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Entry exclusion in F-like plasmids requires intact TraG in the donor that recognizes its cognate TraS in the recipient

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The mating pair stabilization (Mps) protein of the F plasmid, TraG, is unique to F-like type IV secretion systems. TraG is a polytopic inner-membrane protein with a large C-terminal periplasmic domain that is required for piliation and Mps, whereas the N-terminal region is sufficient for pilus synthesis. The C-terminal region of TraG is thought to be cleaved by the host signal peptidase I to give a fragment called TraG* that is responsible for Mps. Using mutational analysis and cell localization studies, it was shown that TraG* is most probably an artifact caused by non-specific degradation. TraS (173 aa in F), which is involved in entry exclusion (Eex), blocks redundant conjugative DNA synthesis and transport between donor cells, suggesting that it interferes with a signalling pathway required to trigger DNA transfer. Using the F and R100 plasmids, TraG in the donor cell was found to recognize TraS in the recipient cell inner membrane, in a plasmid-specific manner. This activity mapped to aa 610–673 in F TraG, the only region that differs significantly from R100 TraG. Expression of *traG* or *traG** in a recipient cell did not affect mating ability or Eex. These results suggest that TraG may be translocated to the recipient cell, where it contacts the inner membrane, initiating transfer, a process that is blocked by TraS.

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INTRODUCTION

Bacterial conjugation is the transfer of plasmid DNA from a donor to a recipient cell, thereby distributing new genetic elements and other adaptations for survival in unique environments (Ochman *et al.*, 2000; Wilkins & Frost, 2001). Although conjugative plasmids have been identified in several bacterial species, the F plasmid from *Escherichia coli* remains a paradigm for the study of bacterial conjugation (Lawley *et al.*, 2004). The 100 kb F plasmid encodes all genes necessary for conjugation in the 33.3 kb transfer (*tra*) region, including genes for pilus synthesis, mating pair formation (Mpf) and DNA transfer (Frost *et al.*, 1994). Conjugative systems have recently been classified as a subset of the type IV secretion system (T4SS), due to the considerable similarity between the genes involved in both processes (Lawley *et al.*, 2003).

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Abbreviations: Eex, entry exclusion; Mpf, mating pair formation; Mps, mating pair stabilization.

The conjugative transfer of DNA is a plasmid-driven process that involves an intimate association between a donor and recipient cell, termed a mating pair (Lederberg, 1956; Achtman, 1975; Durrenberger *et al.*, 1991). This is followed by the transfer of plasmid ssDNA through a membrane-associated, supramolecular structure known as the Mpf complex. In F-like systems, mating pair stabilization (Mps) allows cells to mate more efficiently in liquid media, and to more readily resist disaggregation by mechanical forces (Bradley *et al.*, 1980; Manning *et al.*, 1981). Initially, Mps was thought to be mediated through the F pilus alone. However, further analysis has shown that, whereas the F pilus is important for initiating Mpf, the TraN and TraG proteins are responsible for the phenotypes of resistance to SDS and shear forces (Achtman *et al.*, 1977). Kingsman & Willetts (1978) have shown that mutations in *traN* and *traG* allow the initiation of conjugative DNA replication in the donor, but not the transfer of radiolabelled F plasmid DNA to the recipient cell. Another F protein, TraU, has been shown to be involved in DNA transfer, but not pilus synthesis, and is tentatively grouped with the Mps proteins (Moore *et al.*, 1990; Lawley *et al.*, 2003). F TraN is a cysteine-rich, outer-membrane protein that requires OmpA in the outer membrane of the recipient cell for efficient mating in liquid cultures (Klimke & Frost, 1998; Klimke *et al.*, 2005). TraN has also been suggested to interact with TraG,

although this remains unproven (Firth & Skurray, 1992; Klimke *et al.*, 2005). A counterpoint to Mps is provided by two processes involved in the prevention of redundant DNA transfer between donor cells. The first is surface exclusion (Sfx), encoded by *traT*, which impedes contact between donor cells carrying the F plasmid (Achtman *et al.*, 1977; Harrison *et al.*, 1992; Klimke *et al.*, 2005). The second process is entry exclusion (Eex), encoded by *traS*, which prevents the transfer of DNA between equivalent donor cells (Kingsman & Willetts, 1978). The Eex systems of the closely related plasmids F and R100 are plasmid-specific, whereby the transfer apparatus in the donor cell recognizes, via TraG, the cognate TraS in the recipient cell (Anthony *et al.*, 1999). Recently, the integrating conjugative elements SXT and R391 from *Vibrio cholerae* and *Providencia rettgeri*, respectively, have been shown to behave in a similar fashion to TraG, recognizing its cognate TraS during Eex (Marrero & Waldor, 2005).

At 102.5 kDa, F TraG is one of the largest proteins encoded by the *tra* region of the F plasmid (Frost *et al.*, 1994). Mutations that map to the N-terminal half of F TraG (e.g. *traG106*, frameshift) affect pilus assembly, whereas mutations in the C-terminal half of F TraG, such as *traG101* (frameshift) and *traG79* (amber), affect Mps but permit pilus synthesis, as monitored by phage infection (Willetts & Achtman, 1972; Fig. 1). Antibodies directed towards the C-terminal domain of F TraG detect two products: full-length F TraG and, within the periplasm, a 50 kDa product named TraG* (Firth & Skurray, 1992). This is not to be confused with the TraGp* fusion product that results from in-frame cloning of the proximal end of *traG* to a protein coding sequence in pRS29 via an *Eco*RI site (Manning *et al.*, 1981). Sequence analysis of TraG predicts the presence of a signal peptidase I cleavage site after residue 451, generating a 52.7 kDa product that could be TraG*, which may be released into the periplasmic space and contribute to Mps (Firth & Skurray, 1992).

In the current study, we confirmed the localization and topology of F TraG, and determined that the middle portion of TraG was responsible for TraS recognition during Eex. TraG* is likely to be an artifact caused by degradation during cell disruption and protein isolation. We demonstrated that Mps required the presence of full-length TraG, rather than TraG* (aa 452–938), within the donor cell. Finally, we noted that Eex was achieved through interaction of TraG and TraS *in trans*, as co-expression of TraG and TraS in recipient cells did not interfere with mating efficiency or Eex.

METHODS

Bacterial strains, plasmids and growth conditions. Cells were grown in Luria–Bertani (LB) broth (BDH) at 37 °C with appropriate antibiotics on a tube roller. Glucose was added to cultures to a final concentration of 0.4 % to repress expression from an IPTG- or arabinose-inducible promoter. Antibiotics (Sigma) were added to the following final concentrations: ampicillin 50 μg ml^{-1}; chloramphenicol 20 μg ml^{-1}; kanamycin (Km) 25 μg ml^{-1}; spectinomycin (Sp) 100 μg ml^{-1}; streptomycin (Sm) 200 μg ml^{-1}; tetracycline 10 μg ml^{-1} and nalidixic acid (Nal) 16 μg ml^{-1}. Arabinose was added to 0.1 % for expression from pBAD vectors. *E. coli* XK1200 (F^- Nal^r) and MC4100 (F^- Sm^r) have been described previously (Anthony *et al.*, 1994), as has ED24 (Sp^R, F^- Lac^-) (Achtman *et al.*, 1971). Vectors included pBAD24 and pBAD33 (Guzman *et al.*, 1995), pCR-Blunt-TOPO (Invitrogen), pALTER-Ex2 (Promega) and pET26b (Novagen). Conjugative plasmids used included F*lactraG79*, F*lactraG106*, F*lactraG101* (Achtman *et al.*, 1972) and pOX38::Tc (Anthony *et al.*, 1994). pOX38::TcΔGKm was constructed by replacing the *Pst*I/*Pst*I fragment in *traG* of pOX38::Tc with a Km resistance cassette using recombination, as described by Klimke *et al.* (2005).

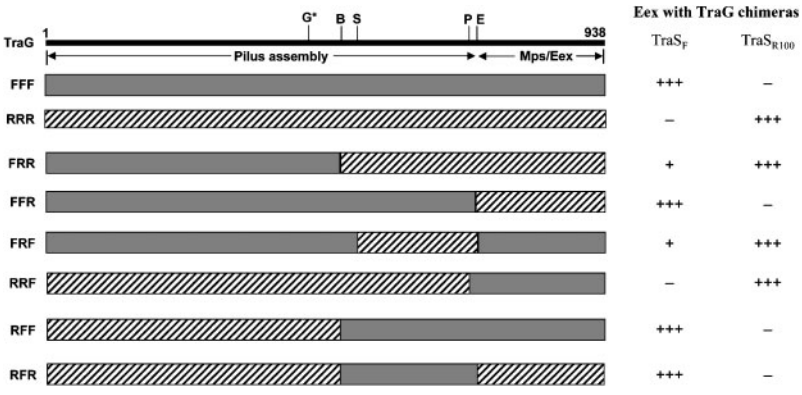

Fig. 1. Depiction of F TraG/R100 TraG chimeras. Chimeras were generated from the 5′ (nt 0–1600), middle (nt 1600–2160) and 3′ (nt 2160–2813) coding regions of F TraG (F) or R100 TraG (R). Restriction endonuclease sites for generation of the chimeras are *Bst*EII (B, nt 1541/aa 514), *Sma*I (S, nt 1600/aa 533), *Pst*I (P, nt 2154/aa 717) and *Eco*RI (E, nt 2160/aa 720). Regions of the chimeras arising from F TraG are in solid grey, and regions from R100 TraG are hatched. The predicted signal peptidase cleavage site (G*) is at residue 451, and the regions of TraG involved in pilus assembly and Mps/Eex, according to Frost *et al.* (1994), are indicated below the line. Beside each chimera are the relative Eex indices observed when the TraG chimeras are present in XK1200/pOX38::TcΔKm, and F or R100 TraS is present in recipient cells (detailed in Table 3). Eex levels are shown as + + + (wild-type), + (partial) and – (no Eex).

IS*phoA*/in insertions into F *traG*. A 3.0 kb fragment containing F *traG* was subcloned into pBSII SK+, which also eliminated the *Bam*HI site in the multiple cloning site of the vector. This construct, named pBK20, was used to insert IS*phoA*/in into *traG*, according to previously described procedures (Manoil & Bailey, 1997; Klimke *et al.*, 2005). DNA isolated from IS*phoA*/in derivatives of *traG* was treated with *Bam*HI, and the larger DNA fragment was religated to give mutants of *traG* with 31 aa epitopes fused in-frame in the *traG* coding sequence.

Construction of ppelBTraG*. nt 1357–1717 of the coding region of TraG* (aa 452–938) were amplified by PCR with Vent polymerase (New England Biolabs), using forward primer LFR193 (5′-CCATGGCAGGCAGTGTGG-3′) containing an *Nco*I site at the 5′ end, and reverse primer G21120 (5′-GCAGTGACTCTGTCACTGG-3′), and cloned into pCR-Blunt-TOPO. After *Nco*I and *Sma*I digestion, the 5′ proximal portion (front) of *traG* was moved into a pBAD24 construct carrying the back half (*Sma*I to 3′ end; nt 1718–2814) of *traG* to produce pTraG*. The *traG** fragment of this construct was cloned using *Nco*I and *Hin*dIII into pET26b immediately downstream of the pelB leader sequence. The pelB leader sequence and the TraG* encoding sequence were then recloned into pBAD24, using *Xba*I and *Hin*dIII, generating the plasmid ppelBTraG*.

Generation of *traG* point mutations. The *Pst*I/*Pst*I fragment from the F *traG* gene was cloned into pALTER-Ex2. A mutation was induced in this fragment using the Altered Sites II *in vitro* Mutagenesis Systems kit (Promega), according to the manufacturer's instructions. The base at position 1353 was changed from G to C, resulting in an amino acid change from alanine to proline at residue 452. Additional mutations at A449 (A449D) and A451 (A451E) were similarly constructed. The altered DNA was reintroduced into full-length *traG* in pBAD24 by restriction digest and ligation, and called pTraGA452P, pTraGA451E and pTraGA449D, respectively.

Construction of chimeric F and R100 TraG and TraS clones. R100 and F *traG* chimeras were constructed using conserved restriction sites in the two genes (Fig. 1). The chimeras were named by referring to the source of the DNA in the 5′ gene fragment (nt 1–1599), middle section (nt 1600–2160) and the 3′ end (nt 2161–2814), using F and R for the F and R100 plasmids, respectively. All constructs with F sequences in the 5′ region were in the pBAD24 vector, while chimeras starting with the R100 sequence were in pT7.3.

Construction of *traS* vectors. pTraS-F was prepared by amplification of F *traS* using forward primer LFR183 (5′-CCA-TGGGTAGGGTATGGAG-3′) containing a 5′ *Nco*I site, and reverse primer LFR184 (5′-AAGCTTTTATTTTACTCTTGATAAC-3′) with a 5′ *Hin*dIII site. The PCR product was ligated into pCR-Blunt-TOPO, digested from this vector with *Nco*I and *Hin*dIII, and ligated into pBAD24 digested with the same enzymes. pTraS-R100 was prepared in a similar manner using the forward primer LFR191 (5′-CCATGGCTGTTTTTGGGAGATG-3′) with a 5′ NcoI site, and reverse primer LFR192 (5′-AAGCTTAAAACGTCATAATATAC-3′) containing a 5′ *Hin*dIII site. A six-histidine sequence (His6 tag), which was fused to the N or C terminus of F TraS, was similarly constructed, but with the coding sequence for the six histidines included in the primers used for amplification.

Membrane separation by density flotation. Density flotation of cellular membranes was done according to the procedure of Grahn *et al.* (2000), and isolated cell membranes were resuspended in 2 ml 55 % sucrose. All sucrose solutions (w/v) were made up in 10 mM Tris/HCl, pH 7.5, and 5 mM EDTA; gradients were prepared in a stepwise fashion with layers of 0.5 ml 60 % sucrose, 2 ml sample, 2 ml 50 %, 3 ml 45 %, 3 ml 40 %, 1 ml 35 % and 0.5 ml 30 % sucrose. Gradients were centrifuged in a Beckman SW41 rotor at 200 000 ***g*** for 72 h. Fractions of 750 µl were collected from the top, the protein content of the samples was measured using a Bradford assay (data not shown), and 15 µl aliquots of each fraction were analysed by Western blotting for TraG, TraD, OmpA and CpxA, as follows. Proteins from SDS-PAGE were transferred to PVDF membranes, according to the manufacturer's instructions. Identification of TraG, TraD, CpxA and OmpA was done using standard procedures and employed rabbit polyclonal primary antibodies at the following dilutions: anti-TraG, 1 : 5000; anti-TraD, 1 : 15 000; anti-OmpA, 1 : 100 000; and anti-CpxA, 1 : 5000. Chemiluminescent detection employed a goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Amersham) at a dilution of 1 : 20 000.

Detection of periplasmic TraG. pBAD24 constructs containing *traG*, *traGA452P*, *pelBtraG** and a vector control were transformed into XK1200/pOX38::TcΔGKm. Cultures (2 ml) of these transformants were grown in LB broth to mid-exponential phase, and induced with 0.1 % arabinose for 1 h at 37 °C. Periplasmic shock preparation proceeded as follows. Cell pellets from 1.5 ml chilled, induced cultured cells were washed twice in 0.8 ml chilled 10 mM Tris/HCl (pH 7.5) and 30 mM NaCl. The washed cells were pelleted and resuspended in 20 % sucrose and 33 mM Tris (pH 7.5), and then EDTA was added to a concentration of 1 mM. After incubation at room temperature with gentle rocking for 10 min, the cells were pelleted and resuspended in a small amount of supernatant, and chilled. The resuspended cells were introduced into 10 ml cold 0.5 mM $MgCl_2$, containing Complete Mini Protease Inhibitors–EDTA (Roche), and stirred gently for 10 min. The resulting shock fluid was cleared of cells by centrifugation, and 0.5 ml aliquots were TCA-precipitated for SDS-PAGE and Western blotting analysis, which proceeded as described above. Samples of induced whole cells (OD_{600} 0.1) were also included for comparison.

Mating assays. Mating assays were performed as described by Anthony *et al.* (1994). Briefly, 2 ml cultures were grown to mid- to late-exponential phase in LB broth with appropriate antibiotics. pBAD constructs were induced with 0.1 % arabinose for 1 h at 37 °C. Cells were pelleted, washed once with LB broth to remove antibiotics, and resuspended in an equal volume of LB broth. One hundred microlitres each of donor and recipient cells (typically 1×10^7 to 4×10^8 cells ml^{-1} were used in each mating assay) were mixed with 0.8 ml LB broth and 0.1 % arabinose for pBAD constructs, and allowed to mate for 45 min at 37 °C. The mating mixtures were vortexed vigorously and put on ice to prevent further mating. Mating cultures were serially diluted 10-fold in 4 °C SSC buffer (150 mM sodium chloride, 15 mM sodium citrate, pH 7.0) five times, and 10 µl of each dilution was spot-plated separately on agar plates selective for donors, recipients or transconjugants. Colonies were counted after overnight incubation at 37 °C, and the number of transconjugants per 100 donor cells was calculated.

Bioinformatics software. Sequence alignments of F and R100 TraG and TraS were performed using CLUSTALW (Thompson *et al.*, 1994). Figs. 4 and 5 were prepared with minor manual editing of the CLUSTALW alignments. Transmembrane helices were predicted using TMHMM (Krogh *et al.*, 2001; http://www.cbs.dtu.dk/services/TMHMM-2.0/).

RESULTS

Characterization of TraG

Earlier studies using proteolysis of spheroplasts have shown that TraG localizes within the inner membrane (Firth & Skurray, 1992). TraG is predicted to contain three (aa 54–73,

327–385 and 407–447; Firth & Skurray, 1992) or five (aa 54–73, 327–348, 364–385, 407–426 and 428–447; Frost *et al.*, 1994) transmembrane α-helices, as well as a putative C-terminal fragment (TraG*, aa 452–938) in the periplasm (Achtman *et al.*, 1972; Manning *et al.*, 1981; Firth & Skurray, 1992). TraG was confirmed to localize to the inner membrane, using density flotation gradients to separate inner and outer membranes. The fractions were analysed by immunoblotting using antibodies to TraG, as well as known inner (CpxA, TraD; Weber & Silverman, 1988; Panicker & Minkley, 1992) and outer membrane proteins (OmpA; Sonntag *et al.*, 1978) (Fig. 2).

The presence of TraG* in the periplasmic space (Firth & Skurray, 1992) was an intriguing idea that merited further exploration, since it would explain the need for the C-terminal region of TraG for Mps. TraG was detected as a faint band in single-copy F+ cells, but TraG* could have been below the limits of detection (data not shown). The existence of TraG* was further assessed in periplasmic fractions from cells carrying pBAD24 derivatives of either wild-type TraG or a signal peptidase-resistant mutant (TraGA452P) (Fig. 3). These were compared to pelBTraG*, which is a fusion of the putative TraG* sequence to the PelB signal sequence to ensure transport to the periplasm. In cells expressing pelBTraG*, the TraG-derived polypeptide was present in the periplasmic fraction, and acted as a size marker for the putative TraG* protein (52.7 kDa). When cellular lysates or periplasmic shock fractions were probed with an anti-TraG antibody, full-length TraG and associated degradation products were found in cell lysates containing TraG or the TraGA452P mutant, whereas no TraG-derived bands were found in the periplasm. We conclude that TraG* does not exist under normal circumstances. Immunoblots of mating cells did not reveal a band that could be interpreted as TraG* (data not shown), suggesting that it is not generated during Mpf.

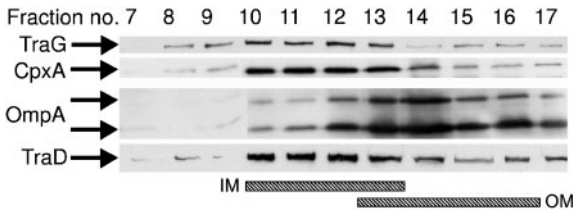

Fig. 2. Localization of TraG in the inner membrane. Inner (IM) and outer (OM) membranes were separated by sucrose density flotation, and proteins were detected by Western blot analysis. TraG was observed to reside predominantly in fractions containing known inner-membrane proteins CpxA and TraD, but not the outer-membrane protein OmpA. The fraction numbers from the gradient are shown along the top, and the positions of the test proteins are shown on the left. The two arrows for OmpA indicate the two bands that were detected with OmpA antiserum.

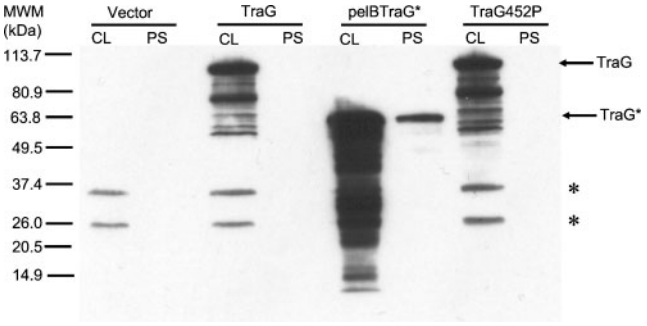

Fig. 3. TraG* was not found in the periplasmic space. Western blot analysis of XK1200/pOX38::TcΔKm plus pBAD24 (vector) constructs for TraG, TraG* (pelBTraG*) and TraGA452P in cell lysates (CL) and periplasmic shock fluid (PS) were performed using a polyclonal anti-TraG antibody. The positions of TraG (102.5 kDa) and TraG* derived from pelBTraG (52.7 kDa) are shown on the right, and molecular mass markers (MWM) are on the left. Non-specific bands present in all cleared lysates are marked with asterisks.

The predicted topology of TraG (Firth & Skurray, 1992; Frost *et al.*, 1994) was examined using IS*phoA*/in insertions that can be converted to in-frame 31 aa epitope fusions, as described by Manoil & Bailey (1997). Twelve separate IS*phoA*/in insertions into TraG were obtained in four unique sites in TraG [aa D159 (one insertion), N163 (six insertions), L716 (one insertion) and E739 (four insertions)]. D159 and N163 resided in the first predicted periplasmic loop (aa 74–326), and L716 and E739 were within the C-terminal domain of TraG that extended into the periplasm (Fig. 4). No inserts were isolated for the second predicted periplasmic domain (aa 386–406), possibly because the target was too small (60 bp), or because there are three, not five, transmembrane helices. Only the insert at codon E739 (pBKG739) could be converted to a stable 31 aa fusion. Using a complementation assay with pOX38::TcΔGKm, pBKG739 had a mating efficiency that was 15 % of that of the wild-type, suggesting that insertion of the 31 aa in the periplasmic domains greatly affected TraG function.

The nature of the mutations originally described by Willetts & Achtman (1972) was characterized by sequencing clones of each of the mutant *traG* genes. The mutations in *traG106* and *traG101* were +1 frameshifts in runs of Gs or Cs starting at nt 19795 and 21606 (aa T131 and T735), respectively, according to the sequence presented by Frost *et al.* (1994) (Fig. 4). The mutation in *traG79* was a C to T transition at nt 21341, resulting in a glutamine (CAG, Q647) changing to an amber codon (Fig. 4).

Mps requires full-length TraG

We asked whether TraG* alone, expressed as *pelBtraG** (ppelBTraG*), could complement a frame-shift mutant capable of synthesizing pili (F*lactraG101*), with

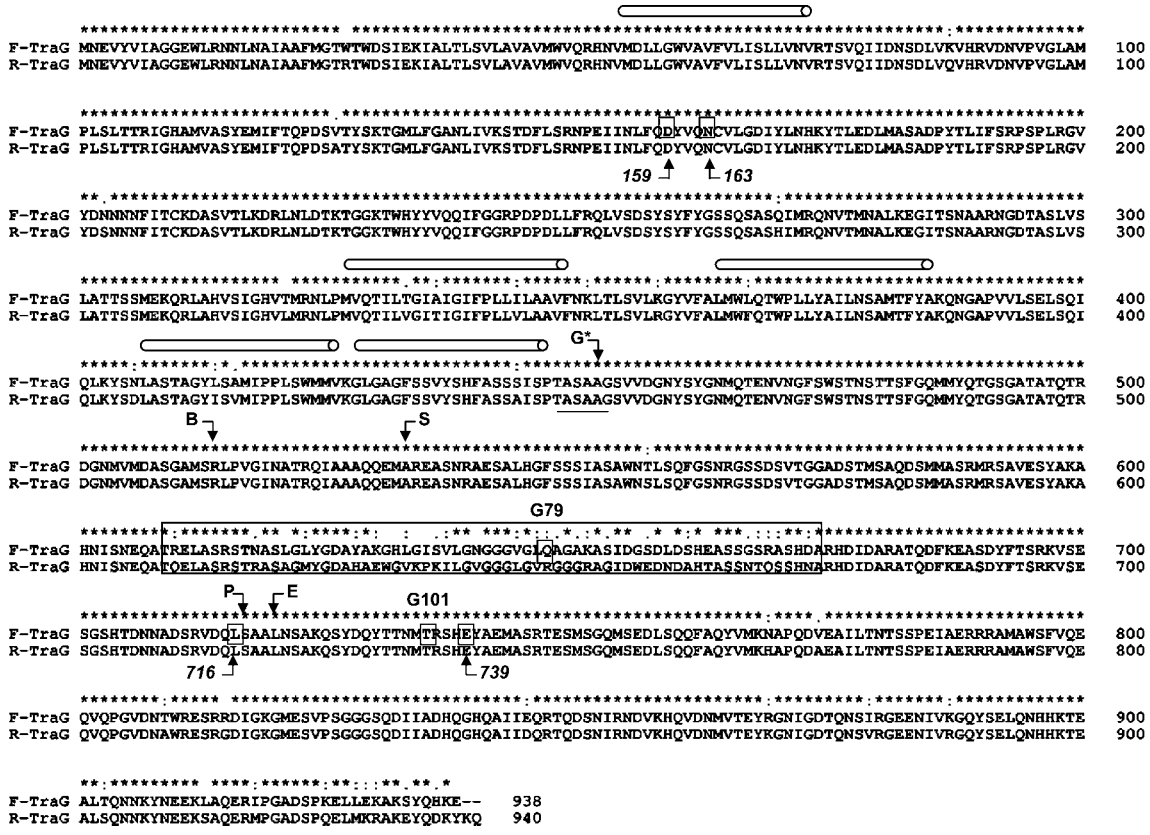

Fig. 4. Sequence alignment of the TraG proteins from the F and R100 plasmids. Alignments of the complete F TraG (NP_061478) and R100 TraG (NP_052976) proteins are shown, with strictly, highly and moderately conserved residues indicated by the symbols '*', ':' and '.', respectively. The region between residues 610 and 673 (boxed) displays the greatest dissimilarity between the two proteins, at 55.7 % identity versus 93 % overall identity between the two proteins. Restriction sites in the corresponding DNA sequence for *Bst*EII (B, nt 1541/aa 514), *Sma*I (S, nt 1600/aa 533), *Pst*I (P, nt 2154/aa 717) and *Eco*RI (E, nt 2160/aa 720), used to generate the chimeras, are indicated by arrows above the sequence. The five transmembrane helices (aa 54–73, 327–348, 364–385, 407–426 and 428–447) are indicated by cylinders above the sequences. The predicted signal peptidase cleavage site (G*; Frost *et al.*, 1994) is at residue 451, and the residues forming the −3 to +1 peptidase region (aa 449–452) are underlined. Highlighted amino acids are boxed. T131, Q647 and T735 are the amino acids affected by the *traG106*, *traG79* and *traG101* mutations, respectively; 159, 163, 716 and 739 (italic type) are the sites of IS*phoA*/in insertions at the codons for D159, N163, L716 and E739, respectively.

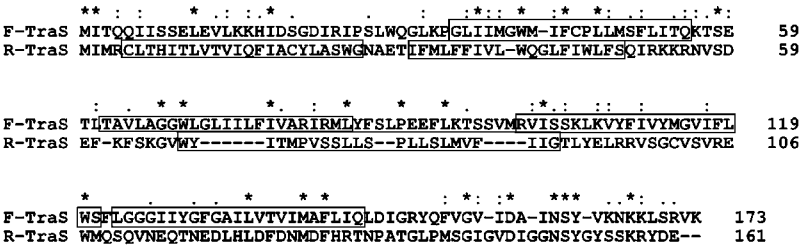

Fig. 5. Sequence alignment of the TraS proteins from the F and R100 plasmids. F TraS (NP_061479) and R100 TraS (NP_052977) showed an overall sequence identity of 17 %, with no single region displaying greater identity. Strictly, highly and moderately conserved residues are indicated by the symbols '*', ':' and '.', respectively. Predicted transmembrane helices are boxed.

Table 1. Mps requires full-length TraG

ND, Not determined.

Donor plasmid†	Complementing plasmid	Transconjugants‡§ (cells ml^{-1})	Mating efficiency§‖	R17 phage sensitivity
pOX38::TcΔGKm	None	0	0	−
pOX38::TcΔGKm	pBAD24	0	0	−
pOX38::TcΔGKm	pTraG	2×10^7	8	+++
pOX38::TcΔGKm	ppelBTraG*	0	0	−
pOX38::TcΔGKm	pTraG-A452P	0	0	++
pOX38::TcΔGKm	pTraG-A449D	2×10^7	6.3	++
pOX38::TcΔGKm	pTraG-A451E	1×10^7	2.9	++
F*lac traG101*	None	0	0	+++
F*lac traG101*	pBAD24	0	0	ND
F*lac traG101*	pTraG	1×10^6	1.0	ND
F*lac traG101*	ppelBTraG*	4×10^2	0.0004	ND
F*lac traG101*	pTraG-A452P	0	0	ND
F*lac traG101*	pTraG-A449D	ND	ND	ND
F*lac traG101*	pTraG-A451E	ND	ND	ND

†Donor cells were *E. coli* XK1200/pOX38::TcΔGKm or XK1200/F*lac traG101* with the constructs listed in the next column, and cell concentrations were 1.0×10^8–3.5×10^8 cells ml^{-1}.

‡Recipient cells were *E. coli* MC4100. 0 indicates no transconjugants in a 10^{-2} dilution.

§An average of two to four mating experiments were performed for each construct.

‖Mating efficiency was defined as transconjugants per 100 donor cells. 0 mating efficiency indicates no transconjugants at a 10^{-2} dilution.

pOX38::TcΔGKm acting as a control. Using mating experiments, only clones expressing full-length TraG could complement both pOX38::TcΔGKm and F*lactraG101* donors (Table 1). ppelBTraG* partially complemented F*lactraG101* at very low levels in some assays, which was thought to be due to reversion of the frameshift mutation in F*lactraG101*.

Based on studies of the amino acid requirements at the signal peptidase I cleavage site (von Heijne, 1983), mutations that are predicted to severely alter peptidase activity were introduced into TraG by site-directed mutagenesis at positions −3, −1 and +1 of the predicted cleavage site [residues 449–452 (ASA-A); G*, Figs 1 and 4]. Thus +1 alanine was converted to proline (A452P), −1 alanine to glutamic acid (A551E), and the −3 alanine to aspartic acid (A449D). These mutations were introduced into pTraG and used in complementation assays with pOX38::TcΔGKm and F*lactraG101*, which synthesizes F pili (Table 1). The lack of complementation by pTraGA452P suggested either that the proline altered the conformation of TraG or that A452 was indeed important for TraG function. However, two other point mutations, TraGA449D and TraGA451E, complemented pOX38::TcΔGKm and gave 40–80 % of wild-type results for pOX38::TcΔGKm/pTraG (Table 1). We conclude that there is no signal peptidase I cleavage site in the middle of TraG, and that the proline at position 452 likely introduced a kink into the periplasmic domain that affected TraG function in Mps. Thus, full-length TraG is required for Mps with both the N- and C-terminal regions participating in establishing stable mating junctions.

Eex involves plasmid-specific recognition between TraG and TraS

Eex is a plasmid-specific process, defined by TraS in the recipient cell, which blocks DNA transport between cells carrying the same conjugative plasmids (Ou, 1975; Kingsman & Willetts, 1978) by recognizing its cognate TraG in the donor cell (Anthony *et al.*, 1999; Marrero & Waldor, 2005). While the F and R100 TraS clones were being constructed, we realized that the sequences originally presented for R100 TraS (Ogata *et al.*, 1982) and F TraS (Jalajakumari *et al.*, 1987) were incorrect. The correct accession numbers are NP_061479 (F TraS; gi398520) and NP_052977 (R100 TraS; gi5036330), based on more recent sequence data (Fig. 5). TraS was fused to a His6 tag at both the N and C termini, and tested for Eex using mating efficiency assays. Both fusions gave Eex comparable to wild-type TraS (Table 2). Using anti-His6 antibodies, the presence of TraS, expressed from a pBAD24 clone that was induced with 0.05 % arabinose, was confirmed to be in the inner membrane by flotation density gradient analysis (data not shown).

The specificity of putative TraG–TraS interactions was examined using a series of F TraG/R100 TraG chimeras (Fig. 1) that were assessed for their ability to restore Eex in *traG*-deficient donor cells (pOX38::TcΔGKm; Table 3). When native TraG ($TraG_{FFF}$ or $TraG_{RRR}$) was assessed, only

Table 2. Eex through TraG and TraS occurs *in trans*

Donor cells were *E. coli* XK1200/pOX38::Km (1×10^8–4×10^8 cells ml^{-1}).

Recipient†	Transconjugants‡ (cells ml^{-1})	Mating efficiency§	Eex index‖
pBAD24	4.5×10^6	1.8	0
pBAD24-TraS$_F$	4.5×10^3	0.0015	3.0
pBAD24-TraS$_F$6H	4.0×10^3	0.0018	3.05
pBAD24-6HTraS$_F$	2.0×10^3	0.0011	3.35
pBAD33	1.5×10^7	10	0
pBAD33-TraG$_F$	2.0×10^7	10	0
pBAD33-TraG*	1.5×10^7	8.6	0
pBAD24 + pBAD33	5.0×10^6	4.0	0
pBAD33-TraG$_F$ + pBAD24-TraS$_F$	2.0×10^3	0.0010	3.4
pBAD33-TraG* + pBAD24-TraS$_F$	3.5×10^3	0.0012	3.2

†Recipient cells were *E. coli* MC4100/pBAD24 or its derivatives (1.5×10^8–5×10^8 cells ml^{-1}).
‡Mating experiments were done in duplicate.
§Mating efficiency was defined as transconjugants per 100 donor cells.
‖Eex index was defined as the log of MC4100/pBAD24 (pBAD33) transconjugants minus the log of MC4100/F or R100 TraS transconjugants. Eex <0 was counted as no entry exclusion.

Table 3. Eex by F and R100 TraG chimeras (defined in Fig. 4)

Donor pOX38::TcΔGKm*	Recipient†	Transconjugants‡ (cells ml^{-1})	Mating efficiency‡§	Eex index‡‖
pBAD24	pBAD24	0	0	–
pTraG$_{FFF}$	MC4100	1.7×10^8	68	0
	pTraS$_F$	2.3×10^4	0.006	3.9
	pTraS$_{R100}$	2.2×10^7	11	0.9
pTraG$_{FFR}$	MC4100	1.7×10^8	57	0
	pTraS$_F$	2×10^4	0.005	3.9
	pTraS$_{R100}$	2.3×10^7	4.6	0.9
pTraG$_{RFR}$	MC4100	2×10^8	50	0
	pTraS$_F$	7×10^4	0.018	3.5
	pTraS$_{R100}$	3.5×10^7	8.8	0.8
pTraG$_{RFF}$	MC4100	2×10^8	80	0
	pTraS$_F$	1×10^4	0.005	4.0
	pTraS$_{R100}$	2.8×10^7	14	0.9
pTraG$_{RRR}$	MC4100	1.2×10^8	40	0
	pTraS$_F$	5.6×10^8	187	0.3
	pTraS$_{R100}$	7.7×10^4	0.019	3.2
pTraG$_{RRF}$	MC4100	1.2×10^8	60	0
	pTraS$_F$	5.5×10^6	2.2	1.4
	pTraS$_{R100}$	5.5×10^4	0.018	3.0
pTraG$_{FRF}$	MC4100	2.1×10^7	15	0
	pTraS$_F$	1.8×10^5	0.18	2.1
	pTraS$_{R100}$	9.3×10^3	0.005	3.4
pTraG$_{FRR}$	MC4100	5.5×10^7	27.5	0
	pTraS$_F$	9.5×10^6	4.8	0.8
	pTraS$_{R100}$	5.5×10^3	0.003	4.0

*Donor cells were *E. coli* XK1200/pOX38::TcΔGKm (1.5×10^8–4×10^8 cells ml^{-1}).
†Recipients were *E. coli* MC4100 with pBAD24 or its derivatives.
‡An average of three mating experiments were performed.
§Mating efficiency was defined as transconjugants per 100 donor cells.
‖Eex index was defined as the log of MC4100 transconjugants minus the log of MC4100 with F or R100 TraS transconjugants.

the corresponding TraS (F or R100, respectively) in the recipient cell gave Eex. This was narrowed down to the middle region of TraG (aa 533–717 in F), since this region was required for recognition of the cognate TraS in the recipient cell (Table 3). All other TraG chimeras had low to undetectable Eex. With the exception of a single amino acid difference at position 558 (S to T), the only differences in the sequences of aa 533–717 in F and R100 TraG were within aa 610–673 (Fig. 4), which further delimits the region of TraG involved in Eex.

The ability to interfere with TraS function by co-producing TraG or TraG* in the recipient cell was monitored using an Eex assay (Table 2). Neither TraG nor TraG* interfered with Eex, suggesting that TraG and TraS interacts *in trans* between donor and recipient cells. Thus, TraG must be delivered to the recipient cell inner membrane by a process that defines the phenomenon of Mps.

DISCUSSION

The *traG* gene product has been shown to be involved in both pilus synthesis and Mps (Manning *et al.*, 1981). Sequence homology has been noted between the N-terminal domain of TraG and VirB6, a polytopic inner-membrane protein of the Ti plasmid of *Agrobacterium tumefaciens* also involved in T pilus synthesis and T-DNA transport (Lawley *et al.*, 2003). However, unlike Ti VirB6, which is predicted to contain between four and nine transmembrane helices (Christie, 2001; Judd *et al.*, 2005), TraG is predicted by hydropathy plot analysis (Firth & Skurray, 1992; Frost *et al.*, 1994) to span the inner membrane three to five times, with large periplasmic regions at approximately aa 75–325 and 445–938 (C terminus). Our limited number of IS*phoA*/in insertions confirmed these results, and suggested that there were at least three transmembrane domains. Interestingly, the only IS*phoA*/in insertion that could be converted to a stable 31 aa epitope fusion (at E739) is very close to the *traG101* frameshift mutation at T735 that affects Mps but not pilus synthesis. No evidence was observed for TraG*, a periplasmic fragment suggested to be involved in Mps (Firth & Skurray, 1992). Degradation products in the lysate of cells overproducing TraG were approximately correct in size (Fig. 3), but none of these products was found in the periplasm. The observation that TraG, but not TraG*, can fully complement F*lactraG101* suggests that whole TraG is required for biological activity, which also argues against the presence of TraG*.

Examination of the ability of F or R100 transfer proteins to complement F *tra* mutations and affect phage sensitivity or Eex suggests that TraG in the donor cell is specific for its cognate TraS in the recipient cell (Anthony *et al.*, 1999). A comparison of the sequences of TraG from the F and R100 plasmids indicates that they share an overall sequence identity of 93 %. However, the region between residues 610 and 673 of TraG shows the most dissimilarity, with an overall sequence identity of 55.7 % (Fig. 4). This region is within the portion of TraG in the chimeric plasmids (aa 533–717) that exhibits specificity for TraS, and is probably responsible for this phenomenon.

Unlike TraG, TraS from F and R100 plasmids were highly distinct, exhibiting only 17 % sequence identity (Fig. 5). The orientations of F and R100 TraS in the membrane were difficult to predict and could be quite different for the two proteins. Using the TMHMM algorithm, three or four transmembrane helices were predicted for R100 and F TraS, respectively, but in significantly different locations. Thus, there is no limited region of dissimilarity in TraS to aid in predicting the features that are recognized by TraG. Marrero & Waldor (2005), who have studied Eex specificity in the more closely related SXT and R391 systems, have been able to narrow the specificity region of TraG to three amino acids, aa 606–608, which approximates the location of the dissimilar region in F and R100 TraG (aa 610–673). They have also been able to identify the C-terminal half of TraS as being responsible for Eex specificity. Our attempts to construct stable TraS chimeras were unsuccessful, possibly due to incorrect insertion of the chimeras in the membrane.

TraG is involved in pilus synthesis as well as Mps; therefore, it would be expected to interact with other proteins in the donor cell, although none have so far been identified. There has been speculation that TraG might form a transenvelope bridge and contact TraN in the outer membrane (Firth & Skurray, 1992); however, this interaction has not been detected using cross-linking (Klimke *et al.*, 2005). We are currently exploring the possibility that TraG interacts with TraU, which is a putative Mps protein, and is known to be part of an interaction complex that is involved in pilus synthesis, but is not itself required for this process (Moore *et al.*, 1990; Harris & Silverman, 2004).

This report further defines a third function of TraG, that of Eex through interaction with TraS in the recipient cell. This function is plasmid-specific and appears to involve aa 610–673 of TraG. This TraG–TraS interaction occurs *in trans* between a donor (TraG) and recipient cell (TraS); supplying TraG or TraG* in the recipient cell did not affect mating or Eex. As both TraG and TraS are inner-membrane proteins, the mechanism by which they recognize each other is puzzling. Since Mps involves close contact between mating cells that is difficult to disrupt (Achtman, 1975), a process can be envisioned whereby F TraN binds OmpA in the recipient outer membrane and triggers pore formation. TraG would then be thrust into the periplasmic space to establish contact with the inner membrane of the recipient cell. If TraS interferes with this signalling mechanism, conjugative DNA synthesis and transfer are blocked. Attempts to isolate a TraG–TraS complex from mating cells by cross-linking or immunoprecipitation, or to demonstrate interaction using a bacterial two-hybrid system, have been unsuccessful. This appears to be due to the small percentage of the total amount of TraG or TraS involved in a single mating bridge, and possibly a

requirement for higher-order multimeric structures for correct interaction between TraS and TraG.

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REFERENCES

Achtman, M. (1975). Mating aggregates in *Escherichia coli* conjugation. *J Bacteriol* **123**, 505–515.

Achtman, M., Willetts, N. & Clark, A. J. (1971). Beginning a genetic analysis of conjugational transfer determined by the F factor in *Escherichia coli* by isolation and characterization of transfer-deficient mutants. *J Bacteriol* **106**, 529–538.

Achtman, M., Willetts, N. & Clark, A. J. (1972). Conjugational complementation analysis of transfer-deficient mutants of *Flac* in *Escherichia coli*. *J Bacteriol* **106**, 529–538.

Achtman, M., Kennedy, N. & Skurray, R. (1977). Cell-cell interactions in conjugating *Escherichia coli*: role of *traT* protein in surface exclusion. *Proc Natl Acad Sci U S A* **74**, 5104–5108.

Anthony, K. G., Sherburne, C., Sherburne, R. & Frost, L. S. (1994). The role of the pilus in recipient cell recognition during bacterial conjugation mediated by F-like plasmids. *Mol Microbiol* **13**, 939–953.

Anthony, K. G., Klimke, W. A., Manchak, J. & Frost, L. S. (1999). Comparison of proteins involved in pilus synthesis and mating pair stabilization from the related plasmids F and R100-1: insights into the mechanism of conjugation. *J Bacteriol* **181**, 5149–5159.

Bradley, D. E., Taylor, D. E. & Cohen, D. R. (1980). Specification of surface mating systems among conjugative drug resistance plasmids in *Escherichia coli* K-12. *J Bacteriol* **143**, 1466–1470.

Christie, P. J. (2001). Type IV secretion: the *Agrobacterium* BirB/D4 and related conjugation systems. *Biochim Biophys Acta* **1694**, 219–234.

Durrenberger, M. B., Villiger, W. & Bachi, T. (1991). Conjugational junctions: morphology of specific contacts in conjugating *Escherichia coli* bacteria. *J Struct Biol* **107**, 146–156.

Firth, H. & Skurray, R. (1992). Characterization of the F plasmid bifunctional conjugation gene, traG. *Mol Gen Genet* **1**, 145–153.

Frost, L. S., Ippen-Ihler, K. & Skurray, R. A. (1994). Analysis of the sequence and gene products of the transfer region of the F sex factor. *Microbiol Rev* **58**, 162–210.

Grahn, A. M., Haase, J., Bamford, D. H. & Lanka, E. (2000). Components of the RP4 conjugative transfer apparatus form an envelope structure bridging inner and outer membranes of donor cells: implications for related macromolecule transport systems. *J Bacteriol* **182**, 1564–1574.

Guzman, L. M., Belin, D., Carson, M. J. & Beckwith, J. (1995). Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. *J Bacteriol* **177**, 4121–4130.

Harris, R. L. & Silverman, P. M. (2004). Tra proteins characteristic of F-like type IV secretion systems constitute an interaction group by yeast two-hybrid analysis. *J Bacteriol* **186**, 5480–5485.

Harrison, J. L., Taylor, I. M., Platt, K. & O'Connor, C. D. (1992). Surface exclusion specificity of the TraT lipoprotein is determined by single alterations in a five amino acid region of the protein. *Mol Microbiol* **6**, 2825–2832.

Jalajakumari, M. B., Guidolin, A., Buhk, H. J., Manning, P. A., Ham, L. M., Hodgson, A. L. M., Cheah, K.-C. & Skurray, R. A. (1987). Surface exclusion genes *traS* and *traT* of the F sex factor of *Escherichia coli* K-12. Determination of the nucleotide sequence and promoter and terminator activities. *J Mol Biol* **198**, 1–11.

Judd, P. K., Mahli, D. & Das, A. (2005). Molecular characterization of the *Agrobacterium tumefaciens* DNA transfer protein VirB6. *Microbiology* **151**, 3483–3492.

Kingsman, A. & Willetts, N. (1978). The requirements for conjugal DNA synthesis in the donor strain during *Flac* transfer. *J Mol Biol* **122**, 287–300.

Klimke, W. A. & Frost, L. S. (1998). Genetic analysis of the role of the transfer gene, *traN*, of the F and R100-1 plasmids in mating pair stabilization during conjugation. *J Bacteriol* **180**, 4036–4043.

Klimke, W. A., Rypien, C. D., Klinger, B. A., Kennedy, R. A., Rodriguez-Maillard, J. M. & Frost, L. S. (2005). 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. *Microbiology* **151**, 3527–3540.

Krogh, A., Larsson, B., von Heijne, G. & Sonnhammer, E. L. L. (2001). Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *J Mol Biol* **305**, 567–580.

Lawley, T., Klimke, W. A., Gubbins, M. J. & Frost, L. S. (2003). F factor is a true type IV secretion system. *FEMS Microbiol Lett* **224**, 1–15.

Lawley, T., Wilkins, B. M. & Frost, L. S. (2004). Bacterial conjugation in Gram-negative bacteria. In *Plasmid Biology*, pp. 203–226. Edited by B. E. Funnell & G. J. Phillips. Washington, DC: American Society for Microbiology.

Lederberg, J. (1956). Conjugal pairing in *Escherichia coli*. *J Bacteriol* **71**, 497–498.

Manning, P. A., Morelli, G. & Achtman, M. (1981). TraG protein of the F sex factor of *Escherichia coli* K-12 and its role in conjugation. *Proc Natl Acad Sci U S A* **78**, 7487–7491.

Manoil, C. & Bailey, J. (1997). A simple screen for permissive sites in proteins: analysis of *Escherichia coli* lac permease. *J Mol Biol* **267**, 250–263.

Marrero, J. & Waldor, M. K. (2005). Interactions between inner membrane proteins in donor and recipient cells limit conjugal DNA transfer. *Dev Cell* **8**, 963–970.

Moore, D., Maneewannakul, K., Maneewannakul, S., Wu, J. H., Ippen-Ihler, K. & Bradley, D. B. (1990). Characterization of the F plasmid conjugative transfer gene *traU*. *J Bacteriol* **172**, 4263–4270.

Ochman, H., Lawrence, J. G. & Groisman, E. A. (2000). Lateral gene transfer and the nature of bacterial innovation. *Nature* **405**, 299–304.

Ogata, R. T., Winters, C. & Levine, R. P. (1982). Nucleotide sequence analysis of the complement resistance gene from plasmid R100. *J Bacteriol* **151**, 819–827.

Ou, J. T. (1975). Mating signal and DNA penetration deficiency in conjugation between male *Escherichia coli* and minicells. *Proc Natl Acad Sci U S A* **72**, 3721–3725.

Panicker, M. M. & Minkley, E. G., Jr (1992). Purification and properties of the F sex factor TraD protein, an inner membrane conjugal protein. *J Biol Chem* **267**, 12761–12766.

Sonntag, I., Schwarz, H., Hirota, Y. & Henning, U. (1978). Cell envelope and shape of *Escherichia coli*: multiple mutants missing the outer membrane lipoprotein and other major outer membrane proteins. *J Bacteriol* **136**, 280–285.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment

through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.

von Heijne, G. (1983). Patterns of amino acids near signal-sequence cleavage sites. *Eur J Biochem* **133**, 17–21.

Weber, R. F. & Silverman, P. M. (1988). The Cpx proteins of *Escherichia coli* K12: structure of the CpxA polypeptide as an inner membrane component. *J Mol Biol* **203**, 467–476.

Wilkins, B. M. & Frost, L. S. (2001). Mechanisms of gene exchange between bacteria. In *Molecular Medical Microbiology*, pp. 355–400. Edited by M. Sussman. London: Academic Press.

Willetts, N. & Achtman, M. (1972). Genetic analysis of transfer by the *Escherichia coli* sex factor F, using P1 transductional complementation. *J Bacteriol* **110**, 843–851.

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