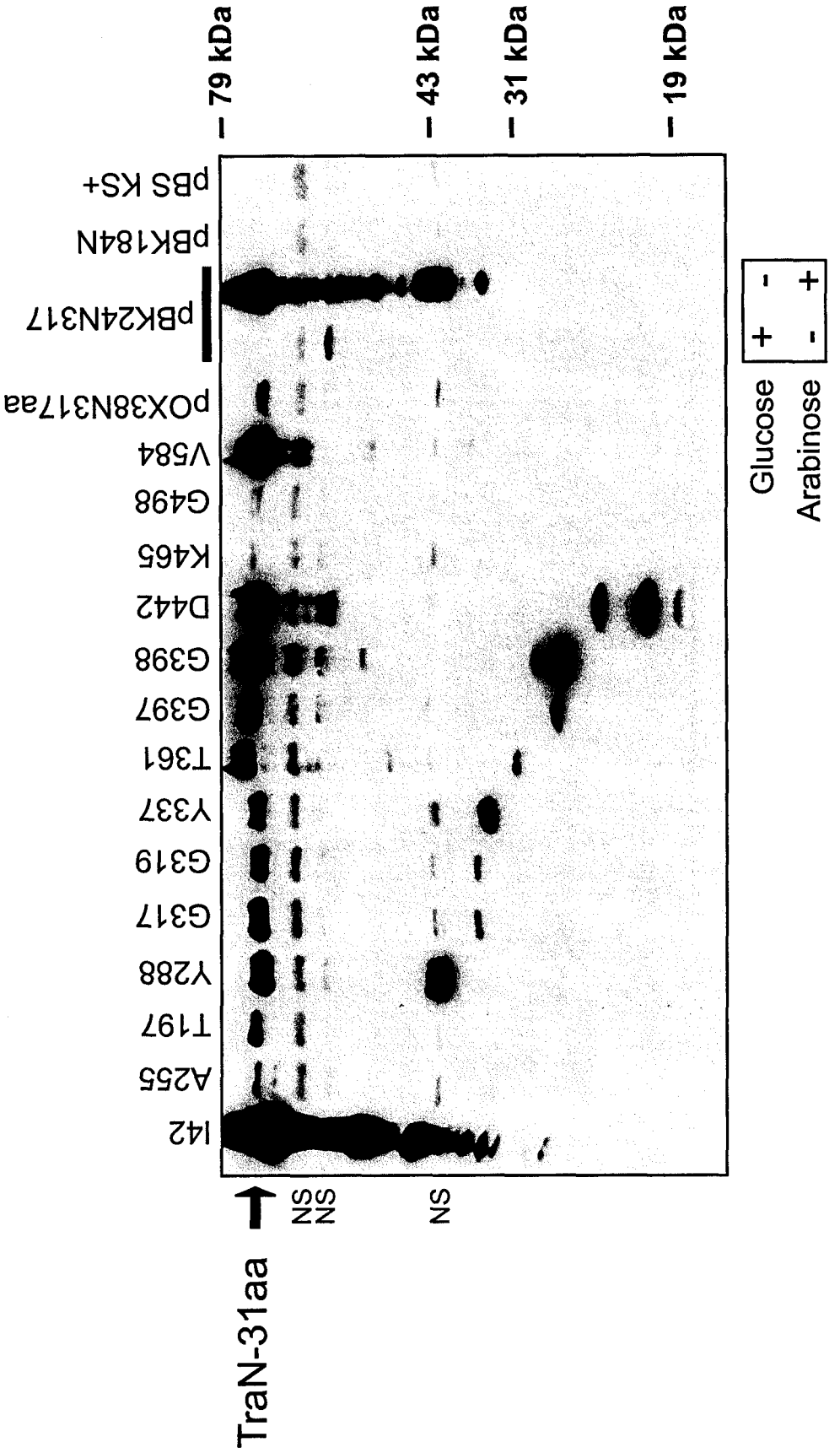
Once correct insertion had been determined, pOX38*N1*::CAT-Km and –Tc were mated into DY330Rif^{R}. PCR was used to amplify DNA containing the 31-aa epitope of pAKN317 and pAKN465 and the resulting DNA fragment was recombined into the pOX38*N1*::CAT derivatives, replacing the CAT cassette with sequence containing the 31-codon insert. Once recombination had taken place, the pOX38 derivatives were mated into MC4100 and tested for mating ability, and the presence of the 31-aa epitope was measured by immunoblot. pOX38N317-Km, pOX38N465-Km, pOX38N317-Tc, and pOX38N465-Tc were generated this way.

In addition, the entire 3 Kb fragment from pAKN317 containing *trbC, traNG317,* and *trbE* was subcloned into the pBAD24 vector. Expression of TraNG317 from the resulting construct, pBK24N317, was controlled by the addition of 0.05% arabinose to the growth medium.

4.2.3. Characterization of 31-aa epitope derivatives of F TraN. The 14 unique 31-aa insertion mutants of F TraN were tested for complementation ability of pOX38N1::CAT/ED24 to MC4100 (Table 4.1). Surprisingly, the only insertion mutant that was defective in complementation was TraNG498. This insertion is in the most highly conserved C-terminal region of TraN, and insertion of the 31-aa epitope probably disrupts function. All other 31-aa insertion mutants, as well as single copy derivatives such as pOX38N317-Km, functioned at wild-type levels.

Just prior to the mating performed above, a sample of cells was taken corresponding to an OD_{600} of 0.1 in order to assess protein levels of the various insertion mutants (Figure 4.1). It is readily apparent that there is a great variation in the levels of TraN31-aa derivatives present in the cells, and that some degradation is occurring. TraNI42 is expressed or stable at the highest level, while TraNK465 and TraNG498 are expressed or stable at very low, yet equivalent levels. The mating efficiency of both mutant derivatives is not dependent on the protein levels however, as TraNK465 supports wild-type levels of transfer while TraNG498 is defective in complementation. The protein levels of TraNG317 expressed from pOX38N317-Km showed the expression levels from within the transfer operon are low as compared to expression from multicopy plasmids. Very little TraN appears to be needed for wild-type complementation, as pBK24N317 grown under repression (1% glucose) showed that no full-length TraNG317 is found under these conditions. A degradation product was present, suggesting a small amount of TraNG317 is expressed and degraded. Once arabinose was added to the medium, the amount of TraNG317 expressed from pBK24N317 increased significantly.

Figure 4.1. Expression of TraN-31-aa epitope-tagged mutants. ED24 cells containing pOX3*8N1*::CAT were transformed with plasmids expressing epitope-tagged TraN mutants (marked above each lane). Cultures were grown to mid-log phase, and cell volumes corresponding to an equivalent number of cells were pelleted. The presence of the 31-aa epitope was determined by immunoblot. Single-copy (pOX38N317-Km), and multicopy (pBK24N317 with 1% glucose or with 0.05% arabinose), expression was also examined. The position of TraN-31-aa is marked with an arrow, and 3 non-specific bands that show up in the negative controls (pBK184N and pBS KS+) are marked with NS. The size of the molecular weight standards (Bio-Rad kaleidoscope markers) is shown on the far right. Presence of arabinose or glucose is marked below. These cells correspond directly with the donor cells in Table 4.1.



There appears to be a unique *in vivo* degradation product for each TraN-31-aa mutant. As the site of 31-aa insertion moved progressively towards the C-terminus of TraN, the *in vivo* degradation product from that mutant became successively smaller. This suggests a correlation between the site of 31-aa insertion, and cleavage that occurs near or at that site. Degradation is discussed below.

4.2.4. Outer membrane localization of TraNG498. The TraNG498 mutant might be defective in outer membrane localization. To assess this, ED24 cells containing pOX38*N1*::CAT were transformed with pBKN498 and pBKN584. Cells were grown to mid-log phase, disrupted by French press, and the inner and outer membranes separated on a density flotation sucrose gradient. Fractions were collected, and every second fraction was analyzed for protein content, sucrose concentration, and NADH oxidase activity, which is an inner membrane enzyme. Antibodies directed against the C-terminal domain of OmpA were used as a marker for outer membrane fractions, and the presence of both OmpA and TraN-31aa were assayed by immunoblot (Figure 4.2). Both TraNG498 and TraNV584 colocalized with OmpA, suggesting that they were in the outer membrane fractions. Thus, the defect in TraN function caused by the insertion at TraNG498 cannot be attributed to a defect in outer membrane localization. A small amount of TraNV584 was found in fractions containing high levels of NADH oxidase activities, suggesting that some of the TraNV584 protein is found in the inner membrane, probably due to the relative abundance of that protein.

4.2.5. *In vivo* degradation is slightly altered in a *degP* mutant. The *in vivo* degradation pattern of two different TraN-31-aa mutants was tested in a *degP* mutant strain. DegP is a periplasmic serine protease that is induced in response to membrane damage (Swamy *et al.*, 1983; Lipinska *et al.*, 1990; Mecsas *et al.*, 1993). Wild-type (MC4100) and *degP* (JMR201) cells containing pOX38*N1*::CAT were transformed with pBKN42 and pBKN288. Cells were grown to mid-log phase and equal cell volumes were pelleted, electrophoresed on SDS-PAGE and the presence of the 31-aa epitope-tagged TraN was examined by immunoblot (Figure 4.3). Degradation still

Figure 4.2. Subcellular localization of TraNG498 and TraNV584 by flotation sucrose density gradient centrifugation. Top, TraN-31-aa derivatives; middle, OmpA; bottom, sucrose content as measured by refractive index (circles), specific activity of NADH oxidase as a percentage of maximum specific activity (squares).

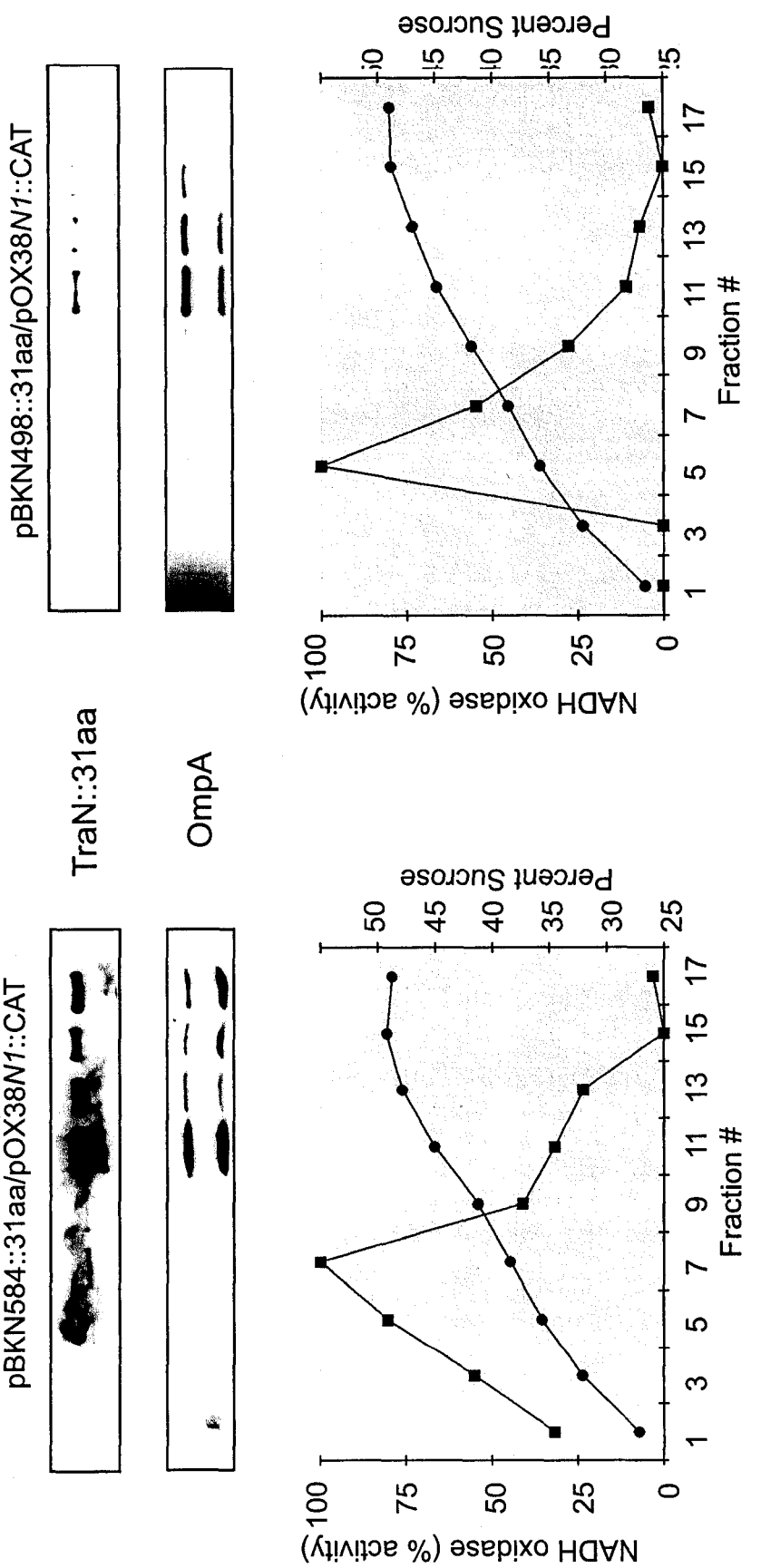
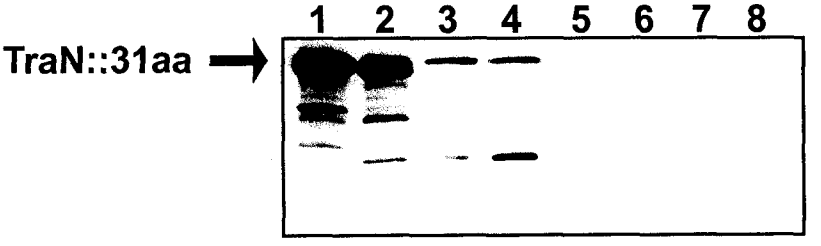


Figure 4.3. *In vivo* degradation of TraN-31-aa is slightly altered in a *degP* mutant. MC4100 (wild-type) and JMR201 (*degP*) cells containing pOX38*N1*::CAT were transformed with pBKN42 and pBKN288 and the presence of TraN-31-aa examined by immunoblot. Lanes 1 and 2, pBKN42; lanes 3 and 4, pBKN288; lanes 5 and 6, pBK184N; lanes 7 and 8, pK184. Lanes 1, 3, 5, and 7 are samples from strain JMR201, and lanes 2, 4, 6, and 8 are from MC4100.



occurred *in vivo* in the *degP* mutant strain, but there was a slight alteration in the degradation pattern. This suggests that DegP might contribute somewhat to TraN-31-aa cleavage, but that it is not the sole determinant of degradation. Other periplasmic or outer membrane proteases might contribute that function.

4.2.6. Topological analysis of 31-aa epitopes of TraN. F TraN is not sensitive to exogenous trypsin when it is expressed in maxicells (Maneewannakul *et al.*, 1992a). The 31-aa epitope is sensitive to trypsin and was used to map insertions in TraD (*Lee et al.*, 1999). The sensitivity of the 31-aa insertion in TraNG317 expressed from pOX38N317-Km was examined initially in whole cells and spheroplasts (Figure 4.4; *Lee et al.*, 1999). The TraNG317 protein was only sensitive to trypsin in spheroplasts and was resistant to trypsin in whole cells. Other TraN-31aa insertion mutants were tested in this manner, but it was found that they were unstable in spheroplasts, and even without exogenous trypsin added, full-length TraN-31aa disappeared.

An alternative method was used to examine trypsin sensitivity by maintaining cell integrity in 10 mM $MgCl_2$, and osmotically sensitizing cells in 0.5 M sucrose (Matos *et al.*, 1996; Merck *et al.*, 1997). Under these conditions, TraN-31aa insertion mutants were intact in osmotically sensitized cells when no exogenous trypsin was added (Figure 4.5). None of the 13 insertion mutants that were tested were digested by exogenous trypsin in whole cells, whereas all were digested in osmotically sensitized cells, suggesting all 31-aa insertions are in periplasmically localized regions of TraN. Cells containing the plasmid pAKN319 did not grow and were not tested. As the R319 insertion site is only 2 amino acid residues away from the G317 insertion site it is likely to be periplasmic as well.

After trypsin cleavage in osmotically sensitized cells, there is a small cleavage product visible on the immunoblot, and the size of this cleavage product did not correspond to the *in vivo* degradation product. This is shown most readily in the TraNG397 insertion mutant that has an *in vivo* degradation product of approximately 30 kDa, but has a trypsin cleavage product of 44 kDa.

Figure 4.4. Trypsin accessibility of TraNG317 expressed from pOX38N317-Km. MC4100 cells containing pOX38N317-Km were grown to mid-log phase. One-half of the cells were treated with lysozyme to form spheroplasts (SP), while the other half was kept intact as whole cells (WC). Cells were treated with 25 μg/ml of trypsin.

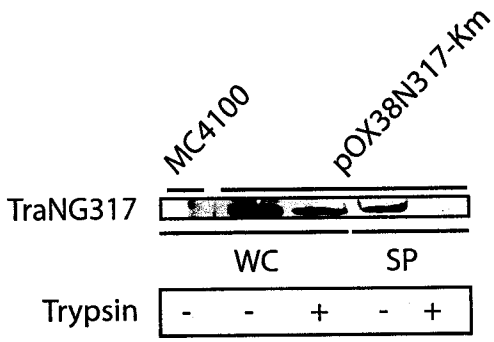
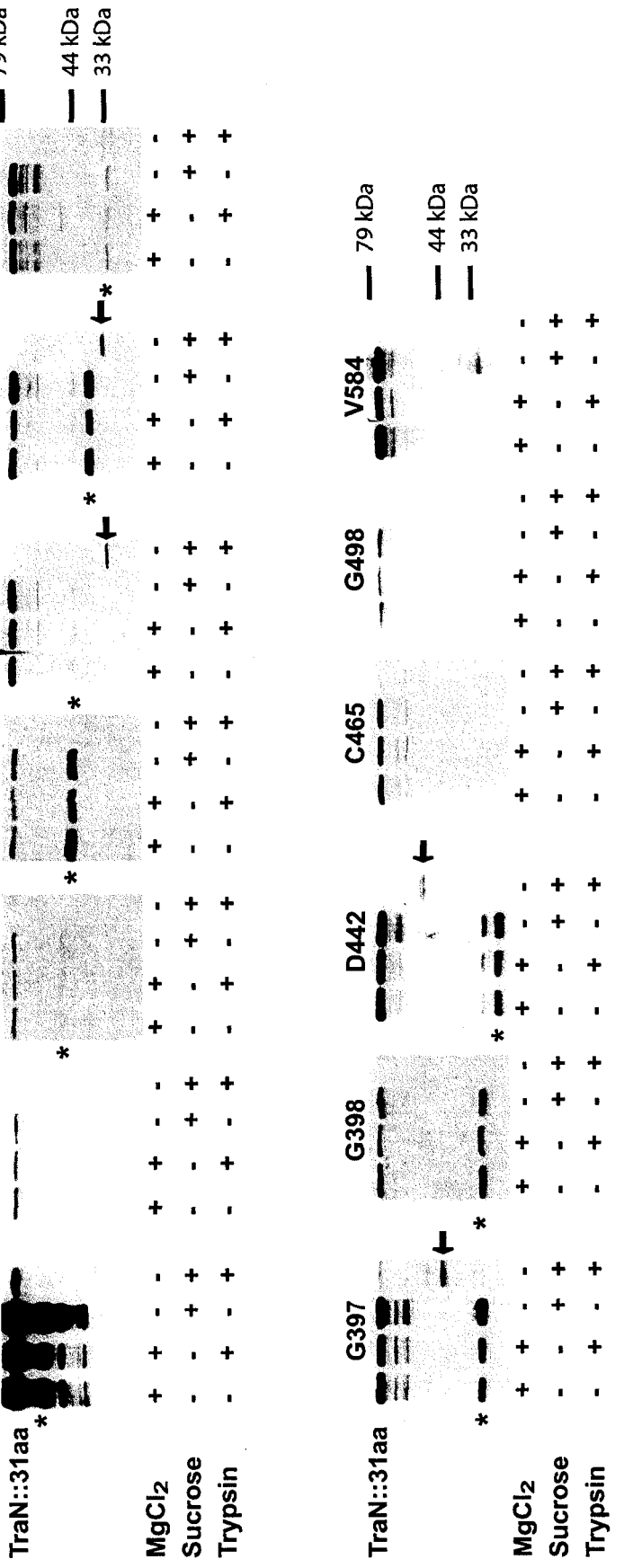


Figure 4.5. Trypsin sensitivity of 31-aa insertion mutants of TraN. Whole cells (10 mM $MgCl_2$) and osmotically sensitized cells (0.5 M sucrose) expressing insertion mutants were treated with trypsin under identical conditions. The band corresponding to the 31-aa epitope-tagged TraN is indicated on the left. Molecular weight markers are shown at the right of each row. Major *in vivo* degradation products (if visible) are marked to the left of each subpanel with an asterisk. The major trypsin cleavage product (if visible) is marked to the right of each subpanel with an arrow.

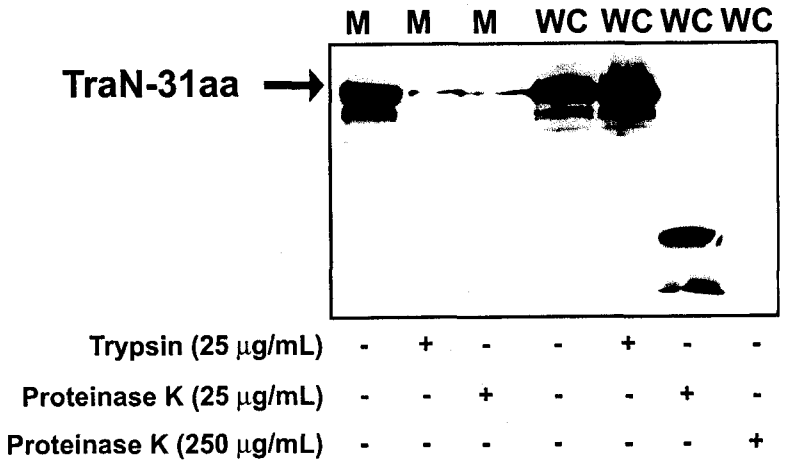


It was expected that the trypsin cleavage product would be identical to the *in vivo* degradation product since the site of trypsin cleavage is thought to be directly within the 31-aa epitope that contains a number of positively charged residues. This data suggested that wild-type TraN was sensitive to trypsin cleavage at periplasmic regions.

In order to test this assumption, wild-type TraN was labeled in an *in vivo* expression system. XK100 cells containing pKI375/pOX38*N1*::CAT or pOX38-*tra715* were grown to mid-log phase, washed and resuspended in minimal media. T7 RNA polymerase (RNAP) production was induced with IPTG. XK100 contains the T7 RNAP gene, and the T7 RNAP enzyme is insensitive to rifampicin. Efficient and specific labeling of TraN with radiolabeled methionine in the presence of rifampicin was achieved. The cells were then tested as above for trypsin sensitivity and after SDS-PAGE, wild-type TraN was visualized by exposure to X-ray film. As expected, wild-type TraN was sensitive to trypsin in osmotically sensitized cells suggesting that the protein contains periplasmic sites that are sensitive to trypsin cleavage (data not shown).

The sensitivity of TraN to exogenous proteinase K was also tested. Maneewannakul *et al.*, (1992a) had used the T7 RNAP expression system described above to label F TraN in maxicells and test the sensitivity of the protein to trypsin and proteinase K. Trypsin did not cleave wild-type TraN, whereas proteinase K digested TraN after 18 hrs with 250 μg/ml of proteinase K. Here, ED24 cells containing pOX38*N1*::CAT were transformed with the plasmid pBKN584. The cells were grown to mid-log phase, and either crude membranes were prepared, or the cells were treated as above to maintain whole cells. Membrane preparations were resuspended in the same buffer as the whole cells then trypsin or proteinase K were added (Figure 4.6). As demonstrated above, TraNV584 was not digested in whole cells by exogenous trypsin, while in the presence of proteinase K, full-length TraNV584 disappeared. With 25 μg/ml of proteinase K, two cleavage products were visible, whereas when 250 mg/ml of proteinase K was added, no immunoreactive TraNV584 products were visible. Crude cell membranes that were treated with trypsin showed digestion of TraNV584, indicating trypsin can cleave in the buffer used.

Figure 4.6. TraNV584 is digested by exogenous proteinase K. ED24 Cells containing pBKN584/pOX38*N1*::CAT were grown to mid-log phase and the cell culture was split into two parts. Crude membrane preparations were derived from one part, while the other part was left intact. Crude membranes were resuspended in the same buffer as the whole cell buffer. Preparations were either left untreated, or treated with 25 μg/ml of trypsin, or treated with 25 or 250 μg/ml of proteinase K. M, membrane preps; WC, whole cells.



4.2.7. Prediction of TraN topology. A number of predictive methods have been used to look at the topology of outer membrane proteins. Many of these methods are confounded by the fact that, unlike in inner membrane proteins, the transmembrane (TM) segments of outer membrane proteins are beta-strands, and are not as easily predicted. Although the externally facing residue at every second position is typically hydrophobic, this is not always the case.

A modified hydrophobicity scale that took into account the propensity for aromatic residues to girdle an outer membrane beta-barrel, as well as the alternating hydrophobic and hydrophilic residues in the beta-strands, was used to incorrectly predict the maltoporin as a 16-stranded beta-barrel, based on the structures of porins that were known at that time (Schirmer and Cowan, 1993). The crystal structure of LamB showed that it was an 18-stranded beta-barrel.

Another method used a more complex set of rules to predict the TM segments of several porins with up to 82% accuracy (Gromiha *et al.*, 1997). Their system examines every amino acid residue with 5 different rules: 1) conformational parameters of the residue, 2) conformational parameters of a 6 residue window, 3) hydrophobicity of a residue, 4), average hydrophobicity of a 6 residue window, and 5) amphipathicity of a 2 residue window. Conformational parameters were the propensity of each amino acid residue to occur in a TM segment of a protein with known structure. Each residue is tested against these 5 parameters, and a binary output of 0 or 1 is then assigned. A 0 for every rule indicates that the residue is unlikely to participate in a TM segment, whereas a 1 indicates it is likely. The 5 rules are combined to give probabilities ranging from 0-5 for every residue. Contiguous segments with high probability for participation in TM segments are scored high, and must be at least 6 residue in length in order to span the bacterial outer membrane. Only when these rules are met is a TM beta-strand predicted to occur (see Appendix).

F TraN had 15.1% beta-sheet and 36.9% alpha-helix as predicted by the consensus method of Biotools Peptool. TraN has 25.4% alpha-helical content, and 24.1% beta-sheet content as predicted by the PHD algorithm. F TraN was analyzed by the algorithm of Gromiha *et al.*, (1997), which was used to predict outer membrane transmembrane segments, and also analyzed for beta-strand prediction by the PHD program, which had been trained on soluble proteins but was used to predict the transmembrane segments of some outer membrane proteins (Rost and Sander, 1993; Paquet *et al.*, 2000; Figure 4.7). As expected, very little of the TraN protein contains predicted TM segments or beta-sheet by either the Gromiha or PHD algorithms respectively. Many of the TM segments are very short, containing only 6 residues.

If the predicted topological model was accepted as is, then it would place several of the 31-aa insertions outside of the cell, and based on the data presented above none of those 31-aa insertions are extracellularly exposed. The topological data obtained with trypsin accessibility of the 31-aa insertions has been combined with the structure prediction by the two algorithms above to form a topological model of TraN in the OM (Figure 4.8). TM segments that contained an insertion site in the middle of the membrane-spanning region were ignored unless the TM segments could be extended towards the N- or C-terminus by at least 6 residues. An even number of TM segments would be expected between two 31-aa insertion sites if any TM segments were predicted to occur between those sites. The final predicted topological map of TraN indicates that it contains 22 TM segments. The average length of the TM segments is 7.6 residues, well within the average range of 7-9 residues for most OMPs (Koebnik *et al.*, 2000). If the TM segments are the only beta-structures in the protein, then the combined TM segments would make up 27.9% of the protein, close to the 24.1% beta-structure predicted by the PHD program. The largest extracellular loop is the first loop between aa 68-128, with the remaining extracellular loops of a very short nature. This implies that the highly divergent region (aa 162-332) of TraN is found mostly in the periplasm, where it may interact with other transfer proteins. The site for OmpA interactions is predicted to be within the small extracellular loops. The periplasmic loops are generally larger than the extracellular loops, with the largest spanning

Figure 4.7. Prediction of transmembrane segments in F TraN. The transmembrane segments (shaded boxes) of F TraN were predicted using the algorithm of Gromiha *et al.*, (1997). Beta-strands (unshaded boxes) were predicted using the PHD program (www.embl-heidelberg.de/predictprotein/predictprotein.html). The 31-aa insertion sites are marked with an arrow and the deletion sites are marked with a straight vertical line.

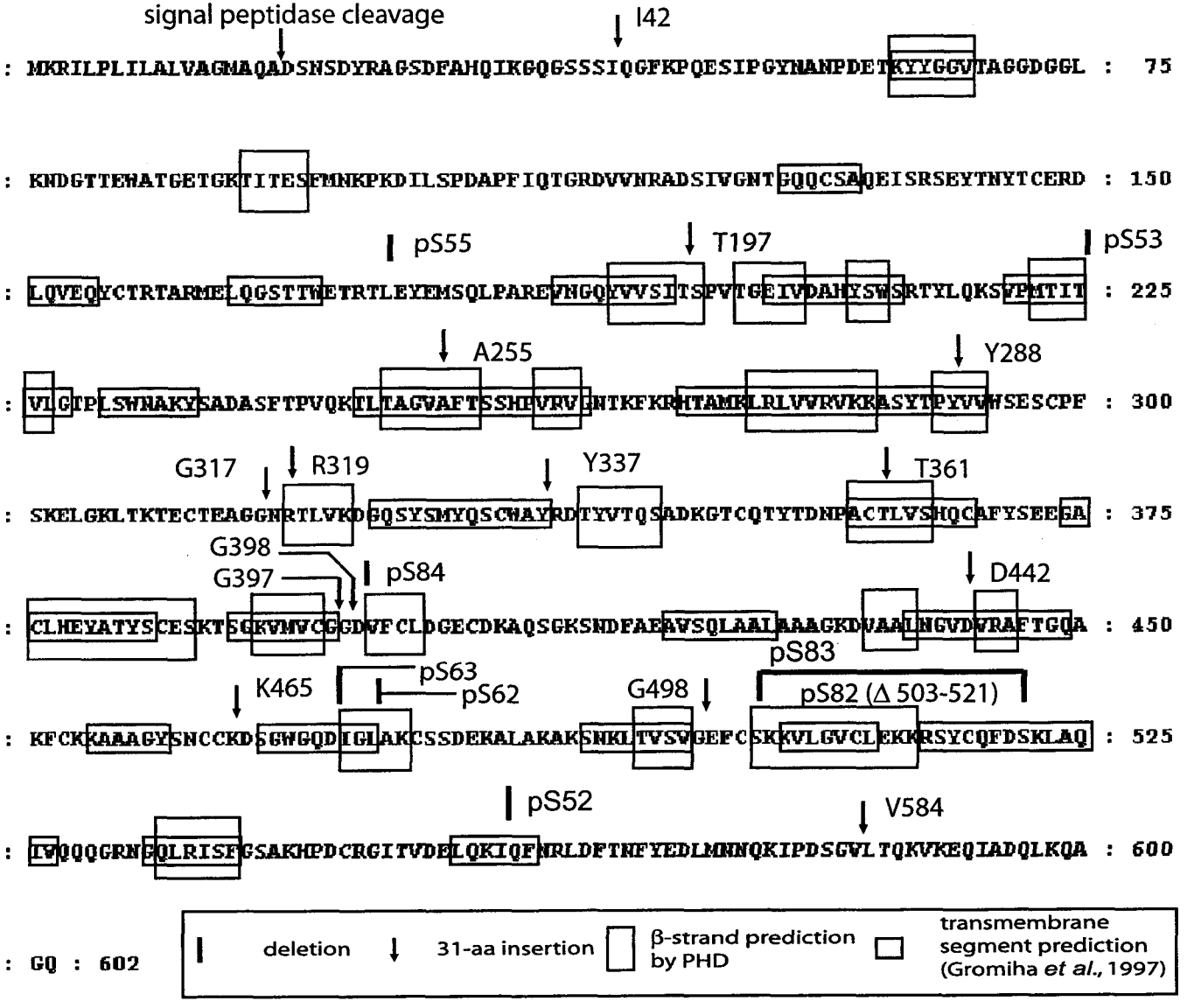
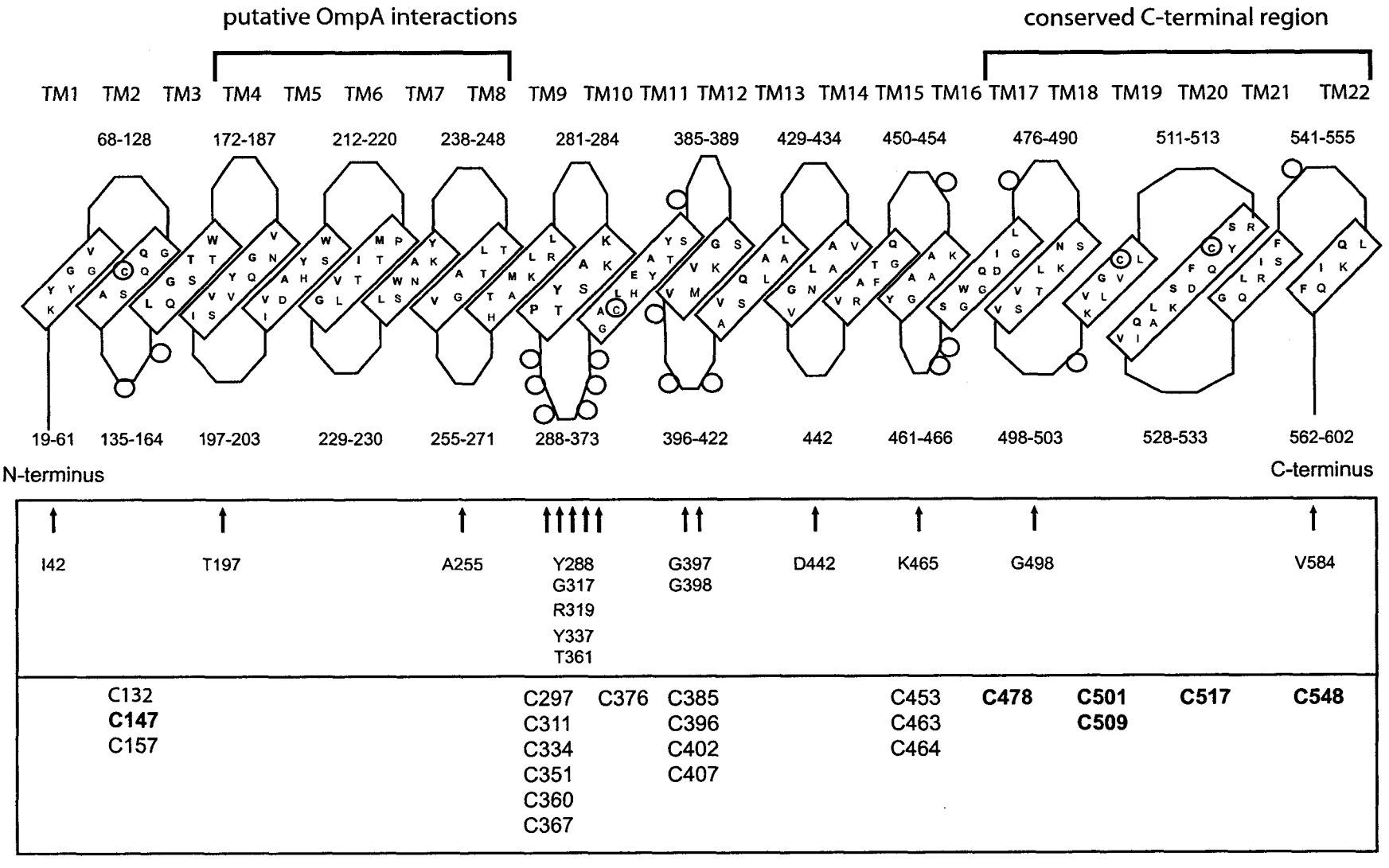


Figure 4.8. Topological model of F TraN. The predicted transmembrane segments and beta-strands were combined with the topological data from the 31-aa insertion mutants to generate the topological map of TraN. Transmembrane segments are boxed, with amino acids predicted to face the hydrophobic external environment in bold type and on the left, and interior-facing residues in normal type and on the right. The 22 TM segments are indicated at the top. Extracellular and periplasmic loops are marked with the range of amino acids, and each 31-aa insertion site is marked below the periplasmic loops. The size of the loops does not correspond with their actual size. The external residues predicted to interact with OmpA, and the conserved C-terminal region are marked. The N-terminus of the mature protein as well as the C-terminus are marked as well. The sites of 31-aa insertions are marked with an arrow below the figure. Conserved cysteines are indicated with a circle, and are marked below, with the 6 absolutely conserved cysteines are marked in bold.



residues 287-375, which contains 5 31-aa insertions. The shortest loop is comprised of a single aa, D442.

4.3. Discussion

The topology of F TraN in the outer membrane was examined using a transposon delivery system to generate in-frame alkaline phosphatase fusions (Manoil and Bailey, 1997). Initial transposon mutagenesis using pKI375 suggested that the fusions were unstable, thus a low-copy derivative, pBK184N was used. Eight unique AP fusions were generated in pKI375, and 12 were generated in pBK184N. Three of the AP fusions in pKI375 were sufficiently stable to be converted to 31-aa insertions, whereas 11 of the 12 in pBK184N were converted to their corresponding epitope insertions.

Thirteen of the 14 insertions supported wild-type levels of transfer when complementing pOX38*N1*::CAT transfer. The only insertion that did not, TraNG498, mapped to a highly conserved region of TraN, suggesting that this region is very important for TraN function. Two of the C-terminal deletions, pS82 and pS83, map to this region as well (Figure 3.1). The pS82 deletion is an in-frame removal of aa 503-521, resulting in a defective protein (Table 3.3). Insertion of the 31-aa at this site either disrupts the function or the structure of TraN. Protein levels of TraNG498 were within the range of other insertion mutants that exhibited wild-type function and localized correctly to the outer membrane fractions.

Apparently very little TraN is required to function in a wild-type capacity as evidenced by the expression of TraNG317 from the pBK24N317 plasmid. This agrees with the result obtained when a promoterless CAT cassette was inserted in the *traN* gene on pOX38, and no chloramphenicol resistant colonies were found (Chapter 3). Expression levels of TraN from pOX38-*tra715* had been found presumably to be low (Maneewannakul K., 1992c). Expression from pOX38N317-Km was lower when compared to many of the insertion mutants expressed from multicopy vectors.

Wild-type TraN is insensitive to trypsin cleavage in whole cells, allowing the trypsin sensitivity of the 31-aa insertion site to be tested. All of the 31-aa insertion mutants tested showed sensitivity to trypsin in osmotically sensitized cells, but not in whole cells, suggesting that all insertion sites map to periplasmically localized regions. This suggests two possibilities, either very little of TraN is exposed extracellularly, or that active PhoA fusions cannot be made to extracellular loops. The two possibilities are not mutually exclusive. Transposon mutagenesis of the FepA protein produces no active PhoA fusions to outer membrane segments (Murphy and Klebba, 1989). A hybrid outer membrane protein consisting of the N-terminal region of lipoprotein fused to OmpA was degraded and unstable when alkaline phosphatase was fused to an extracellular loop of OmpA (Stathopolous *et al.*, 1996). This suggests that PhoA cannot be extracellular and stable at the same time. Only active PhoA fusions were selected in the experiments presented here.

The topology of F TraN was predicted by combining a predictive algorithm for TM segments with that for predicted beta-strands by the PHD program (Rost and Sander, 1993; Gromiha *et al.*, 1997; Paquet *et al.*, 2000). The predicted TM segments were combined with the known periplasmic 31-aa insertion sites to generate a topological model of F TraN in the outer membrane. The topological model suggests that most of the protein is exposed periplasmically and contains very few extracellularly exposed segments. The majority of the divergent region is then predicted to be periplasmically localized, suggesting that the region for OmpA interactions is contained within the small extracellular segments. The divergent segments in the periplasm might be important for interactions with other transfer proteins.

The algorithm used was only 82% accurate when predicting TM segments of porins, and was less accurate when used to predict the TM segments of TolC (Gromiha *et al.*, 1997; data not shown). Unfortunately the 22 cysteine residues in F TraN also presents a problem, since based on the data set of conformational parameters which were generated from known outer membrane structures, cysteines have unassigned values because no cysteines were found in their data set.

It is not known if cysteine residues would participate in TM segments, and accordingly, very few of the predicted TM segments have cysteine residues. TM2, TM11, TM19, and TM20 contain 1 cysteine residue each, with the one in TM11 predicted to face the interior of the protein, and the other three predicted to face the external environment. Perhaps the two externally facing cysteines in adjacent TM strands 19 and 20 form a disulfide bridge. Cysteine residues will be explored in Chapter 5.

The predicted topology does explain some of the results obtained with the deletion mutants presented in Chapter 3. pS84 is the largest deletion that still functions at some capacity. This deletion expresses TraN from aa 1-399, very close to the TraNG397, and TraNG398 insertion mutants, suggesting it is in a periplasmic region of TraN and is therefore stable, resulting in a TraN derivative that contains an even number of TM segments. The deletion mutants pS62 and pS63 express TraN proteins of 475 and 472 aa residues respectively, yet exhibit almost two logs difference in mating efficiency. The deletion pS62 exhibits much higher levels of function, while pS63 is quite deficient. The topological model indicates that the stable deletion pS62 occurs at the end of a TM segment, while the deletion pS63 is in the middle of the TM segment. This probably results in a severe disruption in TraN folding and insertion into the outer membrane, probably contributing to the lack of function. The deletions pS82 and pS83 are also predicted to disrupt TM segments, and the deletion pS52, which only deletes the last 42 aa of TraN, may also occur in the middle of a TM segment. pS52 could not be transformed into cells, suggesting a severe perturbation of TraN function, resulting in cell inviability.

Chapter 5

Stability and multimerization of TraN

5.1. Introduction.

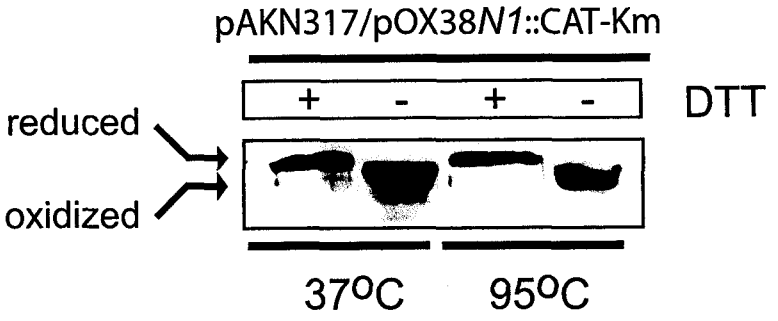
F TraN has 22 cysteines, of which 20 are conserved in at least 50% of the other TraN homologues (Figure 3.2). Due to the highly oxidizing environment of the periplasm, cysteine residues are often involved in intramolecular disulfide bridges (Bardwell, 1994). Disulfide bond formation in the gram-negative periplasm is facilitated by the Dsb enzymes (disulfide bond formation). The key enzyme involved in disulfide bond formation is DsbA, which catalyzes the formation of intramolecular disulfide bridges in periplasmic proteins (Bardwell *et al.*, 1991). DsbA is a disulfide oxidoreductase, and contains a CXXC motif that can form a disulfide bridge itself. DsbA effectively donates its disulfide bond to another protein, resulting in the reduction of the intramolecular bond in DsbA and oxidation of the target protein. DsbA is then recycled to the active form by the DsbB protein. Another Dsb enzyme, DsbC, is involved in the isomerization of incorrectly formed disulfide bridges, and is in turn recycled to the active form by the DsbD protein (Missiakis *et al.*, 1994; Shevchik *et al.*, 1994).

The Dsb system was originally discovered when selection for slow folding of a β-galactosidase-MalF fusion protein was used, resulting in active β-galactosidase due to the complete folding of the protein (Bardwell *et al.*, 1991). The *dsbA* mutation also resulted in F^+ cells becoming resistant to bacteriophage infection, suggesting a defect in F pilus formation (Bardwell *et al.*, 1991).

5.2. Results.

5.2.1.1. F TraN contains intramolecular disulfide bridges. To determine if a protein contains disulfide bonds, the protein can be separated by electrophoresis in the presence and absence of a reducing agent, to determine if there is a shift in mobility. Proteins that contain intramolecular disulfide bonds in the absence of reducing agent will exhibit faster mobility due to the internal covalent linkage of the disulfide bond forming a more compact protein. MC4100 cells containing pOX38*N1*::CAT were transformed with the plasmid pAKN317, and the presence of TraNG317 was examined by immunoblot. Samples were heated at 37°C or 95°C in the presence or absence of the reducing agent dithiothreitol (DTT; Figure 5.1). In the absence of reducing agent, the TraNG317 protein exhibits a faster mobility, suggesting that intramolecular disulfide bonds exist.

Figure 5.1. TraN contains intramolecular disulfide bonds. MC4100 cells containing pOX38*N1*::CAT were transformed with pAKN317. Samples were resuspended in SDS sample buffer with or without the reducing agent dithiothreitol (DTT). Samples were heated at 37°C and 95°C prior to loading onto a 10% SDS polyacrylamide gel.



5.2.1.2. F TraN stability decreases in a *dsbA::kan* strain. Intramolecular disulfide bonds in F TraN might contribute to protein stability. In order to test this, *dsbA::kan* and *dsbC::cam* mutant strains were obtained from Jon Beckwith. pOX38*N1*::CAT-Km was conjugated into *dsbC::cam* and pOX38*N1*::CAT-Tc was conjugated into *dsbA::kan*. The cells were then transformed with pAKN317, and the presence of TraNG317 examined by immunoblot (Figure 5.2). The amount of TraNG317 decreases significantly in the *dsbA::kan* mutant strain, while there was no effect in the *dsbC::cam* and MC4100 wild-type strains. Intramolecular disulfide bonds probably contribute to the stability of F TraN. Since the *dsbA::kan* strain is defective for pilus formation, the effect of a lack of disulfide bond formation on TraN could not be tested (Bardwell *et al.*, 1991).

A lack of disulfide bond formation on outer membrane localization of TraN was tested using wild-type cells and *dsbA::kan* cells containing pOX38*N1*::CAT-Tc or pOX38*N1*::CAT-Km, transformed with pAKN317 (Figure 5.3). The cells were grown to mid-log phase, disrupted by French press, and the crude membranes isolated by high speed centrifugation. The inner and outer membranes were fractionated via flotation sucrose density gradient centrifugation. While there was a clear decrease of TraNG317 in the *dsbA::kan* background, all of the protein appeared to cofractionate with the outer membrane as measured by the presence of OmpA. No TraN was found in the inner membrane fractions as measured by NADH oxidase activities. This suggests that either TraN can insert into the OM without disulfide bonds, or that some small portion of disulfide bonds can form in the absence of DsbA.

The presence of disulfide bonds in the *dsbA::kan* background was measured by separating samples via electrophoresis in the presence and absence of the reducing agent DTT (Figure 5.4). In a *dsbA::kan* background, the small amount of TraNG317 present still contained disulfide bonds as indicated by the increased mobility of the protein in the absence of reducing agent. This effect could be due to either spontaneous disulfide bond formation, or rescue of the *dsbA::kan* mutation

Figure 5.2. TraN stability depends on DsbA. pOX38*N1*::CAT derivatives were conjugated into *dsbC::cam*, *dsbA::kan* and wild-type (MC4100) cells. The plasmid pAKN317 was transformed into these three derivatives, and the protein levels of TraNG317 were examined by immunoblot.

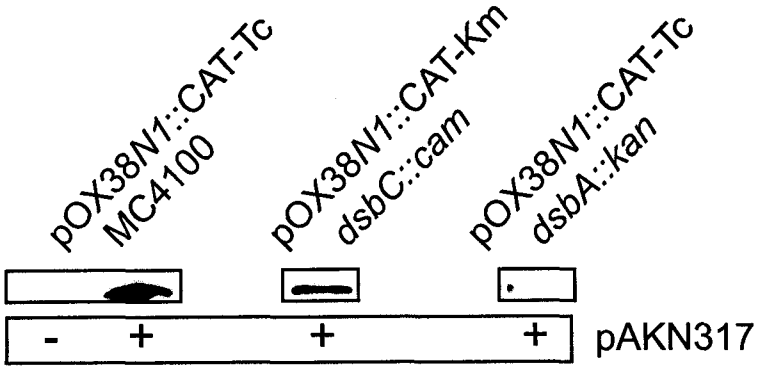


Figure 5.3. Outer membrane localization of TraN in the *dsbA::kan* background. Inner and outer membrane containing fractions were separated by flotation sucrose density gradient centifugation as in Figure 4.2. Top, TraNG317; middle, OmpA, bottom, fractions were tested for NADH oxidase (squares), and sucrose content (circles).

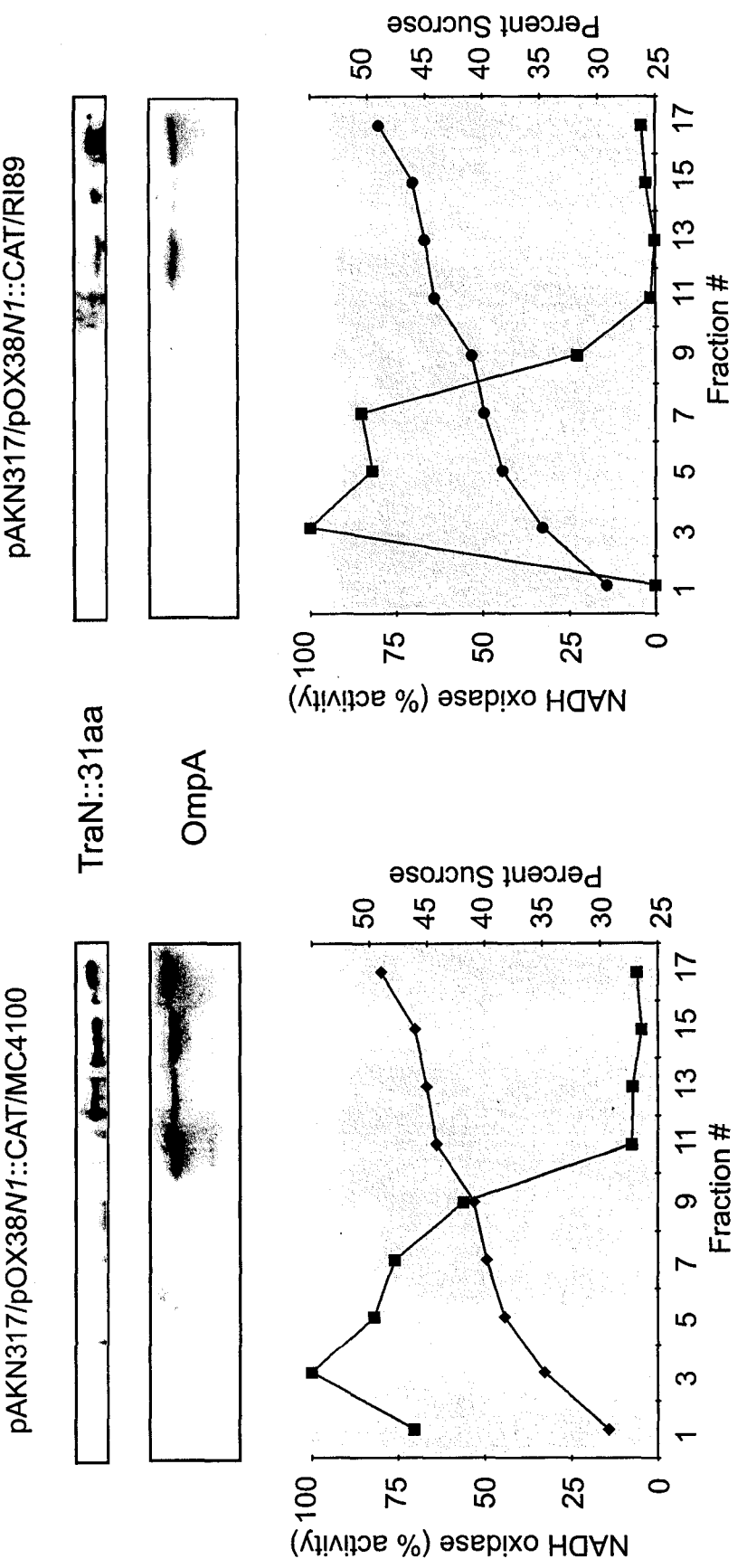
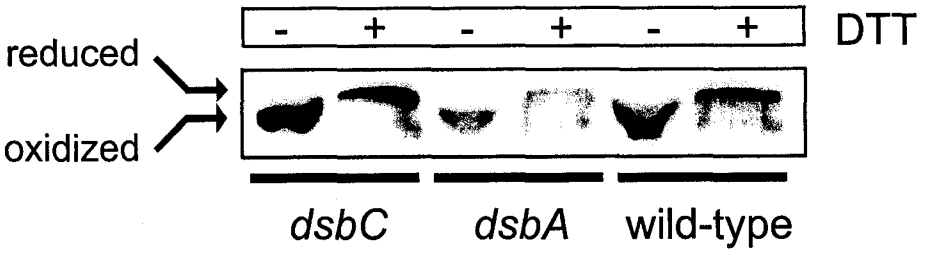


Figure 5.4. Disulfide bond formation still occurs in the *dsbA::kan* background. Cells expressing TraNG317 were treated as in Figure 5.1 and heated at 95°C.



by DsbC which can overcome the defects in disulfide bond formation in a *dsbA* mutant strain, especially when it is overexpressed (Missiakis *et al.*, 1994; Rybin *et al.*, 1996).

5.2.2. Stability of TraN in *tra* mutant backgrounds. TraN probably participates in the transferosome, the multiprotein complex in the cell envelope of the donor cell, and it might interact with other transfer proteins. A mutation in an interacting partner might destabilize TraN if that protein is required for stability, or the mutation might disrupt protein-protein interactions. To test this, the stability of TraNG317 was examined in a wide variety of transfer mutants. ED2149 cells containing various F*lactra* mutants were transformed with pAKN317, and the presence of TraNG317 was examined by immunoblot (Figure 5.5). The only mutation that had a significant effect on TraNG317 levels was F*lactraV569* while a small effect was observed in the F*lactraB2* strain. No change was observed with the *traG* mutants, F*lactraG106* or F*lactraG101*, which disrupt pilus synthesis and mating pair stabilization, respectively. The pOX38N317-Km labeled lane shows the expression levels from a 31-aa-epitope-tagged TraN recombined into the pOX38-Km plasmid.

The effects of the F*lactraV569* were also examined on a minigel, which is more sensitive than large gels for immunodetection of proteins (Manchak *et al.*, 2002). In this case, a small amount of TraNG317 is observed in the *traV569* background (Figure 5.6).

The TraV protein is an outer membrane lipoprotein that is involved in pilus synthesis (Doran *et al.*, 1994). There is another family of outer membrane proteins that require a lipoprotein for outer membrane insertion/stability. The secretins that participate in type II, type III, and filamentous phage secretion form large multimeric pores in the outer membrane. The majority of these require an outer membrane lipoprotein for stability. If TraN acts like a secretin for DNA conjugation, then it might be defective in outer membrane localization in the F*lactraV569* background. Outer and inner membranes of mutant and wild-type cells containing pAKN317 were fractionated via flotation sucrose density gradient centrifugation, and the presence of TraNG317 was examined

Figure 5.5. Stability of TraNG317 in transfer mutant strains. F*lac* or pOX38 transfer mutants were transformed with pAKN317 and the TraNG317 protein levels were examined by immunoblot.

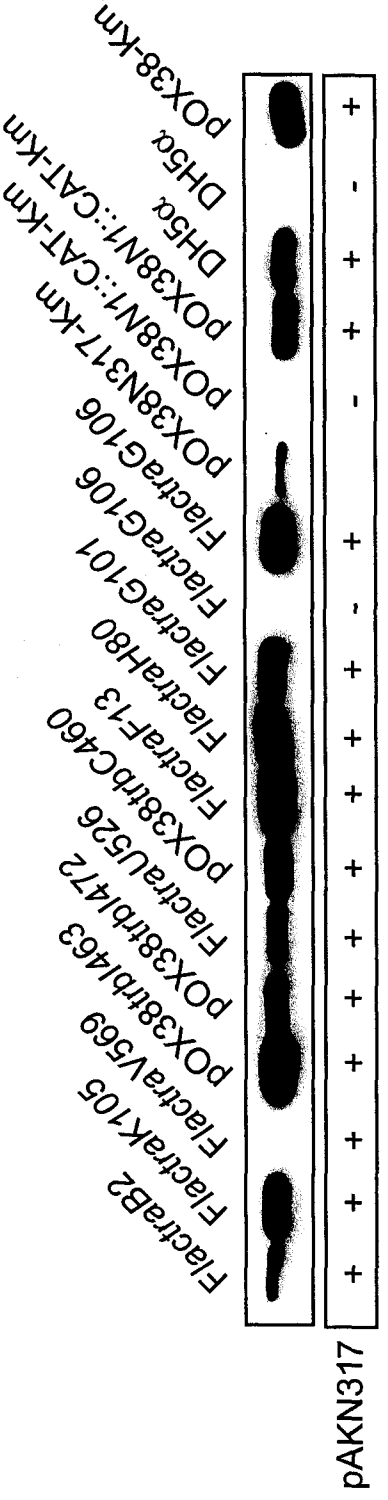
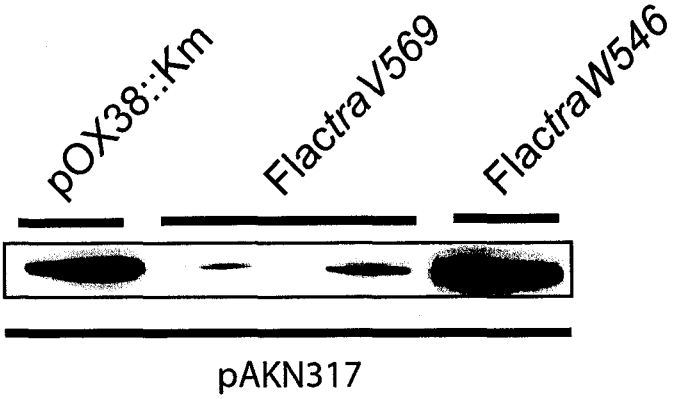


Figure 5.6. Protein levels of TraNG317 in F*lactraV569*. Cells were treated as in Figure 5.5. and samples were separated on a 10% SDS-PAGE minigel, and the levels of TraNG317 were examined by immunoblot.



by immunoblot after separation on a 10% SDS-PAGE minigel (Figure 5.7). As was observed in the *dsbA::kan* background, TraNG317 still fractionates with the outer membrane, even though it is at much lower levels than in the wild-type. TraV is apparently not absolutely required for TraN insertion into the outer membrane, but does aid in stabilization of TraN.

5.2.3.1. Multimerization of TraN. If TraN participates in the transferosome, and interacts with other proteins, multiprotein complexes could be detected by using a chemical crosslinker to form covalent linkages between the interacting proteins, which could be detected as multiprotein complexes after separation on a SDS-polyacrylamide gel. Initial attempts using TraNG317 expressed from either pAKN317 or pOX38N317-Km met with limited success. Upon increasing crosslinker concentrations, using formaldehyde, DSP, or BS3, the monomeric form of TraNG317 disappeared. A large smear of immunoreactive material typically appeared at the interface of the stacking and separating gels (data not shown). The pIV secretin exhibited a similar phenotype (Kazmierczak *et al.*, 1994). When the homobifunctional primary amine crosslinker BS3 was used at concentrations of 0.5 to 2.5 mM, pIV appeared as a large molecular weight complex at the interface of the stacking and separating gels. Individual bands were discerned when BS3 was used at lower concentrations of 20 μM to 0.5 mM.

In an attempt to obtain a clearer picture of TraN protein-protein interactions, in vivo crosslinking of the TraNV584 protein was done with the crosslinker BS3 at concentrations similar to those used with the pIV protein (Figure 5.8). Five distinct TraN-31-aa immunoreactive higher molecular weight bands of 99, 162, 177, 185, and 196 kDa were visible. As the concentration of BS3 in the reaction increased, the monomeric form of TraNV584 disappeared, with more of the protein participating in higher molecular weight complexes. At 2.5 mM, almost all of the monomeric TraNV584 disappeared and shifted to a very large molecular weight complex at the interface of the stacking and separating gels. A sample was taken just prior to the crosslinking experiment which was resuspended in sample buffer without the reducing agent DTT, and electrophoresed

Figure 5.7. Outer membrane localization of TraNG317 in F*lactraV569* cells. Cells were treated as in Figure 4.2 and Figure 5.3. Top, TraNG317; middle, OmpA; bottom, NADH oxidase (squares), and sucrose content (circles).

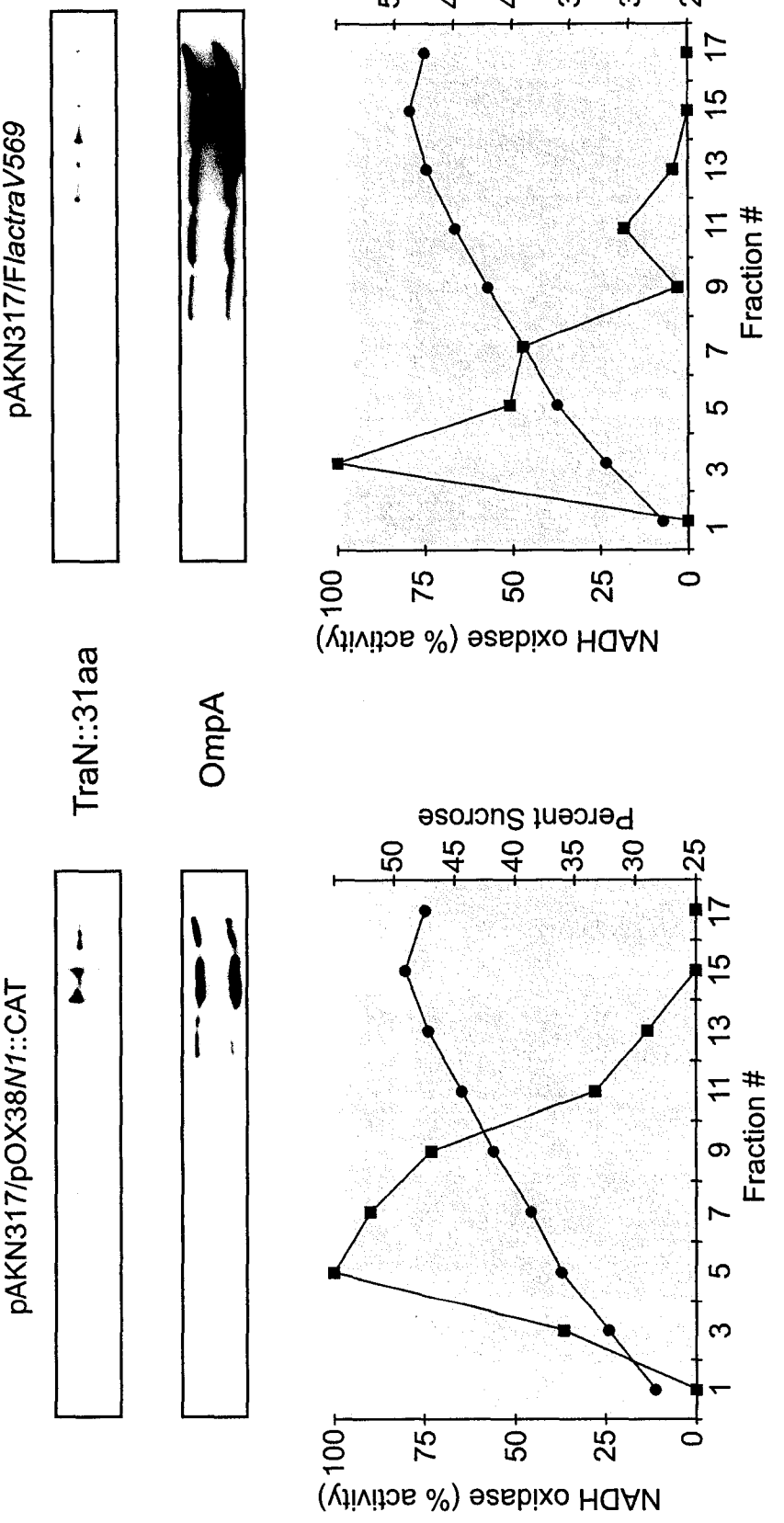
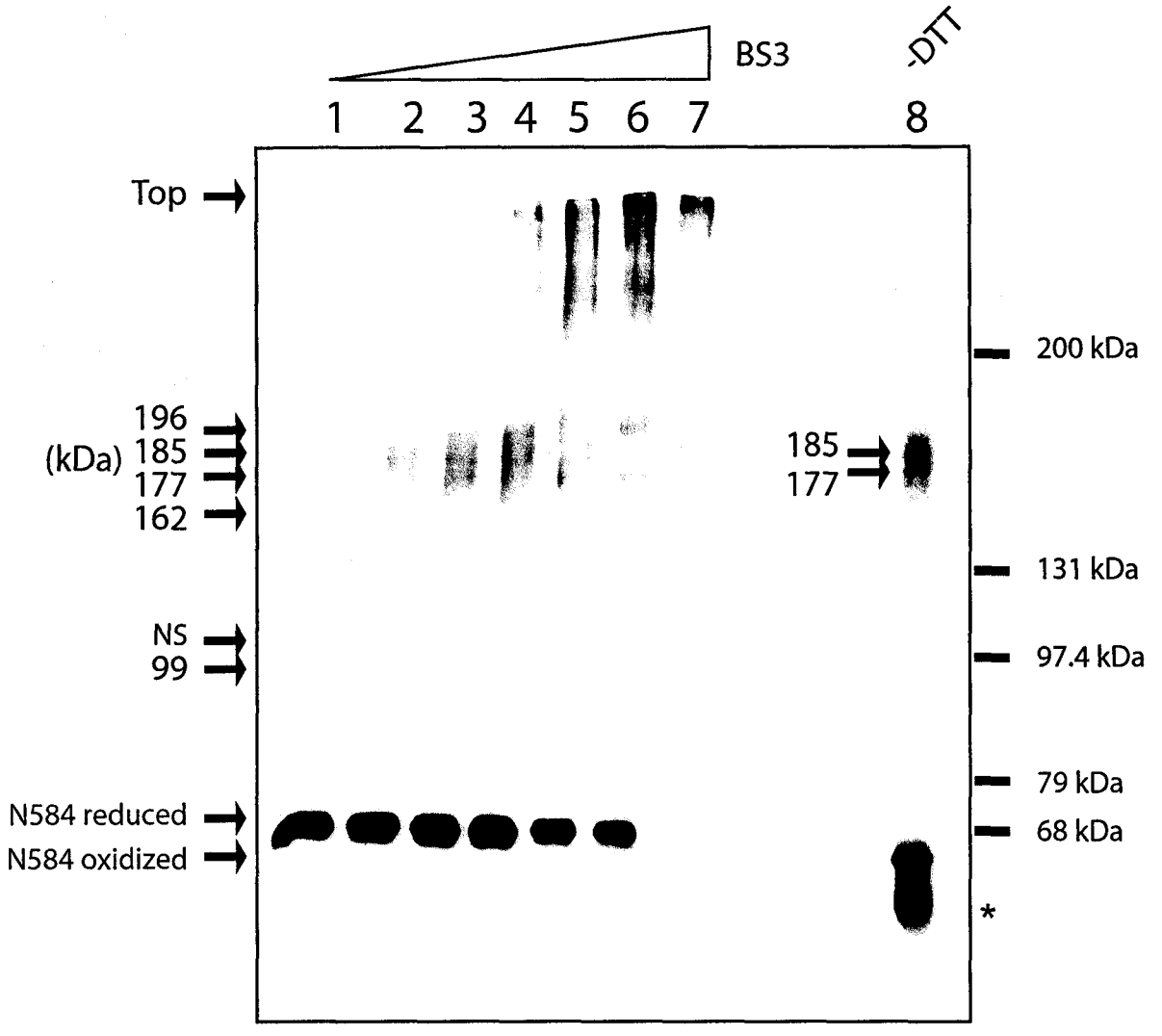


Figure 5.8. Crosslinking of TraNV584. ED24 cells containing pOX38*N1*::CAT were transformed with pBKN584. Cells were grown to mid-log phase, the cells were then washed, and treated with the homobifunctional primary amine crosslinker BS3. Lane 2, 50 μM BS3; lane 3, 100 μM BS3; lane 4, 200 μM BS3; lane 5, 500 μM BS3; lane 6, 1 mM BS3; lane 7, 2 mM BS3. Crosslinking was allowed to proceed for 30 min. at room temperature, and then the reaction was quenched with 50 mM Tris-HCl, pH 7.5. Prior to crosslinking, a sample corresponding to the same cell volume as the crosslinked samples was pelleted. This sample was resuspended in SDS sample buffer without DTT, while the crosslinked samples were resuspended in normal sample buffer. The protein complexes were separated on a 3.5%/7.5% stacking/separating large gel. After transfer to Immobilon-P membrane, the membrane was split at the 79 kDa marker, and the bottom half was probed with anti-31-aa antibodies (1/5000), and anti-rabbit HRP conjugate (1/10 000). The top half was probed with anti-31-aa antibodies (1/2000) and anti-rabbit HRP (1/5000). The oxidized and reduced forms of TraNV584 are indicated. Crosslinked bands are indicated with an arrow and the apparent molecular weight and the interface of the stacking and separating gel is marked top. A non-specific band that shows up in all lanes is marked NS. Molecular weight markers are on the right.



on the same gel. In this lane two higher molecular weight bands appear in the absence of reducing agent that are similar in size to some of the crosslinked species.

It was suspected that some of the degradation products of TraNV584 might be participating in these higher molecular weight complexes, giving multiple crosslinked species, since the *in vivo* degradation products apparently disappeared in the crosslinked samples. This possibility was tested by using a different 31-aa insertion mutant, which exhibited an altered *in vivo* degradation pattern (Figure 4.1). TraNY337 (right panel) and TraNV584 (left panel), which were crosslinked in an identical manner, exhibited the exact same crosslinking pattern (Figure 5.9). These results suggest that the *in vivo* degradation products are not components of the immunoreactive TraN-31-aa higher molecular weight complexes.

5.2.3.2. Identification of interacting proteins. Two potential candidates for protein interactions with TraN are TraG, which participates in mating pair stabilization, and TraV, which was found to be required for TraN stability. If these proteins do interact with TraN, then in a mutant background for each gene, these interactions should be abolished.

In order to test this possibility, F*lactraV569* and F*lactraG106* were transformed with pBKN584, and crosslinked as above (Figure 5.10). The *trav569* mutation decreased monomeric TraNV584 levels significantly, as expected. There were no visible crosslinked species in the *traV569* background, however, it is not known if this was because TraN protein-protein interactions were abolished, or that protein levels decreased by such a significant degree that a TraN-containing complex would not be observed. It is not known why TraNV584 decreased in the F*lactraG106* background during the course of this experiment as the protein levels of TraNG317 did not appear to be affected in the F*lactraG106* background (Figure 5.5). Higher molecular weight complexes in the F*lactraG106* background were visible and were similar to those found in the wild-type control suggesting that the *traG106* mutations does not disrupt any TraN protein-protein interactions.

Figure 5.9. Crosslinking of two different TraN-31-aa derivatives. Cells expressing TraNY337 and TraNV584 were treated with the crosslinker BS3 as in Figure 5.8. Samples were electrophoresed on the same gel. Lanes containing TraNV584 were exposed for 5 minutes to X-ray film, while the lanes containing TraNY337 were exposed for 30 min. in order to visualize crosslinked complexes (marked with an arrow). The positions of TraNY337 and TraNV584 are indicated, and a non-specific band in all lanes is marked NS.

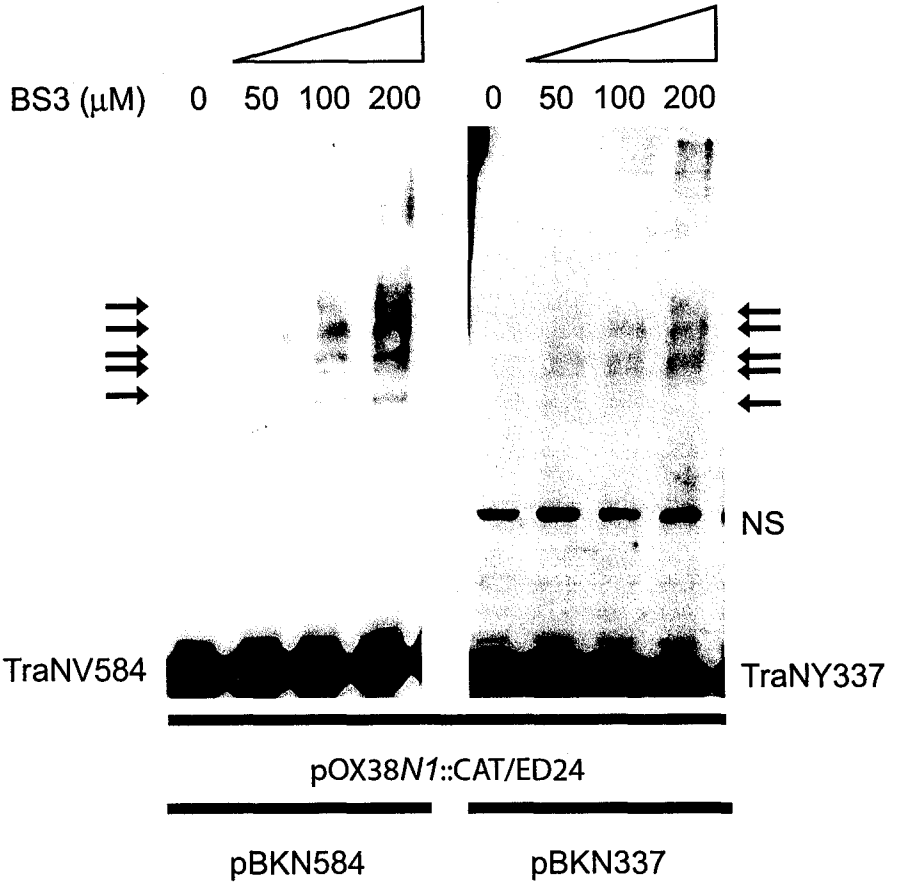
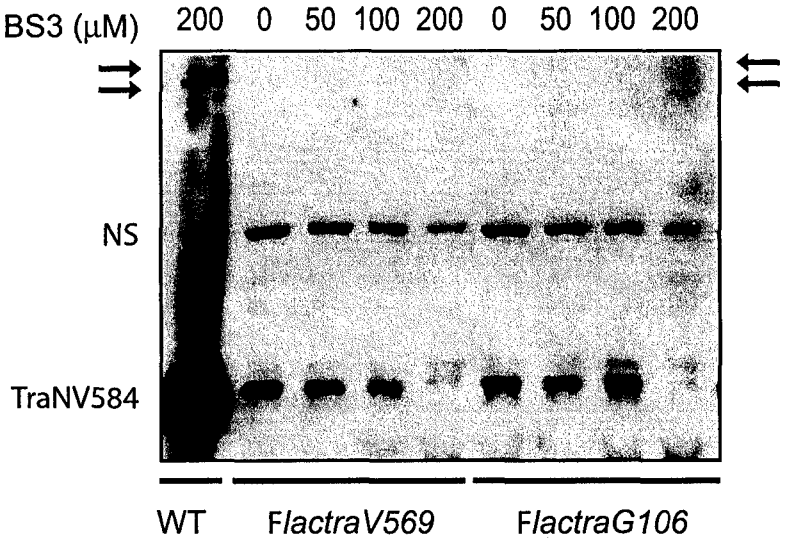


Figure 5.10. Crosslinking of TraNV584 in F*lactraV569* and F*lactraG106* cells. Cells containing wild-type (pOX38*N1*::CAT), or mutant (F*lactraV569* or F*lactraG106*) were transformed and treated with BS3 as in Figure 5.8 and 5.9. After transfer of proteins from the SDS-polyacrylamide gel, the membranes were probed with anti-31-aa antibodies, and anti-rabbit-HRP at 1/5000 and 1/10000 dilution respectively. The position of TraNV584 is marked. BS3 concentrations are indicated. A non-specific band is marked NS. Two visible crosslinked species are marked with an arrow in the wild-type and F*lactraG106*.



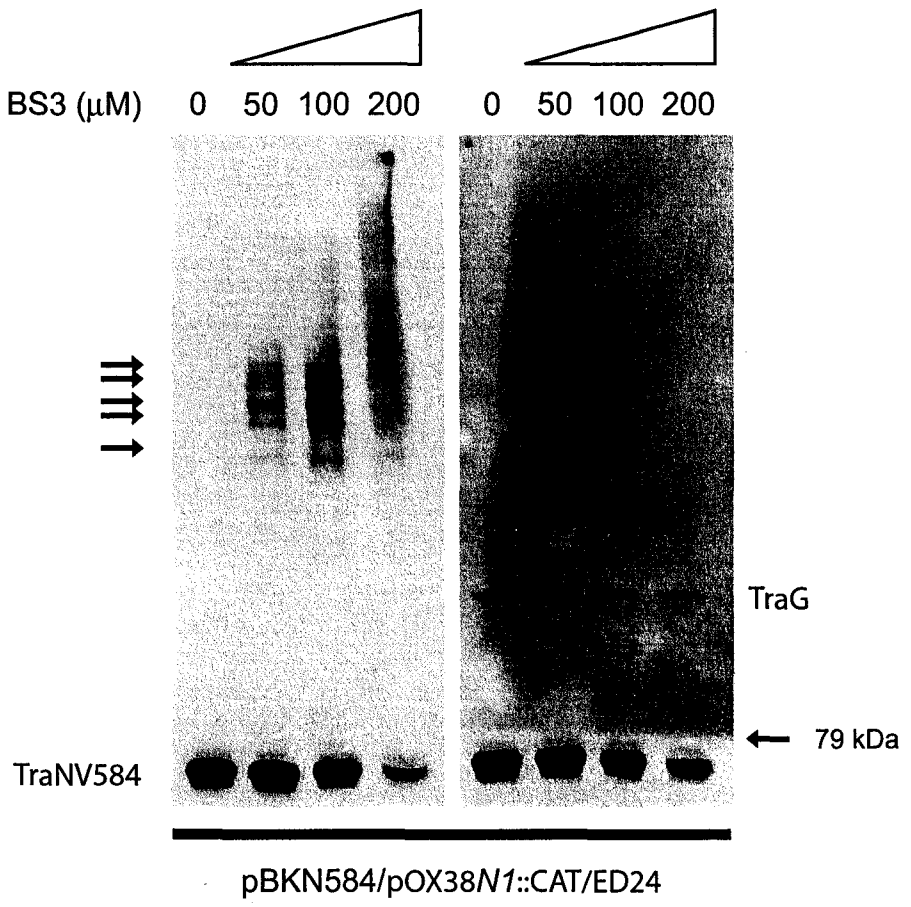
In an attempt to determine if any TraN-TraG interactions existed, cells containing pBKN584 were crosslinked as above and TraG crosslinked species were examined (Figure 5.11). In this case duplicate samples were used and separated on the same gel. After transfer to Immobilon-P, the membrane was split in half vertically, with one-half incubated in anti-31-aa antibodies, and the other half incubated with anti-TraG* antibodies, obtained from Neville Firth (Firth and Skurray, 1992). Although higher molecular weight complexes were observed with the anti-31-aa antibodies, no higher molecular weight complexes were observed with anti-TraG* antibodies, suggesting that TraG does not participate in these higher molecular weight complexes that are comprised, at least in part, of TraNV584.

5.2.4. Purifcation of TraN.

The secretins form large SDS-resistant multimers, a feature that has been exploited for easy purification (Koster *et al.*, 1997; Linderoth *et al.*, 1997; Bitter *et al.*, 1998; Nouwen *et al.*, 1999; Schmidt *et al.*, 2001). When heated in SDS sample buffer at temperatures less than 95°C, the secretins form large multimeric complexes, and are typically found at the interface between the stacking and separating gels on an SDS polyacrylamide gel. As shown in Figure 5.1, TraNG317 did not exhibit multimerization in SDS sample buffer when heated at 37°C and, thus did not behave similarly to the secretin family (data not shown). It was decided that an attempt at TraN purification would be useful in assessing if TraN was involved in a stable multimeric complex.

5.2.4.2. Detergent solubility of TraN. A number of secretins including YscC, InvG, PulD, and XcpQ have been purified via size exclusion chromotography after solubilization in SDS, n-lauroyl sarcosine, or deoxycholate (Koster *et al.*, 1997; Bitter *et al.*, 1998; Daefler and Russel, 1998a). The large dodecameric secretin multimers were easily purified from other protein constituents in the cell envelope because of their large sizes which are in excess of 500 000 MW. TraN had previously been tested in a variety of detergents to measure the solubility characteristics of the protein (Maneewannakul *et al.*, 1992a; Maneewannakul, Ph.D.). The detergents used in that

Figure 5.11. F TraG does not appear to participate in high molecular weight complexes with TraN. Cells containing pBKN584 were treated as in Figure 5.8-5.10. Crosslinked samples were then run in two sets of lanes on SDS-PAGE. After transfer of proteins to Immobilon-P, the membrane was split vertically between both sets of samples, and horizontally at the 79 kDa marker. The bottom half and top left quarter were treated as in Figure 5.8. The top right quarter was incubated with anti-TraG* antibodies (1/500) and anti-rabbit HRP (1/1500).



study were used at lower concentrations than those used to solubilize the secretins. The solubility of TraNG317 was examined in a variety of detergents at the same concentrations as those reported for the purification of secretin multimers.

The plasmid pBK24N317 was used to express TraNG317 after induction with 0.05% arabinose and crude membranes were isolated, resuspended, and solubilized in a variety of detergents (Figure 5.12). The only two detergents that significantly solubilized TraNG317 were SDS and n-lauroyl sarcosine. None of the detergents tested completely solubilized TraNG317, since some TraNG317 was always found in the pellet fraction. Most of the other detergents, including Zwittergent 3-14, sodium cholate, and Triton X-100 were capable of solubilizing TraNG317 to some extent, while octyl-β-D-glucoside was completely unable to solubilize the protein.

N-lauroyl sarcosine was used to solubilize TraN prior to separation by size exclusion chromatography (Koster *et al.*, 1997). Samples of each fraction were precipitated with trichloroacetic acid (TCA), resuspended in sample buffer, and electrophoresed on a 10% SDS-polyacrylamide gel, after which the presence of TraNG317 was determined by immunoblot (Figure 5.13). Calibration of size was obtained by fractionating blue dextran (1000 kDa) and alcohol dehydrogenase (150 kDa) through the S300 column, which were found in fractions 9-10, and 13-14 respectively. TraNG317 was found in fractions 10 and 11, suggesting that it was contained within a very large molecular weight complex. However, contaminating proteins were found in the same fractions as determined by silver staining (data not shown). These results do not correspond to those for the secretin YscC, which, after size exclusion chromatography, was free of contaminating proteins (Koster *et al.*, 1998).

A two-step purification procedure was used to eliminate contaminating proteins from the crude TraN preparation. Some secretins have been purified by anion exchange column chromatography, after solubilization in non-ionic or zwitterionic detergents (Bitter *et al.*, 1998). TraNG317 was solubilized as above in n-lauroyl sarcosine, and then dialyzed in buffer containing

Figure 5.12. Solubilization of TraNG317. Cells containing pBK24N317 were induced with 0.05% arabinose to express TraNG317. Crude membranes were isolated, and equal volumes were solubilized in a variety of detergents. After pelleting the insoluble components, the pellet was resuspended in a volume of buffer equal to the supernatant. Pellet (P) and soluble (S) fractions were loaded at equal volumes. The lane on the far left is a sample of whole cells taken prior to detergent solubilization.

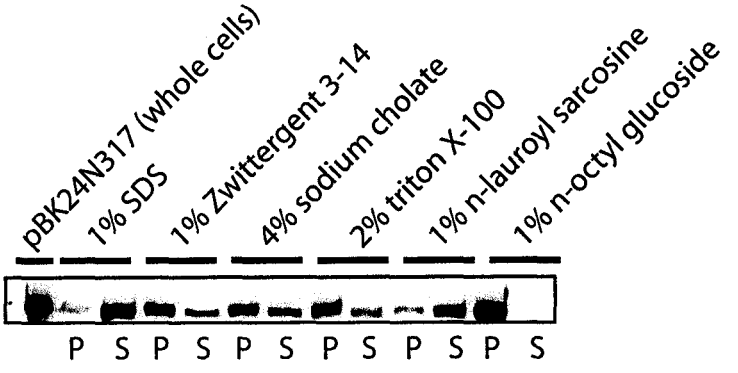
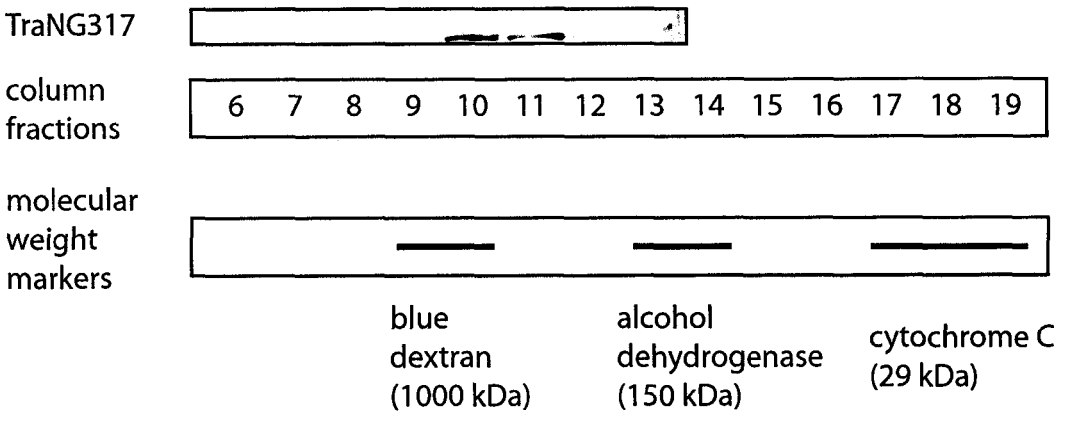


Figure 5.13. Size fractionation of TraN. Cells containing pBK24N317 were inoculated into 200 mL LB and the expression of TraNG317 induced with arabinose. Crude membranes were isolated, and TraNG317 was solubulized in 1% n-lauroyl sarcosine, dialyzed overnight in 0.1% n-lauroyl sarcosine, and fractionated through an S300 size exclusion column equilibrated in the same buffer. The presence of TraNG317 was examined by immunoblot, shown at the top. The corresponding fraction is shown in the middle. The position of the molecular weight markers is shown at the bottom. Only fractions 6 through 13 were examined for the presence of TraNG317 since previous runs had established TraNG317 at the position shown.



0.2% SB-12, a zwitterionic detergent. TraNG317 was loaded onto a Q sepharose anion exchange column, and eluted with a salt gradient. TraNG317 was found to elute with two major peaks between fractions 34-35, and fractions 37-39 (Figure 5.14). Both peaks were pooled, to use as much TraNG317 as possible, concentrated, and loaded onto an S300 column equilibrated in the same buffer. TraNG317 was found in fractions corresponding to a lower molecular weight than was found previously (Figure 5.15). This suggested that TraN had lost a stabilizing element during purification, resulting in the dimeric or trimeric form of TraN dominating.

5.2.4.3. Velocity sedimentation of TraN. The secretin pIV exhibits a large molecular weight *in vivo* as demonstrated by velocity gradient sedimentation (Kazmierczak *et al.*, 1994). TraNV584 was treated in the same manner as pIV in order to assess the *in vivo* multimeric state of TraN. After extracting proteins with the detergent Triton X-100, samples were loaded onto a continuous 5-20% sucrose gradient and centrifuged at 110 000 X g overnight. Fractions were collected, and after TCA precipitation, the presence of TraNV584 was determined by immunoblot (Figure 5.16). In this instance, the TraNV584 was found near the top of the gradient, in fraction 3. Previously, the pIV multimer had been had been found to migrate past all other *E. coli* proteins in the gradient due to its large multimeric state (Kazmierczak *et al.*, 1994). As TraNV584 did not exhibit this same characteristic, it is likely not involved in a stable detergent-resistant complex.

5.2.5. Does F TraN form a pore in the outer membrane? The secretins form large gated pore complexes in the outer membrane. The pIV protein, carrying an S324G mutation, resulted in cells becoming 10-fold more sensitive to the antibiotic vancomycin (Russel, 1994). The BfpB secretin, however, was permeable to pass large compounds such as vancomycin, suggesting that it did not form a gated pore (Schmidt *et al.*, 2001). The possibility that TraN exhibits pore-forming characteristics was examined by testing cells expressing TraN in the presence or absence of other transfer proteins for their sensitivity to detergents and large molecular weight antibiotics (Table 5.1). No increase was found with either the detergents SDS or deoxycholate, or with the

Figure 5.14. Fractionation of TraNG317 on a Q sepharose anion exchange column. TraNG317 was solubilized as in Figure 5.13, and dialyzed in buffer containing 0.2% SB-12. The sample was loaded onto a Q sepharose column, and TraNG317 was eluted with a salt gradient of 0.1-2M NaCl. Fractions were precipitated with TCA, and run on a 10% SDS-PAGE. The presence of TraNG317 was detected by immunoblot. Two peaks were found to contain TraNG317, fraction 34-35, and fractions 37-39.

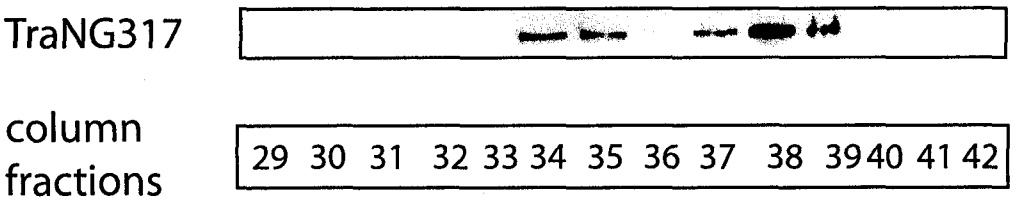


Figure 5.15. *Size fractionation of TraNG317 after anion exchange purification.* Fractions 34-35 from Figure 5.14 were pooled, concentrated, and loaded onto an S300 column equilibrated with the same detergent. Samples were treated as before. The concentrated Q sepharose fractions were analyzed for the presence of TraNG317. The smear at fraction 8 and 9 is not TraNG317.

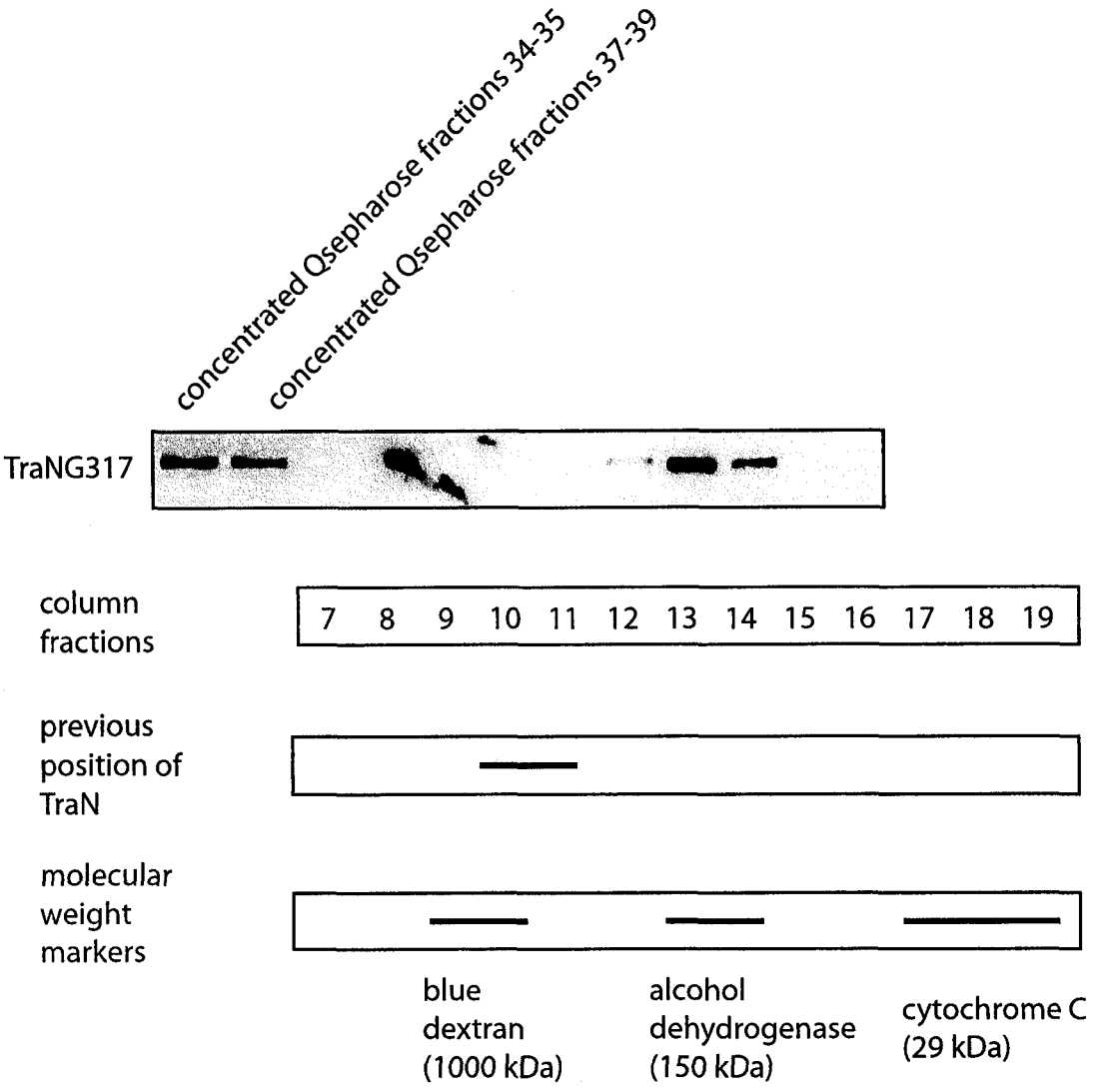


Figure 5.16. Velocity sedimentation of TraN. Cells expressing TraNV584 were extracted with Triton X-100 overnight on ice. After pelleting the insoluble components, the supernatant was loaded onto a continuous 5-20% sucrose gradient, and centrifuged at 110 000 X g for 43 hrs. Twelve 1 ml fractions were collected from the bottom, precipitated by TCA, and run on a 10% SDS-PAGE. The presence of TraNV584 was assayed by immunoblot. Fraction 1 is the top of the gradient, fraction 12 is the bottom.

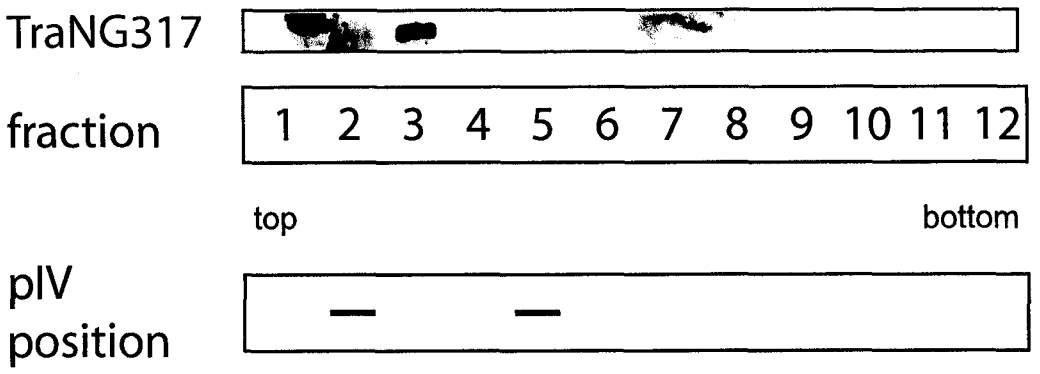


Table 5.1.Sensitivity of cells expressing various TraN derivatives to external agents[a].

Plasmid	Vancomycin 300 μg	Bacitracin 300 μg	SDS 2 mg	Deoxycholate 2 mg
MC4100				
MC4100	0.95	-	1.6	1.15
pOX38-Km	0.9	-	1.5	1.1
pOX38*N1*::CAT-Km	1.05	-	1.6	1.1
pBS SK+	1.1	-	1.6	1.1
pKI375	1.35	-	1.6	1.0
pS84	1.3	-	1.8	0.9
pS63	1.1	faint[b]	1.5	1.0
pS55	0.9	-	1.3/1.8[c]	1.0
pS82	0.9/1.1	-	1.5/1.7	1.0
pOX38*N1*::CAT/MC4100				
pBS SK+	1.1	-	1.6	1.1
pKI375	0.8	-	1.6	1.0
pS84	1.0	-	1.4	1.0
pS63	1.1	faint	1.5	1.1
pS55	1.1/1.5	-	1.5/1.7	0.7
pS82	0.3/1.3	-	1.4/1.6	1.0

a. Cells were grown to mid-log phase, and mixed with top agar to form a lawn of bacteria on an agar plate. Antibiotic or detergent-impregnated discs were placed at regular intervals on the plate. After incubation at 37°C, the diameter of the zone of inhibition was measured.
b. A faint zone of inhibition was barely visible at the outer surface of the disc.
c. Two measurements are given, one for the diameter of the zone of clearing, and one for the diameter of the zone of inhibition.

antibiotics bacitracin or vancomycin. TraN might form a gated pore complex, or it might not be involved in a pore complex whatsoever.

5.3. Discussion.

TraN apparently contains intramolecular disulfide bonds, and these disulfide bonds contribute to the stability of the protein. The sheer number of conserved cysteine residues in TraN suggests that it forms a crosslinked structure, the function of which is not known. Since the *dsbA*::*kan* mutation blocks pilus synthesis, a step that precedes and is required for MPS, the effect of a lack of disulfide bond formation on TraN function could not be ascertained.

When the stability of TraN was examined in the transfer mutant backgrounds, it was discovered that the outer membrane lipoprotein, TraV, which is involved in pilus synthesis, was required for TraN stability (Doran *et al.*, 1994). This was surprising because no previous link between TraN and pilus synthesis had been established. The bifunctional protein, TraG, is involved in both of these aspects, pilus synthesis and MPS. Therefore both MPS proteins have a connection to the pilus. This suggests that the process of MPS is highly linked to the pilus, and perhaps correct localization of the MPS proteins is required for proper mating pair stabilization.

TraN apparently requires an outer membrane lipoprotein for stability. The requirement for a pilot protein for stability and/or membrane insertion is a characteristic of the secretins (Hardie *et al.*, 1996; Daefler *et al.*, 1997a; Daefler *et al.*, 1997b; Daefler and Russel, 1998; Crago and Koronakis, 1998). This pilot protein is often, though not always, a lipoprotein (Schmidt *et al.*, 2001). TraN is not homologous to the secretin family, and did not exhibit the characteristic of SDS- or detergent-resistant multimers that is always associated with these proteins (Koster *et al.*, 1997; Linderoth *et al.*, 1997; *Bitter et al.*, 1998; Nouwen *et al.*, 1999; Schmidt *et al.*, 2001). Since every secretion system that has been intensively studied has been found to require an outer membrane pore complex for the passage of secreted substrates, the possibility that TraN functions analogously during conjugation was examined.

It was not possible to purify TraN using the same procedures used for the secretins. TraN did not exhibit the same characteristics as pIV when centrifuged through a velocity gradient, and finally, TraN did not exhibit any pore forming capabilities. These results together suggest that TraN is not analogous to the secretins found in type II, type III, and filamentous phage secretion.

Crosslinking analysis suggested that TraN does participate in a number of higher molecular weight complexes. If TraN is a large homomultimer, then a stepwise increase in the crosslinked species would be expected. However, this was not the case, since 5 crosslinked species of 99, 162, 177, 185, and 196 kDa were observed, suggesting that TraN is involved in multiheteromeric complexes. The expected size of the dimer and trimer of TraN-31-aa is 134 and 201 kDa respectively. There is no apparent band at 134 kDa, but the 201 kDa is close to the approximately 196 kDa molecular weight complex observed, suggesting it might be a homotrimer of TraNV584. Perhaps TraN forms a complex similar to the TolC and PapC trimeric pore complexes involved in type I secretion and P pilus extrusion respectively (Thanassi *et al.*, 1998; Koronakis *et al.*, 2000).

Surprisingly, when TraNV584 was electrophoresed in the absence of reducing agent, two large molecular weight complexes were also observed that were very close in size to two of the crosslinked species at 177 and 185 kDa. This indicates that TraN not only contains intramolecular disulfide bonds, but also participates in intermolecular complexes via disulfide bridges.

Two proteins which potentially interact with TraN are TraV, which is required for TraN stability, and TraG, which is also involved in MPS. TraV has an apparent molecular weight of 17 kDa, so a TraN-TraV interaction would have an expected MW of 84 kDa, smaller than the 99 kDa crosslinked species that was observed. A TraN-TraG interaction would be approximately 168 kDa since TraG has an apparent molecular weight of 101 kDa (Manning *et al.*, 1981; Firth and Skurray, 1992). TraU has an apparent molecular weight of 34.2 kDa, and a TraN-TraU interaction

would be very close to the 99 kDa band that was observed. A TraN-TraV-TraK-TraB complex would be 160 kDa, very close to the 162 kDa complex that was observed.

Analysis of the TraNV584-containing crosslinked species in both the *traV569* and *traG106* background did not result in identification of any potential interacting partner proteins. Since no higher molecular weight complexes were detected using anti-TraG* antibodies, TraG probably does not participate in the TraN-containing complexes.

The crosslinked species and the disulfide bridged species suggests interactions with either other transfer or host-encoded proteins. When transfer proteins that participate in the transferosome were examined for cysteine residues, it was found that TraB, H, G, N, P, T, U, V and TrbI have 2, 3, 2, 22, 2, 1, 10, 3, and 1 conserved cysteines, respectively, keeping in mind that the N-terminal cysteine in both TraV and TraT is processed by signal peptidase II, resulting in lipid attachment. This was interesting for two reasons, the first being that this conservation suggests that the F plasmid transferosome, and other F-like plasmids, potentially have a highly crosslinked structure. Secondly, it has recently been shown that a TraV-TraK-TraB complex forms a transenvelope complex, and may be the initial scaffolding around which other proteins involved in pilus synthesis are added (Harris *et al.*, 2001). The Vir system has disulfide-linked proteins in the transferosome, with a VirB7-VirB9 heterodimer mediating the stability of a number of other proteins in the complex (Spuddich *et al.*, 1996). TraV has been suggested to be analogous to VirB7 and, therefore, TraN might be analogous to VirB9. Although these specific F- and Vir-encoded proteins exhibit very little similarity, they may function in a similar manner.

Chapter 6

Analysis of the mating pair stabilization protein TraG

6.1 Introduction. TraG is a bifunctional protein that participates both in pilus synthesis and in mating pair stabilization as defined by frameshift mutations (Achtman *et al.*, 1972). *traG106* is a proximal mutation that completely blocks pilus synthesis, while *traG101* is a distal mutation that allows normal pilus expression, but mating pair stabilization function is greatly affected and DNA transfer is inhibited (Achtman *et al.*, 1971; Achtman *et al.*, 1972; Kingsman and Willetts, 1978; Manning *et al.*, 1981).

F TraG was shown to be an inner membrane protein of approximately 100 kDa (Manning *et al.*, 1981). The predicted topology of TraG suggested that it contains 3 transmembrane segments with two periplasmic domains, one in the N-terminal region, and the other being the entire C-terminal region after aa 448 (Firth and Skurray, 1992). Antibodies directed against a C-terminal region of TraG detected two TraG products, a full-length product of 100 kDa, and a 50 kDa product, which corresponded to the C-terminal region, in the periplasm (Firth and Skurray, 1992). It was suggested that there was a signal peptidase I cleavage site at aa 451, which would result in release of the entire C-terminal domain into the periplasm. Since mutations that map to the C-terminal region affect MPS, it was suggested that this cleavage product, TraG*, is the domain involved in MPS, and possibly interacts with TraN (Achtman *et al.*, 1972; Firth and Skurray, 1992).

6.2.1 TraG is a target for the entry exclusion protein, TraS. As noted before, the F and R100 plasmids exhibit a range of allele-specific differences. The surface and entry exclusion proteins, TraT and TraS, respectively, inhibit transfer of their cognate plasmids (Kingsman and Willetts, 1978; Finlay and Paranchych, 1986; Willetts and Maule, 1986). When F TraS is in the recipient cell, it inhibits F plasmid entry, but not pED208 plasmid entry (Finlay and Paranchych, 1986). The conclusion was that a specific F plasmid transfer protein is the target for TraS action. Work in our lab identified TraG as the target for TraS-dependent inhibition (Anthony *et al.*, 1999). F TraS in the recipient cell inhibited entry of the F*lactraG106* plasmid when it was complemented with F TraG, but not when it was complemented with R100 TraG, in the donor.

In order to demonstrate that the reciprocal was true, R100 *traT* and *traS* were cloned as a single fragment into the pT7.3 vector. A surface/entry exclusion test with pOX38-Tc and R100-1 determined that R100 TraT/TraS specifically inhibited R100-1 (Table 6.1). When F*lactraG106* was complemented with F TraG, plasmid transfer was not inhibited by R100 TraS in the recipient cell, but was inhibited when R100 TraG was used to complement F*lactraG106* (Table 6.2). This suggests that the TraS protein, which is found in the inner membrane, specifically interacts with TraG to inhibit conjugation.

6.2.2 Sequence of frameshift mutations in F*lactraG106* and F*lactraG101*. Analysis of the specific mutations in F*lactraG106* and F*lactraG101* might be useful in obtaining information about the function of TraG. Both plasmids were isolated via plasmid purification, and PCR was used to amplify the *traG* regions of both plasmids. Both mutations were sequenced multiple times from independently isolated DNA to ensure that the correct mutation was analyzed. The mutation in *traG106* is a frameshift at position 19798 of the F plasmid transfer operon, resulting in a run of 4 Gs becoming 5 Gs (accesion U01159; Frost *et al.*, 1994). The *traG101* frameshift is at position 21608, with 3 Cs becoming 4 Cs. Both frameshift mutations result in altered reading frames and premature stop codons shortly thereafter.

6.2.3 Comparison of F TraG homologues. A multiple sequence alignment using all TraG homologues was generated in the same manner as for TraN. Two *traG* sequences were obtained from different *Neisseria gonorrhoeae* strains that encode 2 different alleles of *traG* (Hamilton *et al.*, 2001; Dillard and Seifert, 2001). Sequences of *traG*, like *traN*, were found in the unfinished genomes of a number of microbial species.

A multiple sequence alignment was obtained with ten TraG homologues (Figure 6.1). The N-terminal regions exhibit the greatest sequence identity, while the C-terminal region is more divergent. The sequences overall are fairly similar to one another, in size and in sequence length.

Table 6.1. R100 TraT/TraS inhibits R100 transfer.

Plasmid	Mating efficiency R100 *traS*$^{+}$/*traT*$^{+a}$	Mating efficiency vector control[b]	Sfx[c]
pOX38-Km	14.3	21	1.47
R100-1	0.06	66.7	111.2

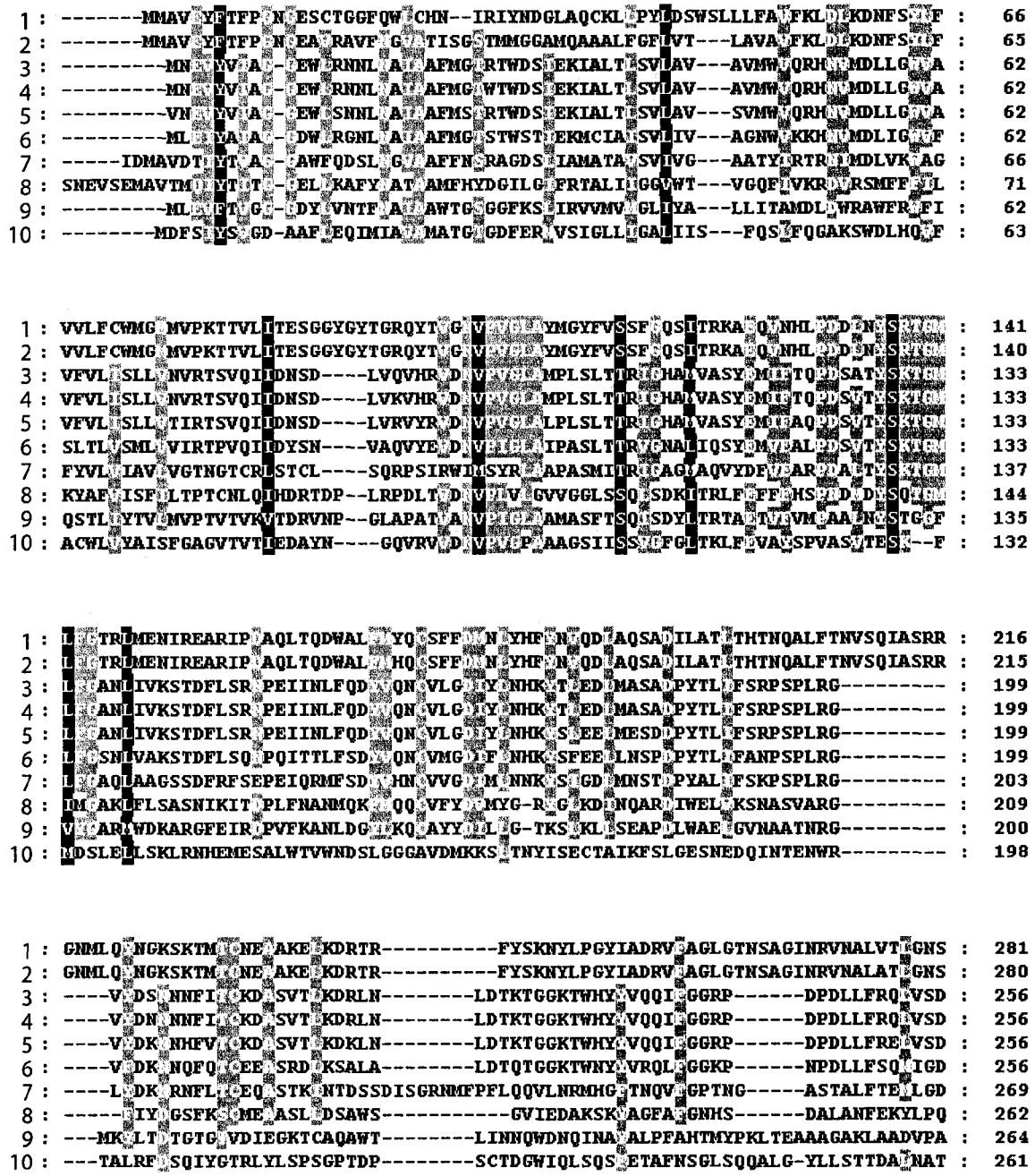
a. Recipient cells containing pBK16 expressing R100 TraT/TraS
b. Recipient cells containing the vector control
c. Surface exclusion index is calculated as in Table 3.6.

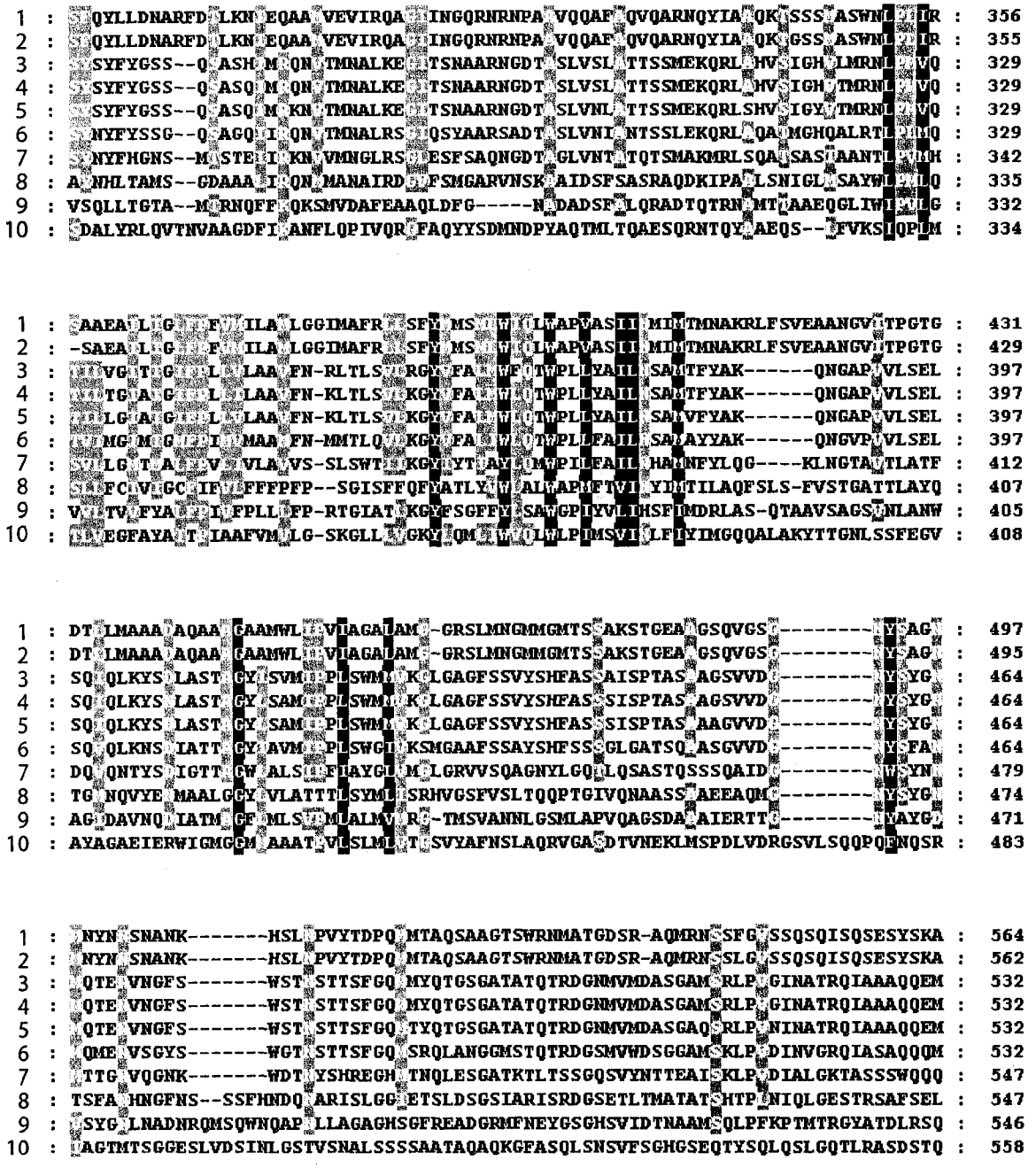
Table 6.2. Specificity of R100 TraS for R100 TraG.

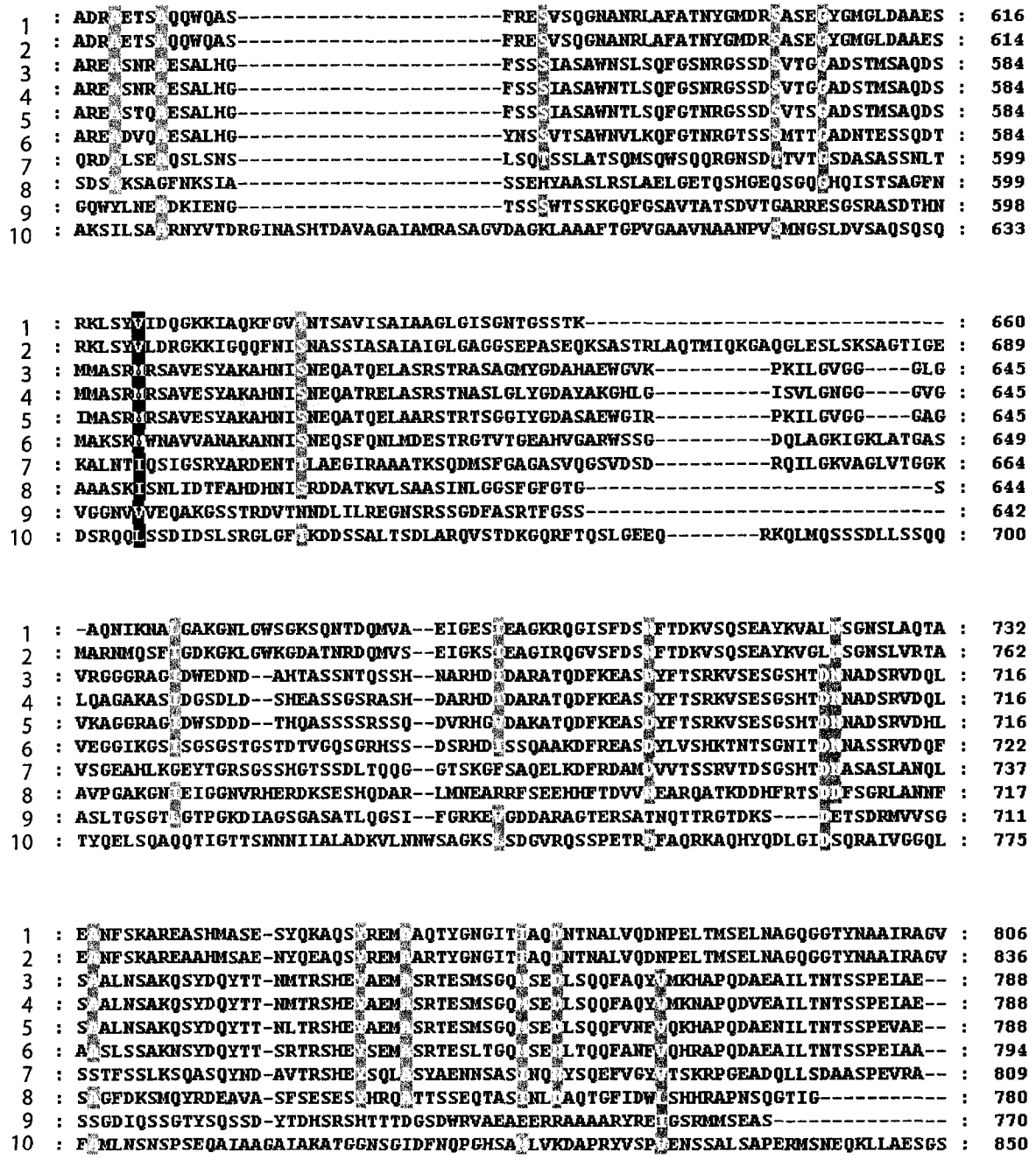
Conjugating plasmid	Relevant genotype	Complementing plasmid	Relevant genotype	Mating[a] efficiency (R100 *traT*$^+$/*traS*$^+$)	Mating[b] efficiency (vector control)	Sfx[c]
pOX38-Tc	F TraG	-	-	1.33	11.25	8.5
R100-1	R100 TraG	-	-	0.02	100	6666.7
F*lactraG106*	*traG106*	pRS1670[d]	F *traG*	3.89	111.7	27.4
F*lactraG106*	*traG106*	pBK20[d]	F *traG*	26.5	111.5	12.6
F*lactraG106*	*traG106*	pKAR-6	R100 *traG*	0.05	22.5	468.8
F*lactraG106*	*traG106*	pBS SK+	-	0	0	ND[e]

a. Recipient cells contained a clone of R100 *traS/traT* in the plasmid pT7.3
b. Recipient cells contained only the vector pT7.3
c. Sfx is calculated as in Table 3.6.
d. Average of two independent mating experiments
e. ND, not determined

Figure 6.1. Multiple sequence analysis of ten TraG homologues. 1) *Neisseria gonorrhoeae* strain MS11 TraG, 2) *Neisseria gonorrhoeae* strain JC1 Sac4, 3) R100 plasmid TraG, 4) F plasmid TraG, 5) *Salmonella typhimurium* LT2 pSLT plasmid TraG, 6) *Klebsiella pneumoniae* TraG, 7) pED208 plasmid TraG, 8) *Legionella pneumophila* TraG, 9) *Novosphingobium aromativoricans* pNL1 plasmid TraG, 10) *Salmonella enterica* TraG. Sequences 6 and 8 are from the Unfinished Microbial Genomes Database. 1, 2, 6, 8, and 10 are found in the chromosome (and not on conjugative or other plasmids). Residues conserved at 100% are shaded black, 80% shaded dark grey, and 60% shaded light grey (allowing for conservative substitutions).







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 4 : -RRRA AWSFVQEQVQ GVDNTWRESRRDIGKGMESVPSGGGSQDIIADHQ-------------GHQAIIEQRTQ :  849
 5 : -RRRA AWAFVQEQVQ GVDNAWRSPR-DIGSGMSTVSGGGDKQDVVADHQ-------------AHQATVEQRTR :  848
 6 : -QREM AREFVKAQVE RIDSSFEQGRNDIGNGMASVSGTPGDQTVVSDFG-------------KHSGDIESMSR :  855
 7 : -ERDR AQAFVEDRMK QLLQEFEQNRGRTGEGMGSVGASSGGGNLQSEYD-------------TQQTQMENRAN :  870
 8 : -----QQAEWLIRHD ELAQSY--ARQFVGEKTAQSISQFQKNHPINESQ-------------VHQKNEGFKSR :  835
 9 : ------YAESHGFQISSDMSNLIQDRYEALQREHPEWHLPDLSNPRLDYRD-------------VTRRDQAITYI :  826
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 6 : NSG KENVRNQVDS VEDNTRINNAGKIN KEQGEGIKQQN---------------------------------- :  896
 7 : AGG R-----QQDD SGDVTDRRRHAESQ NDEKNTVISSG---------------------------------- :  906
 8 : IAGSAG-----VDHALGDYTNTFNDRAKN NAG-SVNKDSV---------------------------------- :  870
 9 : MDD MGDLRAHRIDELSDIAGIAGNGSLGTNTDLGMPQTPS---------------------------------- :  867
10 : KEGFAESFGQSMSK AQMTPEQRDQFIEDTKKGDDYVKQEYGLGGFLAVGAANLGRDIIGVGISGFEAGKEWLTG : 1000

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 3 : SELQNHHKTEALSQN-NKYNEEKSAQERMPGADSPQEL KRAKEYQDKYKQ------------------------ :  940
 4 : SELQNHHKTEALTQN-NKYNEEKLAQERIPGADSPKEL EKAKSYQHKE------------------------- :  938
 5 : SGLQKQYKTEALSQN-NKYNEEKAAQERMPGADSPEEL KRAKEYQDK-------------------------- :  936
 6 : ADFEMLHKKEGNDFN-ERYNKELNGQVNNPLSNTTEEFRKKAEESARKMDGKLP--------------------- :  949
 7 : NTPKKNTIVCRVCMN-RVIKTLVMPRVLKKKGKMIGSV MV-------------------------------- :  946
 8 : SAVDKDFARAKSEMS-----KNVEGIVQDGSQVMQDVI AQANKDVK--------------------------- :  912
 9 : LPSPRAEGLVPSPLEGGTLLAVPNGPVDGAALERFSIS DHIRMN---------------------------- :  912
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 4 : ------------------------------------------------------------ :    -
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 9 : ------------------------------------------------------------ :    -
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Neisseria gonorrhoeae JC1 TraG (Sac4) is 1014 aa, while *Neisseria gonorrhoeae* MS11 TraG is 973 aa (Dillard and Seifert, 2001). *Salmonella enterica* encodes the longest TraG which is 1075 aa in length and is found within a pathogenicity island in the chromosome. F and R100 TraG differ at very few residues, with a small region between aa 610 to 670 of F TraG displaying the most dissimilarity. This suggests that the specificity exhibited by TraS-dependent entry exclusion is found within the C-terminal amino acid residues of TraG, a region that was suggested to produce the cleavage product TraG* that resides in the periplasm.

Also of interest is the putative cleavage site. The AXA motif that was predicted to be a potential signal peptidase I cleavage site with cleavage predicted to occur after the second A at position 451 in F TraG (Firth and Skurray, 1992). The cleavage site is not conserved in the 10 TraG homologues, with only 3 proteins containing the first alanine, and 8 containing the second. This suggests that the TraG* fragment, if it is indeed a cleavage product, must be generated by another means. Perhaps a different periplasmic peptidase functions to cleave within the C-terminal domain of TraG to generate TraG*.

6.2.4 Topological analysis of TraG. A topological analysis of F TraG was done in a similar manner to that for TraN. F TraG was cloned as a PCR fragment into the vector pBS SK+, resulting in elimination of the *Bam*HI site in the vector. This F *traG* clone, pBK20, was fully capable of complementing the F*lactraG106* mutation, indicating that it expressed functional F TraG (Table 6.2). The plasmid was transformed into CC118 cells, which were infected with λTn*phoA*/in, resulting in insertion of the IS*phoA*/in sequence into the pBK20 plasmid.

A total of 12 different IS*phoA*/in insertions were obtained at 4 unique sites in *traG* (Table 6.3). Unfortunately, as was the case for *traN*, multicopy plasmids carrying IS*phoA*/in inserts in *traG* became unstable. The result was that TraGE739 was the only 31-aa insertion recovered. Insertion of 31-aa at position 739 resulted in a TraG construct that was greatly defective as measured by complementation. Interestingly, the site of insertion at 739 is very close to the site of

Table 6.3. Properties of IS*phoA*/in and 31-aa insertions of *traG.*

Insertion	Number of insertions[a]	Converted[b]	Position[c]	Plasmid[d]	Mating efficiency[e]
1	1	No	159	N/A	-
2	6	No	163	N/A	-
3	4	No	716	N/A	-
4	1	Yes	739	pBKG739	0.015

a. Number of independently isolated and sequenced inserts. N/A, not applicable.
b. Only one insertion in pBK20 could be converted to its corresponding 31-aa insertion. Yes, converted; No, unable to be converted; N/A, not applicable.
c. Position is the amino acid directly preceding the 31-aa epitope.
d. Only those IS*phoA*/in inserts that could be converted to 31-aa derivatives are indicated (along with wild-type counterparts). N/A, not applicable.
e. Plasmids containing 31-aa insertion mutants of *traG* were tranformed into F*lactraG106*/JC3272 and the mating efficiency to ED24 was determined. Efficiency is defined as transconjugants/100 donor cells.

the *traG101* frameshift mutation at position 735, suggesting that the region after aa 734 of TraG is very important for function. The 4 sites of IS*phoA*/in insertion agree with the predicted topological model of TraG, with two periplasmic domains contained within the N- and C-terminal regions (Firth and Skurray, 1992). The insertions at aa 159 and 163 are in the first periplasmic domain, while the insertions at aa 716 and 739 are in the second periplasmic domain.

6.2.5.1 Construction of a GFP-TraG fusion. TraG functions in two very critically important elements of conjugation: pilus synthesis and mating pair stabilization, suggesting that these two components are linked (Manning *et al.*, 1981). The F plasmid, which exists at 1 to 2 copies per cell, must interact with the mating apparatus at the time of transfer. This suggests that the position of the transferosome, and the protein components that constitute the apparatus, must be localized coordinately. Information on the *in vivo* localization of a number of proteins has been obtained by using the green fluorescent protein (GFP) as a reporter. GFP contains an internally formed chromophore that can be excited with UV light, creating a fluorescent emission. If the GFP is fused to a protein of interest, information on where that protein exists in the cell can be obtained. For instance a fusion of GFP to the TrhC protein, the homologue of F TraC from plasmid R27, revealed the existence of a number of foci in the cell (Gilmour *et al.*, 2001).

To generate the TraG fusion, PCR was used to amplify the *traG* gene using the pOX38-Km plasmid as a template. The PCR product was initially cloned into the pCR4Blunt-TOPO vector, and then subcloned into the pDSW209 vector to generate pJY1 (Weiss *et al.*, 1999). The resulting fusion places GFP at the N-terminus of TraG, which is predicted to reside in the cytoplasm.

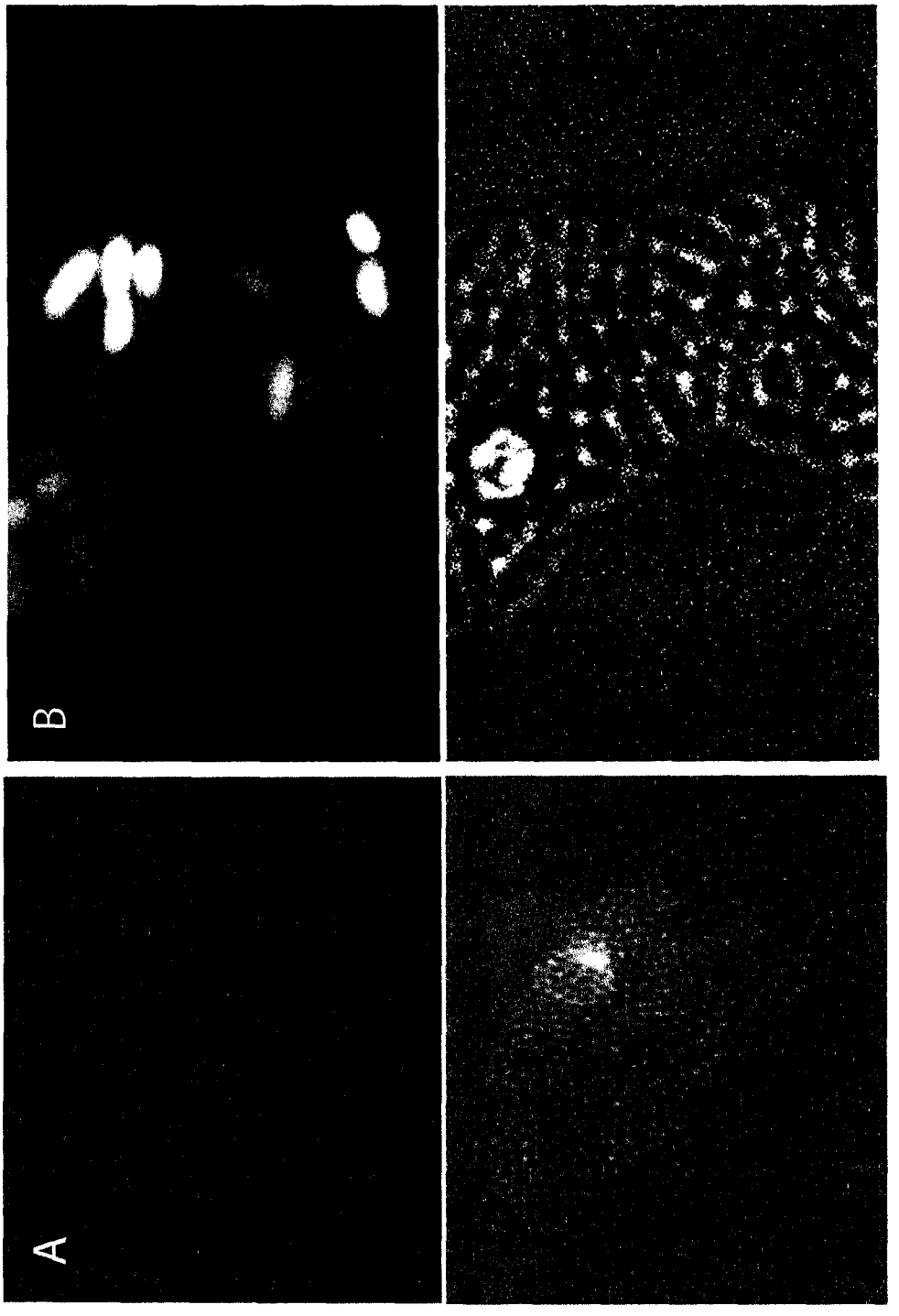
6.2.5.2 Localization of GFP-TraG. The plasmid pJY1 was transformed into JC3272 with or without F*lactraG106*. The cells were grown to exponential phase, and the culture was divided into two aliquots. One of the samples was treated with 50 μM IPTG, while the other was left untreated. After one hour of induction, the cells were washed and placed onto a thin layer of

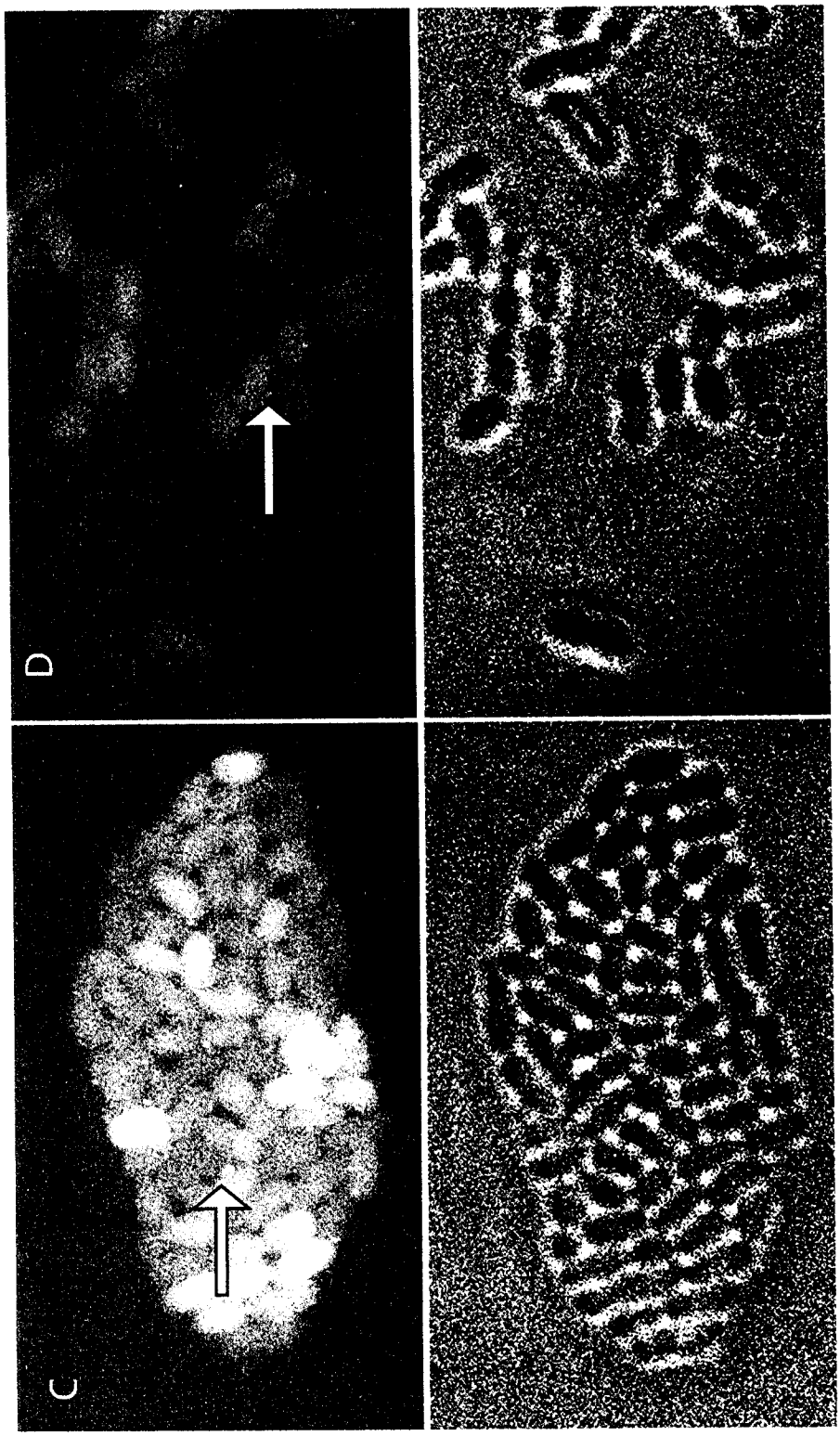
minimal media on a glass slide and GFP was visualized using UV excitation (Gilmour *et al.*, 2001). When cells expressing only wild-type TraG from the pRS1670 plasmid were examined, no fluorescence was observed (Figure 6.2A). In cells containing the vector pDSW209, a number of cells exhibit fluorescence, but no visible foci are present (Figure 6.2B). Cells expressing the GFP-TraG fusion show a number of foci (marked with a white arrow), in the presence or absence of the F plasmid (Figure 6.2C-F). Addition of IPTG to the medium appears to cause an increase in the fluorescence level of all the cells, but did not appear to increase the number of foci present. Cells that were not induced with IPTG were exposed for a longer period of time in order to visualize the GFP. Not all these cells had foci, while a number of cells had multiple foci.

6.2.5.3 Characterization of GFP-TraG fusion and TraGE739. The complementation ability of a number of F TraG expressing plasmids was tested including pJY1, pBKG739, and pBADTraG (Table 6.4). pJY1 was defective in its ability to complement FlactraG106. However, it was capable of supporting R17 phage infection. This indicates that a functional pilus is assembled since R17 binds to the sides of the elongated pilus. Bacteriophage f1 sensitivity was also normal. pBKG739 was also defective in complementation of F*lactraG106* for DNA transfer, albeit not to the same levels as pJY1, while phage sensitivity was normal. Mating efficiency increased 100-fold when pBADTraG was induced with 0.05% arabinose, suggesting that the amount of TraG in the cell might affect transfer efficiency. F*lactraG106* itself produces no detectable transconjugants. Both the fusion of GFP to the N-terminus of TraG and insertion of 31-aa at position 739 of TraG appear to disrupt MPS function of TraG, as pilus synthesis appeared normal, whereas transfer efficiency was greatly reduced, similar to what is observed with the *traG101* mutation.

6.2.6 Detection of TraG derivatives via immunoblot. The cells used for the determination of mating efficiency were also tested for the presence of TraG products via immunoblot. Samples corresponding to an OD_{600} of 0.1 were loaded onto a 10% SDS–polyacrylamide gel, and after transfer to Immobilon-P, the presence of TraG was detected with anti-TraG* antibodies, while the presence of TraGE739 was detected with anti-31-aa antibodies (Figure 6.3).

Figure 6.2. Cellular localization of GFP-TraG. JC3272 cells containing either F*lactraG106*, or no F plasmid, were transformed with wild-type TraG (pRS1670), the GFP vector (pDSW209), or the GFP-TraG fusion construct (pJY1). Cells were grown to mid-log phase and divided into two aliquots. One was induced with 50 μM IPTG while the other was untreated. After induction for one hour, the cells were pelleted, washed, and a drop was placed onto a thin pad of minimal agar medium on a glass slide. Cells were examined for fluorescence (top), or normal visualization via phase contrast (bottom) of the same field. A) pRS1670/F*lactraG106*, B) pDSW209/F*lactraG106* with IPTG, C) pJY1/F*lactraG106* without IPTG, D) pJY1/F*lactraG106* with IPTG, E) pJY1 without IPTG, F), pJY1 with IPTG. Cells were exposed for different times to visualize GFP-TraG foci. White arrows mark representative foci.





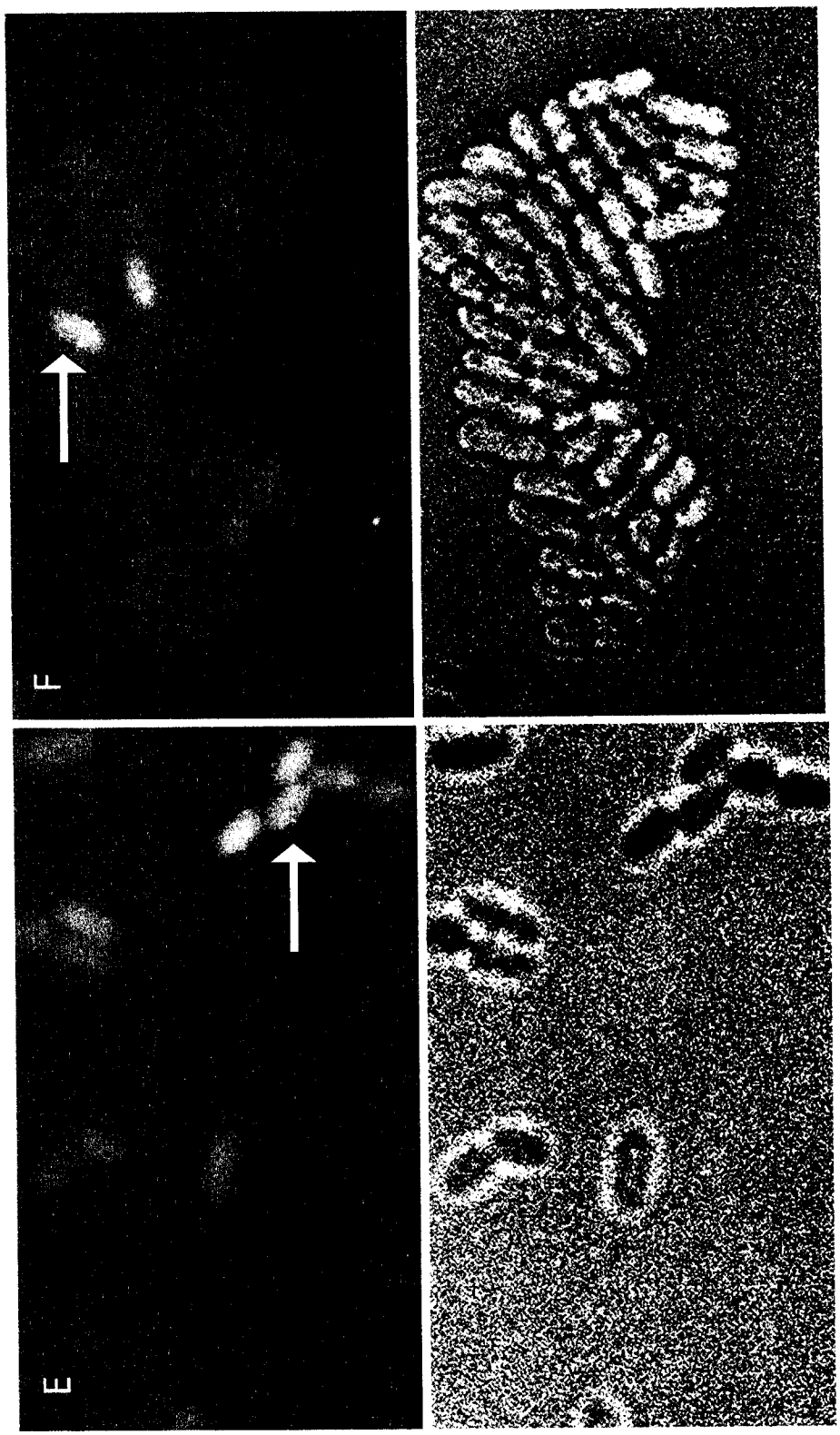
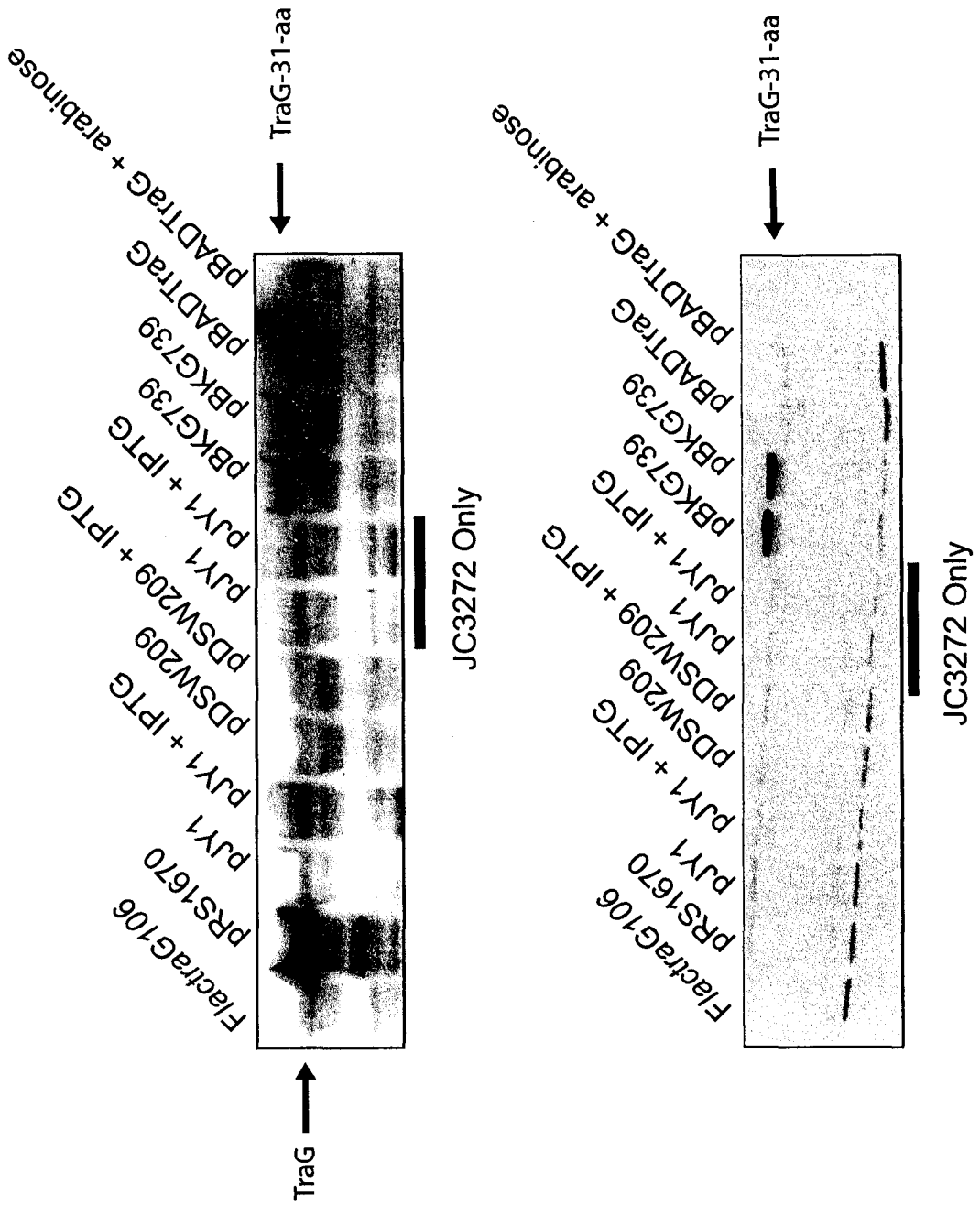


Table 6.4. Complementation ability of F TraG constructs.

Conjugating plasmid	Complementing plasmid	TraG expressed	Mating efficiency[a]	R17[Sb]
F*lactraG106*	pRS1670	F TraG	66.67	S
F*lactraG106*	pBKG739	TraGE739	0.01	S
F*lactraG106*	pJY1	GFP-TraG	0.0053	S
FlactraG106	pJY1 + IPTG	GFP-TraG	0.0091	N/D
F*lactraG106*	pBADTraG	F TraG	0.77	N/D
F*lactraG106*	pBADTraG + arabinose	F TraG	114.3	N/D
F*lactraG106*	pDSW209	-	0	N/D
F*lactraG106*	-	-	0	R

a. Mating efficiency is as described in Table 3.1.
b. Spot test for R17 bacteriophage sensitivity. S=sensitive, R=resistant.

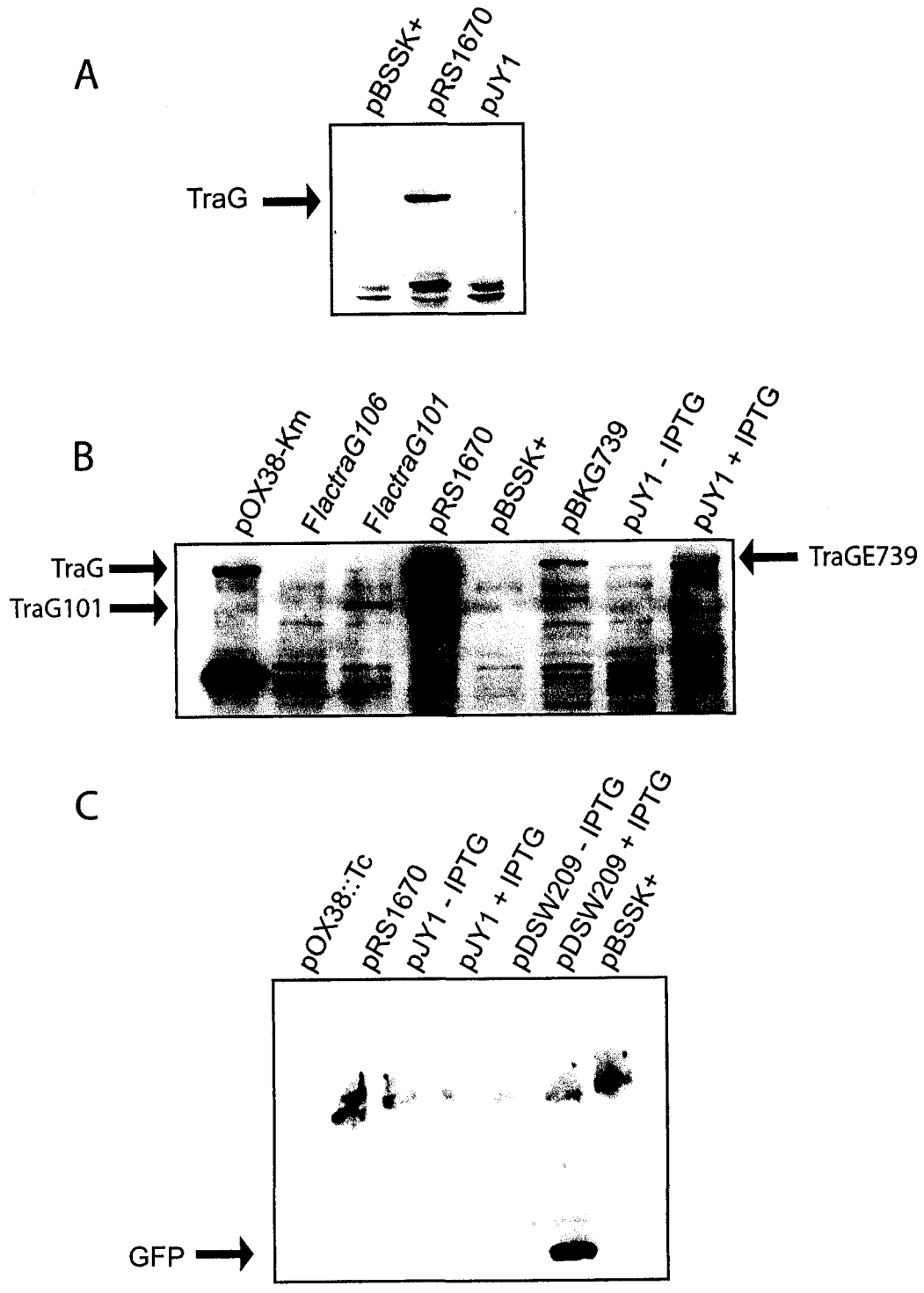
Figure 6.3. Detection of TraG derivatives via immunoblot. JC3272 cells containing either F*lactraG106*, or no F plasmid, were transformed with a variety of TraG constructs. Cells were grown to mid-log phase, and GFP constructs were induced with 50 μM IPTG, and the pBADTraG construct was induced with 0.05% arabinose. Samples corresponding to an OD_{600} of 0.1 were pelleted and electrophoresed on a 10% SDS-PAGE. Top, anti-TraG* antibodies, bottom, anti-31-aa antibodies. The position of wild-type TraG and TraG-31-aa are marked. These cells correspond directly with the cells in Table 6.4.



When only the plasmid F*lactraG106* was present, no immunoreactive TraG band was detected. In the presence of pRS1670, there was an increase in the amount of TraG present in the cells. The GFP-TraG fusion was barely visible in the absence of IPTG, and protein levels increased upon addition of IPTG. Surprisingly, the GFP-TraG fusion appeared to be the same molecular weight as wild-type TraG (see below). The expected size of the GFP-TraG fusion was approximately 130 kDa, while the observed size was 100 kDa. The control lane with only the GFP fusion vector alone shows no immunoreactive band with anti-TraG* antibodies. Insertion of the 31-aa epitope into TraG, resulting in TraGE739, increases the molecular weight of TraG. The TraGE739 protein is easily detected with the anti-31-aa antibodies. The anti-TraG* antibodies were used at a much higher concentration because the GFP-TraG fusion was not easily detected (see below).

Initial attempts to detect the GFP-TraG fusion with lower concentrations of anti-TraG* antibodies were unsuccessful (Figure 6.4A). Although higher amounts of the antibody detected the fusion (Figure 6.4B). No immunoreactive bands were detected when Anti-GFP antibodies were used (Figure 6.4C). The GFP protein was visible when expressed from the pDSW209 vector, and protein levels increased upon IPTG induction. Since the GFP-TraG fusion, when detected with anti-TraG* antibodies, was of the same apparent molecular weight as wild-type TraG, it was assumed that the GFP-TraG fusion is being degraded. Since no immunoreactive band is detected with anti-GFP antibodies, neither the GFP-TraG fusion nor GFP by itself were observed, it is possible that the GFP moiety was cleaved from the fusion and subsequently degraded. Sequence analysis of the GFP-TraG construct demonstrated that the fusion was in the correct reading frame (data not shown). No immunoreactive band is visible in the F*lactraG106* lane, while a smaller product is visible in the F*lactraG101* lane. Since the anti-TraG* antibodies were directed against the C-terminal region of TraG, the truncated product from F*lactraG106* would not have been picked up, while the truncation of TraG due to the *traG101* mutations results in a clearly visible product (Firth and Skurray, 1992).

Figure 6.4. GFP-TraG appears to be degraded. A) Anti-TraG* antibodies were used at a 1/1500 dilution and anti-rabbit HRP conjugate was used at 1/5000 dilution B) Increased concentration of anti-TraG* antibodies detects GFP-TraG fusion. Anti-TraG* was used at a 1/500 dilution and the anti-rabbit HRP conjugate was used at 1/1000 dilution. C) Detection with anti-GFP antibodies. A non-specific band is observed near the top of the gel. The position of TraG, TraGE739, TraG101, and GFP are marked.

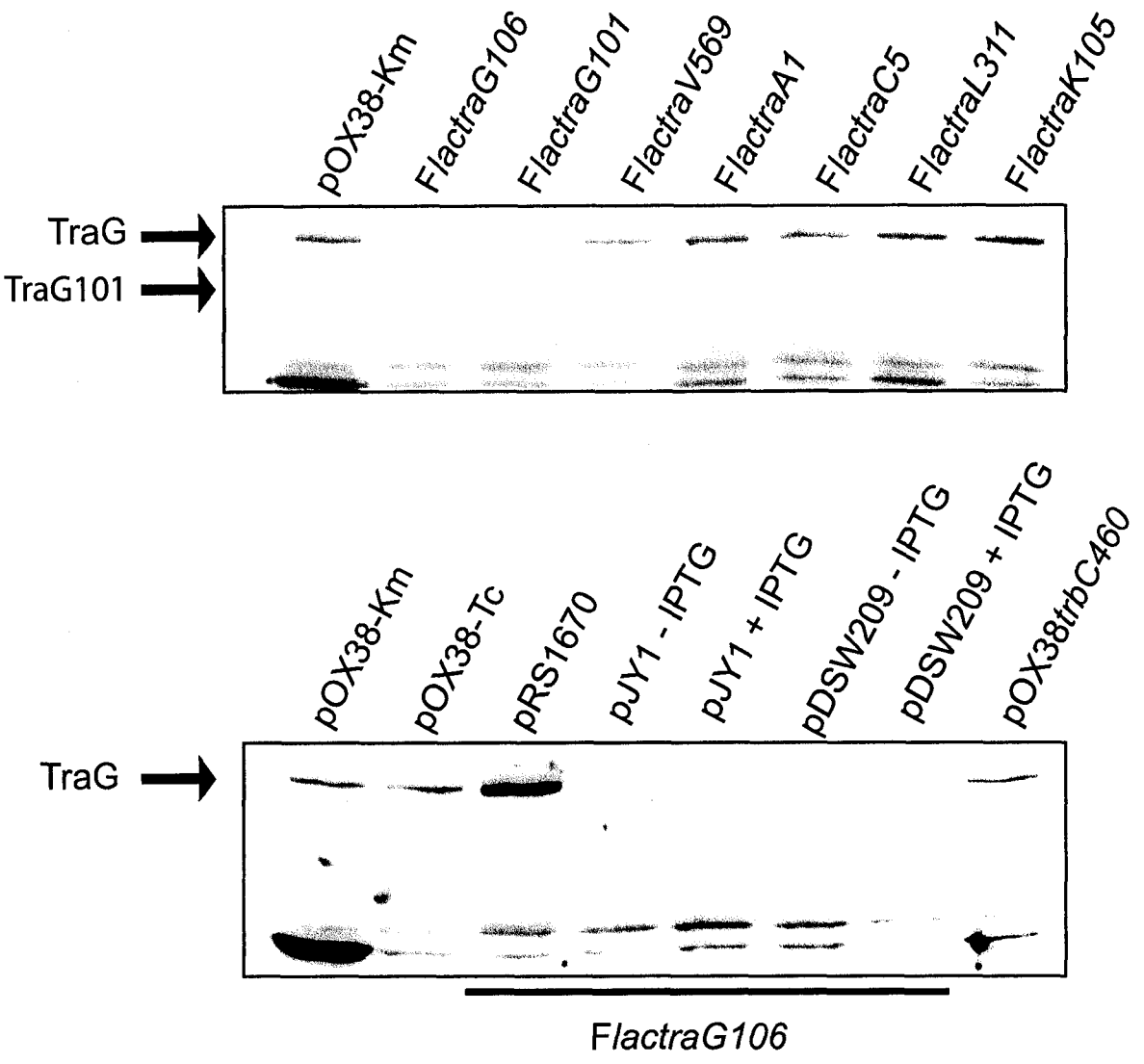


6.2.7 Stability of TraG in transfer mutant backgrounds. The protein levels of TraG were observed in a number of F*lac* and pOX38 *tra* mutant backgrounds as was done for TraN. In this case, no TraG expressing plasmid was required as the anti-TraG* antibodies sufficed to detect native TraG expression. Since it was discovered that only 6 proteins apparently function in the formation of the pilus tip at the cell surface of *E. coli*, those mutant strains were examined along with *traV569* and *trbC460* which do not affect pilus tip formation (Anthony *et al.*, 1999). In this case, no change in TraG protein levels were observed in any strain other than the *traG* mutant strains (Figure 6.5). The smaller TraG101 product is shown. pOX38-Km appears to produce an immunoreactive band of approximately 50 kDa, while the isogenic pOX38-Tc does not. TraG protein levels do not change in the *traV569*, *traA1*, *traC5*, *traL311*, *traK105*, or *trbC460* backgrounds.

6.3 Discussion. An examination of the inner membrane mating pair stabilization protein, F TraG, revealed that entry exclusion mediated by the F TraS protein in the recipient cell was specific for F TraG when it was present in the donor cell (Anthony *et al.*, 1999). Since TraG has two roles, one in pilus synthesis and one in mating pair stabilization, it has been suggested that entry exclusion inhibits the MPS function of TraG. The results presented here confirm the reciprocal nature of the above experiment, with R100 TraS in the recipient cell specifically inhibiting R100 TraG in the donor cell.

An attempt at confirming the predicted topology of TraG using transposon-directed insertion of the *phoA* gene into TraG met with limited success due to the unstable nature of the multicopy constructs. However, all 4 unique IS*phoA*/in insertion sites agreed with the predicted topological model of TraG. The single 31-aa insertion derivative obtained, TraGE739, was severely perturbed in complementation assays of F*lactraG106*. Interestingly, this insertion at position 739 is very close to the *traG101* frameshift mutation that was found at position 735, suggesting that this region of the protein is very important for TraG function.

Figure 6.5. Stability of TraG. Cells containing various F*lac* or pOX38 *tra* mutants were examined for their effect on TraG. Anti-TraG* antibodies were used at 1/1000 dilution, and anti-rabbit HRP conjugate was used at 1/2500 dilution. No change in TraG is found in any mutant background except the *traG* mutants. Multicopy pRS1670 increases the levels of TraG in the cell. An immunoreactive band found with pOX38-Km is not observed in the isogenic pOX38-Tc.



Analysis of the multiple sequence alignment of all TraG homologues revealed that the N-terminal region of TraG is more conserved than the C-terminal region. The region of highest dissimilarity between F and R100 TraG corresponds to aa 610 to 670 of F TraG, suggesting that this region might be important for TraS-mediated plasmid-specific entry exclusion. The C-terminal domain of TraG is predicted to be involved in MPS, suggesting that TraS inhibits the MPS function of TraG and inhibits DNA transfer.

TraG, along with a subset of transfer proteins involved in pilus synthesis, appear to be responsible for formation of the pilus tip at the surface of the cells (Anthony *et al.*, 1999). Mutations in *traG106*, *traA1*, *traC5*, *traL311*, and *traK105*, render the cells resistant to M13KO7 infection. However, none of the mutations in these genes affected the levels of the TraG protein in the cell. Apparently, if TraG interacts physically with any of these proteins, such as interaction is not needed for the stability of TraG.

The GFP-TraG fusion was constructed in an attempt to ascertain the localization of TraG, and potentially, the initial transenvelope complex that forms the pilus. Foci were observed that localized to the cell envelope, however, it was discovered that this GFP-TraG fusion appeared to be unstable. Immunodetection of the GFP-TraG fusion with anti-TraG* antibodies discovered that the full-length product was not observed, and that a product with the same apparent molecular weight as wild-type TraG was observed. This suggested that the fusion was unstable, and degraded at the site of fusion, with the resultant GFP moiety degraded. This fusion was also defective in complementation of the *traG106* mutation in terms of DNA transfer, although the cells exhibited normal R17 phage sensitivity, indicating that the pilus is still produced. It is not known why the fusion was degraded, and may point to an important role for the N-terminal tail of TraG that is predicted to reside in the cytoplasm of the cell.

Chapter 7

Discussion

The research presented here is an attempt to understand the underlying mechanism in F plasmid conjugation. The specific aspect that was studied was the process of mating pair stabilization as mediated by TraN in the outer membrane and TraG in the inner membrane. The F-like plasmid, R100, provides a useful reservoir of allele-specific genes that can function in complementation in F mutant background, while still maintaining a plasmid-specific nature. Our results in Chapter 3 indicate that F TraN is specific for OmpA in the recipient cell during conjugation, while R100 TraN is not. This specificity appears to be encoded in a highly variable region found within the middle one-third of the F TraN protein. These results are contrary to the oft-cited idea that the pilus, or the pilus tip, would be specific for OmpA binding during conjugation. A topological analysis of the TraN protein was done in Chapter 4 in an attempt to obtain information about the supposed cell surface exposed regions of TraN that would interact with OmpA. Surprisingly, very little of the protein appears to be exposed at the cell surface based on our proposed model. However, further work needs to be done to completely characterize the cell surface regions. In Chapter 5, the epitope-tagged TraN derivatives that were used to obtain information on topology were used to determine potential protein-protein interactions amongst the transferosome proteins. It was discovered that the TraV lipoprotein that is involved in pilus synthesis appears to stabilize TraN. TraN was also found to contain intramolecular disulfide bonds, which possibly mediate its stability. TraN may also be involved in intermolecular interactions with other transfer proteins. Finally, in Chapter 6, it was discovered that F and R100 TraS in the recipient cell specifically inhibit the F and R100 TraG proteins, respectively, in donor cells. Localization of a GFP-TraG fusion revealed a number of foci per cell, although these results are inconclusive based on the apparent instability of that fusion.

7.1 Mating Pair Stabilization. The formation of a completely functional and erect pilus marks the starting point for the mating cycle of the F plasmid. Once the pilus contacts a recipient cell and retracts, the unstable wall-to-wall contacts are converted to stable ones by the process of mating pair stabilization (Manning *et al.*, 1981). F pili can interact with ConF$^-$ donors, suggesting that the nature of pilus tip interactions is either for a different component in the recipient cell, one which

was not picked up in any of the mutational screens designed to generate ConF$^-$ recipient cells, or that the nature of pilus tip-recipient cell interactions is non-specific. Since purified F pili cannot bind to *Bacillus* or *Pseudomonas* cells, it does appear that the F pilus can contact something specific to enteric organisms (Achtman and Skurray, 1977). Once the cells are in close wall-to-wall contact, F TraN apparently interacts with the receptor OmpA, possibly in conjunction with lipopolysaccharide, in the recipient cell, to aid in the formation of stable mating contacts, allowing for highly efficient DNA transfer. R100 TraN apparently requires a different outer membrane protein for mating pair stabilization. Alternatively, R100 TraN could interact with multiple and different receptors in the recipient cell, including OmpA, and would only require one receptor in order for function. Although a number of cells that lack various outer membrane proteins were examined for a specific ConR100$^-$ defect, none appeared to inhibit R100-1 mating efficiency more than F mating efficiency. The specific R100 TraN receptor remains unknown.

The region of TraN that interacts with OmpA is apparently encoded, at least in part, within the first 399 aa of the protein, probably in small extracellular loops. The single mutation in OmpA (G154D) that disrupts MPS is in the 4th extracellular loop of the protein, near to a bound and co-crystallized LPS moiety (Ried and Henning, 1987; Pautsch and Schultz, 1998). Since mutations in *waaP* that affect PPEA addition to the inner core of the LPS molecule also affect MPS, this suggests one of two things (Anthony *et al.*, 1994). Either LPS is required for proper OmpA insertion and folding in the outer membrane, or that TraN specifically binds to both OmpA and LPS (Achtman *et al.*, 1978b). Mutations in LPS do affect the levels of OmpA in the OM (Havekes *et al.*, 1976). However, addition of PEA to the medium of mating cells decreased transfer efficiency of the F plasmid, suggesting that this moiety is part of the TraN receptor (Anthony *et al.*, 1994). Although the topological analysis presented here suggests that TraN interacts with OmpA through a number of small extracellular loops, further studies are needed in order to determine the correct topology of TraN. Insertion of a small peptide encoding a trypsin sensitive cleavage site into TraN, and cleavage by exogenous trypsin would allow more definitive assignments of TraN

extracellular segments. A chimeric construct of F and R100 TraN would also aid in analysis of the regions of F TraN that interact with OmpA.

Mating pair stabilization appears to require the C-terminal periplasmic domain of F TraG, although the function of TraG or TraG* in MPS is currently unknown (Manning *et al.*, 1981). TraG did not appear to interact with TraN as determined by crosslinking analysis, although that experiment was done with vegetatively growing cells, and not with donor cells in the process of mating. Potentially the C-terminal domain of TraG or TraG* might still interact with TraN in order to bring about mating pair stabilization.

Three different mating pair stabilization systems appear to exist in conjugative plasmids (Bradley *et al.*, 1980). F-like plasmids encode TraN and TraG proteins that mediate interactions with the recipient cell, as well as flexible/retractable pili that facilitate liquid matings. IncI plasmids encode a type IV adhesive pilus that appears to interact with recipient cell LPS moieties in order to stabilize cells (Ishiwa and Komano, 2000). The IncP systems are surface-dependent mating systems, and have not evolved mating pair stabilization functions.

7.2 TraN and TraG Function in DNA Transport. It is apparent in examining the phenotypes of ConF$^-$ and *traN* and *traG* donor cells, that the defects in mating stabilization functions in the donor cell are quite severe, while the defects in the recipient cell are less severe and can be rescued by mating on a solid surface (Achtman *et al.*, 1978b). *traN* and *traG* mutations are somewhat rescued on a solid surface (approximately 10- to 100-fold; Manning *et al.*, 1981). This suggests that TraN and TraG both have additional functions in DNA transport during conjugation. This was further supported by the finding that both *traN* and *traG* genes appear within a virulence island in the *Neisseria gonorrhoeae* chromosome (Dillard and Seifert 2001; Hamilton *et al.*, 2001; Dillard, personal communication). Disruption of either gene abolishes DNA transport into the medium, as well as having effects on virulence (Dillard, personal communication). This suggests that TraN and TraG function directly in DNA transport since *N. gonorrhoeae* extrudes DNA directly into the

medium, and not into a recipient cell. The DNA in the media is taken up by a separately encoded competence system. The function of TraN and TraG during DNA transport is not known. Possibly they aid in formation of the mating channel that connects the donor and recipient cell, not solely in the process of mating pair stabilization but through a separate mechanism as well.

7.3 Surface Exclusion. Since the surface exclusion protein TraT, appears to inhibit mating aggregate formation when present in the recipient cell, an analysis was done of the plasmid-specific nature of that protein (Achtman *et al.*, 1977). Surprisingly, TraT inhibited pOX38*N1*::CAT transfer equally well whether F TraN or R100 TraN was expressed in the donor cell. This was unexpected as $TraT^+$ cells allow normal pilus tip binding, but prevent stable shear-resistant mating pairs from forming, which suggested that TraT would block the TraN-specific step of MPS (Achtman *et al.*, 1977). TraT specifically inactivated some OmpA-specific phages, suggesting it blocked or inhibited binding of the phage to OmpA. The function of TraT therefore, remains elusive. It may function in a subtle mechanism that is not detectable in the assays used, or it may be specific for another plasmid transfer protein, although what that may be is unknown. It has been suggested that TraT functions in the disaggregation of stable mating pairs once plasmid transfer is complete (Achtman et al., 1978a). If this is true, then an analysis of stable mating pairs with a defective TraT gene should reveal that the mating aggregates persist for a longer period of time. TraT is also associated with serum resistance, suggesting that it prevents formation of the membrane attack complex of complement (Sukulpovi and O'Connor, 1990). Perhaps the two functions are similar. TraT may have evolved primarily as a means of disaggregating the cells after conjugation, and its role in surface exclusion and complement resistance is just an extension of that role.

7.4 Entry Exclusion. The finding that TraG is specific for the TraS protein in the recipient cell was surprising as well (Anthony *et al.*, 1999). The question arises, how does an inner membrane protein in the recipient cell interact with an inner membrane protein or periplasmic product in the donor cell. Recent evidence from our laboratory has suggested that the region of TraG

responsible for TraG-TraS interactions is in the C-terminal domain (Manchak, unpublished data). These results suggest that either TraG is transported into the recipient cell during conjugation, or that TraS is transported into the donor cell during conjugation. Since no other protein appears to be involved in entry exclusion, perhaps functioning as an intermediate in Eex, it is puzzling how this could be accomplished. It is possible that during normal conjugation TraG is moved into the donor cell where it helps establish mating pair formation. TraS, when resident in the recipient cell, interferes with this function, thereby excluding the donor cell from transporting DNA. This becomes all the more puzzling when it is known that entry exclusion blocks events that signal the relaxosome to initiate DNA transfer, while mutations in *traG* had no effect on this process (Kingsman and Willetts, 1978). This suggests that the mating signal is transmitted through TraG to the relaxosome proteins. If TraG must be transported into the recipient cell, where TraS interacts with it, then how does TraG normally transmit the mating signal to the transferosome? Instead, does a mating signal normally transmit through TraG in the cell envelope in the donor cell to the relaxosome, and TraS thereby inhibits this when it is transferred into the donor cell. Since TraS is already resident in the donor cell, expressed from the donor cell F plasmid, this suggests that a much more complex process is going on. Further work on characterizing the interactions of TraG and TraS, perhaps by determining protein-protein interactions by crosslinking during conjugation, or by yeast two-hybrid or bacterial two-hybrid analysis. The transport of either TraG into the recipient cell, or TraS into the donor cell, during conjugation, also remains to be determined.

If TraG is indeed communicating with the relaxosome, then this suggests that TraG must interact with one of the relaxosome proteins in order to signal DNA unwinding, either directly or indirectly. The two proteins that are responsible for DNA unwinding are TraI, the helicase, and TraM, which promotes DNA transfer, but has no effect on the relaxation complex of TraI, IHF, and TraY. TraG may interact with these proteins to signal DNA unwinding, Alternatively TraG could be interacting with the relaxosome through the TraD IM DNA transporter, which is required for DNA transport, and has an effect on donor conjugal DNA synthesis (Kingsman and Willetts, 1978). TraD and

TraM interactions have also been discovered, suggesting that this might be the normal course of events (Disque-Kochem and Dreiseikelmann, 1997). It would be expected that this would be a transient contact during the conjugation process, making the process of elucidating these interactions difficult. The disruption of TraG function by addition of GFP to the N-terminus suggests that the cytoplasmic N-terminal tail of TraG might be important in mediating these interactions. TraG was not found to be involved in any higher molecular weight complexes as the concentrations of BS3 we used in this study. Perhaps by use of alternative crosslinking reagents with different specificities or different spacer lengths, the nature of these interactions can be determined. TraG has not been found to interact with any other transfer protein by yeast two-hybrid analysis (Silverman, personal communication).

7.5 Localization and Limitation of TraG. The localization of TraG with the GFP fusion marker is interesting in that the fusion exhibited cell envelope localization and only a small number of foci appeared. Although absolute conclusions based on the unstable GFP-TraG fusions cannot be made, the results are suggestive that a limited number of TraG containing foci appear in the cell, apparently independently of other transfer proteins. This is unlike the TrhC-GFP fusion that exhibited many more foci per cell than shown here (Gilmour *et al.*, 2001). TrhC is the TraC homologue of the IncH plasmid R27. Based on the analysis of the expression levels from pBADTraG having an effect on mating efficiency levels, this suggests that the levels of TraG may be limiting for transfer efficiency. Since TraG is apparently required for pilus tip formation, this suggests that TraG may be a limiting factor in assembly of the initial complex that is capable of tip formation. Analysis of the protein complex assumed to be formed by the subset of transfer proteins involved in pilus tip synthesis, including TraE, TraL, TraK (TraV-TraK-TraB), and TraC, perhaps by crosslinking analysis or bacterial two-hybrid analysis, should help determine if this is the case. If TraG is limiting, then mutations in the proximal part of *traG* should have effects on the other members of this complex.

7.6 Disulfide Bonds and Protein-Protein Interactions. The sequence analysis of TraN homologues revealed a large number of conserved cysteine residues. As far as we know, no other periplasmic or outer membrane protein contains such a large number of cysteine residues. The discovery that TraN contains both intramolecular disulfide bonds and intermolecular disulfide bonds revealed the stabilizing nature of the crosslinking nature of TraN. These findings suggest that TraN adopts a fairly rigid and crosslinked structure in the outer membrane. A number of other transfer proteins, including most of the proteins involved in the transferosome that reside in the periplasm and outer membrane also contain conserved cysteine residues, suggesting that part of the transferosome might be made up of intermolecular disulfide-bonded proteins. The fact that the Vir system contains a number of disulfide linked proteins suggests that these two type IV systems share one common theme in the transferosome proteins that make up the mating channel.

The fact that TraV is required for TraN stability sheds some new light on the process of MPS as well by indicating that TraN is linked to the pilus synthesis machinery. A TraV-TraK-TraB group of interacting proteins has been discovered by yeast two-hybrid analysis (Harris *et al.*, 2001). This group of proteins has been suggested to form a nucleation complex for other proteins involved in pilus synthesis. Since TraV appears to direct TraN stability, this suggests that TraV interacts with TraN. In the *A. tumefaciens* T-DNA transport system, VirB7 forms homodimers and heterodimers with VirB9, and the heterodimer mediates the stability of a number of other components in the Vir mating bridge (Anderson *et al.*, 1996; Fernandez *et al.*, 1996; Spudich *et al.*, 1996). Recently it has been shown that two conserved cysteines in TraV, which appear to be homologous to the two cysteines in VirB7, affect the insertion of TraN into the outer membrane when mutated (Harris *et al.*, 2001; Silverman, personal communication). This suggests that TraV and TraN are linked directly through a disulfide bond, and this linkage assures proper TraN insertion into the outer membrane. These results also suggest that although the VirB and F plasmid systems do not appear to encode periplasmic and outer membrane proteins that are homologous to each other, that they at least appear to function in an analogous manner. Since TraN appears to be involved

in a number of higher molecular weight complexes, these interactions may help elucidate the additional functions of TraN that are not involved in interactions with OmpA in the recipient cell.

TraN has also been found to be involved in self-interactions in a yeast two-hybrid analysis (Silverman, personal communication). The minimal amount of the protein that was picked up when *traN* was used as a bait corresponds to residues 63-305. This suggests that TraN is involved in homomultimerization, and as indicated by our crosslinking analysis, possibly as homotrimers. This also suggests that the divergent region in TraN might be involved in self-recognition.

7.7 The Type IV Secretin. Since a number of other outer membrane proteins in transport systems require a lipoprotein for stability, namely the secretins, an attempt at seeing if TraN had a number of secretin phenotypes was examined. This was in part prompted by the complete lack of a known outer membrane pore for DNA transport or pilus secretion. Our results suggest that TraN is not the secretin involved in DNA transport. Even though the recent finding that in the type III secretion system, secreted proteins are ejected from the tip of the needle or pilus itself, the pilus must be extruded through the outer membrane secretin (Jin *et al.*, 2001). If DNA can pass through an extended pilus, then the pore complex involved in conjugation is probably involved in pilus synthesis. Candidates include TraV or TraH. TraH is required for pilus elongation, and is outer membrane associated, although it does not appear to be cell-surface exposed (Manwaring, Ph.D.). An analysis of cell membrane envelopes for large multimeric structures that are resistant to denaturation could determine the type IV secretin. Analysis of F mutants that lack this multimeric structure would then pinpoint the exact transfer protein that forms this complex.

7.8 Similarity Between Type III, VirB, and F Plasmid Transporters. Although the TTSS and type IV systems do not share homologous proteins, the morphological similarity between the type III needle complex, the VirB system, and the F system suggests that analogous functions may be found.

The type III secretion system appears to have an ordered stepwise pathway for construction of the transenvelope complex (Kimbrough and Miller, 2000; Sukhan *et al.*, 2001). The first step involves the Sec-dependent lipid modification and outer membrane insertion of InvH. InvH appears to bind InvG and aid in outer membrane localization of the secretin composed of an InvG multimeric complex (Daefler and Russel, 1998). The inner membrane complex of PrgH and PrgK forms, and interacts with the outer membrane secretin, resulting in stabilization of the entire complex. The inner membrane components including the InvC ATPase can associate with PrgH/K and promote the polymerization of the needle subunit PrgI. Once properly polymerized PrgI is processed through the outer membrane secretin, the InvJ protein functions in a transport switch (Kubori *et al.*, 2000). If InvJ does not function, then the needle elongates uncontrollably, leading to a needle complex incapable of functioning in protein secretion. Once the switch is made, the complete transporter translocates effectors that form membrane pore in the host cell (*Yersinia* YopB/D; Tardy *et al.*, 1999). Although needle complexes themselves are capable of penetrating host cell membranes, insertion of YopB/D apparently aids in movement of secreted effectors into the host cell.

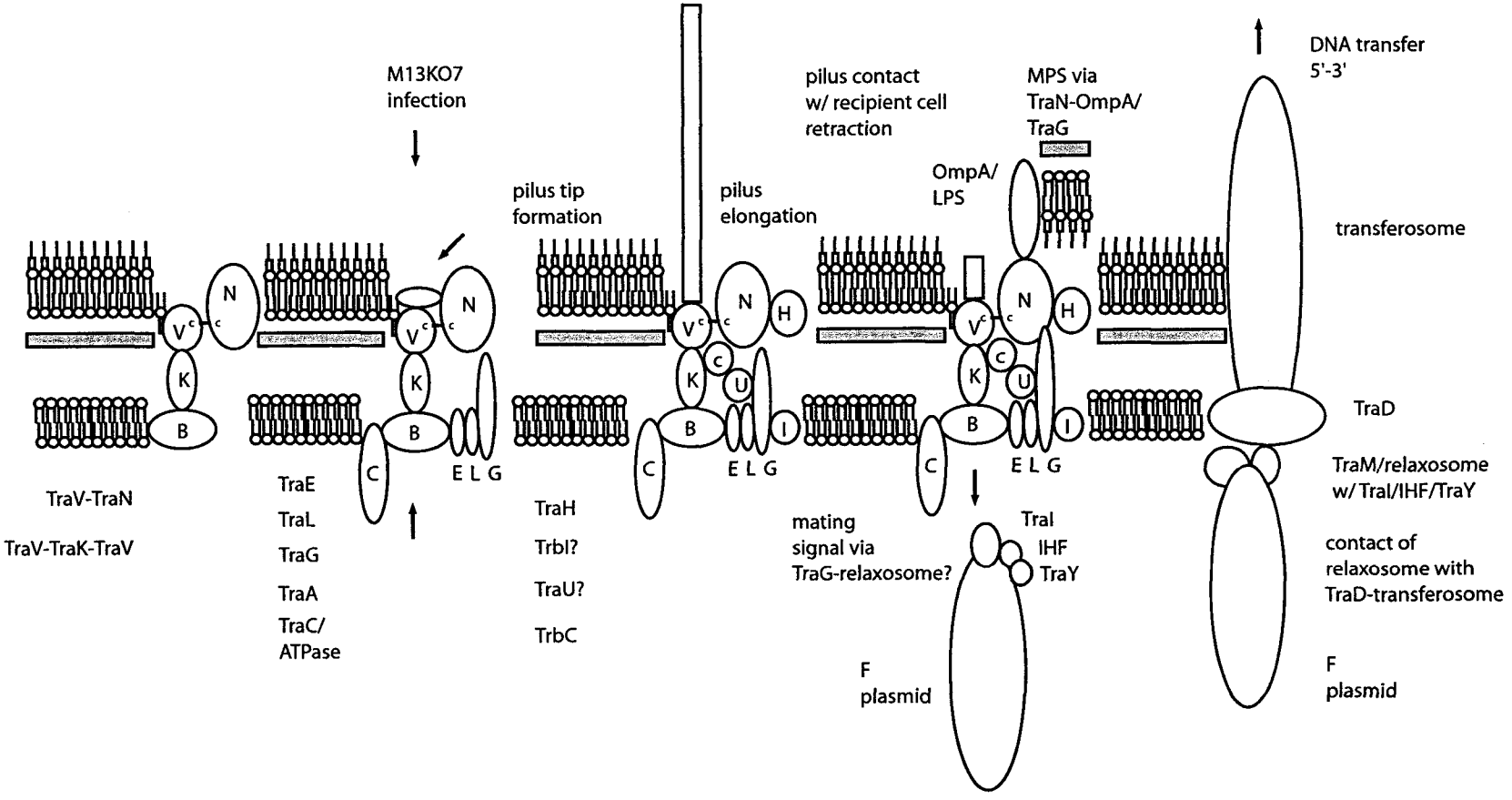
The type IV VirB transporter appears to assemble in a similar fashion (Christie, 2001; Sagulenko *et al.*, 2001; see Introduction). The similarities include the formation of a lipoprotein-outer membrane protein complex (VirB7-VirB9) that stabilizes a number of other proteins. The outer membrane complex then interacts with two inner membrane proteins VirB8 and VirB10 and promotes VirB10 oligomerization. This transenvelope-spanning complex, in conjunction with theVirB4 ATPase, can function as a structural pore based on the following two observations. The homologous proteins ComB4, and ComB7-B10 form a competence structure in the cell envelope that allows the entry of DNA into the cell in *H. pylori* (Hofreuter *et al.*, 2001). This subset of proteins, possibly in conjunction with VirB6, promotes the uptake of the mobilizable plasmid RSF1010 when they are expressed in the recipient *A. tumefaciens* cell (Bohne *et al.*, 1998). Polymerization of the pilus subunit can now occur, possibly when the VirB11 ATPase associates

with the structure. Secretion of VirE2 into plant cell membranes results in pore formation, and the VirD2-T-strand complex is transported by the VirB structure in conjunction with VirD4. Coating of the ss DNA by VirE2 in the plant cell leads to localization of the T-strand to the plant cell nucleus by signals in VirE2 and VirD2.

The model for how the F plasmid transporter forms is also similar to the TTSS needle complex and VirB transporter (Figure 7.1). TraV is lipid modified and interacts with TraN through intermolecular disulfide bonds. TraV interacts with periplasmic TraK and inner membrane TraB, stabilizing them. The inner membrane components TraE, TraL, TraG, and the TraC inner membrane associated ATPase can interact, and form the pilus tip at the outer membrane surface with the pilin subunit. Pilus elongation requires the additional periplasmic proteins TrbC, TraU, and the outer membrane protein TraH. The inner membrane protein TrbI mediates elongation or possibly retraction. The pilus interacts with the recipient cell, and retracts, bringing the cells into close contact. TraN interacts with OmpA/LPS to enable MPS. TraT, if present in the recipient cell, can inhibit this step. TraG or TraG* promotes MPS by an unknown mechanism. A mating signal is sent to the relaxosome, possibly through TraG, promoting DNA unwinding in preparation for DNA transfer, a step that also requires TraM complex formation at the *oriT* region. TraS can inhibit this step if it is present in the recipient cell. DNA transport in conjunction with the IM DNA transporter, TraD, results in ss DNA transferred to the recipient cell in a 5' to 3' manner.

The complex formation of InvH-InvG secretin suggests a similar mechanism in Vir and F, VirB7-VirB9 and TraV-TraN, respectively. Is VirB9 the secretin in VirB secretion? TraN does not appear to encode a similar function in F, suggesting another outer membrane associated protein functions in this respect. Interaction between InvG-PrgH/K and VirB7-B9 and IM B8/B10 suggests that the B8/B10 complex forms the inner membrane rings. Could the TraK/TraB proteins in the F system function in a similar manner?

Figure 7.1. Formation of the F plasmid transferosome and DNA transport. The F plasmid transferosome is assembled in a stepwise fashion. TraV becomes lipidated, and forms an intermolecular disulfide bond with TraN. TraV inserts into the outer membrane, leading to localization of TraN. TraV also associates with periplasmic TraK and TraK associates with TraB in the inner membrane. TraE, TraG, and TraL in the inner membrane also associate with this complex, TraC becomes associated in the presence of the other transfer proteins, and this entire complex in conjunction with the pilin subunit is capable of forming a pilus tip structure at the outer membrane surface. Further association of outer membrane TraH, and periplasmic TrbC and TraU function in the elongation of the pilus. Pilus elongation/retraction is controlled by inner membrane TrbI. The pilus can interact with a recipient cell and retract. Mating pair stabilization occurs through TraN-OmpA-LPS interactions, possibly in association with the C-terminal domain of TraG*. The surface exclusion protein, TraT, when present in the recipient cell can interfere with stabilization mechanisms. A mating signal is now sent, supposedly through the TraG protein, to the relaxosome to signal DNA unwinding. TraS, when present in the recipient cell can interfere with signaling. TraM binds to sbmC and promotes DNA unwinding and DNA transfer, possibly by binding to the inner membrane DNA transporter, TraD. TraD facilitates DNA movement through the transferosome and possibly through the lumen of the now retracted pilus structure still residing in the donor cell envelope. The localization of each protein is based on experimental as well as predicted data. Contact between each subunit does not indicate protein-protein interactions and is merely used for compactness. The only known protein-protein interactions are TraV-TraN, TraV-TraK-TraB, and TraM-TraD. Oligomerization is not indicated.



Filament formation from a polymerized subunit, PrjI, VirB2 pilin, TraA pilin in the TTSS, Vir, and F systems, respectively, now occurs. Filament control is mediated by another protein, InvJ and TrbI in the TTSS and F systems, respectively. The uncontrolled growth of the needle in *invJ* mutants has been suggested to result from the inability to switch between secretion of the needle subunit PrjI, and secreted effectors, while TrbI has been suggested to function in control of pilus elongation or retraction. Perhaps these systems function similarly, and TrbI actually controls polymerization of pilin into the pilus structure. It is not known if a similar mechanism exists in the VirB system.

Secreted effectors can apparently traverse the internal hollow tube of the filament in TTSS. This suggests that pore complex components such as YopB/D and VirE2 can be transported in a similar mechanism. This also suggests that the VirD2-T-strand can be transported through the lumen of the pilus as well. No F plasmid encoded protein has ever been found to be transported during conjugation. Perhaps the F system does transport a pore complex into the recipient cell membrane and this has just not been discovered yet.

It will be very interesting with all of the advances in recent years in our understanding of transenvelope complexes, and all of the sequence data being generated by large scale sequencing projects, to see exactly how widespread transfer genes occur.

Chapter 8

Appendix

8.1. Prediction of transmembrane segments of F TraN.

This algorithm uses 5 primary, and 4 secondary rules (Gromiha *et al.*, 1997). Conformational and surrounding hydrophobicity are presented in Table 8.1.

Primary rules.

P1. Conformational parameters of a single amino acid residue.

if β_i is greater than or equal to 1.0, then prior=1

if β_i is less than 1.0, then prior=0

P2. Average conformational parameter of a 6-residue window.

if average of 6 residues (β_I) is greater than or equal to 1.0, then prior=1

if average of 6 residues (β_I) is less than 1.0, then prior=0

P3. Hydrophobicity of a single amino acid residue.

if $(h_p)_i$ is greater than or equal to 13.34, then prior=1

if $(h_p)_i$ is less than 13.34, then prior=0

P4. Average hydrophobicity of a six residue window.

if average of 6 residues $(h_p)_I$ is greater than or equal to 13.34, then prior=1

if average of 6 residues $(h_p)_I$ is less than 13.34, then prior=0

Table 8.1. Conformational parameters and surrounding hydrophobicity of amino acid residues.

Amino acid	Conformational parameters[a]	Surrounding hydrophobicity[b]
Alanine	0.991	13.85
Asparate	0.680	11.61
Cysteine	0.000[c]	15.37
Glutamate	0.836	11.38
Phenylalanine	1.016	13.93
Glycine	0.844	13.34
Histidine	1.152	13.82
Isoleucine	1.210	15.28
Lysine	1.296	11.58
Leucine	1.139	14.13
Methionine	1.296	13.86
Asparagine	0.591	13.02
Proline	0.192	12.35
Glutamine	1.188	12.61
Arginine	0.960	13.10
Serine	0.981	13.39
Threonine	0.987	12.70
Valine	1.315	14.56
Tryptophan	1.729	15.48
Tyrosine	1.459	13.88

a. Conformational parameters of all 20 amino acid residues was generated by computing the frequency of occurrence of an amino acid residue in the transmembrane segment of an outer membrane protein as compared to the frequency of occurrence in the whole protein (Gromiha *et al.*, 1997).
b. Surrounding hydrophobicity scale was generated as described in Ponnuswamy and Gromiha, 1993.
c. When scanning outer membrane proteins of known structure for the presence of cysteine residues, none were found.

P5. Amphipathic nature of 2 residues.

if average of 2 residues $(h_p)_l$ is 13.34 +/- 0.5, then prior=1

else, then prior=0

Secondary rules

S1. If a residue is priority 5, then a transmembrane segment could exist around that residue. Extend length of segment in both N- and C-terminal directions until two consecutive low priority (prior < 3), or a residue of zero priority occurs.

S2. If the priorities of two consecutive residues is 4, then extend the segment according to S1. If three residues of high priority are consecutive, then consider a short segment of 6 residues.

S3. If the priorities of 3 consecutive residues is greater than or equal to 3, then examine surrounding residues. If there are more than 3 residues in the high range, then consider a segment around that residue.

S4. If the segment extends more than 20 residues, cut it into smaller segments are the residue with highest hydrophobicity. If the segment is 10-14 residues, then make it 16 residues to correlate the 6 and 2 residue window rules.

Prediction of F TraN

The five primary rules were applied to F TraN (Figure 8.1). The predicted transmembrane segments were calculated using the secondary rules, and are shown in Figure 4.7.

Figure 8.1. Prediction of F TraN transmembrane segments. The algorithm of Gromiha *et al.*, (1997) was used to calculate the propensity of a given amino acid residue to participate in a transmembrane segment according to the 5 primary rule sets. F TraN reads from aa 1 in the upper left, to aa 602 in the bottom right. The total of the 5 rules is given to the right of each amino acid residue. The 4 secondary rules were applied and generate the output present in Figure 4.7.

FtraN	Total	I	4	D	1	V	4	I	4	A	3	K	3	N	0	D	0	G	3	L	3	F	3
M	4	P	1	I	3	E	1	V	5	F	3	L	4	P	2	K	2	Y	4	E	1	N	1
K	2	G	2	L	3	Q	3	D	2	T	2	T	1	A	3	A	3	S	2	K	2	R	1
R	1	Y	3	S	2	Y	4	A	4	S	3	K	1	C	4	Q	2	N	1	K	2	L	3
I	4	N	1	P	0	C	2	H	4	S	3	T	0	T	3	S	2	C	1	R	3	D	0
L	4	A	2	D	0	T	2	Y	5	H	4	E	0	L	4	G	1	C	2	S	3	F	3
P	2	N	0	A	3	R	2	S	3	P	2	C	2	V	4	K	1	K	2	Y	3	T	1
L	4	P	0	P	2	T	2	W	4	V	5	T	0	S	4	S	2	D	1	C	3	N	1
I	4	D	1	F	4	A	3	S	3	R	1	E	0	H	5	N	0	S	3	Q	3	F	3
L	4	E	1	I	2	R	2	R	2	V	3	A	2	Q	4	D	0	G	3	F	3	Y	3
A	3	T	1	Q	1	M	3	T	2	G	3	G	2	C	3	F	4	W	4	D	1	E	0
L	4	K	3	T	2	E	0	Y	4	N	2	G	3	A	2	A	2	G	3	S	3	D	1
V	4	Y	4	G	3	L	4	L	4	T	1	N	2	F	2	E	1	Q	3	K	4	L	3
A	4	Y	5	R	0	Q	4	Q	2	K	2	R	2	Y	3	A	3	D	2	L	4	M	4
G	2	G	3	D	2	G	3	K	2	F	3	T	2	S	1	V	4	I	4	A	4	N	1
M	3	G	2	V	3	S	3	S	2	K	2	L	3	E	0	S	4	G	4	Q	3	N	0
A	2	V	3	V	3	T	1	V	4	R	2	V	4	E	1	Q	4	L	4	I	4	Q	1
Q	2	T	1	N	2	T	1	P	2	H	4	K	2	G	3	L	4	A	1	V	4	K	2
A	1	A	2	R	3	W	4	M	5	T	2	D	1	A	3	A	3	K	1	Q	1	I	4
D	0	G	2	A	3	E	0	T	2	A	3	G	4	C	3	A	2	C	2	Q	1	P	0
S	2	G	1	D	1	T	2	I	4	M	4	Q	4	L	3	L	4	S	2	Q	2	D	0
N	1	D	0	S	2	R	2	T	1	K	4	S	4	H	3	A	1	S	1	G	2	S	4
S	1	G	2	I	3	T	2	V	3	L	5	Y	5	E	1	A	2	D	0	R	1	G	2
D	0	G	2	V	2	L	3	L	4	R	3	S	4	Y	4	A	3	E	1	N	2	V	4
Y	3	L	3	G	2	E	1	G	3	L	4	M	4	A	3	G	2	K	2	G	4	L	4
R	1	K	1	N	1	Y	3	T	1	V	3	Y	5	T	2	K	2	A	2	Q	4	T	1
A	2	N	0	T	2	E	0	P	2	V	4	Q	4	Y	4	D	2	L	3	L	5	Q	2
G	2	D	1	G	3	M	4	L	5	R	2	S	4	S	3	V	3	A	2	R	1	K	3
S	2	G	3	Q	2	S	2	S	3	V	4	C	3	C	2	A	2	K	2	I	4	V	4
D	2	T	1	Q	3	Q	2	W	4	K	2	W	4	E	0	A	2	A	2	S	3	K	2
F	4	T	1	C	4	L	3	N	2	K	1	A	2	S	2	L	4	K	2	F	4	E	1
A	4	E	2	S	3	P	1	A	2	A	4	Y	4	K	2	N	2	S	3	G	2	Q	3
H	4	W	3	A	3	A	2	K	2	S	4	R	1	T	2	G	3	N	1	S	2	I	3
Q	2	A	2	Q	2	R	0	Y	4	Y	5	D	1	S	4	V	5	K	4	A	1	A	2
I	4	T	1	E	1	E	2	S	2	T	2	T	2	G	3	D	1	L	5	K	1	D	1
K	2	G	1	I	4	V	5	A	1	P	3	Y	4	K	4	V	5	T	2	H	3	Q	3
G	2	E	1	S	3	N	3	D	0	Y	4	V	4	V	4	R	1	V	4	P	0	L	4
Q	4	T	2	R	1	G	4	A	3	V	4	T	1	M	3	A	3	S	2	D	0	K	2
G	4	G	2	S	2	Q	4	S	2	V	4	Q	2	V	3	F	4	V	3	C	4	Q	3
S	4	K	2	E	1	Y	4	F	3	W	2	S	2	C	3	T	2	G	1	R	2	A	3
S	4	T	1	Y	4	V	4	T	0	S	1	A	1	G	3	G	3	E	1	G	1	G	3
S	3	I	4	T	1	V	3	P	2	E	0	D	0	G	2	Q	3	F	3	I	3	Q	2
I	2	T	0	N	1	S	2	V	4	S	2	K	2	D	1	A	2	C	3	T	2		
Q	2	E	1	Y	3	I	3	Q	2	C	2	G	3	V	3	K	2	S	2	V	4		
G	2	S	2	T	1	T	1	K	2	P	1	T	2	F	2	F	3	K	2	D	1		
F	2	F	2	C	1	S	2	T	3	F	4	C	3	C	1	C	2	K	4	E	1		
K	1	M	3	E	1	P	1	L	5	S	2	Q	1	L	3	K	2	V	4	L	5		
P	0	N	0	R	1	V	5	T	3	K	2	T	1	D	0	K	3	L	5	Q	2		
Q	1	K	1	D	2	T	1	A	4	E	1	Y	3	G	1	A	3	G	2	K	3		
E	0	P	0	L	4	G	1	G	3	L	4	T	0	E	0	A	2	V	3	I	4		
S	2	K	1	Q	3	E	3	V	4	G	2	D	0	C	2	A	3	C	2	Q	2		

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