Interaction between the co-inherited TraG coupling protein and the TraJ membrane-associated protein of the H-plasmid conjugative DNA transfer system resembles chromosomal DNA translocases

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Bacterial conjugation is a DNA transfer event that requires three plasmid-encoded multi-protein complexes: the membrane-spanning mating pair formation (Mpf) complex, the cytoplasmic nucleoprotein relaxosome complex, and a homo-multimeric coupling protein that links the Mpf and relaxosome at the cytoplasmic membrane. Bacterial two-hybrid (BTH) technology and immunoprecipitation were used to demonstrate an interaction between the IncH plasmid-encoded transfer protein TraJ and the coupling protein TraG. TraJ is essential for conjugative transfer but is not required for the formation of the conjugative pilus, and is therefore not regarded as an Mpf component. Fractionation studies indicated that TraJ shared a similar cellular domain to that of TraG at the cellular membrane. Protein BLAST analyses have previously identified TraJ homologues encoded in a multitude of plasmid and chromosomal genomes that were also found to encode an adjacent TraG homologue, thus indicating co-inheritance. BTH analysis of these TraJ and cognate TraG homologues demonstrated conservation of the TraJ–TraG interaction. Additional occurrences of the *traJ*–*traG* module were also detected in genomic sequence data throughout the Proteobacteria, and phylogenetic comparison of these IncH-like TraG proteins with the coupling proteins encoded by other conjugative transfer systems (including IncP, IncW and IncF) that lack TraJ homologues indicated that the H-like coupling proteins were distinct. Accordingly, the IncP, IncW and IncF coupling proteins were unable to interact with TraJ, but were able to interact with IncH plasmid-encoded TrhB, an Mpf component known to complex with its cognate coupling protein TraG. The divergence of the IncH-type coupling proteins may partly be due to the requirement of TraJ interaction, and notably, TraG and TraJ cumulatively represent the domain architecture of the known translocase family FtsK/SpoIIIE. It is proposed that TraJ is a functional part of the IncH-type coupling protein complex required for translocation of DNA through the cytoplasmic membrane.

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INTRODUCTION

Bacterial conjugation is a mechanism of horizontal gene transfer, which has both evolutionary and medical implications. Plasmids can represent a major contribution to overall genetic content, and conjugation is the principal route for the dissemination of antibiotic resistance determinants

(Mazel & Davies, 1999). The conjugative pilus initiates plasmid transfer from the donor cell by recruitment of recipient cells. A membrane-spanning protein complex called the mating pair formation (Mpf) complex, which constitutes the greater part of the conjugative type IV secretion system (T4SS), enables the production of conjugative pili, and facilitates the transfer of plasmid DNA from donor to recipient bacteria (for reviews, see Cascales & Christie, 2003, 2004; Lawley *et al.*, 2003a). Plasmid DNA is processed to a transfer intermediate in the cytoplasm by the relaxosome, a DNA–protein complex

Abbreviations: BTH, bacterial two-hybrid; Mpf, mating pair formation; T4SS, type IV secretion system; TM, transmembrane; UPGMA, unweighted pair group method with arithmetic mean.

containing the relaxase and accessory proteins (Furste *et al.*, 1989). The T4SS also includes the coupling protein at the cytoplasmic membrane, and by serving as the link between the relaxosome and Mpf complex, the transferring DNA strand contacts the cell envelope and then proceeds onwards to the recipient cell.

The incompatibility group HII plasmid (IncHII) R27 is a large (180 kb) self-transmissible resistance plasmid (Sherburne *et al.*, 2000). There has been some confusion over the years as to the origin of R27, but Datta & Olarte (1974) have clearly distinguished it as a plasmid characteristic of *Salmonella enterica* serovar Typhi. However, R27 and a related plasmid Tp117 have been isolated from *Salmonella typhimurium* strain 1M1407 (Lawn *et al.*, 1967; Smith *et al.*, 1973). The proteins required for R27 transfer are located within two separate transfer regions, Tra1 and Tra2 (Lawley *et al.*, 2002, 2003b). Encoded within Tra1 are the relaxosome, the coupling protein and part of the Mpf complex, and the remainder of the Mpf is encoded in Tra2. Interactions between the relaxosome and coupling protein have been identified in the IncW, IncP and IncF plasmid families (Disque-Kocher & Dreiseikelmann, 1997; Llosa *et al.*, 2003; Schroder *et al.*, 2002). The coupling protein then interacts with the Mpf/T4SS at the cytoplasmic membrane through the TraB/VirB10 protein (Gilmour *et al.*, 2003; Llosa *et al.*, 2003). The R27 coupling protein is termed TraG, and this family of proteins has been structurally characterized. The hexameric ring structure of the IncW plasmid-coupling protein TrwB resembles the tertiary structure of a number of P-loop ATPases, such as the F1 ATPase, suggesting that this complex is actively involved in DNA translocation (Gomis-Ruth *et al.*, 2001).

Three other essential genes encoded in the Tra1 region (*traI*, *traH* and *traJ*) are not required for the production of the H-conjugative pilus, and consequently are not components of the Mpf complex (Lawley *et al.*, 2002). The TraI protein shares homology with the relaxase family of proteins, whereas TraH contains a DNA-binding motif and an N-terminal coiled-coil domain (Lawley *et al.*, 2002). TraJ homologues from a variety of genomic sources have been identified (Gilmour *et al.*, 2004), and an alignment of these homologues has indicated that the predicted four transmembrane (TM) domains are well-conserved among this family of proteins. A conserved module of *traI*–*traG*–*traJ* has been identified in each of the genomic sources that encode a TraJ homologue (Gilmour *et al.*, 2004). Few of the TraJ-encoding genomes encode R27-like Mpf genes, suggesting that the module of *traI*–*G*–*J* genes can be inherited independently from other transfer determinants.

The objective of the present study was to identify additional R27-encoded interaction partners for the coupling protein TraG. Here, we identified an interaction between TraJ and TraG, and propose that because of co-localization at the cytoplasmic membrane, binary heterologous interaction, and co-inheritance with conserved domain organization of known DNA translocases, TraJ is a protein which associates

with the coupling protein TraG and is involved in DNA translocation.

METHODS

Bacterial strains and growth conditions. *Escherichia coli* strains used in this study are listed in Table 1. All strains were grown in Luria–Bertani (LB) broth, Lennox formulation (Difco) at 37 °C with shaking at 200 r.p.m., unless otherwise stated. Antibiotics used in this broth were as follows: ampicillin (100 µg ml^{−1}), kanamycin (50 µg ml^{−1}), tetracycline (10 µg ml^{−1}), rifampicin (50 µg ml^{−1}), nalidixic acid (20 µg ml^{−1}) and trimethoprim (25 µg ml^{−1}). For the bacterial two-hybrid (BTH) assay, conjugative transfer proteins were fused to catalytic domain fragments of adenylate cyclase from *Bordetella pertussis* by cloning into the BTH vectors pKT25 and pUT18C (Karimova *et al.*, 1998). Plasmids encoding fused proteins were co-transformed into competent BTH101 cells, processed and analysed as previously described (Gilmour *et al.*, 2003). Liquid conjugative matings and complementation experiments with a derepressed derivative of R27, which contains an insertion in the *htdA* gene that causes a derepressed phenotype, were performed as described previously (Lawley *et al.*, 2002; Taylor & Levine, 1980). Complementation experiments with the F plasmid were performed similarly, with the exception that donor and recipient cells were grown at 37 °C, and mating experiments were performed at 37 °C for 16 h. For solid matings, conjugative transfer of RP4 and R388 plasmids was performed as described by Bradley *et al.* (1980).

DNA manipulation and cloning. Standard recombinant DNA methods were used, as described by Sambrook *et al.* (1989). PCR products were amplified with terminal restriction enzymes added within the primer sequences (Table 2). Amplified DNA was initially cloned into pGEM-T (Promega), digested with appropriate enzymes, and subcloned into the expression vector pMS119EH/pMS119HE or the adenylate cyclase fusion vectors pKT25 and pUT18C. Restriction enzyme endonucleases were used according to the manufacturer's specifications, and digested products were resolved on agarose gels.

Production of coupling protein homologues. A His₆ tag was incorporated into the primer design for coupling protein amplification, such that each protein contained a C-terminal histidine tag for detection of the recombinant proteins. *E. coli* strain DH5 α , containing pMS119EH encoding each tagged coupling protein, was grown at 28 °C to OD₆₀₀ 0.5–0.7. The bacteria were induced for 1 h at 28 °C with IPTG at a final concentration of 0.4 mM. Whole-cell lysates were resolved on 10% SDS-PAGE, and following transfer to nitrocellulose membranes, a primary mouse anti-His₆ mAb (Invitrogen) and a secondary goat anti-mouse–horseradish peroxidase (HRP) antibody conjugate were used to probe for protein synthesis.

Immunoprecipitation. Immunoprecipitation of R27 transfer proteins was performed as described by Couturier *et al.* (2006). Briefly, *E. coli* DH5 α cells containing the plasmid pUT18C–TraJ were co-transformed with pBAD33–TraG_{FLAG} or pBAD33–TrhB_{FLAG} for the interaction studies. Cells were induced at mid-logarithmic growth phase (OD₆₀₀ 0.5–0.7) with 0.4 mM IPTG and 0.2% arabinose for 1 h prior to cell lysis. Cells were harvested by centrifugation and resuspended in a threefold volume of lysis buffer [150 mM PBS, 7.15% (w/v) sucrose, 1% Nonidet P-40, 1 mM PMSF, 1/66 vol. 15 µg ml^{−1} lysozyme]. The cells were frozen and thawed three times, and lysed cells were removed by centrifugation at 14 000 r.p.m. for 10 min. The supernatant was removed and anti-FLAG M2 agarose (Sigma) beads were added. The beads had been incubated at 4 °C overnight with rotation in a 5% BSA solution to avoid non-specific precipitation. The supernatant and beads were incubated at 4 °C with rotation for 2 h. The supernatant was removed after centrifugation at 14 000 r.p.m. for 30 s, and the beads

Table 1. Bacterial strains and plasmids used in this study

Abbreviations: Nal^r, nalidixic acid resistance; Rif^r, rifampicin resistance; Tc^r, tetracycline resistance; Kan^r, kanamycin resistance; Amp^r, ampicillin resistance; Str^r, streptomycin resistance; Cm^r, chloramphenicol resistance; Tp^r, trimethoprim resistance.

Strain or plasmid	Relevant genotype, phenotype or characteristic	Source or reference
<i>E. coli</i> strains		
DH5 α	<i>supE44 lacU169</i> (Φ 80 <i>lacZ</i> Δ M15) <i>hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	Invitrogen
DY330	W3110 Δ <i>lacU169 gal-490 λcI857 Δ(cro-bioA)</i>	Yu <i>et al.</i> (2000)
DY330R	Temperature-resistant revertant; Rif ^r	Lawley <i>et al.</i> (2002)
DY330N	Temperature-resistant revertant; Nal ^r	Lawley <i>et al.</i> (2002)
BTH101	F <i>cya-99 araD139 galE15 galK16 rpsL1</i> (Str ^r) <i>hsdR2 mcrA1 mcrB1</i>	Karimova <i>et al.</i> (1998)
RG912	<i>ara leu lac</i>	Taylor (1983)
DT1942	RG192 (Rif ^r) containing pDT1942	Maher <i>et al.</i> (1991)
Plasmids		
pBAD33	P _{BAD} expression vector; P _{ara} ; origin of replication; Cm ^r	Guzman <i>et al.</i> (1995)
pBAD33-traG _{FLAG}	2.1 kbp <i>EcoRI/BamHI</i> -digested PCR product in pBAD33; Cm ^r	This study
pBAD33-trhB _{FLAG}	1.9 bp <i>XbaI/SphI</i> -digested PCR product in pBAD33; Cm ^r	This study
pMS119EH	Expression vector; P _{tac} - <i>lacI</i> ^q ; pMB1 origin of replication; Amp ^r	Strack <i>et al.</i> (1992)
pMS119EH-traG ^H _{His}	2.1 kbp <i>EcoRI/BamHI</i> -digested R27 (IncH) PCR product in pMS119EH; Amp ^r	This study
pMS119EH-traG ^P _{His}	1.9 kbp <i>EcoRI/BamHI</i> -digested RP4 (IncP) PCR product in pMS119EH; Amp ^r	This study
pMS119EH-traD ^F _{His}	2.2 kbp <i>HindIII/EcoRI</i> -digested F (IncF) PCR product in pMS119EH; Amp ^r	This study
pMS119EH-trwB ^W _{His}	1.5 kbp <i>EcoRI/BamHI</i> -digested R388 (IncW) PCR product in pMS119EH; Amp ^r	This study
pKT25	p15A origin of replication encoding CyaA ₁₂₂₄ ; Kan ^r	Karimova <i>et al.</i> (1998)
pUT18C	ColE1 origin of replication encoding CyaA ₂₂₅₃₉₉ ; Amp ^r	Karimova <i>et al.</i> (1998)
pKT25-zip	Leucine zipper of GCN1 (BTH positive control); Kan ^r	Karimova <i>et al.</i> (1998)
pUT18C-zip	Leucine zipper of GCN1 (BTH positive control)	Karimova <i>et al.</i> (1998)
pUT18C-trhB	1.4 kbp <i>BamHI/KpnI</i> -digested PCR product in pUT18C; Amp ^r	Gilmour <i>et al.</i> (2003)
pUT18C-traJ	660 bp <i>PstI/BamHI</i> -digested PCR product in pUT18C; Amp ^r	Gunton <i>et al.</i> (2005)
pUT18C-traG ^{R27}	2.1 kbp <i>PstI/BamHI</i> -digested R27 (IncH) PCR product in pUT18C; Amp ^r	Gilmour <i>et al.</i> (2003)
pUT18C-traG ^{RP4}	1.9 kbp <i>PstI/BamHI</i> -digested RP4 (IncP) PCR product in pUT18C; Amp ^r	This study
pUT18C-traD ^F	2.2 kbp <i>PstI/BamHI</i> -digested F (IncF) PCR product in pUT18C; Amp ^r	This study
pUT18C-trwB ^{R388}	1.5 kbp <i>PstI/BamHI</i> -digested R388 (IncW) PCR product in pUT18C; Amp ^r	This study
pUT18C-traG ^{R478}	2.1 kbp <i>PstI/BamHI</i> -digested R478 (IncH) PCR product in pUT18C; Amp ^r	This study
pUT18C-traD ^{R391}	1.8 kbp <i>PstI/BamHI</i> -digested R391 (IncJ) PCR product in pUT18C; Amp ^r	This study
pUT18C-5177 ^{Pfl}	2.1 kbp <i>PstI/BamHI</i> -digested <i>P. fluorescens</i> PCR product in pUT18C; Amp ^r	This study
pKT25-trhB	1.4 kbp <i>BamHI/KpnI</i> -digested PCR product in pKT25; Kan ^r	This study
pKT25-traJ ^{R27}	660 bp <i>PstI/BamHI</i> -digested R27 (IncH) PCR product in pKT25; Kan ^r	This study
pKT25-traJ ^{R478}	662 bp <i>PstI/BamHI</i> -digested R478 (IncH) PCR product in pKT25; Kan ^r	This study
pKT25-36 ^{R391}	644 bp <i>PstI/BamHI</i> -digested R391 (IncJ) PCR product in pKT25; Kan ^r	This study
pKT25-5176 ^{Pfl}	732 bp <i>PstI/BamHI</i> -digested <i>P. fluorescens</i> PCR product in pKT25; Kan ^r	This study
pDT1942	Derepressed transfer mutant of R27; R27::Tn <i>lacZ</i>	Maher <i>et al.</i> (1991)
pDT2989	pDT1942 with mini::Tn10 inserted into <i>traG</i>	Lawley <i>et al.</i> (2002)
pOX38	F plasmid (IncF)::mini-Tn10; Tc ^r	Anthony <i>et al.</i> (1996)
pOX38::TraD 411	Kanamycin-resistance cassette insertion in TraD of pOX38; Kan ^r	Maneewannakul <i>et al.</i> (1996)
pDB126	Essential transfer proteins of RP4 (IncP); Cm ^r	Balzer <i>et al.</i> (1994)
pDB127	Derivative of pDB126 with a deletion of TraG; Cm ^r	Balzer <i>et al.</i> (1994)
R388	IncW plasmid; Kan ^r	Cabezón <i>et al.</i> (1994)
pSU1456	72 bp insertion in <i>trwB</i> of R388; Tp ^r	Cabezón <i>et al.</i> (1994)

were washed three times with lysis buffer. The immunoprecipitate fraction was collected by resuspending the beads in 50 μ l 1 $\times$ Laemmli sample buffer (5% β -mercaptoethanol). Upon resolution of the cleared lysate and immunoprecipitation by 10% SDS-PAGE, proteins were transferred to nitrocellulose and probed with the anti-adenylate cyclase mAb 3D1 (List Biological Laboratories). This mAb is specific for the distal portion of the adenylate cyclase catalytic domain (aa 377–399) (Lee *et al.*, 1999).

Membrane preparations. Overnight culture (20 ml) of *E. coli* DH5 α (pUT18C-TraJ) with ampicillin was used to inoculate 500 ml LB–Lennox broth. Cells were grown to mid-exponential phase at 28 °C and 225 r.p.m., and induced with 0.4 mM IPTG for 20 min. Bacterial cells were harvested by centrifugation (7000 g, 7 min), resuspended in 10 ml buffer A [10 mM Tris/HCl, pH 7.6, 5 mM MgCl₂, one Complete Protease Inhibitor Cocktail tablet (Roche), 1 mM PMSF, 100 U micrococcal nuclease, 1 mg RNase]. One milligram of

Table 2. Primers used in this study

Primer	Sequence (5'–3')*	Predicted product size (bp)
<i>traG</i> -FLAG-f	TATATCTAGAATGACAAAATCAAAAAGAACCAAC	2109
<i>traG</i> -FLAG-r	TATAAAGCTTTTACTTGTTCATCGTCGTCCTTGATGTCATGCAATTCCTTAGATATTTATTTA	2109
<i>traG</i> -His-R27-f	TATATGAATTCAATGACAAAATCAAAAAGAAC	2103
<i>traG</i> -His-R27-r	TATATGGATCCCTTAATGGTGATGGTGATGGTGCGCATGCAATTCCTTAGATATT	2103
<i>traG</i> -His-RP4-f	TATAGAATTCAATGAAGAAACCCGAAACAACGCCGTG	1926
<i>traG</i> -His-RP4-r	TATAGGATCCTCAATGGTGATGGTGATGGTGATCGTGATCCCCCTCCCCTTC	1926
<i>traD</i> -His-F-f	TATAGAATTCAATGAGTTTAAACGCAAAAGGATATGAC	2172
<i>traD</i> -His-F-r	TATAAAGCTTTCAATGGTGATGGTGATGGTGGAATCATCTCCCGGCTCAAC	2172
<i>trwB</i> -His-W-f	TATAGAATTCAATGCATCCAGACGATCAAAAG	1542
<i>trwB</i> -His-W-r	TATAGGATCCTCAATGGTGATGGTGATGGTGGAATAGTCCCCTCAACAAAGGC	1542
<i>trhB</i> -18C-f	TATAGGATCCAGACATTAAAAAGGCCTGGGAAAAATAAAC	1359
<i>trhB</i> -18C-r	TATAGGTACCGCGCGGCTTGTCAGGTTTGTAG	1359
<i>traG</i> -18C-R27-f	TATAGTGCAGGACAAAATCAAAAAGAACCAAC	2085
<i>traG</i> -18C-R27-r	TATAGGATCCTCAATGCAATTCCTTAGAT	2085
<i>traG</i> -18C-RP4-f	TATAGGATCCCAAGAACCAGAAACAACGCCGTG	1908
<i>traG</i> -18C-RP4-r	TATAGGTACCGTATCGTGATCCCCCTCCCCTTC	1908
<i>traD</i> -18C-F-f	TATAGGATCCCGAGTTTAAACGCAAAAGGATATG	2154
<i>traD</i> -18C-F-r	TATAGGTACCGGAAATCATCTCCCGGCTCAAC	2154
<i>trwB</i> -18C-W-f	TATACTGCAGGCATCCAGACGATCAAGAAAG	1524
<i>trwB</i> -18C-W-r	TATAGGATCCTTAGATAGTCCCCTCAACAAAGGC	1524
<i>trhB</i> -25K-f	TATAGGATCCCCGACATTAAAAAGGCCTGGGAAAAATAAAC	1359
<i>trhB</i> -25K-r	TATAGGTACCGCGCGGCTTGTCAGGTTTGTAG	1359
<i>traJ</i> -R27-25K-f	TATACTGCAGGCGCTGCGGATAATTCTGCTCGTG	663
<i>traJ</i> -R27-25K-r	TATAGGATCCTCACATAAGTTTTTGAAAGTTAGAC	663
<i>traJ</i> -R478-25K-f	TATACTGCAGGCGCTGCGGATAATACAAGCAG	663
<i>traJ</i> -R478-25K-r	TATAGGATCCTCACATGAGTTTTTGAAAGTTAGAC	663
<i>36-R391-25K-f</i>	TATACTGCAGGCTTTGGCATGAGCAAGCATAAG	645
<i>36-R391-25K-r</i>	TATAGGATCCTCATATCTTCTCTGCAAGTGAC	645
<i>5176-P.flo.-25K-f</i>	TATACTGCAGGCGCGACTTCCGCCAGAACACG	732
<i>5176-P.flo.-25K-r</i>	TATAGGATCCTCAGAGATATTTCTTAAATGTACTTGC	732
<i>traG</i> -R478-18C-f	TATACTGCAGGTCAGATTGGAAGAGGACTAAC	2085
<i>traG</i> -R478-18C-r	TATAGGATCCTCAATGCAACTTCCTTAGAAATTTA	2085
<i>traD</i> -R391-18C-f	TATACTGCAGGAAAGAAAACGCATACGAAATGC	1821
<i>traD</i> -R391-18C-r	TATACTGCAGGAAAGAAAACGCATACGAAATGC	1821
<i>5177-P.flo.-18C-f</i>	TATATCTAGAGGCCGAGCATGCAATGGAGTCCAAG	2106
<i>5177-P.flo.-18C-r</i>	TATAGGTACCTCACCCACCTGATCCAGATCGAAG	2106

*Underlined sequences denote restriction enzyme cleavage sites.

lysozyme was added to cells for 30 min on ice, and cells were lysed using sonication for 3 min (Fisher model 300 sonicator). Unlysed cells were removed by centrifugation (twice at 6800 g for 10 min), and this preparation was called the whole-cell lysate. The cytoplasmic/periplasmic fraction was obtained by ultracentrifugation (100 000 g for 2 h at 4 °C) and the supernatant fraction was transferred to a new tube. The resulting pellet containing the membrane fraction was resuspended in 1 ml buffer B [55 % sucrose (w/v), 10 mM Tris/HCl, pH 7.6, 5 mM EDTA].

Immunodetection of proteins. Protein preparations were boiled for 10 min and separated using 10 % SDS-PAGE, transferred to nitrocellulose membranes, and blocked overnight in 1 % (w/v) skimmed milk and 0.1 % Tween 20 in PBS. Membranes were probed with the anti-adenylate cyclase mAb 3D1, anti-DnaK mouse antibody (Stressgen) or anti-CpxA rabbit antibody (Raivio *et al.*, 1999) for 1 h

at room temperature, washed, and the secondary anti-mouse IgG or anti-rabbit IgG (Sigma) was applied. Detection was performed using the ECL kit (Amersham Biosciences) according to the manufacturer's instructions.

Phylogenetic analysis. Protein sequences were aligned using CLUSTAL_W (Gonnet matrix, gap penalty=10, extension penalty=0.2) (Thompson *et al.*, 1994), and phylogenetic trees were constructed using the unweighted pair group method with arithmetic mean (UPMGA) algorithm of MEGA3 (Kumar *et al.*, 2004). To test the statistical significance of the tree, 500 bootstrap samples were generated from the alignment data.

Web-based computer programs. PSI-BLAST (<http://www.ncbi.nlm.nih.gov/blast/>), ScanProsite (<http://au.expasy.org/tools/scanprosite/>), TMHMM (<http://www.cbs.dtu.dk/services/>)

Fig. 1. *In vivo* BTH interactions between the R27 coupling protein TraG and R27 transfer protein TraJ. Conjugative proteins were fused with adenylate cyclase domains and co-produced in BTH101. The BTH controls were BTH101 cells containing pUT18C and pKT25 (negative control), and pUT18C-leucine zipper with pKT25-leucine zipper (positive control). Liquid cultures of transformed BTH101 cells were analysed for β -galactosidase activity using a Miller assay (Miller, 1972). Results of a representative experiment are shown.

TMHMM-2.0/), CLUSTAL_W (<http://www.ebi.ac.uk/clustalw/>) and EMBOSS pairwise local alignment (Blosum40 matrix, gap penalty = 10, extension penalty = 0.5; <http://www.ebi.ac.uk/emboss/align/>) were used.

RESULTS AND DISCUSSION

Although it has been difficult to identify interacting partners within the membrane-associated components of the conjugative apparatus because of the extracytoplasmic locations of the interacting proteins, BTH studies with the IncH plasmid R27 have previously demonstrated an interaction between the coupling protein TraG and VirB10 homologue TrhB (Gilmour *et al.*, 2003). Elegant studies have identified similar interactions between TrwB (coupling protein) and TrwE (VirB10 homologue) from the IncW plasmid R388, as well as VirB10 homologues in IncN and IncX plasmids (Llosa *et al.*, 2003). In this study, BTH technology was used to investigate TraG and potential interactions with non-Mpf

Fig. 2. Co-immunoprecipitation of an IncH R27 TraJ–TraG complex. The cellular lysates (CL) of *E. coli* DH5 α cells expressing TraJ_{AC18} and TraG_{FLAG} were mixed with M2 anti-FLAG affinity gel beads, washed, and resolved with 10% SDS-PAGE. After transfer to nitrocellulose, the samples were probed with the anti-adenylate cyclase mAb 3D1, which detected TraJ in the immunoprecipitate samples (IP). As a control for non-specific precipitation of TraJ_{AC18}, cellular lysates containing TraJ_{AC18} and TrhB_{FLAG}, or TraJ_{AC18} alone were mixed with M2 anti-FLAG affinity gel beads, and resolved as described above.

proteins TraJ, TraH and TraI, located contiguously in the TraI region of R27 (Lawley *et al.*, 2002).

In vivo detection of the R27 TraG and TraJ interaction

The R27 relaxase gene *traI*, non-Mpf gene *traH* and non-Mpf gene *traJ* (encoding a putative membrane-associated transfer protein) were each cloned into the BTH vector pUT18C. The R27 coupling protein gene *traG* was cloned in the appropriate reading frame of the pKT25 vector, and the resulting gene product TraG_{AC25} was a fusion between the 78 kDa coupling protein and the C-terminal 25 kDa region of the adenylate cyclase catalytic domain. Screening for pairwise interactions using the BTH system did not identify an interaction that involved R27 relaxase TraI or TraH. However, co-production of R27 TraJ_{AC18} with TraG_{AC25} allowed for functional complementation of the *B. pertussis* adenylate cyclase domains, which were initially screened by plating transformed BTH101 cells onto media containing X-Gal. More than 99% of the colonies containing TraJ_{AC18} with TraG_{AC25} constructs were blue within 48 h of plating. The BTH positive control (vectors containing leucine

Fig. 3. TraJ_{AC18} localizes to the cellular membrane. Bacteria containing pUT18C–TraJ were fractionated into a cytoplasmic and periplasmic soluble fraction (S), and a total membrane fraction (M). Whole-cell lysate (L) samples were also run on SDS-PAGE, and transferred to nitrocellulose membranes. Membranes were individually probed with (A) anti-AC 3D1 to detect TraJ_{AC18}, (B) anti-DnaK and (C) anti-CpxA. There was evidence of non-specific banding with the application of the polyclonal anti-CpxA antibody.

Fig. 4. BTH data for R27 transfer protein TraJ or the Mpf protein TrhB produced with the coupling protein from IncH (R27), IncF (F plasmid), IncP (RP4) and IncW (R388) plasmids. The BTH plasmid pKT25 was used to produce IncH R27-adenylate cyclase fusion proteins TraJ_{AC25} and TrhB_{AC25}. The coupling protein homologues were cloned into pUT18C, resulting in the expression of TraG^H, TraD^F, TraG^P and TrwB^W. Protein interaction was determined by assaying for the β -galactosidase activity produced by co-expression of (A) TrhB^H or (B) TraJ^H with TraG^H, TraD^F, TraG^P and TrwB^W. The BTH controls were BTH101 cells containing pUT18C and pKT25 (negative control), and pUT18C-leucine zipper with pKT25-leucine zipper (positive control). Results of a representative experiment are shown.

zippers) showed similar blue colonies, whereas the BTH101 cells containing empty BTH vectors were white. A standard Miller assay was used to quantify β -galactosidase activity (Fig. 1). The *in vivo* interaction between TraJ and TraG was found to produce comparable levels of β -galactosidase activity to those of the BTH leucine zipper-positive control (Fig. 1).

Both TraG and TraJ contain multiple, well-conserved predicted TM domains (Gilmour *et al.*, 2004), and to ensure that this interaction was not a non-specific interaction of membrane-spanning domains caused by

overexpression of R27 transfer proteins, the R27 *virB10* homologue *trhB* was cloned into the BTH vector pUT18C. The VirB10 family of proteins are cytoplasmic membrane-associated conjugative proteins that have been shown to interact with coupling proteins (Gilmour *et al.*, 2003; Llosa *et al.*, 2003). When TraJ_{AC18} was co-produced with TrhB_{AC25} in BTH101, >99% of the colonies were white after 48 h incubation on media containing X-Gal. The inability of TrhB to interact with TraJ implies that the productive TraG–TraJ interaction was not simply due to predicted co-localization of transfer proteins at the cytoplasmic membrane.

Immunoprecipitation of a TraG and TraJ complex

To confirm the interaction between the R27 coupling protein and TraJ, we purified R27-tagged protein complexes using an immunoprecipitation technique. The C termini of TraG and TrhB were fused with a FLAG octapeptide Asp-Tyr-Lys-Asp-Asp-Asp-Lys tag. Cell lysates were prepared after co-production of TraJ_{AC18} and TraG_{FLAG}, and in the presence of Sepharose beads conjugated with anti-FLAG antibody, the TraJ_{AC18} construct was precipitated by TraG_{FLAG} (Fig. 2). The mAb 3D1, which recognizes an epitope in the 18 kDa adenylate cyclase fragment of *B. pertussis* (Lee *et al.*, 1999), was used to detect precipitated TraJ_{AC18}. When TraG_{FLAG} was omitted from the immunoprecipitation experiment, no TraJ_{AC18} was recovered by the anti-FLAG bead matrix (Fig. 2), ensuring that TraJ was not bound non-specifically by a component of the immunoprecipitation reaction. Additionally, to ensure that the TraG and TraJ interaction was not due to overproduction of two membrane-associated proteins, TrhB was included in an immunoprecipitation experiment with TraJ. Although TraJ_{AC18} was soluble and detectable in the cleared lysate prior to immunoprecipitation, TrhB_{FLAG} did not precipitate TraJ_{AC18} (Fig. 2). Furthermore, the inability of TraJ to interact with TrhB confirmed that the interaction of TraJ_{AC18} and TraG_{FLAG} was not due to non-specific interaction between the 18 kDa adenylate cyclase domain and TraG_{FLAG}. The precipitation of a TraG–TraJ complex confirmed the BTH data, which showed that the R27 coupling protein interacted with the non-Mpf protein TraJ.

TraJ localizes to the cellular membrane

Sequence alignments of R27-encoded TraJ and TraJ homologues have identified that this group of proteins are similar in size (~220 aa), and contain four conserved hydrophobic TM regions (Gilmour *et al.*, 2004). To determine if the predicted TM regions correlated with localization of TraJ to the cellular membrane, a fractionation study was performed on *E. coli* containing pUT18C-TraJ. Separation of the cytoplasmic membrane and outer membranes (M fractions) from the soluble cytoplasmic and periplasmic fractions (S fractions), and subsequent Western blot analysis revealed that TraJ_{AC18} associated exclusively with the membrane fraction (Fig. 3A).

Table 3. Distribution of the *traJ*–*traG* module in gamma- and beta-Proteobacteria

The criteria for selection were gene products that were similar to R27-encoded TraG and TraJ (by protein sequence identity by BLAST, similar size in amino acids and similar number of predicted TM domains), and were also encoded by adjacent or nearly adjacent coding sequences.

Organism	Genome location*	TraG homologue					TraJ homologue			
		Protein	Accession no.	Size (aa)	No. of TM†	Distance between <i>traG</i> and <i>traJ</i> (bp)‡	Protein	Accession no.	Size (aa)	No. of TM†
<i>Salmonella enterica</i> serovar Typhimurium§	R27	TraG	NP_058332	694	2	1089	TraJ	NP_058330	220	4
<i>Serratia marcescens</i>	R478	TraG	NP_941281	694	2	1095	TraJ	NP_941283	220	4
<i>Vibrio cholerae</i>	SXT element	TraD	AAL59680	599	0	557	s043	AAL59721	211	4
<i>Providencia rettgeri</i>	R391 element	TraD	AAM08004	606	0	547	ORF36	AAM08039	214	4
<i>Pseudomonas resinovorans</i>	pCAR1	TraG	NP_758665	609	0	372	ORF124	NP_758667	212	4
<i>Proteus vulgaris</i>	Rts1	ORF202	NP_640162	655	0	382	ORF204	NP_640164	219	4
<i>Polaromonas</i> sp. JS666	Plasmid 1	Bpro_5224	YP_551983.1	650	1	619	Bpro_5225	YP_551984.1	212	4
<i>Polaromonas</i> sp. JS666	Plasmid 2	Bpro_5471	YP_552223.1	635	1	696	Bpro_5469	YP_552221.1	214	4
<i>Rhodospirillum rubrum</i> T118	Plasmid 1	Rfer_4266	YP_515951.1	629	0	630	Rfer_4264	YP_515949.1	209	4
<i>Ralstonia eutropha</i> H16	pHG1	TraG	NP_943001.1	621	1	785	PHG369	NP_943004.1	205	4
<i>Ralstonia metallidurans</i> CH34	Plasmid 2	Rmet_6309	YP_582118.1	622	1	787	Rmet_6312	YP_582121	205	4
<i>R. metallidurans</i> CH34	Chromosome 1	Rmet_2336	YP_584482.1	729	2	–4	Rmet_2337	YP_584483.1	249	5
<i>Acidovorax avenae</i> subsp. citrulli AAC00-1		AaveDRAFT_0947	EAT93286.1	730	1	–4	AaveDRAFT_0946	EAT93285.1	249	4
<i>Acidovorax</i> sp. JS42		AjsDRAFT_3704	ZP_01381120.1	666	1	–4	AjsDRAFT_3703	ZP_01381119.1	207	4
<i>Azoarcus</i> sp. EbN1		ebA2421	YP_158384.1	718	2	–4	ebA2420	YP_158383.1	249	5
<i>Burkholderia pseudomallei</i> S13		BpseS_03003063	ZP_01328838	640	0	19	BpseS_03003064	ZP_01328839.1	248	5
<i>Burkholderia vietnamiensis</i> G4		Bcep1808DRAFT_6269	EAM32001.1	622	1	736	Bcep1808DRAFT_6271	EAM32003.1	201	4
<i>Burkholderia xenovorans</i> LB400	Chromosome 1	Bxe_A1195	YP_559810.1	728	2	–4	Bxe_A1194	YP_559811.1	249	5
<i>Congregibacter litoralis</i> KT71		KT71_18006	EAQ99589.1	673	0	1	KT71_18001	EAQ99588.1	243	4
<i>Dechloromonas aromatica</i> RCB		Daro_2307	YP_285513	635	1	–4	Daro_2308	AAZ47044.1	207	4
<i>D. aromatica</i> RCB		Daro_2598	YP_285798.1	635	1	–4	Daro_2599	AAZ47329.1	207	4
<i>Erwinia carotovora</i> SCRI1043		ECA0560	YP_048676.1	698	1	–3	ECA0561	YP_048677.1	251	3
<i>Haemophilus somnus</i> 129PT		Hsom02001885	ZP_00123135.1	743	0	334	Hsom02001883	ZP_00123133.1	229	4
<i>Marinobacter aquaeolei</i> VT8		MaquDRAFT_1771	ZP_00817611.1	664	0	3	MaquDRAFT_1770	ZP_00817610.1	242	4
<i>Nitrosomonas eutropha</i> C71		NeutDRAFT_1853	EA018140	724	2	–4	NeutDRAFT_1852	EA018139.1	249	4
<i>Polaromonas naphthalenivorans</i> CJ2		PnapDRAFT_0335	EAQ18260	637	3	1689	PnapDRAFT_0337	EAQ18262.1	211	4
<i>Pseudomonas aeruginosa</i> 2192		Paer2_01002163	ZP_00974781	687	0	–4	Paer2_01002164	ZP_00974782.1	249	5
<i>P. aeruginosa</i> SG17M		SG68	AAN62290.1	724	2	–4	SG67	AAN62289.1	249	4
<i>P. fluorescens</i> PfO-1		Pfl_3012	ABA74750.1	701	1	90	Pfl_3013	ABA74751.1	243	4
<i>Pseudomonas putida</i>	pWWO	p081	NP_542873.1	718	2	–4	p080	NP_542872.1	249	5
<i>Rubrivivax gelatinosus</i> PM1		Rgel02003420	ZP_00242291	718	2	–4	Rgel02003421	ZP_00242292.1	249	5
<i>S. Typhi</i> CT18		STY4562	NP_458648	696	1	–4	STY4563	NP_458649.1	252	5

Table 3. cont.

Organism	Genome location*	TraG homologue				Distance between <i>traG</i> and <i>traJ</i> (bp)‡	TraJ homologue			
		Protein	Accession no.	Size (aa)	No. of TM†		Protein	Accession no.	Size (aa)	No. of TM†
<i>Verminephrobacter eiseniae</i> EF01-2		VeisDRAFT_0985	EAT72023	635	1	−4	VeisDRAFT_0986	EAT72024.1	210	4
<i>Xanthomonas axonopodis</i> 306		XAC2259	NP_642576.1	565	0	−4	XAC2260	NP_642577	249	4
<i>Xanthomonas campestris</i> 8004		XC_2040	YP_243121.1	729	1	−4	XC_2039	YP_243120.1	249	5
<i>X. campestris</i> 85-10		XCV2377	YP_364108.1	730	2	−4	XCV2376	YP_364107.1	249	5
<i>Yersinia pestis</i> biovar Orientalis IP275		Ypesb_01003257	ZP_01175344.1	621	0	552	Ypesb_01003259	ZP_01175346.1	209	5
<i>Yersinia pseudotuberculosis</i> 32777		api27	CAF28501.1	706	0	−1	api28	CAF28502.1	250	4
<i>Y. pseudotuberculosis</i> IP 31758		Ypsel_01002746	ZP_00793949.1	706	1	−1	Ypsel_01002747	ZP_00793950.1	250	4

*If no genome location is indicated, it is either chromosomal or part of a draft genomic sequence which has not been fully annotated.

†TM, transmembrane helices predicted by TMHMM.

‡Overlapping coding sequences are marked with a negative value.

§R27 was isolated from *S. typhimurium* IM1407 (Lawn *et al.*, 1967; Smith *et al.*, 1973; see Introduction).

To ensure the efficacy of the fractionation procedure, the M and S fractions were probed with antibodies specific for cytoplasmic and membrane-associated proteins. DnaK is a well-characterized cytoplasmic heat shock protein that is essential for λ replication (Liberek *et al.*, 1988), and an anti-DnaK antibody identified this protein exclusively in the S fraction (Fig. 3B). CpxA is a component of the Cpx pathway involved in the envelope stress response (see Raivio, 2005, for a review), and anti-CpxA antibody detected this protein exclusively in the M fraction (Fig. 3C).

Immunofluorescent analysis of the R27 coupling protein TraG has previously demonstrated a similar membrane localization (Gunton *et al.*, 2005). Fluorescently labelled R27 coupling protein was visualized as discrete foci in the periphery of the cell. The discovery that TraJ is membrane bound and able to interact with the membrane-associated R27 coupling protein indicates that TraJ may act as an interacting partner for the coupling protein during conjugation.

Non-cognate coupling protein interactions with the IncH Mpf

Complementation of conjugative plasmids containing coupling protein mutations by non-cognate coupling proteins has indicated that the Mpf-coupling protein interaction is non-specific (Cabezon *et al.*, 1994; Hamilton *et al.*, 2000; Llosa *et al.*, 2003). Furthermore, recent studies on the coupling proteins and VirB10 homologues from the IncW R388 plasmid, IncN plasmid pKM101 and IncX plasmid R6K have demonstrated that each coupling protein is able to interact with cognate and heterologous VirB10 proteins (Llosa *et al.*, 2003). To expand upon these studies, the coupling proteins from the conjugative plasmids R27 (IncH), RP4 (IncP), F (IncF) and R388 (IncW) were cloned into the pUT18C BTH vector. When each coupling protein was co-produced in BTH101 cells with the R27 Mpf construct TrhB_{AC25}, there were successful interactions between each coupling protein and the VirB10-homologue TrhB (Fig. 4A). The values obtained in the BTH studies of the interactions between the coupling proteins and TrhB were similar to those produced by the BTH leucine-zipper positive control. This seemed remarkable, given the low level of overall similarity shared between members of the coupling protein family. The coupling proteins from the RP4, R388 and F plasmids had similar levels of sequence identity with TraG^{R27}: TraG^{RP4}, 19.6 % identity over an alignment length of 749 aa; TraD^F, 19.5 % identity over an alignment length of 845 aa; and TrwB^{R388}, 20.1 % identity over an alignment length of 750 aa.

As the interface between coupling protein and Mpf is proposed to occur at the cytoplasmic membrane, an interaction was also investigated between the membrane-associated protein TraJ and the non-cognate coupling proteins TraG^{RP4}, TraD^F and TrwB^{R388}. These BTH studies should have indicated if the lack of specificity in the coupling protein–TrhB interaction extended to the coupling

Fig. 5. TraJ protein phylogeny. The bootstrap support values of the phylogenetic tree are from the UPGMA method. Accession numbers and other details are indicated in Table 3. The distance score scale bar is shown at the bottom.

proteins and TraJ, although a limitation of the BTH analysis is that the adenylate cyclase fusions may create steric interference that prevents interactions. The only *in vivo* interaction that was detected using the BTH technology was between the R27 coupling protein and the R27 TraJ protein (Fig. 4B). These data suggest that the coupling protein–TraJ interaction is specific to conjugative systems that encode *traJ*.

Because the coupling proteins TraG^{RP4}, TraD^F and TrwB^{R388} did not interact with TraJ in a BTH screen, and because TraJ is essential for conjugation (Lawley *et al.*,

2002), it is unlikely that these non-cognate coupling proteins would be functionally interchangeable with the R27 coupling protein TraG^{R27} in context with the remainder of the R27 conjugative apparatus. To investigate this, *E. coli* DY330R cells containing an R27 *traG* insertional mutant were transformed with the expression vector pMS119 expressing C-terminal His-tagged TraG^{R27}, TraG^{RP4}, TraD^F and TrwB^{R388}. Immunoblot analysis confirmed expression of each coupling protein (data not shown), and RP4, R388 and F plasmids encoding coupling protein mutations were successfully complemented by the constructs expressing their cognate coupling proteins (data not

Fig. 6. TraG protein phylogeny. The bootstrap support values of the phylogenetic tree are from the UPGMA method. Accession numbers and other details are indicated in Table 3. The distance score scale bar is shown at the bottom.

shown). The TraG coupling protein from R27 was the only protein that restored conjugative transfer of the R27 *traG* mutant.

Functional co-inheritance of TraJ and TraG homologues

Bioinformatic analyses of the IncHI2 plasmid R478 have revealed that *traG* and *traJ* homologues are encoded by a

number of genomic sources, including plasmids, conjugative elements and chromosomes (Gilmour *et al.*, 2004). Similar genomic analyses were repeated, and adjacent determinants encoding proteins similar to TraG and TraJ were observed in 38 gamma- and beta-proteobacteria strains, including many members of the order *Burkholderiales* (Table 3). The newly observed TraJ homologues were all of a similar size (201–252 aa), and typically encoded four or five predicted TM domains.

Fig. 7. BTH data for TraJ and TraG homologues. The *traJ* homologous genes from R27, R478, R391 and *P. fluorescens* (Pfl) were cloned into the BTH plasmid pKT25. The *traG* homologous genes from the same genomic sources were cloned into pUT18C, and co-transformed into BTH101 in a pairwise combination with each pKT25–TraJ construct. The BTH controls were BTH101 cells containing pUT18C and pKT25 (negative control), and pUT18C-leucine zipper with pKT25-leucine zipper (positive control). Liquid cultures of BTH101 containing the BTH vectors were analysed for β -galactosidase activity using a Miller assay (Miller, 1972). Results of a representative experiment are shown.

Phylogenetic analysis of TraJ homologues encoded within this co-inherited module revealed that the majority of the plasmid-encoded determinants clustered on a different node from the chromosomal determinants (Fig. 5). Not all of the presented sequences were fully annotated, so the genomic location (chromosome versus plasmid) was uncertain in some instances, although the majority of TraJ homologues encoded from a chromosomal source had an additional ~30 aa at the N terminus of the protein, and the *traJ* and *traG* coding sequences overlapped (Table 3). The protein sequences similar to TraG were also examined, and a tree with very similar topology to that of TraJ was observed, except that IncP, IncF and IncW coupling proteins, and Ti plasmid-coupling plasmid orthologue VirD4 were included, and these were divergent from the IncH TraG-like sequences (Fig. 6). This comparison of coupling protein homologues indicates that the IncP, IncW and IncF, and Ti plasmid sequences are distinct from the IncH-type coupling proteins, and the requirement for TraJ as an interacting partner may partly explain the divergence of the IncH-type coupling proteins.

To ascertain if an interaction between the coupling protein and TraJ was a common feature within this module of *traG*–*traJ* genes, we selected representative TraJ homologues from different nodes in the TraJ comparison. TraJ homologues encoded on R27, R478, R391 and *Pseudomonas fluorescens* chromosomal DNA were cloned into the BTH vector pKT25. The coupling protein homologues encoded on each respective genomic source were cloned into the BTH vector

pUT18C. The *in vivo* BTH interaction data demonstrated that the cognate TraJ–TraG interaction from each distinct genomic source was conserved (Fig. 7). The diversity of this TraJ–TraG interaction was further characterized by using the BTH technology to determine if non-cognate TraJ homologues interacted with each coupling protein homologue. As expected, the highly related IncH plasmids R27 and R478 encoded TraJ homologues that were interchangeable in interactions with their coupling proteins (Fig. 6). The R391 plasmid-encoded TraJ and TraG homologues were not able to interact with the respective proteins from the other three genomic sources. Intriguingly, the coupling protein homologue from the *P. fluorescens* genome showed a strong interaction with TraJ from R27. In contrast, there was no evidence of an interaction with the opposite configuration, in which TraG from R27 was tested with the *P. fluorescens* TraJ homologue.

Architectural evidence that TraJ is a functional part of the IncH-type coupling protein complex

During the search of GenBank for homologues of TraJ, we identified that TraJ has sequence identity to the FtsK/SpoIIIE family of proteins. The sequence and structural similarity of coupling proteins to FtsK/SpoIIIE-type proteins is already known (Errington *et al.*, 2001; Wu *et al.*, 1995). These DNA translocase ATPases are essential for coordinating chromosome segregation with cell division (FtsK; Weiss, 2004), or for mediating DNA partitioning during the process of sporulation (SpoIIIE; Wu & Errington,

Fig. 8. Schematic representation of the homology shared by R27-encoded TraG and TraJ proteins with an FtsK DNA translocase, *Sh. oneidensis* MR-1 (NP_717901). Arrows indicate the region of FtsK with which the R27 transfer proteins share the greatest level of homology. The level of this homology is represented as a percentage identity with the alignment length represented in parentheses, as determined by EMBOSS pairwise local alignment. TM regions are indicated by hatched regions, as predicted by TMHMM. Walker A boxes determined by ScanProsite are on the left and Walker B boxes with the motif (R/K-X(7-8)-h(4)-D) (Walker *et al.*, 1982) are on the right. A scale bar for amino acids is indicated.

1994). The similarity to conjugative coupling proteins is located primarily within the region surrounding the Walker A and B boxes, which are essential for nucleoside triphosphate binding and hydrolysis (Walker *et al.*, 1982). At the N terminus of the DNA translocases, R27-encoded TraJ is 27 % identical over 110 aa, and has 20.1 % identity over an alignment length of 249 aa to *Streptococcus pneumoniae* SpoIIIE (GenBank accession no. NP_345365). This region of the DNA translocases contains between four and five TM domains that target SpoIIIE and FtsK to the site of cell division (Bath *et al.*, 2000; Sharp & Pogliano, 2003; Wang & Lutkenhaus, 1998). This coincides with the well-conserved TM domains of TraJ, and an alignment of R27-encoded TraJ and TraG to FtsK of *Shewanella oneidensis* illustrates the domains with which the conjugative transfer proteins share homology (TraJ, 21.4 % identity over an alignment length of 243 aa; TraG, 21.1 % identity over an alignment length of 913 aa) (Fig. 8).

The co-inheritance of the *traG-traJ* genes (and interacting gene products) within this module across multiple genetic elements suggests that both plasmid and chromosomal sequences share a common ancestor, but the cellular function of the chromosomal sequences has not been determined. Notably, many of these sequences are present in genomes that do not encode IncH-type Mpf components, indicating that *traG-traJ* forms a module that is inherited independently from the Mpf genes, and that the chromosomal determinants may be involved in functions other than conjugation. The majority of conjugative DNA transfer systems do not encode a TraJ homologue; however, in genetic elements producing both TraG and TraJ, the latter protein may play an essential role in the function of the coupling protein. We propose that the domains comprising TraG and TraJ cumulatively resemble the domain architecture of the FtsK/SpoIIIE DNA translocase proteins. This study confirms that TraJ and TraG interact *in vivo*, and it is possible that these proteins function together

to transport plasmid DNA molecules through the cytoplasmic membrane.

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