Subcellular localization and functional domains of the coupling protein, TraG, from IncHI1 plasmid R27

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Bacterial conjugation is a horizontal gene transfer event mediated by the type IV secretion system (T4SS) encoded by bacterial plasmids. Within the T4SS, the coupling protein plays an essential role in linking the membrane-associated pore-forming proteins to the cytoplasmic, DNA-processing proteins. TraG is the coupling protein encoded by the incompatibility group HI plasmids. A hallmark feature of the IncHI plasmids is optimal conjugative transfer at 30 °C and an inability to transfer at 37 °C. Transcriptional analysis of the transfer region 1 (Tra1) of R27 has revealed that *traG* is transcribed in a temperature-dependent manner, with significantly reduced levels of expression at 37 °C as compared to expression at 30 °C. The R27 coupling protein contains nucleoside triphosphate (NTP)-binding domains, the Walker A and Walker B boxes, which are well conserved among this family of proteins. Site-specific mutagenesis within these motifs abrogated the conjugative transfer of R27 into recipient cells. Mutational analysis of the TraG periplasmic-spanning residues, in conjunction with bacterial two-hybrid and immunoprecipitation analysis, determined that this region is essential for a successful interaction with the T4SS protein TrhB. Further characterization of TraG by immunofluorescence studies revealed that the R27 coupling protein forms membrane-associated fluorescent foci independent of R27 conjugative proteins. These foci were found at discrete positions within the cell periphery. These results allow the definition of domains within TraG that are involved in conjugative transfer, and determination of the cellular location of the R27 coupling protein.

Received 9 June 2005

Revised 19 August 2005

Accepted 22 August 2005

INTRODUCTION

Horizontal gene transfer enables the rapid dissemination of genetic material between prokaryotic organisms. The primary mechanism of this genetic exchange is conjugation (Cascales & Christie, 2003), and the proteins that are essential for conjugative transfer are encoded on plasmids or conjugative transposons. The successful transfer of plasmid DNA from donor to recipient cell is due to the assembly of protein complexes within the cytoplasm, periplasm, cytoplasmic membrane and outer membrane of the donor cells. The cytoplasmic relaxosome is a nucleoprotein complex composed of plasmid DNA and plasmid-encoded proteins, and is responsible for DNA processing prior to transfer of plasmid DNA to the membrane-associated type IV secretion system (T4SS) (Lanka & Wilkins, 1995; Pansegrau *et al.*, 1990). The

T4SS, also referred to as the mating pair formation complex (Mpf), is composed of 12–15 membrane-associated proteins which span the inner and outer membrane of the donor cell (Grahn *et al.*, 2000; Lawley *et al.*, 2003). A multimeric protein, the coupling protein, is responsible for bridging the relaxosome and Mpf complexes, thereby facilitating the conjugative transfer of plasmid DNA (Gilmour *et al.*, 2003; Llosa & de la Cruz, 2005; Llosa *et al.*, 2003). There is a current lack of consensus in the literature regarding whether or not the coupling protein is a component of the T4SS (Lawley *et al.*, 2003; Schroder & Lanka, 2005).

Coupling proteins are polytopic, inner-membrane proteins containing two transmembrane regions near the N-terminus of the proteins; the N- and C-terminal regions of the proteins are located within the cytoplasm, and a periplasmic region separates the two transmembrane domains (Das & Xie, 1998; Gomis-Ruth *et al.*, 2001; Kumar & Das, 2002; Lee *et al.*, 1999; Okamoto *et al.*, 1991; Schroder *et al.*, 2002). The 3D

Abbreviations: BTH, bacterial two-hybrid; drR27, deprepressed R27; T4SS, type IV secretion system(s); WT, wild-type.

structure of the cytoplasmic domain of TrwB, a coupling protein encoded on the incompatibility (Inc) group W plasmid R388, revealed a hexameric, spherical particle with a central channel that ranges in diameter from 7–8 Å (0.7–0.8 nm) at the cytoplasmic side to 22 Å at the membrane end (Gomis-Ruth *et al.*, 2001). Biochemical analysis of coupling proteins from R388, RP4 (IncP α) and F (IncFI) plasmids have revealed that this family of proteins are able to bind in a non-specific fashion to DNA (Hormaeche *et al.*, 2002; Moncalian *et al.*, 1999; Schroder *et al.*, 2002). In addition, coupling proteins have conserved motifs such as the Walker-type nucleoside triphosphate (NTP)-binding domains. Recent analysis of TrwB has revealed that this coupling protein is a DNA-dependent ATPase (Tato *et al.*, 2005). Mutational analyses of the Walker motifs of TraG, the coupling protein from RP4, indicated that the Lys and Asp residues, at positions 187 and 449 of RP4, were essential for conjugation of the IncP plasmid (Balzer *et al.*, 1994).

The most recent advance in the study of coupling proteins has been the identification of the Mpf component to which the coupling protein binds. The coupling proteins TraG and TrwB from the R27 and R388 plasmids, respectively, were both independently determined to interact with the VirB10-like proteins, TrhB and TrwE, from R27 and R388 (Gilmour *et al.*, 2003; Llosa *et al.*, 2003). In the Ti plasmid of *Agrobacterium tumefaciens*, VirB10 is a well-conserved T4SS component and it has been recently proposed to be the ATP energy sensor of the T4SS (Cascales & Christie, 2004a).

The cellular location of the coupling protein has only been demonstrated in *A. tumefaciens*. VirD4 was found to associate with the poles of *A. tumefaciens*, and this polar localization was determined to occur in the absence of other essential conjugation proteins (Kumar & Das, 2002). Subsequent localization experiments with *A. tumefaciens* revealed that a T4SS protein, VirB6, co-localized with VirD4 at the poles of the cell (Jakubowski *et al.*, 2004; Judd *et al.*, 2005). In contrast, a GFP fusion to the T4SS protein TrhC from R27 revealed that this VirB4 homologue was found at random positions around the periphery of the cell (Gilmour *et al.*, 2001). The fluorescent foci formed when TrhC was labelled with GFP resulted in membrane-associated protein complexes which required a number of R27 T4SS proteins (Gilmour & Taylor, 2004).

Our goal was to characterize the coupling protein TraG from the IncHI1 resistance plasmid R27, originally isolated from *Salmonella enterica* serovar Typhi (Lawley *et al.*, 2002; Sherburne *et al.*, 2000). The IncHI plasmids are unique in that conjugative transfer is optimal at 30 °C, and at 37 °C transfer is significantly reduced (Taylor & Levine, 1980). We have determined that *traG* expression is reduced at 37 °C, in comparison with the expression profile observed at 30 °C. Site-specific mutagenesis within *traG* demonstrated that well-conserved residues in the NTP-binding Walker regions are essential for R27 conjugation. In addition, bacterial two-hybrid and immunoprecipitation interaction data determined that substitutions in the four periplasmic-spanning

residues of TraG prevented an interaction with the VirB10 homologue, TrhB. Notably, these periplasmic substitutions did not completely abolish conjugative transfer of the R27 plasmid. Finally, immunofluorescence microscopy was used to determine the subcellular location of TraG. In contrast to the VirD4 polar localization in *A. tumefaciens*, TraG formed fluorescent foci at random positions in the membrane of *Escherichia coli*. The position and number of foci formed by TraG are similar to the distribution of TrhC within the cell.

METHODS

Bacterial strains, growth conditions and plasmids. *E. coli* strains used in this study are listed in Table 1. All strains were grown in Luria–Bertania (LB) broth (Difco) at 37 °C with shaking at 200 r.p.m. unless otherwise stated. Antibiotics used in this study are listed with final concentrations: 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).

RNA extraction. A flask containing 100 ml LB was inoculated from an overnight culture of *E. coli* harbouring either wild-type R27 or derepressed R27 (drR27) grown at either 30 °C or 37 °C (Maher *et al.*, 1991). The 100 ml culture was grown at the same temperature as the overnight culture and the cells were harvested when the culture reached an OD₆₀₀ of 0.3. Total bacterial RNA was purified using the RNeasy Midi Kit (Qiagen), in accordance with the manufacturer's directions. To ensure that the RNA was devoid of contaminating DNA, the preparation was treated with Turbo DNase (Ambion). The RNA was precipitated with ethanol and dissolved in diethyl-pyrocyanate-treated water. RNA levels were quantified using an Ultraspec 4000 spectrophotometer (Pharmacia).

RT-PCR. For RT-PCR amplification of *traG* transcripts, 2 µg total RNA was used as a template. RNA was retrotranscribed into cDNA utilizing the SuperScriptII RT (Invitrogen) with the random hexamers included in the kit. A 1/10 dilution of each retrotranscription was subjected to PCR using the primer pairs specific for the two adjacent genes (Table 2). A negative control containing no template and a negative control with total RNA that had not been retrotranscribed were included in each PCR reaction. A positive control of R27 template DNA was prepared for each primer pair and PCR reactions generated products of predicted sizes. The *E. coli* *pfkA* housekeeping gene, which encodes phosphofructokinase, was included as a positive control for the bacterial transcripts. Amplified products were resolved on a 1% agarose gel stained with ethidium bromide. RT-PCR amplifications were performed at least twice with total RNA preparations obtained from a minimum of three independent extractions. Similar results were obtained in all experiments.

Site-directed mutagenesis of Walker A and B motifs of TraG.

Site-specific mutagenesis of the R27 coupling protein was performed as described previously (Gilmour & Taylor, 2004). Briefly, the template for the site-directed mutagenesis experiments was pJEG217 (pMS119EH-TraG_{His6}). Complementary pairs of oligonucleotides were designed to mutate the Walker A region and Walker B region of the *traG* gene (Table 2). Similarly, primer pairs were used to mutate the periplasmic region (5'-ATGGGCTTTCGTTTCAGATGGGGTG-3') of *traG*, where the underlined region indicates the nucleotides that were substituted (Table 2). *Pfu Turbo* polymerase (Stratagene) was used in a thermocycling reaction with the Walker A or Walker B complementary primers. The parental DNA template of wild-type *traG* gene was digested with *DpnI* (Invitrogen). Newly synthesized DNA containing mutations within the Walker A or B motifs was transformed into XL-1 Blue *E. coli* ultracompetent cells (Stratagene) for recircularization and propagation. The pJEG217

Table 1. Bacteria and plasmids

Bacterial strain or plasmid	Relevant genotype, phenotype or characteristic*	Source or reference
<i>E. coli</i>		
DH5 α	<i>supE44 lacU169</i> (ϕ 80 <i>lacZ</i> Δ <i>M15</i>) <i>hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	Invitrogen
XL-1 Blue	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac</i> [F' <i>proAB lac</i> ^r Δ <i>M15</i> Tn10]	Stratagene
DY330	W3110 Δ <i>lacU169 gal-490 λ</i> c1857 Δ(<i>cro-bioA</i>)	Christie & Vogel (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) with pDT1942	Maher <i>et al.</i> (1991)
Plasmids		
pMS119EH	Expression vector, P _{lac} - <i>lacI</i> ^r , pMB1 origin of replication, Amp ^r	Strack <i>et al.</i> (1992)
pJEG217	<i>traG</i> , 2.1 kb <i>EcoRI/BamHI</i> -digested R27 (IncH) PCR product in pMS119EH, Amp ^r	This study
pJEG217-G210D	pJEG217 with G210D substitution, Amp ^r	This study
pJEG217- K211R	pJEG217 with K211R substitution, Amp ^r	This study
pJEG217-ΔGK	pJEG217 with ΔGK (aa residues 210–211) deletion, Amp ^r	This study
pJEG217-D510S	pJEG217 with D510S substitution, Amp ^r	This study
pJEG217-E511K	pJEG217 with E511K substitution, Amp ^r	This study
pJEG217-ΔDE	pJEG217 with ΔDE (aa residues 510–511) deletion, Amp ^r	This study
pMWG385	pJEG217 with residues FRSD substituted for SSSS at aa positions 40–43, Amp ^r	This study
pKT25	p15A origin of replication, encodes CyaA _{1–224} , Km ^r	Karimova <i>et al.</i> (1998)
pUT18C	ColE1 origin of replication, encodes CyaA _{225–399} , Amp ^r	Karimova <i>et al.</i> (1998)
pKT25- <i>zip</i>	Leucine zipper of GCN1 (BTH positive control), Km ^r	Karimova <i>et al.</i> (1998)
pUT18C- <i>zip</i>	Leucine zipper of GCN1 (BTH positive control)	Karimova <i>et al.</i> (1998)
pUT18C- <i>trhB</i>	1.4 kb <i>PstI/BamHI</i> -digested R27 (IncH) PCR product in pUT18C, Amp ^r	Gilmour <i>et al.</i> (2003)
pUT18C- <i>traJ</i>	660 bp <i>PstI/BamHI</i> -digested R27 PCR product in pUT18C, Amp ^r	This study
pKT25- <i>traG</i>	2.1 kb <i>BamHI/KpnI</i> -digested PCR product in pKT25, Kan ^r	Gilmour <i>et al.</i> (2003)
pKT25- <i>traG</i> :S4	2.1 kb <i>BamHI/KpnI</i> -digested PCR product in pKT25 with residues FRSD substituted for SSSS at aa positions 40–43, Kan ^r	This study
pMS119EH- <i>trhB</i> _{FLAG}	1.4 kb <i>EcoRI/BamHI</i> -digested PCR product in pMS119EH, Amp ^r	Gilmour <i>et al.</i> (2003)
pDT1942	Derepressed transfer mutant of R27: R27::Tn <i>lacZ</i>	Maher <i>et al.</i> (1991)
pDT3152	pDT1942 with a trimethoprim-resistance dihydrofolate reductase gene (<i>dhfrIIIc</i>) inserted into <i>traG</i> , Tp ^r	This study

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

constructs containing Walker A or B mutations were sequenced within the appropriate motifs, and restriction digestion was used to confirm the size of the *traG* constructs. Immunoblot analysis was used to ensure that the mutations within the TraG_{His6} constructs did not affect the stability or expression of the coupling protein. A monoclonal anti-His antibody (Invitrogen) was used to probe the nitrocellulose membrane after transfer, following resolving the whole-cell lysate on a 10% SDS-PAGE. For detection purposes, a secondary rabbit anti-mouse antibody conjugated to horseradish peroxidase (HRP) was used.

Insertional mutagenesis of *traG* on drR27. A gene disruption in *traG* was created using the *E. coli* recombination system, as described previously (Lawley *et al.*, 2002; Yu *et al.*, 2000). A trimethoprim-resistance dihydrofolate reductase cassette, *dhfrIIIc*, was inserted into *traG* of R27. A linear DNA construct was created using PCR primers that amplified *dhfrIIIc* and also contained ~40 bp terminal arms

homologous to *traG* at positions 528–563 and 1545–1585 (Table 2). Presumptive *traG* mutants were screened using PCR to detect a 237 bp size increase in *traG*.

Conjugation. Complementation experiments with R27 were performed as described previously (Lawley *et al.*, 2002; Taylor & Levine, 1980). Briefly, DY330R cells containing drR27 plasmid or an R27 plasmid containing a mutation within the coupling protein gene, *traG*, were grown at 28 °C. DY330N recipient cells were also grown at 28 °C. pDT3152, an R27 plasmid containing a *traG* mutation, was complemented by transforming donor cells with pJEG217 or variants. Upon reaching an OD₆₀₀ of 0.5–0.7, donor cells containing the complementation constructs were induced for 1 h with IPTG at a final concentration of 0.4 mM. Donor and recipient cells were incubated for 16 h at 28 °C. Transconjugants and donors were then plated on selective media after serial dilutions. The transmission frequency was expressed as transconjugants per donor.

Table 2. Primers used for RT-PCR analysis of the transcriptional profile and mutational analyses of the *traG* gene of R27

Primer ID	Primer name	Sequence (5'–3')*	Predicted product size (bp)
1-8	<i>traI-traG-f</i>	AATAGGGGTGTGCCGTTTTAC	523
	<i>traI-traG-r</i>	TGCCACCATGCTCCACCAA	
1-9	<i>traG118-2-f</i>	TCCTGCCATTTCGGTAAACTTCA	405
	<i>traG118-2-r</i>	CGCATAATAACTTCTCTGGGTCAA	
Control	<i>pfkA-f</i>	GTGGCGGTACGTTCTCGGTTCT	762
	<i>pfkA-r</i>	TTTTTCGCGCAGTCCAGCCAGTC	
TraG : Tp ^r	<i>traG-Gil163-f</i>	GGGCAATAACGCCATCTTTGCTGTTGACCGGCGGGCACCCCTTCGCTGCTGCCCAAGGTTG	237
	<i>traG-Gil164-r</i>	ACGACCCCTGTGCGAGTAAGTTTATCATTGAATTGTTTATGGTGCACTCAACCGTGACTTC	
TraG : G210D	<i>traG-Gun46-f</i>	CGTCGGTACCGMTAAAACGGTAC	2085
	<i>traG-Gun47-r</i>	GTACCGTTTAKCGGTACCGACG	
TraG : K211R	<i>traG-Gun44-f</i>	CGGTACCGGTABAACGGTACTTC	2085
	<i>traG-Gun45-r</i>	GAAGTACCGTTVTACCGGTACCG	
TraG : ΔGK	<i>traG-Gun54-f</i>	CCTCATTACCGGAAACGTCGGTACCGTACTTCAGCGCTTACTGAGCATC	2085
	<i>traG-Gun55-r</i>	GATGCTCAGTAAGCGCTGAAGTACGGTACCGACGTTTCCGGTAATGAGG	
TraG : D510S	<i>traG-Gun48-f</i>	GTATTTTCGTCACVCGAAGCACAC	2085
	<i>traG-Gun49-r</i>	GTGTGCTTCGBAGACGAAAATAC	
TraG : E511K	<i>traG-Gun50-f</i>	GTATTTTCGTCGACMAAGCACACTC	2085
	<i>traG-Gun51-r</i>	GAGTGTGCTTKGTCGACGAAAATAC	
TraG : ΔDE	<i>traG-Gun52-f</i>	GAATCAGTATTTTCGTCGCACACTCTGCCATAAAC	2085
	<i>traG-Gun53-r</i>	GTTTATGGCAGAGTGTGCGACGAAAATACTGATTTC	
TraG : S4	<i>traG-Gil169-f</i>	CGTCATAATGCTGGTTATGGGCTCTAGTTCATCTGGGGTGAATATTGCCCC	2085
	<i>traG-Gil170-r</i>	GGGGCAATATTCACCCAGATGAACTAGAGCCCATAACCAGCATTATGACG	

*Modified nucleotides (B, K, M, V) were incorporated into primer sequences to generate pJEG217 substitutions. These modifications correspond to mixtures of nucleotides, where B = T + C + G, K = T + G, M = A + C, V = A + C + G. Sequencing reactions confirmed the single nucleotide which was used for the substitution reaction.

Immunofluorescence microscopy. *E. coli* cells containing pJEG217 were processed for immunofluorescence microscopy according to Kumar & Das (2002). Briefly, *E. coli* cells were grown at 30 °C, unless stated otherwise, to an OD₆₀₀ of 0.5–0.7, then treated for 1 h with IPTG at a final concentration of 0.4 mM. After washing with phosphate-buffered saline (PBS), cells were fixed with 4 % paraformaldehyde for 1 h on ice. The cells were washed three times with PBS and resuspended in 300 µl 25 mM Tris/HCl, 1.8 % glucose and 10 mM EDTA pH 8.0. The resuspension was mixed for 10 min on ice with lysozyme at a final concentration of 2 mg ml⁻¹. Aliquots (50 µl) of the treated cells were spotted onto wells of poly-L-lysine-coated slides and allowed to adhere for 15 min at room temperature. Excess liquid was aspirated from the slides, and the dry slides were washed 10 times with PBS before the addition of 2 % bovine serum albumin (BSA) blocking solution. Slides were incubated with BSA at 37 °C for 30 min, prior to the addition of mouse anti-His antibodies (Invitrogen) and incubation at 4 °C overnight. After washing 10 times with PBS, the slides were incubated with goat anti-mouse antibody conjugated to Alexa Fluor 488 for 1 h at room temperature. After further washing with PBS, the slides were mounted with an antifade mounting medium (*p*-phenylenediamine in 40 % glycerol). Images were collected with a Leica DMI 6000 B microscope equipped with a Hamamatsu Orca ER camera. Image analysis was performed using OpenLab 4.0.2 software.

Bacterial two-hybrid (BTH) system. The conjugative proteins were fused to the catalytic domain of adenylate cyclase from

Bordetella pertussis via cloning into the BTH vectors, pKT25 and pUT18C (Karimova *et al.*, 1998). Plasmids encoding fused proteins were co-transformed into competent BTH101 cells and plated on LB plates with ampicillin, kanamycin and X-Gal at a final concentration of 100 µg ml⁻¹. Cultures were grown at 30 °C for 40–48 h or until the colonies expressing the leucine-zipper-positive control became dark blue in colour due to the degradation of the chromogenic substrate X-Gal. For each interaction, two colonies were streaked on to the above medium and grown at 30 °C for 16 h. These colonies were used to inoculate a 20 ml volume of LB with ampicillin and kanamycin selection and grown at 30 °C for 16 h at 250 r.p.m. The β-galactosidase activity of each culture was measured using the Miller assay (Miller, 1972).

Immunoprecipitation. Immunoprecipitation was performed as described previously with minor modifications (Gilmour *et al.*, 2003). Briefly, DH5α cells containing pKT25-TraG or pKT25-TraG:S4 were co-transformed with pMS119EH-*trh*_{FLAG}. Cells were induced at mid-exponential growth phase (OD₆₀₀ 0.5–0.7) with 0.4 mM IPTG for 1 h before lysis. Harvested cells were resuspended in 1 ml lysis buffer [PBS pH 7.4, 150 mM NaCl, 1 % NP-40, 7.15 % sucrose, 0.2 mg lysozyme ml⁻¹, 1× anti-protease cocktail (Complete; Boehringer Mannheim)] freeze-thawed three times and sonicated for 3 min (30 s pulses, 10 s breaks, Fisher 300 sonicator). The cell lysates were mixed with 40 µl ANTI-FLAG M2-agarose affinity gel (Sigma). The affinity gel was pre-blocked with 5 % BSA for 16 h. The cellular lysates and affinity gel were rotated at 4 °C for 16 h.

The gel was then washed three times with 1 ml lysis buffer. Proteins were eluted from the gel by boiling in Laemmli sample buffer + DTT and resolved on a 10 % SDS-PAGE, transferred to nitrocellulose and blocked with 10 % (w/v) skimmed milk powder, 0.1 % Tween 20 in PBS. A primary antibody of anti-adenylate cyclase [rabbit serum #L24023, specific for the catalytic domain (D. Ladant)] was applied to the nitrocellulose, washed, and a secondary antibody (anti-rabbit HRP, Sigma) was applied for 1 h.

Web-based computer programs. PSI-BLAST (<http://www.ncbi.nlm.nih.gov/blast/>); CLUSTALW (Gonnet matrix; gap penalty 10, extension penalty 0.2; <http://www.ebi.ac.uk/clustalw/>) and TMHMM (<http://www.cbs.dtu.dk/services/TMHMM-2.0/>) were used.

RESULTS

Temperature-dependent transcription of *traG*

TraG is encoded in the Tra1 region of R27, which contains genes encoding five Mpf proteins, two relaxosome proteins, a coupling protein accessory protein, and the coupling protein (Lawley *et al.*, 2002). The origin of transfer (*oriT*) is also located in the Tra1 region of R27 and the *oriT* separates Tra1 into divergently transcribed genes. A single operon in Tra1, the H operon, encodes the following genes: *traH*, *orf121*, *traI*, *traG*, *orf118*, *traJ*, *orf116* and *orf115* (Alonso *et al.*, 2005). To determine the effect of temperature on the expression of the H operon, RT-PCR was performed. Total RNA was extracted from cells harbouring R27 and drR27 at both 30 °C and 37 °C. For the RT-PCR, primers were selected that would amplify *traG* and the adjacent genes in the H operon, *traI* and *orf118* (Table 2, Fig. 1a). For each reaction, DNA template controls were included to confirm the accuracy of the primer sets, 1-8 and 1-9. When reverse transcriptase was excluded from the RT-PCR, there was no evidence of H operon transcript amplification with the 1-8 primer set. The total RNA preparation did not contain DNA that was detected with the 1-8 primer set; however, trace amounts of DNA contamination within the 1-9 primers may be responsible for the faint band present in *traG*-118 lane B.

The *E. coli* housekeeping gene *pfkA* (encoding phosphofructokinase) was selected as an internal control for the RT-PCR temperature analysis. Primers specific for *pfkA* were included in the RT-PCR analysis (Fig. 1a).

The amplification of RNA from both *traI*-*traG* and *traG*-*orf118* was markedly different when an RNA template was extracted from cells grown at 30 °C or 37 °C (Fig. 1b). The RT-PCR is only a semi-quantitative technique; however, the difference in the transcript levels indicates that the effect of temperature on conjugation results from regulation at the transcriptional level. The 1-8 and 1-9 primer sets indicate that there is transcriptional activity of the H operon at both 30 °C and 37 °C. The transcript levels of *pfkA*, the RT-PCR control, were similar when the template was RNA extracted from cells grown at 30 °C or 37 °C (Fig. 1b).

Fig. 1. (a) RT-PCR analysis of the co-transcription of the R27 *traG* gene with the adjacent genes, *traI* and *orf118*. For each primer set, three lanes are shown: A, RT-PCR using an RNA template isolated from cell harbouring R27 grown at 30 °C; B, negative control of the same RNA template with no reverse transcriptase; C, positive control of a DNA template from cells containing R27. *pfk* corresponds to phosphofructokinase (internal control). (b) Characterization of the transcriptional profile of R27 *traG* gene using RT-PCR with RNA from cells harbouring the following plasmids: A, WT R27 grown at 30 °C; B, WT R27 grown at 37 °C; C, derepressed R27 grown at 30 °C; D, derepressed R27 grown at 37 °C. The *pfk* control represents RT-PCR of RNA extracted in the above conditions, demonstrating the presence of equivalent levels of RNA in each sample.

Generation of NTP-binding motif mutations in *traG*

We have previously cloned *traG* into the expression vector pMS119EH, with a His₆ tag on the 3' end of the gene, to form the construct pJEG217 (Table 1). The His-tagged coupling protein contains a Walker A motif (GxxGxGKS/T) and a Walker B motif (hhhhDE; where h is a hydrophobic residue) commencing at amino acid positions 205 and 506, respectively (Fig. 2a, b) (Schneider & Hunke, 1998). The Walker motifs are involved with NTP binding and hydrolysis, and these motifs represent the region with the highest level of homology among coupling proteins (Fig. 2a, b) (Schneider & Hunke, 1998). Site-specific mutagenesis was performed on the Walker A and Walker B motifs of pJEG217, and the resultant plasmid constructs were transformed into DY330R cells harbouring pDT3152 (drR27 with a *traG* mutation). The residues that were substituted for the well-conserved Gly and Lys residues (GK) of Walker A, and the Asp and Glu residues (DE) of Walker B, were selected using a matrix

Fig. 2. (a, b) Alignment of Walker A (a) and Walker B (b) boxes from coupling proteins from R27 (TraG; GenBank accession no. NP_058332), R388 (TrwB; GenBank accession no. CAA44852), F (TraD; GenBank accession no. BVECAD) and RP4 (TraG; GenBank accession no. S22999). The numbers indicate the amino acids preceding the Walker A and B boxes. Motifs were aligned using CLUSTAL W and shaded in GeneDoc using the conservation mode at level 2. Asterisks indicate the conserved residues selected for substitution mutagenesis. (c) Schematic representation of the TraG protein from R27, where the cross-hatched boxes indicate transmembrane regions and the Walker A and Walker B regions have been expanded. Arrows point to the amino acids that were selected for the site-specific mutagenesis studies.

designed for creating ‘safe substitutions’ that minimized protein instability (Fig. 2c) (Bordo & Argos, 1991). Within the Walker A region, the small, non-polar Gly was substituted with the negatively charged, polar Asp (G210D). The positively charged, polar Lys was replaced with the large, positively charged Arg residue (K211R). A complete deletion of the Walker A subregion Gly-Lys (Δ GK) was also created in pJEG217. Within the Walker B region, the Asp residue with an acidic side chain was substituted with the small, non-polar Ser (D510S). The negatively charged, polar Glu residue was replaced with the positively charged, polar Lys (E511K). Finally, the Walker B subregion (Δ DE) was deleted from pJEG217.

Mutations in the NTP-binding motifs of TraG inhibit plasmid transfer

To determine the effect of the Walker A or B mutations on the functionality of the R27 coupling protein, a *traG* mutation was created in the drR27 plasmid. The drR27 construct was named pDT3152 (Table 1). Insertion of a trimethoprim-resistance cassette into the 5′ region of *traG* abolished the conjugative ability of drR27 (Table 3). In the characterization of the TraI region of R27, a mini::Tn10 cassette had been randomly inserted into the 3′ end of *traG*, creating the drR27 construct pDT2956 (Lawley *et al.*, 2002). A recent study on the Walker A and B motifs of TrhC from R27, however, revealed that a mini::Tn10 insertion in the 3′ end of *trhC* downstream of the Walker A motif may have

Table 3. Complementation of pDT3152 with pJEG217 or Walker A or B mutant derivatives of pJEG217

Walker mutation*	pJEG217 mutations†	Transmission frequency‡
None	Wild-type	1.3×10^{-4}
	Vector alone	$< 1 \times 10^{-7}$
A	G210D	$< 1 \times 10^{-7}$
	K211R	$< 1 \times 10^{-7}$
	Deletion of GK	$< 1 \times 10^{-7}$
B	D510S	$< 1 \times 10^{-7}$
	E511K	$< 1 \times 10^{-7}$

*The mutations correspond to the conserved residues of the Walker A (GK) and Walker B (DE) motifs of TraG.

†The pMS119EH vectors expressing wild-type or mutated TraG protein were transformed into DY330R cells containing an R27 *traG* mutant (pDT3152). Transformants were assayed for the ability to transfer to DY330N recipient cells.

‡Transmission frequency expressed as transconjugants per donor. Values represent the mean frequency of two independent experiments.

allowed the production of a TrhC peptide containing a wild-type Walker A region (Gilmour & Taylor, 2004). To eliminate the possibility of production of partial peptides of TraG containing Walker motifs from pDT2956, we created the *traG* mutant pDT3152, which contains a trimethoprim-resistance dihydrofolate reductase cassette situated ~60 bp upstream of the Walker A encoding region.

The functionality of the TraG constructs containing Walker A or B mutations was determined using a standard conjugative transfer complementation experiment. DY330R cells containing pDT3152 were transformed with pJEG217 and its mutant derivatives, and the conjugative ability of the R27 *traG* mutant was determined. The plasmid pJEG217 containing wild-type (WT) Walker A and B motifs was able to complement pDT3152, albeit at a frequency of 1.3×10^{-4} transconjugants per donor. This frequency was decreased by 3 logs compared to drR27 transfer ($\sim 10^{-1}$ transconjugants per donor) (data not shown). The lower level of complementation could be due to polar effects of the insertional mutagenesis in *traG* on the H operon (Lawley *et al.*, 2002). There was no complementation of pDT3152 with the six TraG Walker A or B mutants within the detection limits of the conjugation assay, $< 1 \times 10^{-7}$ transconjugants per donor (Table 3). These data suggest that the NTP-binding motifs are essential for the conjugative transfer of R27. Our findings are consistent with results obtained in Walker A and B mutagenic studies on the coupling protein, TraG, from RP4 (Balzer *et al.*, 1994), and Walker A mutations in the *A. tumefaciens* coupling protein VirD4 (Kumar & Das, 2002).

Production and stability of TraG_{His6} containing mutations within the Walker A and B regions

The inability of pJEG217 derivatives containing Walker A or B mutations to complement pDT3152 could be a result of a

mutation causing instability in the mutant coupling protein. To ensure that the substitutions and deletions within the Walker A or B motifs of the R27 coupling protein did not affect protein expression or stability, the steady-state levels of WT and mutant proteins were compared by semiquantitative immunoblot analyses. When equivalent amounts of protein were loaded in an SDS-PAGE and transferred to a nitrocellulose membrane, an anti-His antibody revealed similar levels of protein expression for four pJEG217 mutants, G210D, ΔGK, D510S and E511K (Fig. 3). Degradation products were detected in the cell extract of cells harbouring pJEG217; however, three mutants, ΔGK, D510S and E511K, appeared to contain significantly higher levels of degradation products. Although the expression levels of these three pJEG217 variants were similar to those of WT pJEG217, the mutations may have slightly altered the stability of the coupling protein. There was a partial reduction in the amount of coupling protein detected in the cell extract of DY330R containing pDT3152 and pJEG217 with a K211R mutation (Fig. 3). Notably, the deletion of the Asp and Glu residues (ΔDE) of the Walker B region of TraG in pJEG217 created an unstable protein, as there was no detectable protein in the immunoblot (Fig. 3).

The TraG periplasmic residues are essential for an interaction with TrhB

Using the bacterial two-hybrid (BTH) technique we have recently demonstrated that the coupling protein, TraG, of R27 interacts with its cognate Mpf component TrhB (Gilmour *et al.*, 2003). N- and C-terminal truncations of TrhB revealed that the first 220 amino acids were sufficient to interact with TraG. To identify the domain of TraG which enables this coupling protein–Mpf interaction, we again employed the BTH technique. As the majority of TrhB (418

Fig. 3. Production and stability of pJEG217 (TraG_{His6}) and pJEG217 constructs containing mutations within the Walker A or Walker B regions, expressed from DY330R harbouring pDT3152 (drR27 with a *traG* mutation). A mouse anti-His antibody was used in the immunoblot to detect the TraG coupling proteins after induction with IPTG. The lanes indicate amino acid substitutions and deletions of the WT sequence from the Walker A (GK) and Walker B (DE) boxes. Approximately equal amounts of total protein were loaded from each cell lysate, as the amount of sample used was equalized based on optical density measurements of sample cultures prior to processing the lysate. Coomassie staining of the SDS-PAGE used to resolve WT and substituted coupling proteins confirmed that equal protein amounts were loaded in each lane (data not shown).

of 452 aa; 93 %) is predicted to be within the periplasm of the cell, we investigated the role of TraG's periplasmic-associated residues in the TraG–TrhB interaction. Computational analysis of TraG by TMHMM (<http://www.cbs.dtu.dk/services/TMHMM-2.0/>) predicted that only four residues, two of which are charged, Phe-Arg⁺-Ser-Asp⁻, separate the two transmembrane domains of the R27 coupling protein. Through site-specific mutagenesis we replaced the Phe, Arg and Asp residues with the small, polar Ser residue, thereby creating a TraG protein with a periplasmic spanning domain of Ser-Ser-Ser-Ser (Fig. 4). This triple Ser mutation was made in *traG* cloned in the BTH vector, pKT25, thus creating the construct pKT25-*traG*:S4.

Co-transformation of the BTH vectors pUT18C-*trhB* and pKT25-*traG* in BTH101 resulted in functional complementation of the 18 kDa and 25 kDa domains of the *B. pertussis* adenylate cyclase protein. Adenylate cyclase activity resulted in the conversion of ATP to cAMP, which activates the expression of catabolic genes such as *lacZ*, encoding β-galactosidase (Karimova *et al.*, 1998). Within 48 h, >99 % BTH101 colonies containing pUT18C-*trhB* and pKT25-*traG* were blue, when plated on LB plates containing the chromogenic substrate X-Gal. BTH101 colonies containing pUT18C-*trhB* and pKT25-*traG*:S4 were >90 % white, with a small number showing faint blue centres. The β-galactosidase activity of the faint blue colonies was assayed using the Miller assay (Miller, 1972). The BTH positive control (leucine zipper) and negative control (empty vectors) as well as BTH101 cells containing vectors encoding WT TraG and TrhB were also included in the Miller assay. Whereas the TraG–TrhB interaction showed levels similar to the BTH positive control, the TraG:S4–TrhB interaction did not exceed the negative control levels of the BTH empty vectors (Fig. 5). These data suggest that the periplasmic-spanning residues, FRSD, of the coupling protein TraG are involved in the interaction with the Mpf protein, TrhB.

To confirm that the TraG:S4 mutation did not interfere with the stability or expression of the coupling protein, we assayed the protein encoded by pKT25-*traG*:S4 for the ability to interact with the pUT18C-*traJ*-encoded protein. The TraJ protein is an essential protein for R27 transfer and it has recently been characterized and shown to be an accessory protein to the coupling protein of the R27 plasmid (Lawley *et al.*, 2002; J. Gunton & D. E. Taylor, unpublished). Using the BTH system, we documented an interaction between TraG and TraJ from R27. This interaction enabled us to determine the stability and functionality of the TraG:S4 construct. The BTH data indicated that both the WT TraG and TraG:S4 were able to interact with TraJ; colonies of BTH101 co-transformed with pUT18C-*traJ* and pKT25-*traG*:S4, or pUT18C-*traJ* and pKT25-*traG*, were >99 % blue (data not shown). These data suggest that the domain through which TraG interacts with TraJ is not shared with the periplasmic domain required for the interaction with TrhB.

Fig. 4. The N-terminus of the coupling proteins from R27 (TraG; GenBank accession no. NP_058332), R388 (TrwB; GenBank accession no. CAA44852), F (TraD; GenBank accession no. BVECAD) and RP4 (TraG; GenBank accession no. S22999). The predicted topology of the coupling proteins is indicated by solid lines (cytoplasmic) and dotted lines (periplasmic). Residues that are predicted to be in the periplasm are in bold. The transmembrane regions are shown as cylinders and were predicted using the TMHMM program. Asterisks indicate the residues of TraG that were substituted.

To verify the data from the *in vivo* BTH experiments, in which substitutions in the periplasmic domain of TraG abrogated an interaction with TrhB, we attempted to precipitate a complex of epitope-tagged TrhB and TraG : S4. Whereas TrhB containing a C-terminal FLAG epitope (Asp-Tyr-Lys-Asp-Asp-Asp-Lys) precipitated TraG_{AC}, the anti-adenylate cyclase antibody did not detect TraG : S4_{AC} precipitated by TrhB_{FLAG} (Fig. 6). This biochemical analysis confirms that the periplasmic residues of the R27 coupling protein are essential for a successful interaction with the Mpf protein TrhB.

Fig. 5. *In vivo* bacterial two-hybrid interaction data. A substitution of the R27 TraG periplasmic residues, FRSD, with SSSS abrogated the ability of TraG to interact with the R27 mating pair formation protein, TrhB. Conjugative proteins were cloned into BTH vectors pUT18C or pKT25, to create fusions with adenylate cyclase domains, and then co-produced in *E. coli* BTH101. The BTH controls are BTH101 cells containing pUT18C and pKT25 (negative control), and pUT18C-leucine zipper with pKT25-leucine zipper (Zip, positive control). Liquid cultures of transformed BTH101 cells were analysed for β -galactosidase activity using a Miller assay (Miller, 1972). A representative experiment is shown.

To determine if the substitution of Phe-Arg-Ser-Asp to Ser-Ser-Ser-Ser within the periplasmic region of TraG enabled a protein functional for conjugative transfer to be produced, a complementation experiment was conducted. The 4-Ser periplasmic substitution was made in pJEG217, thus creating the vector pMWG385, which encoded TraG : S4_{His6}. DY330R cells harbouring drR27 containing a *traG* mutation (pDT3152) were transformed with pMWG385. Immunoblot analysis of pMWG385 in pDT3152 with the anti-His antibody showed TraG : S4 expression levels similar to the expression of WT TraG from pJEG217 in pDT3152 (data not shown). Interestingly, the TraG : S4 construct was able to complement an R27 *traG* mutant, albeit at lower levels than a WT TraG supplied *in trans* (Table 4).

The R27 coupling protein forms membrane-associated fluorescent foci

A GFP fusion to the R27 Mpf protein TrhC revealed that in the presence of R27 conjugative proteins, fluorescent foci

Fig. 6. Co-immunoprecipitation of TraG and not a TraG : S4 construct by the R27 Mpf protein, TrhB. Cellular lysates of DH5 α cells expressing TraG_{AC} and TrhB_{FLAG}, and TraG : S4_{AC} and TrhB_{FLAG}, were mixed with M2 anti-FLAG affinity gel beads, washed, and resolved by 10% SDS-PAGE. After transfer to nitrocellulose, proteins precipitated by the TrhB protein were probed with an anti-adenylate cyclase polyclonal antibody. The immunoprecipitate (IP) and cleared lysate (CL) of cells expressing TraG_{AC} and TrhB_{FLAG}, or TraG : S4_{AC} and TrhB_{FLAG}, are shown, with arrows indicating the WT and periplasmic-substituted R27 coupling protein.

Table 4. Complementation of pDT3152 with pJEG217 and a TraG:S4 construct, pMWG385

R27 plasmid	TraG pMS119EH*	Transmission frequency†
pDT1942	NA	5.8×10^{-1}
pDT3152	Vector alone	$< 1 \times 10^{-7}$
pDT3152	Wild-type	1.8×10^{-3}
pDT3152	TraG:S4	3.5×10^{-4}

*The pMS119EH vectors expressing wild-type or mutated TraG protein were transformed into DY330R cells containing an R27 *traG* mutant (pDT3152). Transformants were assayed for the ability to transfer to DY330N recipient cells.

†Transmission frequency expressed as transconjugants per donor. Values represent the mean frequency of two independent experiments.

randomly localized at the periphery of the cell (Gilmour & Taylor, 2004; Gilmour *et al.*, 2001). These TrhC-dependent foci are proposed to represent aggregates of R27 proteins at discrete positions within the cell membrane. Further characterization of these foci indicated that their formation was temperature dependent: at 37 °C TrhC-GFP-containing cells were confluent green in colour whereas at 30 °C, in the presence of additional R27 proteins, discrete foci were visible (Gilmour *et al.*, 2001).

To determine if TraG localized to the same position as these Mpf complexes, we used immunofluorescence microscopy to probe for the location of TraG_{His6} proteins. Interestingly, these studies revealed that DY330R cells harbouring pJEG217 had fluorescent foci at the cell membrane, resembling the foci formed by TrhC-GFP. Furthermore, unlike TrhC, these discrete foci were present at both 30 °C and 37 °C, and were found in both the presence and absence of any other drR27 proteins (Fig. 7). When TrhC-GFP was

expressed from the expression vector, pMS119EH, foci could not be formed at 30 °C or 37 °C in the absence of drR27 proteins (Gilmour *et al.*, 2001).

To determine the cellular position of the TraG foci, each cell was divided into six equal domains, with the first and sixth domain representing the polar positions of the cell. The second and fifth domain represented the quarter cell position and the third and fourth domain were combined to represent the mid-cell region. The polar, quarter and mid-cell positions each formed one-third of the entire cell length. In each condition tested, at 30 °C and 37 °C, and in the presence and absence of any other drR27 proteins, foci were found in the poles, quarter and mid-cell positions (Table 5). The distribution of the foci was slightly more in the polar position (34–36 % of foci at this position), and quarter-cell position (35–38 %) as compared to the mid-cell position (27–29 %). The mean number of foci per cell in DY330R cells containing only pJEG217 was 5.9 and 5.3 at 30 °C and 37 °C, respectively. In the presence of pDT3152 and pJEG217, DY330R cells contained 5.6 foci at both 30 °C and 37 °C (Table 5).

DISCUSSION

Bacterial conjugation is a primary mechanism for horizontal gene transfer. The role of the coupling protein in conjugation had been originally proposed as merely a required connection between the membrane-associated Mpf proteins and the cytosolic relaxosome complex (Cabezon *et al.*, 1997). The resolution of the hexameric, pore-forming structure of the coupling protein TrwB, in conjunction with the presence of well-conserved NTP-binding motifs in the coupling protein family suggests that coupling proteins may play a more complex role in conjugation (Gomis-Ruth *et al.*, 2001). The recently proposed 'shoot and pump' model suggests that the coupling protein secretes the relaxase into

Fig. 7. Subcellular localization of TraG_{His6} from R27, determined by immunofluorescence microscopy. The fluorescent foci were observed in DH5α harbouring pJEG217 at (a) 30 °C and (b) 37 °C. *E. coli* containing pDT3152, a *traG* mutant, showed similar foci when transformed with pJEG217 and grown at (c) 30 °C and (d) 37 °C. Arrows indicate foci on the periphery of the cell.

Table 5. Immunofluorescence data representing the effect of temperature and additional R27 proteins on the subcellular localization of TraG_{His6} encoded by pJEG217

Temp. (°C)	Plasmid(s) (no. of cells)*	Percentage of foci at the cell position:			Foci per cell (no. of foci)
		Polar	Quarter cell	Mid-cell	
30	pJEG217 alone (101)	34	38	28	5.9 (589)†‡
	pJEG217 + pDT3152 (104)	36	35	29	5.6 (578)‡
37	pJEG217 alone (106)	34	37	29	5.3 (561)†§
	pJEG217 + pDT3152 (98)	35	38	27	5.6 (548)§

*The plasmids correspond to the vectors present within the DY330R cells; 'no. of cells' indicates total cells counted for that condition.
†In the comparison of foci per cell of pJEG217 alone at 30 °C versus pJEG217 alone at 37 °C, the *P*-value is 0.001. A two-sample, two-tailed *t* test was performed to determine the *P*-value. A *P*-value of <0.05 indicates that a value is statistically significant.
‡With the comparison of the foci per cell of pJEG217 alone versus pJEG217 and pDT3152 at 30 °C the *P*-value is 0.028.
§With pJEG217 alone versus pJEG217 and pDT3152 at 37 °C the *P*-value is 0.078.

the conduit created by the T4SS and subsequently threads the DNA portion of the substrate through a channel that is formed by the hexameric coupling protein (Llosa & de la Cruz, 2005). Furthermore, the *A. tumefaciens* coupling protein, VirD4, has been found to require functional NTP-binding motifs for bridging the inner and outer subassemblies of VirB/D4 T4SS (Cascales & Christie, 2004a). Clearly, the coupling protein plays a vital role in the transfer of plasmid DNA from donor to recipient cells.

In our characterization of the transfer regions of the IncHI1 plasmid R27 we determined that the coupling protein, TraG, is essential for conjugative transfer (Lawley *et al.*, 2002). In this study, we further characterized the coupling protein encoded on the R27 plasmid. A unique feature of the IncHI family of plasmids is that their conjugative transfer is significantly reduced at 37 °C (Taylor & Levine, 1980). Electron microscopic analysis of cells harbouring R27 plasmid grown at 37 °C revealed an absence of H-pili on the cell surface (Maher *et al.*, 1993). As pili are required to bring the donor and recipient cells into close proximity, the lack of pili explains the inhibition of transfer at 37 °C. Transcriptional analysis of the *Mpf* gene, *trhC*, indicated that transcription was inhibited at 37 °C, thus explaining the lack of H-pili observed during the electron microscopy studies (Gilmour *et al.*, 2001). The transcription analysis of *traG*, and the adjacent *traI* and *orf118* genes, at both 30 °C and 37 °C revealed a decrease in the transcript levels at 37 °C, as compared to the RNA levels present at 30 °C. Whereas *traG* is encoded in the *TraI* region, *trhC* is encoded within the *Tra2* region of R27. The decreased transcript levels of an operon in *TraI* encoding non-Mpf proteins would indicate that a global temperature regulator is controlling R27 conjugation genes in the *TraI* and *Tra2* region. A recent analysis of the effect of temperature on R27 transcription has revealed that H-NS and HhaA proteins have an inhibitory effect on the transcription of a number of *TraI* and *Tra2* genes at 33 °C (Forns *et al.*, 2005). A comprehensive analysis of the temperature regulation of each R27 transfer gene has been recently completed (Alonso *et al.*, 2005).

The C-termini of the coupling family, including the Walker-type NTP-binding domains, show weak similarity with the DNA translocases FtsK and SpoIIIE from *E. coli* and *Bacillus anthracis*, respectively (Errington *et al.*, 2001). The SpoIIIE protein has DNA-dependent ATP hydrolysis activity that is used to pump DNA during sporulation (Bath *et al.*, 2000). The R388 coupling protein also has DNA-dependent ATP hydrolysis activity, and the Walker motifs of R388, RP4 and F plasmid have been demonstrated to be involved in conjugation (Moncalian *et al.*, 1999; Schroder *et al.*, 2002; Tato *et al.*, 2005). Mutational analyses of the Walker A and B motifs of the RP4 coupling protein, TraG, revealed that only two mutations completely abolished RP4 transfer: K187T in Walker A, and D449N in Walker B (Balzer *et al.*, 1994). NTP-binding studies further determined that the K187T mutation rendered the coupling protein unable to bind ATP (Schroder & Lanka, 2003). Mutational analysis of the *A. tumefaciens* coupling protein, VirD4, demonstrated the importance of the Walker A motif (Kumar & Das, 2002). VirD4 mutants containing the substitutions G151S and K152T were defective in the transfer of both DNA and VirE2 to plant cells. Furthermore, using a transfer DNA immunoprecipitation (TriP) technique, the VirD4 K152T mutant was found to prevent substrate transfer to the *A. tumefaciens* T4SS proteins VirB6 and VirB8 (Atmakuri *et al.*, 2004).

We have expanded the mutational studies of the Walker A and Walker B motifs of the coupling protein family. Substitution of the Gly and Lys residues of the Walker A motif and Asp and Glu residues of the Walker B motifs abolished the capacity of the R27 coupling protein to restore conjugative ability in a complementation assay. A deletion of the Walker A Gly and Lys residues, at positions 210 and 211, had the same effect on the transmission of the R27 plasmid. These results are not unexpected, as alignment of coupling protein homologues indicated that the Gly-Lys and Asp-Glu residues of the Walker A and B motifs, respectively, are found in the majority of coupling proteins (Fig. 2). Notably, the R388 coupling protein TrwB has been

identified as a DNA-dependent ATPase (Tato *et al.*, 2005). The discovery that a coupling protein is able to hydrolyse ATP substantiates the finding that key, well-conserved residues in the Walker A and B regions are necessary for conjugative transfer.

The recent discovery that coupling proteins are able to interact with the VirB10 family of proteins suggests a mechanism by which plasmid DNA moves from the cytoplasm to the membrane-associated T4SS (Gilmour *et al.*, 2003; Llosa *et al.*, 2003). A more detailed contact pathway has been proposed by tracking the movement of plasmid DNA in *A. tumefaciens* using the TrIP technique; the T-DNA transfer intermediate interacts with the coupling protein VirD4 initially, and subsequently contacts VirB11, VirB4, VirB6, VirB8, VirB2 and VirB9 (Cascales & Christie, 2004b). Using non-polar *virB* mutations, the pathway of the T-DNA substrate has been expanded to propose that VirB10 interacts with the T-DNA after the substrate's interaction with VirB6 and VirB8 (Cascales & Christie, 2004b). The R27 Mpf protein TrhB, a VirB10 homologue, is probably bitopic, with the majority of the protein extending into the periplasm. TrhB has only 11 residues preceding the single transmembrane domain, as predicted by computational analysis with TMHMM. A 220 aa N-terminal peptide of TrhB (452 aa) retained the ability to interact with the R27 coupling protein TrhB (Gilmour *et al.*, 2003). Interestingly, a shorter N-terminal peptide of TrhB, 133 aa in length, was not able to bind TraG but was able to interact with full-length TrhB. TrhB contains a proline-rich (17.9%) region between amino acids 135 and 173; such a proline-rich region is a common feature of VirB10 homologues (Gilmour *et al.*, 2003). We had postulated that the proline-rich region of TrhB may be involved in the interaction with the coupling protein, TraG (Gilmour *et al.*, 2003). Within the R27 coupling protein, there are only four residues, Phe-Arg-Ser-Asp, that are predicted to be associated with the periplasm. The BTH screen revealed that a substitution of these residues with four Ser residues was sufficient to abolish a TrhB–TraG interaction. The essential role of periplasmic-associated residues in the interaction with TrhB was verified using a co-immunoprecipitation experiment in which an epitope-tagged TrhB was capable of precipitating only WT TraG and not a TraG:S4 construct. Surprisingly, when this coupling protein containing the periplasmic substitutions, TraG:S4, was supplied *in trans* in a complementation assay with an R27 *traG* mutant, the TraG:S4 construct was able to restore conjugative ability. The complementation level, however, was almost 1 log lower than the transfer levels of an R27 *traG* mutant complemented with WT TraG. The ability of the TraG:S4 construct to complement a *traG* mutation could be explained by the coupling protein interacting with multiple points in the T4SS apparatus, and not solely with TrhB. The BTH and co-immunoprecipitation techniques identify only binary interactions, which is considerably different from the interactions that may occur with the coupling protein and a full complement of R27 T4SS proteins.

The Phe-Arg-Ser-Asp residues of TraG do not represent a common motif within the periplasmic regions of the coupling proteins from the RP4, R388 and F plasmids (Fig. 4). Furthermore, there is significant variability in the number of residues separating the hallmark transmembrane regions of the coupling protein; TraD had the largest number of periplasmic-associated residues, with 62 aa, and TraG of R27 had the smallest number, with only 4 aa separating the transmembrane regions. One common feature of the coupling protein family is the interchangeability of the coupling proteins between non-cognate T4SS (Cabezón *et al.*, 1994; Hamilton *et al.*, 2000). This has been further demonstrated using BTH technology in which the R388 VirB10 homologue, TrwE, was found to interact with non-cognate coupling proteins from the IncN plasmid pKM101 and IncX plasmid R6K (Llosa *et al.*, 2003). The R27 VirB10 homologue, TrhB, has also been found to interact with non-cognate coupling proteins encoded on the F, RP4 and R388 plasmids (J. Gunton & D. E. Taylor, unpublished). The BTH and immunoprecipitation data obtained with the R27 TraG:S4 construct suggest that the periplasmic region is essential for the interaction with TrhB. A shared feature of the VirB10 homologues is the proline-rich region in the N-terminus. This structural feature may be interacting in a non-specific fashion, with the periplasmic residues of the coupling protein, thus allowing for the observed interchangeability of coupling proteins between heterologous T4SS.

Immunofluorescence microscopy was used to determine that R27 TraG_{His6} protein forms multiple fluorescent foci within the periphery of the cell. Similar foci were observed when GFP was fused to the C-terminus of the R27 Mpf protein, TrhC (Gilmour *et al.*, 2001). Notably, DY330R cells containing TraG_{His6} or TrhC-GFP formed the same number of foci in the cellular membrane; there was an average of five to six foci in cells containing the R27 coupling protein or TrhC. With both TraG and TrhC, foci were found throughout the cell periphery, with a moderate bias towards the quarter and polar positions of the cell (Gilmour *et al.*, 2001). The position of R27 protein-dependent foci in the cell is strikingly different from the position of the R27 plasmid DNA; GFP-labelled R27 plasmid DNA localized to discrete positions at the mid and quarter cell position (Gilmour *et al.*, 2001; Lawley & Taylor, 2003). Electron microscopy revealed that the H-pilus, product of the R27 T4SS, can assemble and extend from random positions on the cell membrane and is not limited to this mid or quarter cell position (Gilmour *et al.*, 2001).

As R27 conjugation is influenced by temperature, TraG-dependent formation of fluorescent foci was investigated in cells grown at 37 °C. Although foci were found at both 30 °C and 37 °C there was a statistically significant decrease in the number of foci formed when cells expressing TraG alone were grown at 37 °C instead of at 30 °C, a temperature that is permissive for R27 conjugative transfer ($P=0.001$). Furthermore, there was a significant change in the number

of foci at 30 °C when cells expressing TraG also harboured the R27 plasmid ($P=0.028$). These data suggest that in the absence of essential R27 proteins, the number of TraG-associated foci per cell is slightly higher at 30 °C, as compared to the number of foci formed at 37 °C; temperature may be involved in more than simply transcriptional control of the R27 genome. Additionally, in the presence of R27 proteins, the number of TraG foci decreases when compared to cells expressing TraG alone. As expected, this decrease is not significantly different in the presence and absence of pDT3152 at 37 °C, as at this elevated temperature, transcription of the majority of R27 genes is significantly reduced (Gilmour *et al.*, 2001; Alonso *et al.*, 2005).

The ability of a coupling protein to localize to discrete positions within the cell, in the absence of other T4SS proteins, is not a unique phenomenon. The *A. tumefaciens* coupling protein, VirD4, localized to the poles of the cell, independent of other Vir proteins or a T4SS apparatus (Kumar & Das, 2002). The *A. tumefaciens* T4SS protein VirB6 also localizes to the poles; however, like TrhC from the R27 plasmid, this localization is dependent on the presence of a subset of conjugative proteins, VirB7–VirB11 (Judd *et al.*, 2005). However, this dependence of VirB6 positioning on Ti-plasmid-encoded VirB proteins was not demonstrated in a recent study, suggesting that additional work is required to resolve the architecture and temporal assembly of the Vir T4SS (Jakubowski *et al.*, 2004).

In conclusion, the TraG periplasmic-spanning residues and well-conserved residues in the Walker-type ATP-binding domains have been shown to be involved in the functionality of the R27 coupling protein. Moreover, the independent cellular localization of foci formed by the coupling protein is similar in position and number to that seen for potential T4SS complexes encoded by the R27 plasmid. These data support a model of conjugation in which the coupling protein localizes to the periphery of the cell and associates with the T4SS protein TrhB with periplasmic-spanning residues, thus enabling plasmid DNA to traverse the donor cell envelope.

ACKNOWLEDGEMENTS

We thank Marc Couturier for assistance with fluorescence microscopy, and Daniel Ladant for the generous gift of anti-adenylate cyclase antibody. This work was supported by grant MOP6200 to D.E.T. from the Canadian Institutes for Health Research (CIHR). J.E.G. and M.W.G. are recipients of a training award from the Alberta Heritage Foundation for Medical Research (AHFMR). G.A. is a recipient of a visiting scientist award from AHFMR. D.E.T. is a Scientist with AHFMR.

REFERENCES

- Alonso, G., Baptista, K., Ngo, T. & Taylor, D. E. (2005). Transcriptional organization of the temperature-sensitive transfer system from the IncHI1 plasmid R27. *Microbiology* **151**, 3563–3573.
- Atmakuri, K., Cascales, E. & Christie, P. J. (2004). Energetic components VirD4, VirB11 and VirB4 mediate early DNA transfer reactions required for bacterial type IV secretion. *Mol Microbiol* **54**, 1199–1211.
- Balzer, D., Pansegrau, W. & Lanka, E. (1994). Essential motifs of relaxase (TraI) and TraG proteins involved in conjugative transfer of plasmid RP4. *J Bacteriol* **176**, 4285–4295.
- Bath, J., Wu, L. J., Errington, J. & Wang, J. C. (2000). Role of *Bacillus subtilis* SpoIIIE in DNA transport across the mother cell-prespore division septum. *Science* **290**, 995–997.
- Bordo, D. & Argos, P. (1991). Suggestions for “safe” residue substitutions in site-directed mutagenesis. *J Mol Biol* **217**, 721–729.
- Cabezón, E., Lanka, E. & de la Cruz, F. (1994). Requirements for mobilization of plasmids RSF1010 and ColE1 by the IncW plasmid R388: *trwB* and RP4 *traG* are interchangeable. *J Bacteriol* **176**, 4455–4458.
- Cabezón, E., Sastre, J. I. & de la Cruz, F. (1997). Genetic evidence of a coupling role for the TraG protein family in bacterial conjugation. *Mol Gen Genet* **254**, 400–406.
- Cascales, E. & Christie, P. J. (2003). The versatile bacterial type IV secretion systems. *Nat Rev Microbiol* **1**, 137–149.
- Cascales, E. & Christie, P. J. (2004a). *Agrobacterium* VirB10, an ATP energy sensor required for type IV secretion. *Proc Natl Acad Sci U S A* **101**, 17228–17233.
- Cascales, E. & Christie, P. J. (2004b). Definition of a bacterial type IV secretion pathway for a DNA substrate. *Science* **304**, 1170–1173.
- Christie, P. J. & Vogel, J. P. (2000). Bacterial type IV secretion: conjugation systems adapted to deliver effector molecules to host cells. *Trends Microbiol* **8**, 354–360.
- Das, A. & Xie, Y. H. (1998). Construction of transposon Tn3*phoA*: its application in defining the membrane topology of the *Agrobacterium tumefaciens* DNA transfer proteins. *Mol Microbiol* **27**, 405–414.
- Errington, J., Bath, J. & Wu, L. J. (2001). DNA transport in bacteria. *Nat Rev Mol Cell Biol* **2**, 538–545.
- Forns, N., Banos, R. C., Balsalobre, C., Juarez, A. & Madrid, C. (2005). Temperature-dependent conjugative transfer of R27: role of chromosome- and plasmid-encoded Hha and H-NS proteins. *J Bacteriol* **187**, 3950–3959.
- Gilmour, M. W. & Taylor, D. E. (2004). A subassembly of R27-encoded transfer proteins is dependent on TrhC nucleoside triphosphate-binding motifs for function but not formation. *J Bacteriol* **186**, 1606–1613.
- Gilmour, M. W., Lawley, T. D., Rooker, M. M., Newnham, P. J. & Taylor, D. E. (2001). Cellular location and temperature-dependent assembly of IncHI1 plasmid R27-encoded TrhC-associated conjugative transfer protein complexes. *Mol Microbiol* **42**, 705–715.
- Gilmour, M. W., Gunton, J. E., Lawley, T. D. & Taylor, D. E. (2003). Interaction between the IncHI1 plasmid R27 coupling protein and type IV secretion system: TraG associates with the coiled-coil mating pair formation protein TrhB. *Mol Microbiol* **49**, 105–116.
- Gomis-Ruth, F. X., Moncalian, G., Perez-Luque, R., Gonzalez, A., Cabezón, E., de la Cruz, F. & Coll, M. (2001). The bacterial conjugation protein TrwB resembles ring helicases and F1-ATPase. *Nature* **409**, 637–641.
- 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.
- Hamilton, C. M., Lee, H., Li, P. L., Cook, D. M., Piper, K. R., von Bodman, S. B., Lanka, E., Ream, W. & Farrand, S. K. (2000).

- TraG from RP4 and TraG and VirD4 from Ti plasmids confer relaxosome specificity to the conjugal transfer system of pTiC58. *J Bacteriol* **182**, 1541–1548.
- Hormaeche, I., Alkorta, I., Moro, F., Valpuesta, J. M., Goni, F. M. & De La Cruz, F. (2002). Purification and properties of TrwB, a hexameric, ATP-binding integral membrane protein essential for R388 plasmid conjugation. *J Biol Chem* **277**, 46456–46462.
- Jakubowski, S. J., Krishnamoorthy, V., Cascales, E. & Christie, P. J. (2004). *Agrobacterium tumefaciens* VirB6 domains direct the ordered export of a DNA substrate through a type IV secretion. *Syst J Mol Biol* **341**, 961–977.
- Judd, P. K., Kumar, R. B. & Das, A. (2005). The type IV secretion apparatus protein VirB6 of *Agrobacterium tumefaciens* localizes to a cell pole. *Mol Microbiol* **55**, 115–124.
- Karimova, G., Pidoux, J., Ullmann, A. & Ladant, D. (1998). A bacterial two-hybrid system based on a reconstituted signal transduction pathway. *Proc Natl Acad Sci U S A* **95**, 5752–5756.
- Kumar, R. B. & Das, A. (2002). Polar location and functional domains of the *Agrobacterium tumefaciens* DNA transfer protein VirD4. *Mol Microbiol* **43**, 1523–1532.
- Lanka, E. & Wilkins, B. M. (1995). DNA processing reactions in bacterial conjugation. *Annu Rev Biochem* **64**, 141–169.
- Lawley, T. D. & Taylor, D. E. (2003). Characterization of the double-partitioning modules of R27: correlating plasmid stability with plasmid localization. *J Bacteriol* **185**, 3060–3067.
- Lawley, T. D., Gilmour, M. W., Gunton, J. E., Standeven, L. J. & Taylor, D. E. (2002). Functional and mutational analysis of conjugative transfer region 1 (Tra1) from the IncHI1 plasmid R27. *J Bacteriol* **184**, 2173–2180.
- Lawley, T. D., Klimke, W. A., Gubbins, M. J. & Frost, L. S. (2003). F factor conjugation is a true type IV secretion system. *FEMS Microbiol Lett* **224**, 1–15.
- Lee, M. H., Kosuk, N., Bailey, J., Traxler, B. & Manoel, C. (1999). Analysis of F factor TraD membrane topology by use of gene fusions and trypsin-sensitive insertions. *J Bacteriol* **181**, 6108–6113.
- Llosa, M. & de la Cruz, F. (2005). Bacterial conjugation: a potential tool for genomic engineering. *Res Microbiol* **156**, 1–6.
- Llosa, M., Zunzunegui, S. & de la Cruz, F. (2003). Conjugative coupling proteins interact with cognate and heterologous VirB10-like proteins while exhibiting specificity for cognate relaxosomes. *Proc Natl Acad Sci U S A* **100**, 10465–10470.
- Maher, D., Sherburne, R. & Taylor, D. E. (1991). Bacteriophages for incompatibility group H plasmids: morphological and growth characteristics. *Plasmid* **26**, 141–146.
- Maher, D., Sherburne, R. & Taylor, D. E. (1993). H-pilus assembly kinetics determined by electron microscopy. *J Bacteriol* **175**, 2175–2183.
- Miller, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Moncalian, G., Cabezon, E., Alkorta, I., Valle, M., Moro, F., Valpuesta, J. M., Goni, F. M. & de La Cruz, F. (1999). Characterization of ATP and DNA binding activities of TrwB, the coupling protein essential in plasmid R388 conjugation. *J Biol Chem* **274**, 36117–36124.
- Okamoto, S., Toyoda-Yamamoto, A., Ito, K., Takebe, I. & Machida, Y. (1991). Localization and orientation of the VirD4 protein of *Agrobacterium tumefaciens* in the cell membrane. *Mol Gen Genet* **228**, 24–32.
- Pansegau, W., Balzer, D., Kruff, V., Lurz, R. & Lanka, E. (1990). In vitro assembly of relaxosomes at the transfer origin of plasmid RP4. *Proc Natl Acad Sci U S A* **87**, 6555–6559.
- Schneider, E. & Hunke, S. (1998). ATP-binding-cassette (ABC) transport systems: functional and structural aspects of the ATP-hydrolyzing subunits/domains. *FEMS Microbiol Rev* **22**, 1–20.
- Schroder, G. & Lanka, E. (2003). TraG-like proteins of type IV secretion systems: functional dissection of the multiple activities of TraG (RP4) and TrwB (R388). *J Bacteriol* **185**, 4371–4381.
- Schroder, G. & Lanka, E. (2005). The mating pair formation system of conjugative plasmids – a versatile secretion machinery for transfer of proteins and DNA. *Plasmid* **54**, 1–25.
- Schroder, G., Krause, S., Zechner, E. L., Traxler, B., Yeo, H. J., Lurz, R., Waksman, G. & Lanka, E. (2002). TraG-like proteins of DNA transfer systems and of the *Helicobacter pylori* type IV secretion system: inner membrane gate for exported substrates? *J Bacteriol* **184**, 2767–2779.
- Sherburne, C. K., Lawley, T. D., Gilmour, M. W., Blattner, F. R., Burland, V., Grotbeck, E., Rose, D. J. & Taylor, D. E. (2000). The complete DNA sequence and analysis of R27, a large IncHI plasmid from *Salmonella typhi* that is temperature sensitive for transfer. *Nucleic Acids Res* **28**, 2177–2186.
- Strack, B., Lessl, M., Calendar, R. & Lanka, E. (1992). A common sequence motif, -E-G-Y-A-T-A-, identified within the primase domains of plasmid-encoded I- and P-type DNA primases and the alpha protein of the *Escherichia coli* satellite phage P4. *J Biol Chem* **267**, 13062–13072.
- Tato, I., Zunzunegui, S., de la Cruz, F. & Cabezon, E. (2005). TrwB, the coupling protein involved in DNA transport during bacterial conjugation, is a DNA-dependent ATPase. *Proc Natl Acad Sci U S A* **102**, 8156–8161.
- Taylor, D. E. (1983). Transfer-defective and tetracycline-sensitive mutants of the incompatibility group HI plasmid R27 generated by insertion of transposon 7. *Plasmid* **9**, 227–239.
- Taylor, D. E. & Levine, J. G. (1980). Studies of temperature-sensitive transfer and maintenance of H incompatibility group plasmids. *J Gen Microbiol* **116**, 475–484.
- Yu, D., Ellis, H. M., Lee, E. C., Jenkins, N. A., Copeland, N. G. & Court, D. L. (2000). An efficient recombination system for chromosome engineering in *Escherichia coli*. *Proc Natl Acad Sci U S A* **97**, 5978–5983.