The mating pair stabilization protein, TraN, of the F plasmid is an outer-membrane protein with two regions that are important for its function in conjugation

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Received 10 March 2005

Revised 18 April 2005

Accepted 25 April 2005

F plasmid TraN (602 aa, processed to 584 aa with 22 conserved cysteines), which is essential for F plasmid conjugation, is an outer-membrane protein involved in mating pair stabilization (MPS). Unlike R100 TraN, F TraN requires OmpA in the recipient cell for efficient MPS. The authors have identified three external loops (aa 172–187, 212–220 and 281–284) in the highly divergent region from aa 164 to aa 333 as candidates for interaction with OmpA. These loops were identified using both site-directed and random TnphoA/in mutagenesis to insert epitopes (31-aa or c-myc) into TraN and monitor their effect on sensitivity to external proteases and on mating ability. TraN is a hallmark protein of F-type IV secretion systems as demonstrated by BLAST searches of the databases. The C-terminal region is highly conserved and contains five of the six completely conserved cysteines. Mutation of these residues to serine demonstrated their importance in TraN function. TraN appears to require both intra- and intermolecular disulfide bond formation for its stability and structure as demonstrated by its instability in a *dsbA* mutant and its aberrant migration on SDS-polyacrylamide gels under non-reducing conditions or by cross-linking with bis(sulfosuccinimidyl)suberate (BS3). Thus, F TraN appears to have two domains: the N-terminal region is involved in OmpA interaction with OmpA during MPS; and the C-terminal region, which is rich in conserved cysteine residues, is essential for conjugation.

INTRODUCTION

The F plasmid mediates conjugative transfer of DNA from donor to recipient cells in a process that requires the conjugative pilus and the associated proteins involved in a transenvelope complex that has been grouped with type IV secretion systems (T4SS; Lawley *et al.*, 2003). T4SS are involved in protein translocation, DNA uptake and extrusion as well as conjugation in Gram-negative bacteria (reviewed by Cascales & Christie, 2004).

The F-like plasmids contain all of the genes involved in F-pilus synthesis and DNA transfer in the 33.3 kb transfer (*tra*) region (Frost *et al.*, 1994). The *tra* operon encodes genes for the pilin subunit (*traA*); pilin maturation (*traQ*, -X); the core T4SS proteins involved in pilus assembly and DNA transport [*traB*, -C, -E, -G (N-terminal region), -K, -L, -V]; the essential F-specific genes [*traF*, -G (C-terminal

region), -H, -N, -U, -W, *trbC*]; auxiliary F-specific genes (*traP*, *trbI*, *orf169*); the regulatory genes (*traJ*, -Y, *finO*, -P); DNA processing (*traI*, -M, -Y) and transport (*traD*); mating pair stabilization (*traG*, -N) and exclusion (*traS*, -T); as well as genes of unknown function (*traR*, *trbA*, -B, -D, -E, -F, -G, -H, -I, -J; Lawley *et al.*, 2003). The F-pilus is essential for transfer and initiates contact with a recipient cell, causing pilus retraction and close cell-to-cell contact. Mating pair stabilization is thought to involve both TraN in the outer membrane, and the inner-membrane protein TraG (Manning *et al.*, 1981), possibly by constructing the pore for DNA transfer (Kingsman & Willetts, 1978).

F TraN, which is an essential component for DNA transfer (Klimke & Frost, 1998) is 602 aa in length and is processed to 584 aa during transport to the outer membrane (Maneewannakul *et al.*, 1992b). Genetic analysis has revealed that TraN recognizes lipopolysaccharide in the recipient cell. In addition, F TraN, but not the closely related R100 TraN, apparently interacts with OmpA in the recipient cell, thereby increasing the efficiency of mating. This specificity for OmpA mapped to a central region of F TraN (aa 164–333) that differed significantly from R100

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Abbreviations: BS3, bis(sulfosuccinimidyl)suberate; HRP, horseradish peroxidase; T4SS, type IV secretion system(s).

TraN in sequence (Klimke & Frost, 1998). A small in-frame deletion near the C-terminus of F TraN (aa 503–521) was found to severely affect TraN function (Maneewannakul *et al.*, 1992b; Klimke & Frost, 1998).

The topology of TraN was examined using 31-aa epitope fusions generated by insertion and partial deletion of IS*phoA*/in at permissive sites (Manoil & Bailey, 1997) as well as by the insertion of the c-myc epitope into predicted extracellular loops. Using antibodies directed against these epitopes, the orientation in the outer membrane, disulfide bond formation and participation in multimeric protein complexes were investigated. Mating efficiency data combined with computer alignments of 12 TraN homologues revealed that three loops in the specificity region are exposed on the exterior of the cell and are probably involved in OmpA recognition. A conserved region near the C-terminus of the protein which contains five of the six highly conserved cysteines is important for TraN function.

METHODS

Strains, phages, plasmids, and growth conditions. The bacterial strains, plasmids, vectors and phage used in this study are listed in Table 1. *Escherichia coli* strains were grown in LB broth [Luria-Bertani medium; 1% tryptone (Difco), 0.5% yeast extract (Difco), 1% NaCl (BDH)] with shaking to mid-exponential phase, or left standing overnight, and subsequently diluted 1:100 in fresh medium, and grown at 37 °C with appropriate antibiotics unless otherwise noted. Antibiotics (Sigma) were added at the following concentrations: ampicillin (Amp) 50 µg ml⁻¹; chloramphenicol (Cm) 20 µg ml⁻¹; kanamycin (Km) 25 µg ml⁻¹; spectinomycin (Sp) 100 µg ml⁻¹; streptomycin (Sm) 200 µg ml⁻¹; tetracycline (Tc) 10 µg ml⁻¹; rifampicin (Rif) 50 µg ml⁻¹. Determination of mating efficiency was as described by Klimke & Frost (1998).

Enzymes, reagents, and DNA manipulations. All DNA restriction and modifying enzymes were from Roche. Vent Pol (proofreading +) was from NEBiolabs. Qiagen kits were used to purify plasmid DNA. 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. Primers for amplification of *traN* derivatives for use as recombinant templates were LFR56 (Klimke & Frost, 1998) and LFR110 (5'-GCGGATCCGAGCTGCAGGGGAG-TACCAC-3'). pKI375N1::CAT, pAKN317 and pAKN465 (see Table 2) were used as templates. Sequencing of IS*phoA*/in inserts was performed using primer AKE3, which is identical to primer Tnp*phoA*-I of Manoil & Bailey (1997). Mutagenesis of selected cysteine residues was performed using the QuikChange kit (Stratagene) according to the manufacturer's directions.

Multiple sequence alignment of TraN sequences. Sequences similar to F TraN were found by using the BLAST algorithm to search the NCBI Microbial Genomes Database (<http://www.ncbi.nlm.nih.gov/genomes/static/eub.html>). Fifteen TraN homologues come from finished genomes and plasmids: *E. coli* F TraN, gi 730985; *E. coli* p1658/97 gi 32469994; *E. coli* pC15-1a gi 38606135; *Salmonella typhimurium* R100 TraN, gi 2226076; *S. typhimurium* pSLT gi 17233459; *S. typhimurium* pED208 gi 22086658; *Vibrio vulnificus* pYJ016 gi 37595836; *V. vulnificus* CMCP6 (chromosome II) gi 27367071; *S. typhimurium* DT104 (genomic) gi 12719013; *Providencia rettgeri* R391 gi 20095129; *Pseudomonas resinovorans* pCAR1 gi 27228603; *Proteus vulgaris* Rts1 gi 21233906;

Salmonella typhi R27 gi 10957195; *Serratia marcescens* R478 gi 38347857; *Novosphingobium aromaticivorans* pNL1 gi 10956935, and *Neisseria gonorrhoeae* (genomic) TraN gi 58891388. All TraN sequences underwent multiple alignment with CLUSTALW (<http://clustalw.genome.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).

Construction of F and R100 TraN chimeras. F *traN* was transferred into pBAD24 (Guzman *et al.*, 1995) by amplifying the gene, including the *traN* ribosome-binding site (RBS), from pBK184N by PCR to give pBAD24N. This allowed *EcoRI* and *HindIII* sites to be introduced 5' and 3' to the gene, respectively. A unique *KpnI* site was introduced into pBAD24N at the codons for aa 349 and 350 by Quikchange mutagenesis (Stratagene). Using pBK8-2818 (Klimke & Frost, 1998) as a template, the coding sequence for either the first 365 aa (including the R100 *traN* RBS) or the remainder of R100 TraN (aa 365–617) was amplified by PCR with the appropriate restriction sites incorporated into the primers. These fragments were used to replace the front (aa 1–350) or back (aa 351–602) portions of F TraN in pBAD24N to give pBAD24N-RF and pBAD24N-FR, respectively. The resulting clones were sequenced to ensure their validity. Cells containing pBAD24, pBAD24N (wild-type F TraN) or one of the chimeras was grown to 0.05 OD₆₀₀ and induced with 0.2% arabinose for 1 h before mating.

IS*phoA*/in transposition into *traN*. Insertion of IS*phoA*/in into TraN-expressing plasmids was done according to Manoil & Bailey (1997). *E. coli* CC160 was transformed with either pKI375B⁻ or pBK184N and grown as standing overnight cultures at 37 °C. The OD₆₀₀ was determined and 0.2 ml of cell culture was aliquoted into a fresh tube. λTnp*phoA*/in was added at an m.o.i. of 0.1–0.3 phage per cell. MgSO₄ 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 min at 37 °C and then 0.8 ml 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⁻¹) and Cm (100 µg ml⁻¹) for cells containing pKI375B⁻, or Km (25 µg ml⁻¹) and Cm (100 µg ml⁻¹) for cells containing pBK184N. After 2 days growth at 30 °C, bacteria from the entire plate were collected and 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 (Roche) and either Amp (100 µg ml⁻¹) and Cm (40 µg ml⁻¹) for pKI375B⁻ derivatives or Km (25 µg ml⁻¹) and Cm (40 µg ml⁻¹) 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.

Conversion of IS*phoA*/in insertions to in-frame 31-codon fusions. 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 QIAquick 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.

Homologous recombination to construct pOX38 derivatives. pOX38*traN* derivatives were generated using PCR amplification and recombination of PCR products in strain DY330Rif^R (Yu *et al.*, 2000). Two types of derivatives were made: pOX38N1::CAT-Tc or -Km were initially generated, and then replacements of the CAT cassette in *traN* with DNA amplified from pAKN317 or pAKN465

Table 1. Bacterial strains, plasmids and phage

Strain, plasmid or phage	Properties	Reference/source
<i>E. coli</i>		
ED24	Spc ^R , F ⁻ Lac ⁻	Achtman <i>et al.</i> (1971)
XK100	Spc ^R derivative of BL21(DE3) carrying T7 RNA polymerase under <i>lacUV5</i> control	Studier & Moffat (1986)
DH5 α	<i>supE44</i> Δ <i>lacU16</i> (ϕ 80 <i>lacZ</i> Δ 80M15) <i>hsdR17</i> <i>recA1</i> <i>endA1</i> <i>gyrA96</i> <i>thi-1</i> <i>relA1</i>	Hanahan (1983)
MC4100	Sm ^R , <i>araD139</i> Δ (<i>argF-lac</i>)U169 <i>rpsL150</i> <i>relA1</i> <i>flbB3501</i> <i>deoC1</i> <i>ptsF25</i> <i>rbsR</i>	C. Manoil (lab collection)
JC3272	Sm ^R , F ⁻ <i>lac</i> Δ X74 <i>gal</i> <i>his</i> <i>trp</i> <i>lys</i> <i>rpsL</i> <i>tsx</i>	Achtman <i>et al.</i> (1971)
DY330	W3110 Δ <i>lacU169</i> <i>gal490</i> λ cI857 Δ (<i>cro-bioA</i>)	Yu <i>et al.</i> (2000)
DY330Rif ^R	Rif ^R derivative of DY330	Lawley <i>et al.</i> (2002)
CC118	<i>araD139</i> Δ (<i>ara-leu</i>)7697 Δ <i>lacX74</i> <i>phoA20</i> <i>galE</i> <i>galK</i> <i>thi</i> <i>rpsE</i> <i>rpoB</i> <i>argE_{am}</i> <i>recA1</i>	C. Manoil (lab collection)
CC160	<i>araD139</i> Δ (<i>ara-leu</i>)7697 Δ <i>lacX74</i> <i>phoA20</i> <i>galE</i> <i>galK</i> <i>thi</i> <i>rpsL</i> <i>dam</i>	Manoil & Bailey (1997)
CC245	<i>supF</i> <i>supE</i> <i>hsdR</i> <i>galK</i> <i>trpR</i> <i>metB</i> <i>lacY</i> <i>tonA</i> <i>dam::kan</i>	Manoil & Bailey (1997)
RI89	MC4100 <i>phoR</i> - Δ <i>ara714</i> Leu ⁺	Rietsch <i>et al.</i> (1996)
RI90	RI89 <i>dsbA::kan</i>	Rietsch <i>et al.</i> (1996)
RI179	RI89 <i>dsbC::Cam</i>	Rietsch <i>et al.</i> (1996)
CC277	Km ^R , JC3272 <i>ompA::Tn5</i>	Klimke & Frost (1998)
CS2198	Km ^R , CS180 <i>rfaJ19::Tn_{lacZ}</i>	Klimke & Frost (1998)
Vectors and constructs		
pBAD24	Amp ^R , arabinose-inducible promoter	Guzman <i>et al.</i> (1995)
pBAD24N	F TraN from pBK184N in pBAD24	This study
pBAD24N-FR	F TraN RBS, aa 1–350 fused to R100 TraN aa 366–617	This study
pBAD24N-RF	R100 TraN RBS, aa 1–365 fused to F TraN aa 35–602	This study
pBS-KS+	Amp ^R , cloning vector, 3.0 kb	Stratagene
pK184	Km ^R , 2.4 kb cloning vector, p15a replicon	Jobling & Holmes (1990)
pKI375	Amp ^R , F <i>traN</i> in pBS-KS ⁺	Maneewannakul <i>et al.</i> (1992b)
pBK8-2818	Amp ^R , R100 <i>traN</i> in pBS-KS ⁺	Klimke & Frost (1998)
pKI375N1::CAT	Amp ^R , <i>BbrPI/EcoRV</i> of pKI375 (<i>traN</i>) replaced with CAT cassette	Klimke & Frost (1998)
pKI375B ⁻	pKI375 with unique <i>Bam</i> HI site removed	This study
pBK184N	3.0 kb <i>EcoRI/HindIII</i> of pKI375 containing (<i>trbC</i> , <i>traN</i> , <i>trbE</i>) in pK184	This study
Conjugative plasmids		
pOX38	IncFI, Tra ⁺ , RepFIA ⁺ , fl <i>HindIII</i> fragment of F	Guyer <i>et al.</i> (1980)
pOX38::Km	Km ^R , pOX38 + <i>HindIII</i> fragment of Tn5	Chandler & Galas (1983)
pOX38::Tc	Tc ^R , pOX38::miniTn10	Anthony <i>et al.</i> (1994)
pOX38N1::CAT	Cm ^R , <i>traN1::CAT</i> in pOX38	Klimke & Frost (1998)
pOX38N1::CAT-Km	Cm ^R , <i>traN1::CAT</i> in pOX38-Km	This study
pOX38N1::CAT-Tc	Cm ^R , <i>traN1::CAT</i> of pOX38-Tc	This study
pOX38N317-Km	TraN-G317 crossed into pOX38-Km	This study
pOX38N317-Tc	TraN-G317 crossed into pOX38-Tc	This study
pOX38N465-Km	TraN-K465 crossed into pOX38-Km	This study
pOX38N465-Tc	TraN-K465 crossed into pOX38-Tc	This study
pOX38- <i>tra715</i>	T7 promoter replaces Py	Maneewannakul <i>et al.</i> (1992a)
Phage		
λ Tn ϕ oA/in	IS ϕ oA/in, Cm ^R Neo ^R	Manoil & Bailey (1997)

to form single-copy 31-codon insertion mutants of *traN*. Typically a mating was 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 into cells at concentrations up to 1 mg DNA per transformation. pOX38N1::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 *EcoRI* site is present in the CAT cassette). The pOX38N1::CAT derivatives were

mated out of the DY330Rif^R strain to MC4100 or ED24 and *in trans* complementation with pKI375 was used to demonstrate the correct insertion of the CAT cassette into *traN*. Once correct insertion had been established, the pOX38N1::CAT-Km or -Tc derivatives were mated into DY330Rif^R and selected for on plates containing Cm and Rif. TraN 31-aa insertion mutants in pAKN317 or pAKN465 were amplified by PCR and single-copy recombinants were generated as above, except that after recombination to replace the CAT cassette had taken place, the recombinants were mated into MC4100 cells for 1 h 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.

Insertion of the c-myc epitopes at precise sites in TraN-V548. The modified c-myc epitope (KRRKEQKLISEEDL, c-myc epitope underlined) was introduced into *traN*-V548 using a two-step procedure. Two primers of sequence 5'-19-22 nucleotides-NNN-AAGAGGAAGAAAGAGCAGAAGCTCATCTCTGAAGAGGAC-CTA-3' and 3'-N'N'N'-TTCTCCTTCTTCTCGTCTTCGAGTAGA-GACTTCTCCTGGAT-19-22 nucleotides-5' were used. NNN and N'N'N' are the complementary sequences of the codon immediately preceding the insertion site. The underlined sequences are complementary to each other and represent the KRKK-c-myc coding sequence. The 19-22 nucleotide extensions represent sequences complementary to either side of the site of insertion and have a T_m of approximately 52 °C. For each mutation, the two primers were melted and annealed together. They were then annealed to pBKN584 (see Table 2), following the Quikchange protocol (see above). Potential mutants were sequenced and checked for TraN expression using antibodies to the 31-aa epitope or c-myc (Roche).

Trypsin accessibility experiments. Trypsin digestion of osmotically sensitized or whole cells is a modification of the procedure described by Merck *et al.* (1997). Briefly, *E. coli* ED24 cells containing pOX38N1::CAT and expressing various 31-aa insertion mutants of TraN were grown to an approximate OD₆₀₀ of 0.5. Cells were pelleted at 8000 r.p.m. for 2 min at 4 °C and resuspended at an OD₆₀₀ of 0.5 in 0.5 ml of either 50 mM Tris/HCl, pH 8.0, 10 mM MgCl₂ (whole cells) or 50 mM Tris/HCl, pH 8.0, 0.5 M sucrose (osmotically sensitized cells). Cells were incubated on ice for 20 min; then half the reaction was removed and trypsin (Roche) was added to a final concentration of 25 µg ml⁻¹. Cells were incubated on ice for 20 min, and then 6 µl of a solution of Complete Protease Inhibitor was added [Roche; one pellet per 1.5 ml Milli-Q water (Millipore double-distilled and deionized H₂O)] 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 1 × sample buffer, and 8 µl (equivalent to an OD₆₀₀ of 0.1) was loaded onto a 10% SDS-PAGE gel.

Isolation of cell membranes. Cells were grown to an approximate OD₆₀₀ of 0.5 in 200 ml LB plus appropriate antibiotics. Cells were pelleted at 3000 r.p.m. for 10 min at 4 °C in a Sorvall GSA rotor. Cells were resuspended in 10 mM Tris/HCl, pH 7.5, 50 µg RNase ml⁻¹, 100 U DNase I (Roche), and 100 µl Complete Protease Inhibitor solution. Cells were disrupted by three passages through an Aminco French press at 13 000 p.s.i. (90 MPa). The unbroken cells were removed by centrifugation at 3500 r.p.m. for 15 min in a Sorvall SS34 rotor, and the total membranes were pelleted at 15 000 r.p.m. for 45 min.

Density flotation gradient 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 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 35 000 r.p.m. for 72 h. Fractions of 750 µl were collected from the top. The refractive index, protein content, and NADH oxidase activity levels of all odd-numbered fractions were measured (Osborn *et al.*, 1972).

Antibodies. Antibodies to the C-terminal region of OmpA were a gift from Dr Glen Armstrong, University of Alberta. Antibodies to the C-terminal region of TraG were a gift from Dr Neville Firth,

University of Sydney (Firth & Skurray, 1992). Initial experiments with 31-aa insertion mutants of TraN were performed with anti-31-aa antibodies from Colin Manoil, University of Washington. The peptide CS3317 BbNle-GGTEPFPSIQGDPRSDQET-CONH₂ 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 & Bailey (1997) except for the presence of a glycine linker, and the blocked C-terminus. Polyclonal antiserum was derived from a rabbit immunized with KLH-CS3317 conjugate. Anti-c-myc antibodies were purchased from Sigma.

SDS-PAGE and immunoblots. Proteins were separated by SDS-PAGE and immunoblots were performed as described by Gubbins *et al.* (2002). Anti-31-aa antibodies were used at 1/2000 to 1/7500 dilution in 10% skim milk. Anti-OmpA antibodies were used at 1/10⁶ dilution in 5% BSA (Roche) to avoid cross-reaction. Anti-rabbit IgG HRP conjugate (Amersham Pharmacia Biotech) was used at 1/5000 to 1/10 000 dilution for detection of anti-31-aa antibodies, or 1/30 000 dilution for detection of anti-OmpA antibodies. The membranes were washed three times as above, and bands were detected by adding a chemiluminescent reagent (Western Lighting Chemiluminescence Reagent Plus; NEN Life Sciences) for 2 min. Membranes were exposed to X-ray film (Kodak) for 1 min. The procedure for stripping and reprobing membranes with a different primary antibody was as described by Gallagher *et al.* (1997).

In vivo cross-linking. Cross-linking was performed on pOX38-Km- or pOX38N1::CAT/ED24-containing cells with or without various 31-aa insertion mutants of TraN using the cross-linker BS3 [bis(sulfosuccinimidyl)suberate; Sigma]. Cells were grown to mid-exponential phase and immediately chilled on ice. After 10 min, a volume of cells corresponding to an OD₆₀₀ of 1.0 was pelleted at 8000 r.p.m. for 2 min in a refrigerated microfuge. The cells were washed twice with 1 ml ice-cold 50 mM HEPES (BDH) buffer, pH 8.0, 10 mM MgCl₂. One hundred microlitres 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 1 M Tris/HCl, pH 7.5, for 15 min at room temperature and the cells were pelleted twice at 8000 r.p.m., at 4 °C. The supernatant was removed, and the cell pellets were resuspended in 1 × sample buffer. Proteins were separated on a 3.5%/7.5% stacking/separating SDS-PAGE, and transferred to an Immobilon-P membrane (Kazmierczak *et al.*, 1994). The membrane was cut in half at the 79 kDa marker; the top half was probed with anti-31-aa antibodies (1/2000) and anti-rabbit HRP conjugate (1/5000; Amersham Pharmacia Biotech), and the bottom half was probed with anti-31-aa antibodies (1/5000) and anti-rabbit HRP conjugate (1/10 000; Amersham Pharmacia Biotech). The membrane was reassembled prior to exposure to X-ray film.

RESULTS

The N-terminal region of F TraN is involved in OmpA recognition

Several attempts were made to further delineate the region of F TraN involved in OmpA recognition (Klimke & Frost, 1998) by constructing chimeras of the F and R100 TraN proteins. Most attempts led to plasmid instability, which probably reflects the difficulty of fusing portions of two outer-membrane proteins of unknown structure without disrupting essential motifs for membrane insertion and stability. Two chimeras were successfully constructed using the conserved amino acids (aa) 349 and 350 in F

TraN and aa 364 and 365 in R100 TraN (Klimke & Frost, 1998). The codons for aa 349 and 350 were mutated to introduce a unique *KpnI* site that was used to construct the chimeras (see Methods). pBAD24N-FR and pBAD24N-RF, along with the appropriate controls, were checked for their mating efficiency into cells without (CS2198) or with (CC277) an *ompA* mutation as previously described by Klimke & Frost (1998). pBAD24N-FR, with an F N-terminal sequence, required OmpA for efficient mating whereas pBAD24N-RF, with an R100 N-terminal sequence, did not. This confirmed that the sequences involved in OmpA recognition by F are in the first 350 aa of the protein and that changes in sequence in the C-terminal regions of F and R100 TraN are not important for this function.

Transposon mutagenesis of F *traN*

TnphoA/in transposon mutagenesis was used to generate permissive *PhoA* fusions to TraN (Manoil & Bailey, 1997). This system allows for the conversion of *PhoA* fusions to insertion mutants, which can then be characterized using antibodies directed towards the 31-aa epitope. Initially, a derivative of pKI375 (pKI375B⁻) was used to generate *PhoA* fusions, which tended to be unstable, presumably due to the high copy nature of the parent vector. A 3.0 kb fragment containing *trbC*, *traN* and *trbE* was transferred from pKI375 to pKI184 to create pBK184N, which was subsequently used to generate 45 *ISphoA*/in insertions at 12 unique sites in *traN*, as well as a number of insertions in *trbC* and *trbE* (to be reported elsewhere). Twenty unique insertions in total were generated in *traN* from both plasmids, and of these, 14 were

Table 2. Properties of *ISphoA*/in and 31-aa epitope insertions in *traN*

Plasmid/insertion	Number of		Position/mutation‡	Mating efficiency (%)§
	Insertions*	Converted†		
pOX38N1::CAT	NA	NA	No TraN	0.004
pOX38N317-Km	NA	NA	G317	5.0
pBK184N	NA	NA	Wild-type	69.2
pKI375B ⁻	NA	NA	Wild-type	10.0
pKI375B⁻ derivatives				
NA	1	No	K62	—
NA	1	No	A255	—
NA	1	No	Y331	—
pAKN317	1	Yes	G317	15.3
pAKN319	1	Yes	R319	22.2
pAKN465	1	Yes	K465	5.0
NA	1	No	A543	—
NA	1	No	K544	—
pBK184N derivatives				
pBKN42	1	Yes	I42	35.7
NA	6	No	G66	—
pBKN197	1	Yes	T197	5.0
pBKN255	2	Yes	A255	27.5
pBKN288	3	Yes	Y288	69.2
pBKN337	1	Yes	Y337	39.1
pBKN361	24	Yes	T361	23.0
pBKN397	1	Yes	G397	15.8
pBKN398	1	Yes	G398	10.0
pBKN442	1	Yes	D442	10.0
pBKN498	1	Yes	G498	0.05
pBKN584	3	Yes	V584	27.2

NA, Not applicable.

*Number of independently isolated and sequenced inserts.

†Yes or No, ability to be converted to 31-aa epitope fusions.

‡Position indicates the amino acid in TraN directly preceding the 31-aa epitope.

§pOX38N1::CAT/ED24 plus plasmids expressing TraN-31-aa mutants were the donors; MC4100 was the recipient. Mating efficiency is defined as transconjugants/100 donor cells and represents the mean of three determinations which were within 20 % of each other. Wild-type (pKI375B⁻ or pBK184N) gave 1.5×10^8 transconjugants/ 5×10^8 donor cells, on average.

converted to stable 31-aa epitope insertion mutants of TraN, with four in the N-terminal region and ten in the C-terminal region. These were tested for their ability to complement pOX38N1::CAT in a mating assay (Table 2). Surprisingly, only TraN-G498, which was in a highly conserved C-terminal region of TraN, was deficient for complementation (see below).

Single-copy versions of these mutants were constructed by replacing the CAT cassette inserted into *traN* of two F derivatives, pOX38N1::CAT-Km and pOX38N1::CAT-Tc in *E. coli* DY330(Rif^R), with two different 31-aa insertions from pAKN317 and pAKN465 by gene replacement (Yu *et al.*, 2000). pOX38N317-Km and -Tc as well as pOX38N465-Km and -Tc exhibited wild-type levels of mating efficiency (Table 2 and data not shown) and expressed lower levels of TraN, as expected from single-copy plasmids (data not shown).

Topological analysis of 31-aa insertions of F TraN

TraN in whole cells is normally resistant to trypsin (Maneewannakul *et al.*, 1992b) whereas the 31-aa epitope confers sensitivity (Lee *et al.*, 1999). Thirteen 31-aa insertion mutants were examined for sensitivity to trypsin in whole and osmotically permeabilized cells (Fig. 1). All of the insertion mutant proteins tested were resistant to trypsin when expressed in whole cells, but were digested completely when the cells were osmotically sensitized, suggesting that all of the insertion sites in TraN are located in the periplasm. Cells transformed with pAKN319 grew poorly and were not tested.

Although cells expressing the various insertion mutant proteins were treated identically, there was consistent variation in the levels of the epitope-tagged TraN present (Fig. 1) that did not necessarily correlate with TraN function as measured by complementation assays (Table 2). TraN was not induced in these experiments, as background leaky expression from the constructs was found to be sufficient for complementation. Thus, the presence of the 31-aa insert appeared to affect TraN stability but not necessarily TraN function.

An *in vivo* degradation product associated with the presence of the 31-aa insertion was found for most constructs (marked with an asterisk in Fig. 1). As the site of the 31-aa insertion moved towards the C-terminus (not shown for inserts after D442), the degradation product decreased in size, suggesting that *in vivo* degradation occurred at the site of insertion. However, the major trypsin cleavage product found in osmotically sensitized cells was not related to this *in vivo* degradation product as demonstrated for TraN-G397 (marked with an arrow in Fig. 1).

To test whether wild-type TraN (no 31-aa insertion) is intrinsically sensitive to trypsin cleavage at periplasmic sites, the cleavage pattern of wild-type TraN in osmotically sensitized *E. coli* XK100 cells containing pKI375 (*traN*) or pOX38-*tra715* (the entire *tra* operon; Maneewannakul *et al.*, 1992a) was examined. Cells were labelled with [³⁵S]methionine, in the presence of rifampicin, after which the cells were treated with trypsin as above. TraN was not degraded in whole cells but was degraded by trypsin when the cells were osmotically sensitized, suggesting that wild-type TraN has trypsin-sensitive sites located in the periplasm (data not shown).

Fig. 1. Trypsin sensitivity of TraN-31-aa fusions. Whole cells (10 mM MgCl₂) and osmotically sensitized cells (0.5 M sucrose) expressing 31-aa fusions (indicated above each subpanel) were treated with trypsin under identical conditions. The band corresponding to the 31-aa epitope-tagged TraN is indicated on the left. Molecular mass markers (kDa) are shown on 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.

TraN, expressed from pKI375 in maxicells in the absence of F, was found to be sensitive to proteinase K after 18 h digestion in the presence of 250 µg enzyme (Maneewannakul *et al.*, 1992b). When whole cells expressing TraN-V584, in the presence or absence of pOX38N1::CAT (supplying the rest of the *tra* gene products), were treated with 25 or 250 µg ml⁻¹ of proteinase K, only TraN-V584 in the presence of pOX38N1::CAT was degraded within 20 min (data not shown). Thus the presence of the F transfer region appears to have a role in orienting TraN in the outer membrane such that it is proteinase K-sensitive but trypsin-resistant.

Insertion of the c-myc epitope in TraN-V584

The topology for TraN was predicted using a sliding-window algorithm that measures the propensity of amino acid residues to form the amphipathic β -strands usually found spanning the outer membrane (Gromiha *et al.*, 1997). The topology for both F and R100 TraN was considered and the model also took into account the data suggesting a periplasmic location for the 31-aa epitope fusions. This model was used to predict possible extracellular loops in TraN (data not shown, but see below).

The c-myc epitope (underlined), along with an enhanced trypsin cleavage site (KRKKEQKLISEEDL) was inserted into

TraN-V584, cloned into pBKN584, using QuikChange mutagenesis (Stratagene; see Methods). The c-myc epitope was fused to TraN at ten positions initially predicted to be within extracellular loops, and two positions strongly predicted to be in the periplasm (T201, G316). Inserts at A69, M180, L216, F243, V281, S387 and E555 were stably expressed whereas inserts at T201, G316, A430, A450 and K487 could not be isolated after numerous attempts. Except for pBKN584-A69, all of the c-myc epitope fusions were reduced in their ability to complement pOX38N1::CAT, especially TraN-V584-L216 (Table 3). Insertion of the c-myc epitope did not affect the stability of these derivatives of TraN-V584 as assayed by immunoblotting with anti-31-aa and anti-c-myc antibodies (data not shown). The already low mating efficiencies with these fusions precluded testing the effect of an *ompA* mutation in the recipient cells.

Each derivative of TraN-V584 carrying a c-myc epitope was assayed for its sensitivity to trypsin as described above for the 31-aa fusions. All of the c-myc epitope-tagged TraN mutants were sensitive to trypsin (Fig. 2) except TraN-V584-F243, which was resistant, indicating a possible periplasmic location. The inability to insert a c-myc epitope at T201 and G316, in regions strongly predicted to be in the periplasm, suggested that the elusive inserts at A430, A450 and K487 might also be in the periplasm, a model that is consistent with the 31-aa epitope data. Furthermore, G316,

Table 3. Orientation and mating efficiency of c-myc insertion mutants in TraN-V584

Plasmid	Amino acid position*	Stable isolates†	Orientation in outer membrane‡	Mating efficiency (%)§
pBKN584	No insert	NA	NA	100
pBKN584-A69	A69	Yes	Out*	86
pBKN584-M180	M180	Yes	Out	0.2
NA	T201	No	NA	NA
pBKN584-L216	L216	Yes	Out	0.003
pBKN584-F243	F243	Yes	In	0.02
pBKN584-V281	V281	Yes	Out	0.55
NA	G316	No	NA	NA
pBKN584-S387	S387	Yes	Out	0.36
NA	A430	No	NA	NA
NA	A450	No	NA	NA
NA	K487	No	NA	NA
pBKN584-E555	E555	Yes	Out	0.05

NA, Not applicable.

*Position indicates the amino acid in TraN directly preceding the c-myc epitope fusion.

†Yes or No, ability to be converted to c-myc epitope fusions.

‡Orientation was determined by sensitivity of whole cells to trypsin. 'Out' is exposed on the cell surface; 'In' is exposed to the periplasm.

§pOX38N1::CAT/ED24 plus pBKN584 and its derivatives carrying c-myc fusions were the donors; MC4100 was the recipient. Mating efficiency is defined as transconjugants/100 donor cells and represents the mean of three determinations which were within 20% of each other. Wild-type (pBKN584) gave 0.4×10^8 transconjugants/ 5×10^8 donor cells, on average.

Fig. 2. Stability and trypsin sensitivity of TraN fused to the c-myc epitopes. Whole cells (10 mM MgCl₂) expressing TraN-c-myc fusions (indicated above each subpanel) were treated with trypsin under conditions identical to those described in Fig. 1. The amount of trypsin in each lane is shown below the figure.

A430, A450 and K487 were in cysteine-rich regions, suggesting a role for disulfide bond formation.

Disulfide bond formation in TraN

Previously, a comparison of the sequences of F and R100 TraN revealed three main regions, with the N- and far C-terminal regions being highly conserved (aa 1–163, and 334–602 in F), and a central divergent region (aa 164–333). The presence of 20 conserved cysteines between F and R100 was also noted (Fig. 3a; Klimke & Frost, 1998). Using BLAST analysis of the Microbial Genomes Database, 12 TraN homologues from the Proteobacter group of Gram-negative organisms were aligned, with the idea that highly conserved regions might be important for function (Fig. 3b).

The three distinct regions described for F and R100 TraN were present in all homologues; however, the extreme C-terminal region (aa 584–602 of F TraN) was not universally conserved. All 22 cysteine residues in F TraN were conserved in a majority of the other homologues, with each cysteine found at the same position in 50 % or more of all TraN proteins. Nineteen of the 22 conserved cysteines were found in the latter half of the protein. Six of the cysteines (C147, C478, C501, C509, C517 and C548) are absolutely conserved, with five residing in the most highly conserved C-terminal region (Fig. 3a). Interestingly, the site of insertion of the 31 aa epitope at G498, which perturbed its function, mapped to this region, as did the deleterious in-frame deletion reported by Maneewannakul *et al.* (1992b).

The presence of such a large number of cysteines suggested a highly cross-linked structure, which could require DsbA and DsbC, the periplasmic disulfide oxidoreductase and isomerase enzymes, respectively (Bardwell *et al.*, 1991; Missiakas *et al.*, 1994; Shevchik *et al.*, 1994). The presence of disulfide linkages in TraN was determined by analysing TraN-G317 with or without the reducing agent DTT via SDS-PAGE. The mobility of TraN-G317 monomer was slightly increased under nonreducing conditions, suggesting a more compact conformation due to intramolecular disulfide bonds (data not shown, but see Fig. 4). Higher molecular mass complexes were also observed for the highly expressed TraN-V584 that are indicative of intermolecular disulfide bonds (see below). The role of DsbA and DsbC in disulfide bond formation was examined by immunoblot analysis of the TraN-G317 protein in *dsbA* or *dsbC* strains. The levels of TraN-G317 were diminished significantly in the *dsbA* but not the *dsbC* strain (data not shown). Therefore, disulfide bonds in TraN appear to require DsbA, which contributes significantly to the stability of the protein, whereas DsbC, which is important in rearranging disulfide bonds, is not required.

Mutational analysis of six conserved cysteines in TraN

Based on evidence from BLAST analyses of TraN homologues as well as lower complementation ability of deletion mutants (Klimke & Frost, 1998) and some 31-aa epitope-tagged

Fig. 3. Conserved regions and cysteine residues, and sites of 31-aa and c-myc fusions in TraN. (a) Schematic diagrams of F TraN. The N- and C-termini are marked. In the top half of the diagram, the positions of cysteines in F TraN are indicated above and below the line, with the six absolutely conserved cysteines marked by asterisks. The highly conserved C-terminal region is shaded grey. In the bottom half of the diagram, the conserved regions of F TraN (black) and the divergent regions (grey), as compared to R100 TraN, are indicated below the figure. Fifteen sites of insertion of *TnpH* are indicated below the line, with long black arrows indicating successful conversion to TraN-31-aa fusions; the long clear arrow (G66) indicates a site of unsuccessful conversion of a low-copy-number mutant of pBK184N. The 12 sites chosen for c-myc epitope insertion are indicated above and below (F243) the line. The short black arrows indicate the seven sites of successful insertion and the short clear arrows indicate insertion sites that could not be isolated. Arrows above the line represent epitopes that were predicted or shown to be exposed outside the cell; those below the line indicate sites predicted or shown to be within the periplasm. (b) Multiple sequence alignment. The highly conserved C-terminal region of 16 homologues displaying less than 95 % identity to each other were chosen and multiply aligned with CLUSTALX. TraN homologues found in the chromosome (and not on a plasmid or known integrative element such as R391) are shown in bold. The five absolutely conserved cysteines in this region are marked with asterisks. The positions of the TraN-G498 and TraN-V584 insertions are marked with arrows. Residues conserved at 100 % are shaded black, those at 80 % are shaded dark grey, and those at 60 % are shaded light grey (allowing for conservative substitutions). The gi numbers for each entry are given in Methods.

TraN mutants, six conserved cysteines in TraN-V584, at positions 147, 478, 501, 509, 517 and 548, were converted to serine using QuikChange (Stratagene) mutagenesis. All of the mutants expressed comparable levels of protein (data not shown) and were tested for their ability to complement pOX38N1::CAT in a mating efficiency assay (Table 4). All of the mutants had reduced complementation ability, with TraN-V584C548S showing the greatest reduction. Double mutants involving C501, C507, C509 and C517 were also constructed, with the double mutant TraN-V584C509S/C517S exhibiting the greatest reduction in complementation ability. Thus the highly conserved cysteines in TraN are

important for TraN function, possibly by engaging in disulfide bond formation with TraN itself or with other F- or host-encoded proteins.

Outer-membrane localization of TraN

The subcellular localization of the insertion mutants TraN-G498, which had a reduced ability to complement pOX38N1::CAT, and TraN-V584 was examined by flotation density-gradient centrifugation to separate the inner and outer membranes (data not shown). Both TraN-G498 and TraN-V584 were found to co-localize with the

Table 4. Complementation ability of TraN-V584 carrying C to S mutations

pOX38N1::CAT plus	Relevant genotype	Mating efficiency*	Percentage wild-type†
Alone	—	0·0003	0·001
pBKN584	TraN-V584	14·4	100
Single C to S mutants			
pBKN584C147S	TraN-V584C147S	0·25	1·7
pBKN584C478S	TraN-V584C478S	0·2	1·5
pBKN584C501S	TraN-V584C501S	0·2	1·7
pBKN584C509S	TraN-V584C509S	0·8	5·4
pBKN584C517S	TraN-V584C517S	0·05	0·4
pBKN584C548S	TraN-V584C548S	0·01	0·1
Double C to S mutants			
pBKN584C501S/C509S	TraN-V584C501S/C509S	0·3	2·3
pBKN584C501S/C517S	TraN-V584C501S/C517S	0·05	0·3
pBKN584C509S/C517S	TraN-V584C509S/C517S	0·0006	0·004

*Mating efficiency (ME) is expressed as transconjugants/100 donors.
†Percentage wild-type is calculated as: (ME_{TraN mutant}/ME_{TraN wild-type}) × 100.

outer-membrane marker OmpA. Thus, the effect of the 31-aa insertion on TraN function at position G498 cannot be attributed to a defect in outer-membrane localization. A small amount of TraN-V584 protein was found in the inner membrane fractions, as detected by NADH oxidase activity, probably due to the relative abundance of the TraN-V584 protein.

The localization of the small amounts of TraN present in *dsbA::kan* cells was also examined to determine whether the instability of TraN was due to defective localization of TraN in the outer membrane. All of the TraN-G317 protein was found in the outer-membrane fractions in cells carrying the *dsbA::kan* mutation and pOX38N1::CAT/pAKN317 (data not shown), suggesting that *dsb::kan* mutation affected the stability but not the cellular localization of TraN.

Multimerization of TraN-V584

The *in vivo* multimeric state of a number of 31-aa fusions was examined, including TraN-I42, TraN-Y337 and TraN-V584, using a variety of cross-linking reagents, such as formaldehyde, DSP [dithiobis(succinimidyl propionate)] and BS3. Under most conditions, monomeric TraN disappeared, and a large molecular mass complex was found at the interface of the stacking and separating gels (data not shown). Using the insertion mutant TraN-V584, which expressed large amounts of TraN, and lower concentrations of the membrane-impermeant cross-linker BS3, putative multimeric complexes were observed (Fig. 4). At low concentrations of BS3 (50–100 µM), five bands migrating with approximate molecular masses of 99, 162, 177, 185 and 196 kDa were detected, whereas at higher concentrations of BS3 (200 µM to 2 mM) a band appeared at the interface of the stacking and separating gels. Lower-mobility bands did not appear in controls that

Fig. 4. *In vivo* cross-linking of TraN-V584. (a) ED24 cells containing pBKN584/pOX38N1::CAT were grown to mid-exponential phase and treated with various concentrations of the cross-linker BS3. Lanes 1–7, increasing BS3 concentration: 1, no BS3; 2, 50 µM BS3; 3, 100 µM; 4, 200 µM; 5, 500 µM; 6, 1 mM; 7, 2 mM. Lane 8, cells taken prior to cross-linking and resuspended in sample buffer without DTT. The molecular mass markers are indicated on the right. Higher molecular mass bands found with BS3 cross-linking, or without reducing agent, are marked with an arrow along with their estimated molecular mass (kDa) in italics. The arrow marked 'Top' indicates the interface between stacking and separating gels. A non-specific band is designated NS. The degradation product of TraN-V584 is marked with an asterisk. The positions of the reduced and oxidized forms of TraN-V584 monomer are indicated on the left and right of the figure, respectively.

lacked TraN-V584 upon treatment with BS3 (data not shown).

It was possible that the five bands observed were various combinations of TraN-V584 and its degradation products. Examination of two other insertion mutants (TraN-I42 and TraN-Y337), which displayed different *in vivo* degradation patterns (Fig. 1), revealed the same cross-linking pattern, indicating that the degradation products probably do not contribute to the cross-linked species (data not shown).

Under non-reducing conditions during SDS-PAGE (Fig. 4, lane 8), most of the TraN-V584 monomer exhibited a faster mobility, as seen previously for TraN-G317 (data not shown). Because of the greater abundance of TraN-V584, two bands of high molecular mass, similar to the bands at approximately 177 and 185 kDa found in the BS3-cross-linked samples, were also observed. The presence of these bands was also confirmed for two other 31-aa epitope-tagged derivatives, TraN-Y337 and TraN-I42 (data not shown), suggesting the presence of both intramolecular and intermolecular disulfide bonds.

Attempts to identify the members of the large multimeric species were unsuccessful. Cross-linking experiments using TraN-V584 in the presence of various *Flac* mutants gave the same results as described above. Similarly, immunoblot analysis using available antiserum (anti-TraG, -V, -B) did not identify these proteins in the complexes. We conclude that these complexes are composed of TraN associated with other unidentified transfer or host proteins.

DISCUSSION

TraN is an outer-membrane protein that is essential for F-mediated bacterial conjugation. Based on genetic evidence, F TraN in the donor cell recognizes OmpA in the recipient cell and could act as a cell-surface adhesin during mating pair stabilization. The region of F TraN involved in OmpA recognition has been previously mapped to the first 350 aa, whereas the region of OmpA involved in this process maps to the fourth extracellular loop (Manoil & Rosenbusch, 1982). This study was aimed at better defining the topology of TraN, using genetic and computer-aided analyses, in order to delineate which regions of the protein are involved in OmpA recognition, mating pore formation and TraN structure and stability.

Genome sequencing projects have revealed a number of genetic elements that are homologous to the F transfer genes in conjugative plasmids, mobile genomic elements and virulence or pathogenicity islands in the chromosomes of many Gram-negative organisms in the Proteobacter group (Lawley *et al.*, 2003, 2004). TraN, along with the associated genes TraF, -G (C-terminal region), -H, -U, -W and TrbC, are useful markers for F-like T4SS. TraN and TraG have also been implicated in DNA transport in F-mediated conjugation, as demonstrated by Kingsman & Willetts (1978). Interestingly, the F-like transfer genetic island in *N.*

gonorrhoeae is involved in excretion of DNA into the medium (Hamilton *et al.*, 2005), with mutations in the homologue of *traG*, a mating pair stabilization protein in F, affecting this process (Hamilton *et al.*, 2001). *Flac* donors carrying mutations in *traN* and *traG* form loose cell-to-cell contacts and are able to initiate conjugative DNA synthesis in the donor although no detectable plasmid transfer occurs (Kingsman & Willetts, 1978). Thus TraN (and TraG) are probably involved in formation of the conjugative pore, with their role in mating pair stabilization being, in part, a byproduct of this process.

Using *ISphoA/in* insertions to identify permissive PhoA fusion sites in TraN, 14 fusions were converted to stable 31-aa epitope insertion mutants. Although *ISphoA/in* insertions in pBK184N were more stable than those in pKI375B⁻, several of the 31-aa insertions in pBK184N resulted in cell stress and plasmid instability. Cells expressing TraN-T197 (and TraN-M180-c-myc) were very mucoid, suggesting induction of capsular polysaccharide (*cps*; data not shown). This suggests that the presence of additional amino acids near aa 190 might affect the permeability of the outer membrane, leading to a generalized stress response and induction of *cps* expression.

Since F TraN appears to recognize OmpA in some way, it is reasonable to assume that at least a small region of TraN is exposed on the surface of the cell. This was previously demonstrated by the sensitivity of TraN to extracellular proteinase K (Maneewannakul *et al.*, 1992b; unpublished data). Wild-type TraN in whole cells is insensitive to exogenous trypsin, as were all the derivatives of TraN containing 31-aa epitopes, suggesting they were located in the periplasm. This is in accordance with the findings of Stathopoulos *et al.* (1996), who found that in-frame fusions of PhoA to an extracellular loop of OmpA resulted in internalization of the PhoA–OmpA fusion and degradation of the hybrid protein. Similarly, transposon mutagenesis of the outer-membrane protein FepA also produced no active PhoA fusions to outer surface regions of the protein (Murphy & Klebba, 1989). Thus stable PhoA fusions in TraN are a good predictor of a periplasmic location.

Six of the seven stable c-myc fusions were sensitive to trypsin, suggesting that they were exposed on the cell surface. All of the c-myc fusions except A69 had reduced mating efficiencies, that of the L216 fusion being reduced 10 000-fold to nearly undetectable levels. Since the L216 fusion, along with the M180 and V281 fusions that also affected mating efficiency, are proposed to be in external loops within the region of divergent sequence (aa 164–333 in F; Fig. 3a, b), these loops are good candidates for being involved in OmpA recognition. Other mutations or insertions reduced mating efficiency by 100–1000-fold, indicating that more conserved regions of TraN are also important for its function.

The topology of TraN is difficult to predict based on computer algorithms alone. Whereas structural studies of

outer-membrane proteins have revealed a β -barrel structure for that portion of the protein traversing the outer membrane, methods for predicting these regions have been of limited usefulness since the results did not agree with our 31-aa or c-myc fusion data (data not shown). The significant number of cysteine residues present in TraN also confounded predictive methods for outer-membrane segments, since very few outer-membrane proteins of known structure contain many cysteine residues, and the presence of disulfide bonds in transmembrane segments has not been documented.

A topological map of TraN is shown in Fig. 5, with the region from aa 1 to aa 390 being consistent with 31-aa and c-myc fusion data. The topology for the C-terminal third of the protein is harder to predict with confidence since fewer insertions or fusions were obtained for this region. Both the N- (I42) and C- (V584) termini are predicted to be in the periplasm. There appear to be at least four extracellular loops near A69, M180, L216 and V281 within the divergent region (Fig. 3a). Interestingly, the IS*phoA*/in insertion at G66 could not be converted to a 31-aa fusion, probably because it is in an extracellular loop. I42, T197, F243 and A255 appear to be located in the periplasm, as do a large

number of 31-aa fusions from Y288 to T361. Thus, there appears to be a large periplasmic loop starting near V281 and extending to S387, the site of the next extracellular c-myc fusion. It is plausible that one or more large periplasmic loops starting near G397 and extending to near E555, which is extracellular, might also be present. The ability to isolate IS*phoA*/in fusions and the inability to isolate c-myc insertions (except for the E555) in the last third of the protein agree with this model. Thus, these large periplasmic loops, within approximately Y288–S387 and G397–C548, would contain 19 of the 22 cysteines, suggesting a complex pattern of disulfide bonds in the periplasm.

Conserved cysteine residues in a periplasmic or outer-membrane protein suggest the presence of disulfide bonds that would contribute to a more rigid structure. Consistent with this, TraN appears to have intramolecular and intermolecular disulfide bonds as demonstrated by non-reducing SDS gel electrophoresis. Formation of these disulfide bonds appears to require the host DsbA protein since TraN is unstable in a *dsbA::kan* background. Recently, transfer gene products such as TraF and TrbB have been predicted to contain thioredoxin folds (Lawley *et al.*, 2003), with TrbB but not TraF having DsbC-like

Fig. 5. Topological model of TraN. Residues in the membrane portion of the TraN sequence are shown as diamonds, with residues predicted to be exposed to the membrane shaded grey. Residues in the periplasmic and extracellular loops are omitted for clarity, with the range of amino acids in each given above or below. Permissible 31-aa and c-myc epitope insertions are listed with arrows or arrowheads, respectively. The regions involved in OmpA recognition could involve the exterior loops containing the M180, L216 and V281 c-myc insertions with 14, 10 and 4 amino acid differences, respectively, between F and R100 TraN. The conserved region from aa 164 to aa 333 is indicated, as are the external loops (grey boxes) with the aa residues in each loop above the boxes. See text for details.

isomerase activity (T. Elton, S. Holland, L. S. Frost & B. Hazes, unpublished data).

Using the cross-linker BS3 with whole cells, TraN was found in multimeric complexes of unknown composition, although TraN multimers are the most plausible model. Nonreducing conditions also revealed higher molecular mass complexes involving TraN. TraN–TraN interactions were detected using TraN as prey in a yeast two-hybrid screen, with the N-terminal half of the protein being involved (P. Silverman, personal communication). The F plasmid transfer apparatus probably contains many intermolecular disulfide bridges, since the *dsbA* mutation was originally identified for its effect on F-pilus synthesis (Bardwell *et al.*, 1991). A comparison of the F transfer proteins with their homologues in GenBank has revealed conserved cysteine residues in nine transfer proteins including TraB (2), -H (3), -G (2), -N (22), -P (2), -T (1), -U (10), -V (3) and -TrbI (1), keeping in mind that the N-terminal cysteine in TraV and the lone cysteine in TraT are the sites of lipid attachment during lipoprotein maturation. Whereas disulfide bonds have been shown to be important in the VirB system of the Ti plasmid of *Agrobacterium tumefaciens* (reviewed by Baron *et al.*, 2002), and in the F core T4SS protein TraV (Harris & Silverman, 2002), proteins with high cysteine content seem to be a feature of F-like T4SS.

Attempts were made to purify TraN complexes from cells using methodologies developed for other outer-membrane proteins including secretins (Kazmierczak *et al.*, 1994; Koster *et al.*, 1997). TraN was monomeric in SDS and was found in large complexes of inconsistent size in other detergents such as Sarkosyl (data not shown). When Sarkosyl was used to solubilize crude membrane preparations followed by purification by either size exclusion on an S300 column or velocity sucrose gradient sedimentation, TraN dissociated into monomers (data not shown).

The picture that emerges of TraN is one of an intercellular adhesin that is associated with the T4SS complex in some way. TraN does not have obvious homology to other β -barrel proteins (Koebnik *et al.*, 2000), to TolC with its long α -helical extensions into the periplasm (Koronakis *et al.*, 1997), or to pertactin, an outer-membrane adhesin in *Bordetella pertussis* that has an extended β -helix (Emsley *et al.*, 1996). The presence of numerous cysteines suggests a complex pattern of disulfide bond formation which is now the subject of further research. Although TraN is essential for conjugation, overexpression of *traN* does not increase mating efficiency, suggesting that mating pair stabilization is not affected by the amount of TraN. Since F, a very low-copy-number plasmid, expresses only 1–2 pili per cell, TraN might only multimerize when associated with an extended pilus. Attempts to demonstrate TraN–OmpA interactions by two-hybrid techniques were unsuccessful, as was co-immunoprecipitation from mating cells, probably due to the small number of interacting partners in a mating

pair. TraN appears to have a number of regions exposed on the cell surface that are involved in mating pair stabilization, since the insertion of the c-myc epitope interfered with conjugation, possibly by blocking OmpA or lipopolysaccharide recognition. The exterior loops near M180, L216 and V281 are the best candidates for interacting with OmpA since they differ in sequence from R100 TraN in a region known to be involved in OmpA recognition (aa 164–333 in F). The C-terminal region is also important for TraN function, with disulfide bond formation probably playing an important role. Contact with OmpA could mediate the formation of the conjugative pore whereby a complex containing TraG, and perhaps other transfer proteins, forms the channel between the cytoplasm of the donor and recipient cells, as suggested by the elegant experiments of Kingsman & Willetts (1978).

ACKNOWLEDGEMENTS

The authors wish to thank Joseph Dillard and Hank Seifert for releasing the sequence of *Neisseria gonorrhoeae* TraN before publication. Anti-OmpA antibodies were from Glen Armstrong, University of Alberta. Anti-TraG antibodies were from Neville Firth, University of Sydney. Anti-31-aa antibodies and λ TnphoA/in phage were from Colin Manoil, University of Washington. Anti-TraV and -TraB antibodies were from Philip Silverman, Oklahoma Medical Research Foundation. The authors acknowledge Jody Yu for contributions to this project. This work was supported by NSERC; William Klimke was the recipient of AHFMR and NSERC Studentships.

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