JOURNAL OF BACTERIOLOGY, Mar. 2000, p. 1541–1548 Vol. 182, No. 6
0021-9193/00/$04.00+0
Copyright © 2000, American Society for Microbiology. All Rights Reserved.

TraG from RP4 and TraG and VirD4 from Ti Plasmids Confer Relaxosome Specificity to the Conjugal Transfer System of pTiC58

CLAIRE M. HAMILTON,[1]† HYEWON LEE,[2] PEI-LI LI,[1] DAVID M. COOK,[1]‡ KEVIN R. PIPER,[3] SUSANNE BECK VON BODMAN,[1]§ ERICH LANKA,[4] WALT REAM,[2] AND STEPHEN K. FARRAND[1,3]*

Departments of Crop Sciences[1] and Microbiology,[3] University of Illinois at Urbana-Champaign, Urbana, Illinois 61801; Department of Microbiology, Oregon State University, Corvallis, Oregon 97331[2]; and Max-Planck-Institut für Molekulare Genetik, Dahlem, D-14195 Berlin, Germany[4]

Received 26 August 1999/Accepted 21 December 1999

Plasmid conjugation systems are composed of two components, the DNA transfer and replication system, or Dtr, and the mating pair formation system, or Mpf. During conjugal transfer an essential factor, called the coupling protein, is thought to interface the Dtr, in the form of the relaxosome, with the Mpf, in the form of the mating bridge. These proteins, such as TraG from the IncP1 plasmid RP4 ($TraG_{RP4}$) and TraG and VirD4 from the conjugal transfer and T-DNA transfer systems of Ti plasmids, are believed to dictate specificity of the interactions that can occur between different Dtr and Mpf components. The Ti plasmids of *Agrobacterium tumefaciens* do not mobilize vectors containing the *oriT* of RP4, but these IncP1 plasmid derivatives lack the *trans*-acting Dtr functions and $TraG_{RP4}$. *A. tumefaciens* donors transferred a chimeric plasmid that contains the *oriT* and Dtr genes of RP4 and the Mpf genes of pTiC58, indicating that the Ti plasmid mating bridge can interact with the RP4 relaxosome. However, the Ti plasmid did not mobilize transfer from an IncQ relaxosome. The Ti plasmid did mobilize such plasmids if $TraG_{RP4}$ was expressed in the donors. Mutations in *traG*$_{RP4}$ with defined effects on the RP4 transfer system exhibited similar phenotypes for Ti plasmid-mediated mobilization of the IncQ vector. When provided with VirD4, the *tra* system of pTiC58 mobilized plasmids from the IncQ relaxosome. However, neither $TraG_{RP4}$ nor VirD4 restored transfer to a *traG* mutant of the Ti plasmid. VirD4 also failed to complement a *traG*$_{RP4}$ mutant for transfer from the RP4 relaxosome or for RP4-mediated mobilization from the IncQ relaxosome. $TraG_{RP4}$-mediated mobilization of the IncQ plasmid by pTiC58 did not inhibit Ti plasmid transfer, suggesting that the relaxosomes of the two plasmids do not compete for the same mating bridge. We conclude that $TraG_{RP4}$ and VirD4 couples the IncQ but not the Ti plasmid relaxosome to the Ti plasmid mating bridge. However, VirD4 cannot couple the IncP1 or the IncQ relaxosome to the RP4 mating bridge. These results support a model in which the coupling proteins specify the interactions between Dtr and Mpf components of mating systems.

Plasmid conjugation conceptually can be divided into two functions. In the first, the DNA is processed by a complex of proteins, one of which introduces a single-strand nick at the *nic* site within the *oriT* recognition sequence. Called the relaxosome, the proteins of this complex are coded for by genes of the Dtr (DNA transfer and replication) component of the transfer system. In the second, the nucleoprotein transfer intermediate comprised of the nicked strand covalently linked at the 5′ end to the relaxase is secreted from the donor directly into the recipient via a bridge that forms between the mating pair. This translocation apparatus is a complex membrane-associated structure coded for by the Mpf (mating pair formation) genes.

The relaxosome of one conjugal plasmid may or may not be transferrable by the Mpf system of another. Specificity is conferred, in part, by a single protein which is thought to couple the relaxosome with the mating bridge (8, 29). These specificity determinants, exemplified by TraG of the IncP plasmid RP4 ($TraG_{RP4}$), comprise a family of related proteins (29). All contain two conserved regions, and many contain N-terminal secretion signals (Fig. 1 and reference 29). While essential for conjugal transfer, where examined, these proteins are not required for construction of the transport complex. For example, $TraG_{RP4}$, encoded by the Tra1 region (Fig. 2 and reference 58), is required for conjugal transfer but not for Mpf-dependent pilus production or sensitivity to Mpf-specific bacteriophages such as PRD1, pf3, and PRR1 (24, 30, 53).

The IncRh1 Ti plasmids of *Agrobacterium tumefaciens* contain two transfer systems. One, coded for by *vir*, transfers a discrete portion of the plasmid, called the T region, from the bacterium to the plant (14). The Dtr functions of this system are coded for by the *virD* and *virC* operons (23, 48, 50) located in the *vir* regulon of the Ti plasmid (41, 47). The nicking sites, called borders, flank and define the T region, and are closely related at the nucleotide sequence level to the *nic* site within *oriT* of RP4 (37). Furthermore, VirD2, the border-specific strand transferase, is related to TraI, the *oriT*-specific relaxase of RP4 (37, 52). On the other hand, the Ti plasmid *vir* mating bridge coded for by the *virB* operon is only distantly related to Tra2, the locus coding for the Mpf functions of RP4. Components of the VirB Mpf system are most closely related to those of the IncN plasmid pKM101 (39) and to *vir* and *ptl*, which code for transporters known or thought

* Corresponding author. Mailing address: Department of Crop Sciences, University of Illinois at Urbana-Champaign, 240 Edward R. Madigan Laboratory (ERML), 1201 West Gregory Dr., Urbana, IL 61801. Phone: (217) 333-1524. Fax: (217) 244-7830. E-mail: stephenf@uiuc.edu.

† Present address: Clontech, Inc., Palo Alto, CA 94303.

‡ Present address: MJ Research, Inc., Indian Village, NV 89451.

§ Present address: Department of Plant Science, University of Connecticut, Storrs, CT 06269.

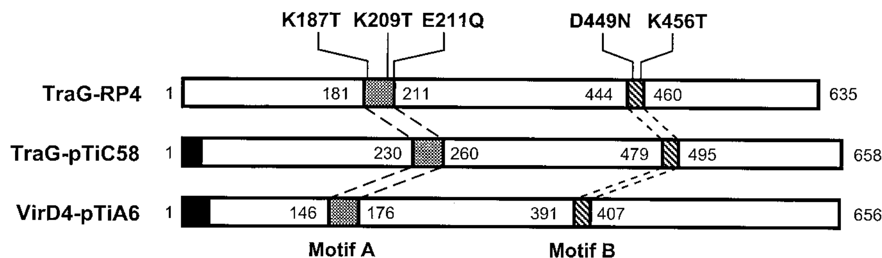

FIG. 1. Structure and relatedness of coupling proteins from the RP4 and Ti plasmid transfer systems. The length of each protein in amino acid residues is indicated by the numbers at each end. Filled regions indicate amino acid sequences with properties of N-terminal secretion signals. The stippled and cross-hatched regions and their coordinates delimit the two motifs conserved among the members of the TraG family (19, 29). The amino acid substitution mutants of TraG_{RP4} are indicated by the single-letter designation for the wild-type residue followed by the position number and the mutant residue.

to be required for the secretion of virulence factors by *Brucella suis* and *Bordetella pertussis*, respectively (35, 55; reviewed in reference 57; see also reference 12).

The second Ti plasmid conjugation system mediates transfer of the entire plasmid from donors to bacterial recipients (reviewed in reference 18). This system is composed of two distantly linked units; *tra*, a set of two operons divergently transcribed from an intergenic region that contains the *oriT* (1, 11, 19); and *trb*, an operon of 12 genes that codes for the conjugal mating bridge (1, 31). The *tra* system also is chimeric; the *oriT* and Dtr genes encoded by *tra* are related to those of the IncQ plasmid RSF1010 (1, 11, 19), while the Mpf functions, coded for by the *tra* and *trb* operons, are closely related to those of the Tra2 genes of RP4 (1, 31).

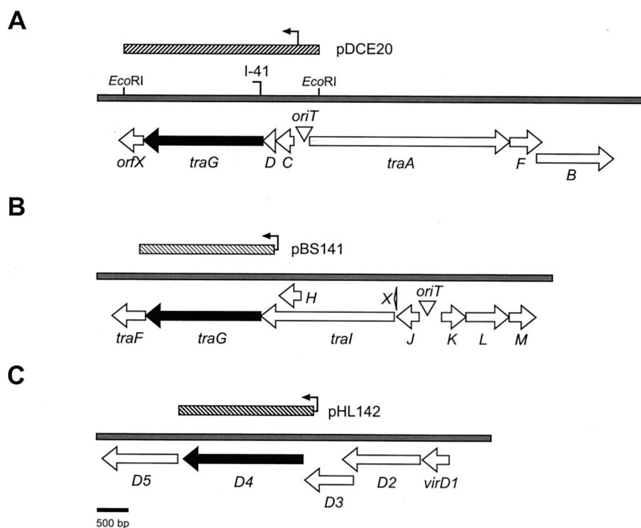

FIG. 2. Gene organization of the Dtr regions of the transfer systems from RP4 and pTiC58. The black-filled arrows represent genes coding for the coupling proteins of the three transfer systems. (A) *tra* locus of the Ti plasmid *tra* system. Locations of genes within the two *tra* operons, as well as the *oriT* sequence, are indicated by the large arrows. The flagstaff represents the site of the Tn*3*HoHo1 insertion located in *traG* of the mutant Ti plasmid pDCKI41. The diagonally hatched bar represents the *Eco*RI fragment from pTiC58 containing *traG* cloned in pDCE20. The small arrow represents the location and direction of transcription of the native TraR-dependent promoter responsible for expression of the *traCDG* operon. (B) Tra1 core region of RP4. The locations of the essential Dtr genes and the *oriT* are shown by large arrows. The diagonally hatched bar depicts the fragment containing the *traG* gene cloned in pBS141. The arrow represents the Tac promoter, provided by the vector, that drives expression of the gene. (C) *virD* operon of the Ti plasmid. The five genes of the *virD* operon are shown by the large arrows. The diagonally hatched bar depicts the fragment containing the *virD4* gene cloned in pHL142. The arrow represents the *lac* promoter, provided by the vector, that drives expression of the gene.

The two Ti plasmid transfer systems each contain a coupling component related to TraG_{RP4} (Fig. 1). The *vir* element, called VirD4, is coded for by the *virD* operon (Fig. 2) and is essential for transfer of the T strand, the processed form of the T region, to plant cells (33, 40). Like TraG_{RP4}, the protein localizes to the cell membrane complex, although both lack canonical membrane-spanning domains (13, 36; V. L. Waters, E. Lanka, and D. G. Guiney, Abstr. 95th Gen. Meet. Am. Soc. Microbiol. 1995, abstr. H-123, p. 513, 1995). The *tra* component, also called TraG (Fig. 1), has not been localized but is essential for conjugal transfer (19). The gene that codes for this component is located in the *traCDG* operon which flanks the Ti plasmid *oriT* (Fig. 2 and reference 19).

The IncQ plasmid RSF1010 contains an *oriT* (16), a site-specific relaxase, MobA (15), and additional relaxosome proteins, MobB and MobC (45, 46). However, the plasmid lacks genes for Mpf functions and a coupling protein (46) and, while mobilizable by other plasmids (56), is not self-conjugal. Mobilization requires the Mpf functions and the coupling protein of the conjugal helper plasmid. For example, mobilization of RSF1010 by RP4 requires the Tra2 locus, *traG*, and *traF*, the Tra1 gene coding for the pilin processing enzyme (17), but not *traI* or other components of the RP4 relaxosome (24, 30, 53). Similarly, the Ti plasmid *vir* system mobilizes RSF1010 to plant cells (6) and also to bacterial recipients (3). Both transfer processes require the *virB*-encoded Mpf system and VirD4 (20, 21) as well as the RSF1010 *oriT* and relaxosome components (6). On the other hand, although the Ti plasmid *oriT* and its associated relaxase, TraA, are related to the analogous components of the RSF1010 *mob* system (11, 19), the Ti plasmid *tra* system does not transfer the IncQ plasmid (11). This failure to transfer is surprising considering that TraG and the Mpf system of the Ti plasmid are closely related to their homologs of RP4, which mobilizes RSF1010 at high frequency. Thus, we undertook a study to determine what factors account for the inability of the Ti plasmid conjugal transfer system to mobilize RSF1010.

MATERIALS AND METHODS

Bacterial strains and plasmids. Strains of *A. tumefaciens* and *Escherichia coli* and the plasmids used in this study are listed in Table 1. pDB127, which is derived from pDB126, contains an in-frame deletion in *traG* and is nonconjugal (2). Transfer of this plasmid is restored by providing *traG* of RP4 in *trans* (2). pDCKI41, which contains a Tn*3*HoHo1 insertion in *traG*, is a transfer-minus (Tra^-) derivative of the transfer-constitutive (Tra^c) Ti plasmid pTiC58Δ*accR* (19). pDCE20 is a recombinant plasmid in which *Eco*RI fragment 20 of pTiC58 is inserted in the IncP1 vector pRK415 (11, 27). This clone contains the entire *traCDG* operon and its native TraR-dependent promoter (19). pDSK519, which codes for resistance to kanamycin (27), and pMMB67HE, which codes for resistance to ampicillin and carbenicillin (22), are mobilizable vectors derived from RSF1010. Both retain *oriT* and *mobA*, *mobB*, and *mobC*, the three genes required to form the RSF1010 relaxosome.

TABLE 1. Bacteria and plasmids used in this study

Bacterial strain or plasmid	Relevant genotype, phenotype, or characteristic[a]	Source or reference
A. tumefaciens		
NT1	Ti plasmid-cured derivative of C58, contains pAtC58	54
C58C1RS	Ti plasmid-cured derivative of C58; Rif^r Str^r; contains pAtC58	49
C58C1EC	Ti plasmid-cured derivative of C58; Ery^r Chl^r; contains pAtC58	32
UIA5	Ti plasmid-cured derivative of GMI9017; Rif^r Str^r; lacks pAtC58	11, 42
NT1(pZLR4)	AAI bioindicator strain; Cb^r	9
E. coli		
DH5α	*supE44* Δ*lacU169* (ϕ80*lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	43
HB101	*hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44* Str^r	43
HB101Nx	*hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44* Str^r Nal^r	2
HB101Rif^R	*hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44* Str^r Rif^r	11
S17-1	*pro* (r^- m^+) Mob$^+$ Amp^r Chl^r Tmp^r Str^r	19
Plasmids		
pTiC58Δ*accR*	*accR* deletion mutant of pTiC58; Tra^c *acc*^{c}	11
pDCKI41	*traG*::Tn*3*HoHo1 insertion mutant of pTiC58Δ*accR*; Tra$^-$	19
pJB3	IncP1 cloning vector; Mob$^+$ Ap^r Cb^r	5
pRK415	IncP1 cloning vector; Mob$^+$ Tc^r	27
pDSK519	IncQ cloning vector; Mob$^+$ Km^r	27
pMMB67HE	IncQ cloning vector; Mob$^+$ Ap^r Cb^r	22
pVK225	Cosmid clone of *virCDE* region from pTiA6NC	26
pDB126	Tra1 core region and Tra2 region of RP4 cloned in ColD vector; Tra$^+$ Cm^r	2
pDB127	*traG* deletion mutant of pDB126; Tra$^-$ Cm^r	2
pBS141	*traG* of RP4 cloned in pMMB67HE; IncQ Ap^r Cb^r	2
pBS141K187T	K187→T mutant of *traG* in pBS141; IncQ Ap^r Cb^r	2
pBS141K209T	K209→T mutant of *traG* in pBS141; IncQ Ap^r Cb^r	
pBS141E211Q	E211→Q mutant of *traG* in pBS141; IncQ Ap^r Cb^r	2
pBS141D449N	D449→N mutant of *traG* in pBS141; IncQ Ap^r Cb^r	2
pBS141K456T	K456→T mutant of *traG* in pBS141; IncQ Ap^r Cb^r	2
pDCE20	*traCDG* operon of pTiC58 cloned in pRK415; IncP1 Tc^r	19
pHL142	*virD4* of pTiA6NC cloned in pDSK519; IncQ Km^r	This study
pPL*trb*	*traI*/*trb* region of pTiC58 cloned in pJB3; IncP1 Ap^r Cb^r	This study
pPL*trb*-DB	Tra1 core region of RP4 cloned into pPL*trb*; IncP1 Ap^r Cb^r	This study
pZLQR	*traR* of pTiC58 cloned in pBBR1MCS2; Inc? Km^r	34
pZLQRF	*traF* of pTiC58 cloned into pZLQR; Inc? Km^r	This study

[a] Antibiotics: Amp and Ap, ampicillin; Cb, carbenicillin; Chl and Cm, chloramphenicol; Ery, erythromycin; Km, kanamycin; Nal, nalidixic acid; Rif, rifampin; Str, streptomycin; Tc, tetracycline; Tmp, trimethoprim. Other symbols: *acc*^{c}, constitutive expression of *acc*; Inc?, unknown incompatibility group; Mob, mobilizable; Tra$^+$, conjugation proficient; Tra$^-$, conjugation deficient; Tra^c, constitutive for conjugation.

Media, chemicals, and growth conditions. *A. tumefaciens* strains were grown at 28°C in Luria-Bertani (LB) broth (43), in ABM minimal medium (10), or on nutrient agar plates (Difco Laboratories, Detroit, Mich.). *E. coli* strains were grown at 28°C or 37°C in LB medium. All liquid cultures were grown with shaking to ensure aerobic conditions. Antibiotics were included in media at the following concentrations: for *A. tumefaciens*, carbenicillin, 100 or 200 μg/ml; chloramphenicol, 100 μg/ml; erythromycin, 150 μg/ml; kanamycin, 50 or 100 μg/ml; nalidixic acid, 50 μg/ml; rifampin, 50 μg/ml; and streptomycin, 200 μg/ml; for *E. coli*, ampicillin, 100 μg/ml; kanamycin, 50 μg/ml; nalidixic acid, 30 μg/ml; and rifampin, 30 μg/ml. AT minimal medium containing nopaline (Sigma Chemical Co., St. Louis, Mo.) as the sole carbon source was prepared as described elsewhere (38).

Plasmid constructions. pHL142, which contains *virD4* from pTiA6NC expressed from the *lac* promoter of pDSK519, was constructed as follows. The gene was amplified by PCR from pVK225 (26) using as the 5′ primer 5′-GGC TCTAGAGGTGAAGTC*ATG*AATTCCAGC-3′, which contains an *Xba*I site (underlined) and the start codon of *virD4* (italics), and as the 3′ primer 5′-CG GGTACC*TCA*TTTCGCAGGCTGTGCCGG-3′, which contains a *Kpn*I site (underlined) and the termination codon of *virD4* (italics). The PCR product was digested with the two enzymes and cloned into similarly digested pDSK519. pPL*trb* was constructed by cloning the *trb* operon of pTiC58 as a *Bgl*II-*Xba*I fragment into the IncP1 vector pJB3 (5). The plasmid confers resistance to ampicillin and carbenicillin. pPL*trb*-DB (Fig. 3) was constructed by cloning the core Tra1 region of RP4 as a *Kpn*I fragment from pDB126 into pPL*trb*. pPLQRF was constructed by cloning *traF*, which codes for the putative pilin-processing enzyme from pTiC58, into pZLQR (34) as an *Eco*RI-*Hin*dIII fragment. This clone, which also expresses *traR*, is derived from pBBR1MCS2, a vector that is compatible with IncP, IncQ, and IncRh1 plasmids (28).

Plasmids were introduced into *A. tumefaciens* strains by mobilization from *E. coli* S17-1 or by electroporation as described previously (19) and into *E. coli* strains by $CaCl_2$-mediated transformation (43).

Matings. Matings between *A. tumefaciens* donors and *A. tumefaciens* or *E. coli* recipients were conducted at 28°C, while those between *E. coli* donors and recipients were conducted at 37°C. All matings were performed on nitrocellulose filters placed on the surface of ABM agar, nutrient agar, or LB agar plates as described by Cook and Farrand (11). In all cases, donors and recipients were grown to late exponential phase, and matings were allowed to proceed for 2- to 24-h intervals before the mixes were recovered from the filters, diluted, and plated onto selective media. The titer of the donor population was determined by dilution plating just prior to mixing with recipients. Frequencies of transfer are expressed as transconjugants recovered per input donor. Coinheritance of pTiC58 or its derivatives was assessed by picking a minimum of 100 transconjugant colonies to AT nopaline plates containing antibiotics selective for the recipient. Coinheritance of the RSF1010-based plasmids was assessed by picking a similar number of transconjugant colonies to nutrient agar or LB agar plates supplemented with the appropriate antibiotics.

AAI assays. Production of AAI [*N*-(3-oxo-octanoyl)-L-homoserine lactone] was assessed by a semiquantitative plate assay using NT1(pZLR4) as the bioreporter as described by Cha et al. (9). NT1(pTiC58Δ*accR*) was used as the positive control.

RESULTS

Ti plasmid *trb* can transfer the RP4 relaxosome. Although the *trb* region of pTiC58 and Tra2 of RP4 clearly are of the same phylogenetic lineage (31), the Ti plasmid does not mo-

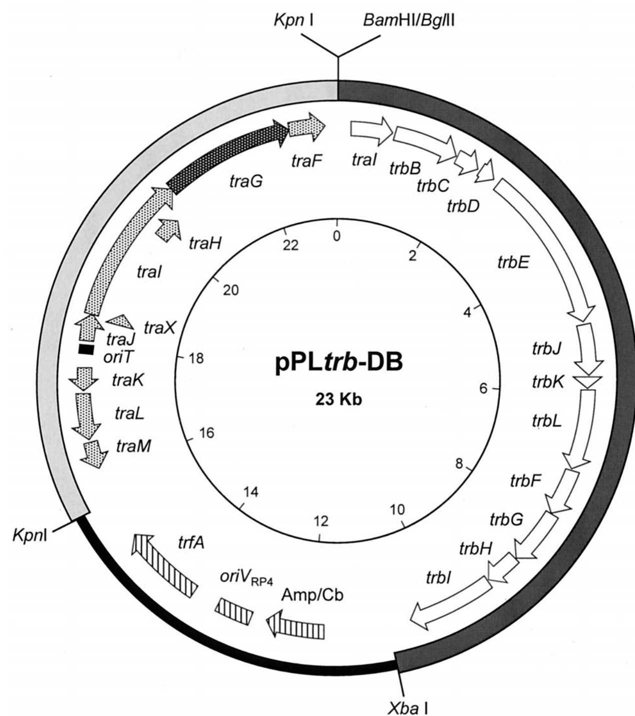

FIG. 3. Genetic structure of pPL*trb*-DB. This plasmid, based on the IncP1 vector pJB3 (thin black line) (5), contains the entire *trb* region from pTiC58 (open arrows) and the Tra1 core region from RP4 (light shaded arrows). *traG*, which codes for the RP4-coupling protein, is indicated by the dark shaded arrow. *oriV*, origin of vegetative plasmid replication; *trfA*, the gene coding for the replication initiation protein of RP4; Amp/Cb, the *bla* gene coding for resistance to ampicillin and carbenicillin. See Materials and Methods for details.

bilize vectors that contain the RP4 *oriT* but lack the Tra1 genes required for a functional relaxosome (11). This is not surprising; the *oriT* of RP4 is unrelated to that of the nopaline- and octopine-type Ti plasmids (1, 11), making it unlikely that the Ti plasmid Dtr functions recognize and process at the RP4 *nic* site. However, it is conceivable that if allowed to form, the RP4 relaxosome would be recognized by the Ti plasmid Mpf system. Thus, we determined whether a plasmid encoding the RP4 Tra1 Dtr system but lacking the IncP1 Mpf functions could be mobilized from an *A. tumefaciens* donor when provided with a copy of the Ti plasmid *trb* system. To do this, we constructed pPL*trb*-DB, which contains the core Tra1 region, including *oriT*, of RP4 and the entire *trb* operon of pTiC58 (Fig. 3). Expression of *trb* requires TraR, the quorum-sensing transcriptional activator (25, 31). We provided this gene, as well as *traF*, the putative pilin-processing enzyme from pTiC58, in *trans* on pPLQRF. Donors harboring pPL*trb*-DB and pPLQRF transferred the former plasmid at a frequency of 1.3×10^{-6} per input donor. Transfer was dependent on *traR* and *traF*; donors lacking pPLQRF failed to transfer pPL*trb*-DB (data not shown). Thus, the RP4 relaxosome can interface with the Ti plasmid *trb* complex.

On the other hand, RSF1010 contains an *oriT* remarkably similar to that of the Ti plasmids (11) and encodes its own relaxosome components (46). One such component, MobA, is the cognate relaxase and is related to TraA of the Ti plasmid *tra* system (19, 45). RSF1010, while mobilized at high frequency by RP4 (56), is not mobilized at detectable levels by pTiC58 (11). Taken together, these results indicate that the Ti

TABLE 2. TraG of RP4 allows pTiC58 to mobilize IncQ plasmids[a]

IncQ plasmid[b]	*traG* allele[c]	Mobilization frequency[d]	Ti plasmid coinheritance[e]
pDSK519	None	$<10^{-9}$	NA
pMMB67HE[f]	None	$<10^{-9}$	NA
pBS141	Wild type	3×10^{-7}	0.04
pBS141K187T	K187T	$<10^{-9}$	NA
pBS141K209T	K209T	1.6×10^{-8}	0.14
pBS141E211Q	E211Q	$<10^{-9}$	NA
pBS141D449N	D449N	$<10^{-9}$	NA
pBS141K456T	K456T	5.5×10^{-7}	0.28

[a] *A. tumefaciens* UIA5(pTiC58Δ*accR*) was used as the donor in filter matings with strain C58C1EC as described in Materials and Methods.
[b] All plasmids are derivatives of RSF1010 and contain the IncQ *oriT* and mobilization genes *mobA*, *mobB*, and *mobC*.
[c] See Fig. 2.
[d] Expressed as transconjugants obtained per input donor. The full set of matings was repeated twice with indistinguishable patterns of results. The data from a single set of matings conducted in parallel on the same day are presented.
[e] Assessed by picking 100 transconjugant colonies to AT minimal medium containing nopaline as the sole carbon source as described in Materials and Methods. NA, not applicable.
[f] Vector used for construction of the pBS141 series of plasmids.

plasmid mating bridge can interact with the relaxosome of RP4 but not with that of RSF1010.

$TraG_{RP4}$ allows pTiC58 to mobilize RSF1010. Interaction between the relaxosome and the Mpf complex of RP4 is thought to be mediated by TraG (30). The TraG proteins of RP4 and pTiC58 are closely related (1, 19) (Fig. 1) and essential for conjugal transfer (2, 19), and that of RP4 is required for mobilization of RSF1010 (30). Based on these observations, we hypothesized that $TraG_{RP4}$, but not $TraG_{pTiC58}$, could couple the relaxosome of RSF1010 with the Ti plasmid Mpf complex. To test this hypothesis, we determined whether pTiC58Δ*accR* can mobilize IncQ plasmids if provided with $traG_{RP4}$. We also tested five single amino acid substitution mutants of $traG_{RP4}$ (Fig. 1), as well as two RP4-mobilizable vectors based on the RSF1010 replicon. To exclude any influence of pAtC58 we used *A. tumefaciens* UIA5, which lacks this plasmid (Table 1), as the donor in this set of matings. As shown in Table 2, pTiC58Δ*accR* failed to mobilize pDSK519 and pMMB67HE, the two RSF1010-derived vectors. However, the Tra^c Ti plasmid mobilized pBS141, the pMMB67HE clone that contains wild-type $traG_{RP4}$, at a low but easily detectable frequency (Table 2).

The Ti plasmid also mobilized one point mutant of $TraG_{RP4}$, pBS141K456T, at frequencies similar to that of the wild-type plasmid, pBS141 (Table 2). This allele, with a K-to-T substitution at position 456, complements a $traG_{RP4}$-null mutant, pDB127, to near-wild-type levels of transfer (2). Three mutants, pBS141K187T, pBS141E211Q, and pBS141D449N, were not mobilized by the Ti plasmid at detectable frequencies, while the remaining mutant, pBS141K209T, was mobilized but at a frequency approximately 20-fold lower than the wild-type parent (Table 2). The K187T, E211Q, and D449N mutations do not complement the $traG_{RP4}$ null mutation while the K209T mutation results in a partially active protein (2). Thus, $TraG_{RP4}$ conferred on the Ti plasmid the capacity to mobilize RSF1010. Moreover, mutations in the gene exhibited phenotypes for Ti plasmid-mediated mobilization of RSF1010 that mimicked those observed for complementation of a *traG* null mutation in the RP4 transfer system (2).

Mobilization of RSF1010 may restrict cotransfer of the Ti plasmid to the same recipient. We assessed transconjugants selected for acquisition of pBS141 or its derivatives for coin-

TABLE 3. $TraG_{RP4}$-mediated mobilization from the IncQ relaxosome does not interfere with Ti plasmid transfer but may restrict transfer to the same recipient

IncQ plasmid	Transfer frequency[a] of:			
	pTiC58Δ*accR*[b]	Coinheritance of IncQ plasmid[c]	IncQ derivative[d]	Coinheritance of Ti plasmid[e]
None	7.1×10^{-3}	NA	NA	NA
pDSK519	6.7×10^{-2}	<0.01	$<10^{-8}$	NA
pBS141	1.2×10^{-2}	<0.01	3×10^{-6}	0.12

[a] Expressed as transconjugants per input donor. The experiment was repeated once with similar patterns of results. NA, not applicable.
[b] Transconjugants were selected on AT minimal medium supplemented with chloramphenicol and erythromycin and containing nopaline as the sole carbon source.
[c] Assessed by picking 100 transconjugant colonies to nutrient agar containing kanamycin (pDSK519) or carbenicillin (pBS141) in addition to chloramphenicol and erythromycin.
[d] Transconjugants were selected on nutrient agar containing kanamycin (pDSK519) or carbenicillin (pBS141) in addition to chloramphenicol and erythromycin.
[e] Assessed by picking 100 colonies to AT minimal agar containing nopaline as the sole carbon source in addition to chloramphenicol and erythromycin.

heritance of the Ti plasmid. Frequencies of cotransfer ranged from 4 to 28% (Table 2). However, we previously reported that RSF1010 has no effect on the frequency of *tra*-mediated conjugal transfer of pTiC58 (11). We considered the possibility that by allowing the Ti plasmid to mobilize RSF1010, $traG_{RP4}$ influences the conjugal transfer of the Ti plasmid itself. To test this, we mated UIA5(pTiC58Δ*accR*) harboring either pDSK519 or pBS141 with C58C1EC and selected independently for transfer of the Ti plasmid and the IncQ plasmid. As before, the Ti plasmid mobilized pBS141 but not the empty vector (Table 3). However, neither pDSK519 nor pBS141 detectably inhibited transfer of the Ti plasmid itself. Transconjugants selected for inheritance of pBS141 again showed low coinheritance of pTiC58Δ*accR* (Table 3). As expected from the 10^4-fold differences between the transfer and mobilization frequencies, none of the tested transconjugants selected for acquiring the Ti plasmid had coinherited pDSK519 or pBS141 (Table 3).

$TraG_{RP4}$ cannot substitute for $TraG_{pTiC58}$ for Ti plasmid transfer. $TraG_{RP4}$ allows pTiC58 to mobilize RSF1010, suggesting that this protein and $TraG_{C58}$ are functionally interchangeable. We assessed this possibility by determining whether $traG_{RP4}$ could restore transfer to pDCKI41, a Tra^- *traG* mutant of pTiC58Δ*accR* (Fig. 2 and reference 19). We first determined if the mutation in this plasmid is complementable by $traG_{C58}$. pDCE20, which codes for this gene as well as the upstream TraR-dependent promoter region (Fig. 2), restored conjugal transfer of pDCKI41 to wild-type levels (data not shown). On the other hand, neither the vector pMMB67HE nor pBS141, the $traG_{RP4}$-containing recombinant clone, detectably complemented the *traG* mutation in the Ti plasmid (data not shown). However, while pMMB67HE was not transferred at a detectable level, pBS141, was mobilized by the *traG* mutant Ti plasmid at a frequency of 2.2×10^{-6} per input donor. Thus, while $TraG_{RP4}$ will not substitute for $TraG_{pTiC58}$ for Ti plasmid transfer, it will substitute for mobilization of the IncQ vector.

VirD4 can replace $TraG_{pTiC58}$ for RSF1010 mobilization but not for Ti plasmid transfer. Given the relatedness between VirD4 and the TraG family (Fig. 1 and reference 29), we determined whether this *vir* component can restore transfer to pDCKI41, the *traG* mutant of pTiC58. To test this possibility,

TABLE 4. VirD4 couples transfer of an IncQ plasmid but not the Ti plasmid to the Ti plasmid mating bridge

Source of coupling protein[a]	Coupling protein	Frequency of transfer[b] of:		
		pDCKI41 to *A. tumefaciens*	IncQ plasmid to:	
			A. tumefaciens	*E. coli*
None	None	$<10^{-8}$	NA	NA
pDCE20	$TraG_{pTiC58}$	2.3×10^{-3}	NA	NA
pDSK519	None	$<10^{-8}$	$<10^{-8}$	$<10^{-8}$
pHL142	VirD4	$<10^{-8}$	1.9×10^{-5}	1.4×10^{-6}

[a] All *A. tumefaciens* donors harbored pDCKI41 (*traG*::Tn*3*HoHo1) in addition to the recombinant plasmid providing the coupling protein. NA, not applicable.
[b] Expressed as transconjugants obtained per input donor. The experiment was repeated three times with indistinguishable patterns of results.

we introduced pHL142, which contains *virD4* expressed from the *lac* promoter of pDSK519 (Fig. 2), into NT1(pDCKI41). The *A. tumefaciens* donor mobilized pHL142 to *A. tumefaciens* and *E. coli* recipients but failed to transfer the mutant Ti plasmid to the *A. tumefaciens* recipient at a detectable frequency (Table 4). Donors harboring the empty vector, pDSK519 failed to transfer either pDCKI41 or the IncQ plasmid. VirD4 also failed to complement the $traG_{RP4}$ mutation in pDB127; *E. coli* donors harboring the mutant $\Delta traG_{RP4}$ plasmid and pHL142 failed to transfer either element at a detectable frequency to *E. coli* recipients (data not shown).

DISCUSSION

We conclude from our studies that the failure of pTiC58 to mobilize RSF1010 results from the inability of the Ti plasmid TraG protein to recognize the relaxosome of the IncQ plasmid. The IncQ Dtr complex can be transferred by the Ti plasmid mating bridge but requires $TraG_{RP4}$ or VirD4 as the coupling protein (Tables 2 and 4). Furthermore, mutations in $TraG_{RP4}$ that affect function in the RP4 transfer system exert a similar effect on Ti plasmid-mediated mobilization from the IncQ relaxosome (Table 2). This observation suggests that $TraG_{RP4}$ interacts with the transfer systems of RP4 and pTiC58.

The Ti plasmid mating bridge will transfer the RP4 relaxosome, albeit at a low frequency. Transfer is dependent on one or more *trans*-acting components of the RP4 Dtr system; the Ti plasmid will not mobilize plasmids that contain only the RP4

TABLE 5. Interactions between Mpf and Dtr systems mediated by IncRh1 and IncP coupling proteins

Coupling protein	Mating bridge[a] (comment[b])			Relaxosome
	IncRh1 *trb*	IncP	IncRh1 *vir*	
$TraG_{pTiC58}$	+ (C)	− (32)		IncRh1 *tra*
				IncP
	− (11)			IncQ
			− (33)	IncRh1 *vir*
$TraG_{RP4}$	− (TS)	− (32)		IncRh1 *tra*
	+ (TS)	+ (C)		IncP
	+ (TS)	+ (56)		IncQ
			− (33, 47)	IncRh1 *vir*
VirD4	− (TS)			IncRh1 *tra*
		− (TS)		IncP
	+ (TS)	− (TS)	+ (3, 6, 20, 21)	IncQ
			+ (C)	IncRh1 *vir*

[a] +, effective interface; −, ineffective interface.
[b] C, cognate system; TS, this study. Numbers denote references in which interactions were observed or can be inferred from the data.

oriT (11). Furthermore, transfer is dependent on TraR and AAI, which are required for expression of the Ti plasmid *trb* operon (31). Dependence on the quorum-sensing regulatory components rules out the possibility that transfer of the RP4 relaxosome is mediated by some other conjugal system in the *A. tumefaciens* donor. Transfer of the RP4 Dtr complex by the Ti plasmid Mpf system is not surprising considering the degree of relatedness between the Mpf proteins of the two conjugation systems (31). However, the components of the two Mpf systems are not interchangeable; the IncP1 Tra2 system will not restore transfer to Ti plasmids with mutations in any one of the nine essential *trb* genes (32). Furthermore, failure of such Ti plasmid *trb* mutants to transfer from donors that also harbor an IncP1 plasmid indicates that neither $TraG_{IncP1}$ nor $TraG_{pTiC58}$ can interface the Ti plasmid relaxosome with the IncP1 mating bridge. Our studies show that the IncP1-coupling protein will not restore conjugation to a Ti plasmid *traG* mutant. Thus, while $TraG_{RP4}$ productively interfaces with the Ti plasmid mating bridge, it cannot interact with the Ti plasmid relaxosome.

These results, as summarized in Table 5, are reminiscent of those reported by Cabezón et al. (7), who concluded that specificities between the relaxosomes and mating bridges of RP4 and the IncW plasmid R388 are conferred by the respective coupling proteins, TraG and TrwB. $TraG_{RP4}$ can function with the Mpf system but not the relaxosome of R388 (7). Similarly, $TraG_{RP4}$ allowed pTiC58 to mobilize an IncQ plasmid but did not complement a $traG_{pTiC58}$ mutant for transfer of the Ti plasmid. Furthermore, Cabezón et al. reported that maximum transfer frequencies occurred only in combinations that included cognate Dtr, Mpf, and coupling proteins (7). Similarly, while RP4 mobilizes RSF1010 at high frequency (30, 56), $TraG_{RP4}$-mediated mobilization of the IncQ plasmid via the Ti plasmid mating bridge occurs at a low frequency (Table 2). This difference in frequency suggests that the IncP1-coupling protein does not efficiently interface the RSF1010 relaxosome to the Ti plasmid Mpf system.

Remarkably, VirD4, the TraG homolog from the Ti plasmid *vir* system, can couple relaxosomes to the Ti plasmid conjugal mating bridge. Like $TraG_{RP4}$, VirD4 exhibits specificity in this interaction, coupling the relaxosome of RSF1010 but not that of the Ti plasmid to the Ti plasmid Mpf system (Table 4). Furthermore, VirD4 failed to complement an RP4 *traG* mutation for transfer from the RP4 relaxosome. We conclude from these results that VirD4 can productively recognize the Mpf complex of the Ti plasmid conjugal transfer system, and also the RSF1010 relaxosome, but not the relaxosomes of the Ti plasmid or RP4 (Table 5). Similarly, VirD4 will couple the IncQ relaxosome to the Ti plasmid mating bridge but not to that of RP4 (Table 5). This observation is surprising given the close phylogenetic relationships between the Mpf systems of the two plasmids. On the other hand, neither TraG from an IncP plasmid nor that from pTiC58 will complement mutations in VirD4 for VirB-mediated transfer of T strands to plant cells (33, 47). Whether either TraG protein can interact with VirD2-border complexes, the T-strand equivalent of the relaxosome, remains to be determined.

Taken together, the results point to these coupling proteins as determinants of recognition and specificity. Thus, TraG of the Ti plasmid does not recognize the relaxosomes of RSF1010 or RP4. $TraG_{RP4}$, on the other hand, can couple its own relaxosome and that of RSF1010 to the Ti plasmid mating bridge (Table 5). But, based on its inability to restore transfer to a *traG* mutant of pTiC58, $TraG_{RP4}$ does not recognize the relaxosome of the Ti plasmid. In this regard, most of the available data indicate that specificity is conferred through interactions between the coupling protein and the relaxosome. However, two experimental observations point to specificities with respect to the coupling protein and the mating bridge. First, IncP1 plasmids do not restore transfer to *trb* mutants of pTiC58Δ*accR* (31, 32). Thus, the Ti plasmid TraG protein apparently cannot couple the Ti plasmid relaxosome to the IncP1 Mpf complex. Second, VirD4 can interface the RSF1010 relaxosome to the Ti plasmid mating bridge (Table 4) and also to the *vir* mating bridge (6), but VirD4 does not allow a *traG* mutant of the RP4 system to mobilize the IncQ plasmid (data not shown). This observation suggests that VirD4 can couple the IncQ relaxosome to the Ti plasmid Mpf but cannot couple this relaxosome to the RP4 mating bridge.

$TraG_{RP4}$-mediated mobilization of the IncQ plasmid via the Ti plasmid *tra* system does not interfere with conjugal transfer of pTiC58 (Table 3). However, although pTiC58 transfers at frequencies 3 to 4 orders of magnitude higher than pBS141, transconjugants selected for receiving the IncQ plasmid coinherit the Ti plasmid at relatively low frequencies (Tables 2 and 3). These observations suggest that Ti plasmid mating bridges catalyzing $TraG_{RP4}$-mediated transfer of the IncQ plasmid cannot also transfer the Ti plasmid. Thus, we suggest that relaxosome recognition by any given mating bridge is determined by the coupling protein involved in the interaction. This hypothesis could explain why RSF1010 inhibits VirB-mediated T-strand transfer to plants (4, 51) but not *trb*-mediated Ti plasmid transfer to bacteria (11). In the first case, transfer of both nucleoprotein intermediates is mediated by VirD4 only, and the RSF1010 relaxosome competes with the VirD2-T strand complex for VirB mating bridges (4), perhaps through VirD4. In the latter case, we propose that any given mating bridge is associated with $TraG_{pTiC58}$ or $TraG_{RP4}$ but not with both. The former recognizes the relaxosome of the Ti plasmid but not that of RSF1010, while the latter recognizes the relaxosome of RSF1010 but not that of the Ti plasmid. Thus, when both coupling proteins are available, there is no competition between the two relaxosomes for a single mating bridge.

Although there is no direct evidence that the coupling proteins physically interact with the mating bridge, our genetic evidence, as well as that of Cabezón et al. (8), supports this hypothesis. Moreover, should they exist, it is not clear if these interactions are transient, with the coupling protein moving into and out of the complex, or permanent, in which the coupling protein is an integral part of the apparatus itself. However, $TraG_{RP4}$ is not required for Mpf-associated pilus production or for sensitivity to infection by Mpf-dependent bacteriophages (24, 30, 53), suggesting that the coupling protein is not essential for the construction or structural integrity of the mating bridge itself.

Our results support a model in which the coupling protein interfaces the relaxosome with the mating bridge. The specificity of these proteins for any given component dictates whether the substrate, in the form of the nucleoprotein relaxosome, will be recognized and transported by the mating bridge to a recipient cell. Remarkably, there exists considerable latitude in such specificities (7, 8, 44). It remains to be determined which, if any, components of the relaxosome and of the mating bridge interact with the coupling protein. Similarly, although the C terminus of TraD, the F-coupling protein, confers some degree of specificity (44), the domains of these coupling proteins that are involved in these interactions have yet to be identified.

ACKNOWLEDGMENTS

We thank Ingyu Hwang, Fernando de la Cruz, and Stanton Gelvin for helpful discussions over the course of these studies.

This work was supported in part by grants R01 GM52465 from the NIH and AG92-3312-8231 from the USDA to S.K.F., 96-35301-3178 from the USDA to W.R., and SFB344/A8 from the Deutsche Forschungsgemeinschaft to E.L. C.M.H. was the recipient of an undergraduate research award from the Colgate-Palmolive company. P.-L.L. was supported in part by HATCH project 15-0326 to S.K.F.

REFERENCES

1. **Alt-Mörbe, J., J. L. Stryker, C. Fuqua, P.-L. Li, S. K. Farrand, and S. C. Winans.** 1996. The conjugal transfer system of *Agrobacterium tumefaciens* octopine-type Ti plasmids is closely related to the transfer system of IncP and distantly related to Ti plasmid *vir* genes. J. Bacteriol. **178:**4248–4257.
2. **Balzer, D., W. Pansegrau, and E. Lanka.** 1994. Essential motifs of relaxase (TraI) and TraG proteins involved in conjugative transfer of plasmid RP4. J. Bacteriol. **176:**4285–4295.
3. **Beijersbergen, A., A. den Dulk-Ras, R. A. Schilperoort, and P. J. J. Hooykaas.** 1992. Conjugative transfer by the virulence system of *Agrobacterium tumefaciens*. Science **256:**1324–1327.
4. **Binns, A. N., C. E. Beaupré, and E. M. Dale.** 1995. Inhibition of VirB-mediated transfer of diverse substrates from *Agrobacterium tumefaciens* by the IncQ plasmid RSF1010. J. Bacteriol. **177:**4890–4899.
5. **Blatny, J. M., T. Brautaset, H. C. Winther-Larson, K. Haugan, and S. Valla.** 1997. Construction and use of a versatile set of broad-host-range cloning and expression vectors based on the RK2 replicon. Appl. Environ. Microbiol. **63:**370–379.
6. **Buchanan-Wollaston, V., J. Passiatore, and F. Cannon.** 1987. The *mob* and *oriT* mobilization functions of a bacterial plasmid promote its transfer to plants. Nature (London) **328:**172–175.
7. **Cabezón, E., E. Lanka, and F. de la Cruz.** 1994. Requirements for mobilization of plasmids RSF1010 and ColE1 by the IncW plasmid R388: *trwB* and RP4 *traG* are interchangeable. J. Bacteriol. **176:**4455–4558.
8. **Cabezón, E., J. I. Sastre, and F. de la Cruz.** 1997. Genetic evidence of a coupling role for the TraG protein family in bacterial conjugation. Mol. Gen. Genet. **254:**400–406.
9. **Cha, C., P. Gao, Y.-C. Chen, P. D. Shaw, and S. K. Farrand.** 1998. Production of acyl-homoserine lactone quorum-sensing signals by gram-negative plant-associated bacteria. Mol. Plant-Microbe Interact. **11:**1119–1129.
10. **Chilton, M.-D., T. C. Currier, S. K. Farrand, A. J. Bendich, M. P. Gordon, and E. W. Nester.** 1974. *Agrobacterium tumefaciens* DNA and PS8 bacteriophage DNA not detected in crown gall tumors. Proc. Natl. Acad. Sci. USA **71:**3672–3676.
11. **Cook, D. M., and S. K. Farrand.** 1992. The *oriT* region of the *Agrobacterium tumefaciens* Ti plasmid pTiC58 shares DNA sequence identity with the transfer origins of RSF1010 and RK2/RP4 and with the T-region borders. J. Bacteriol. **174:**6238–6246.
12. **Covacci, A., J. L. Telford, G. Del Giudice, J. Parsonnet, and R. Rappuoli.** 1999. *Helicobacter pylori* virulence and genetic geography. Science **284:**1328–1333.
13. **Das, A., and Y.-H. Xie.** 1998. Construction of transposon Tn*3phoA*: its application in defining the membrane topology of the *Agrobacterium tumefaciens* DNA transfer proteins. Mol. Microbiol. **27:**405–414.
14. **de la Cruz, F., and E. Lanka.** 1998. Function of the Ti-plasmid Vir proteins: T-complex formation and transfer to the plant cell, p. 281–301. *In* H. P. Spaink, A. Kondorosi, and P. J. J. Hooykaas (ed.), The Rhizobiaceae. Molecular biology of model plant-associated bacteria. Kluwer Academic Publishers, Dordrecht, The Netherlands.
15. **Derbeyshire, K. M., G. Hatfull, and N. Willetts.** 1987. Mobilization of non-conjugative plasmid RSF1010: a genetic and DNA sequence analysis of the mobilization region. Mol. Gen. Genet. **206:**161–168.
16. **Derbyshire, K. M., and N. S. Willetts.** 1987. Mobilization of non-conjugative plasmid RSF1010; a genetic analysis of its origin of transfer. Mol. Gen. Genet. **206:**154–160.
17. **Eisenbrandt, R., M. Kalkum, E.-M. Lai, R. Lurz, C. I. Kado, and E. Lanka.** 1999. Conjugative pili of IncP plasmids and the Ti plasmid T-pilus are composed of cyclic subunits. J. Biol. Chem. **274:**22548–22555.
18. **Farrand, S. K.** 1998. Conjugation in the Rhizobiaceae, p. 199–233. *In* H. P. Spaink, A. Kondorosi, and P. J. J. Hooykaas (ed.), The Rhizobiaceae. Molecular biology of model plant-associated bacteria. Kluwer Academic Publishers, Dordrecht, The Netherlands.
19. **Farrand, S. K., I. Hwang, and D. M. Cook.** 1996. The *tra* region of the nopaline-type Ti plasmid is a chimera with elements related to the transfer systems of RSF1010, RP4, and F. J. Bacteriol. **178:**4233–4247.
20. **Fullner, K. J.** 1998. Role of *Agrobacterium virB* genes in transfer of T complexes and RSF1010. J. Bacteriol. **180:**430–434.
21. **Fullner, K. J., and E. W. Nester.** 1996. Environmental and genetic factors affecting RSF1010 mobilization between strains of *Agrobacterium tumefaciens*, p. 15–29. *In* W. Ream and S. B. Gelvin (ed.), Crown gall. Advances in understanding interkingdom gene transfer. APS Press, St. Paul, Minn.
22. **Fürste, J. P., W. Pansegrau, R. Frank, H. Blöcker, P. Scholz, M. Bagdasarian, and E. Lanka.** 1986. Molecular cloning of the plasmid RP4 primase region in a multi-host range *tacP* expression vector. Gene **48:**119–131.
23. **Ghai, J., and A. Das.** 1989. The *virD* operon of *Agrobacterium tumefaciens* Ti plasmid encodes a DNA-relaxing enzyme. Proc. Natl. Acad. Sci. USA **86:**9154–9158.
24. **Haase, J., R. Lurz, A. M. Grahn, D. H. Bamford, and E. Lanka.** 1995. Bacterial conjugation mediated by plasmid RP4: RSF1010 mobilization, donor-specific phage propagation, and pilus production require the same Tra2 core components of a proposed DNA transport complex. J. Bacteriol. **177:**4779–4791.
25. **Hwang, I., P.-L. Li, L. Zhang, K. R. Piper, D. M. Cook, M. E. Tate, and S. K. Farrand.** 1994. TraI, a LuxI homologue, is responsible for production of conjugation factor, the Ti plasmid *N*-acylhomoserine lactone autoinducer. Proc. Natl. Acad. Sci. USA **91:**4639–4643.
26. **Knauf, V. C., and E. W. Nester.** 1982. Wide host range cloning vectors: a cosmid clone bank of an *Agrobacterium* Ti plasmid. Plasmid **8:**45–54.
27. **Keen, N. T., S. Tamaki, D. Kobayashi, and D. Trollinger.** 1988. Improved broad-host-range plasmids for DNA cloning in Gram-negative bacteria. Gene **70:**191–197.
28. **Kovach, M. E., P. H. Elzer, D. S. Hill, G. T. Robertson, M. A. Farris, R. M. Roop II, and K. M. Peterson.** 1995. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene **166:**175–176.
29. **Lessl, M., W. Pansegrau, and E. Lanka.** 1992. Relationships of DNA-transfer-systems: essential transfer factors of plasmids RP4, Ti and F share common sequences. Nucleic Acids Res. **20:**6099–6100.
30. **Lessl, M., D. Balzer, K. Weyrauch, and E. Lanka.** 1993. The mating pair formation system of plasmid RP4 defined by RSF1010 mobilization and donor-specific phage propagation. J. Bacteriol. **175:**6415–6425.
31. **Li, P.-L., D. M. Everhart, and S. K. Farrand.** 1998. Genetic and sequence analysis of the pTiC58 *trb* locus, encoding a mating-pair formation system related to members of the type IV secretion family. J. Bacteriol. **180:**6164–6172.
32. **Li, P.-L., and S. K. Farrand.** 1999. Essential components of the Ti plasmid *trb* system, a type IV macromolecular transporter. J. Bacteriol. **181:**5033–5041.
33. **Lin, T.-X., and C. I. Kado.** 1993. The *virD4* gene is required for virulence while *virD3* and *orf5* are not required for virulence of *Agrobacterium tumefaciens*. Mol. Microbiol. **9:**803–812.
34. **Luo, Z.-Q., and S. K. Farrand.** 1999. Signal-dependent DNA binding and functional domains of the quorum-sensing activator TraR as identified by repressor activity. Proc. Natl. Acad. Sci. USA **96:**9009–9014.
35. **O'Callaghan, D., C. Cazevieille, A. Allardet-Servent, M. L. Boschiroli, G. Bourg, V. Foulongne, P. Frutos, Y. Kulakov, and M. Ramuz.** 1999. A homologue of the *Agrobacterium tumefaciens* VirB and *Bordetella pertussis* Ptl type IV secretion systems is essential for intracellular survival of *Brucella suis*. Mol. Microbiol. **33:**1210–1220.
36. **Okamoto, S., A. Toyoda-Yamamoto, K. Ito, I. Takebe, and Y. Machida.** 1991. Localization and orientation of the VirD4 protein of *Agrobacterium tumefaciens* in the cell membrane. Mol. Gen. Genet. **228:**24–32.
37. **Pansegrau, W., and E. Lanka.** 1991. Common sequence motifs in DNA relaxases and nick regions from a variety of DNA transfer systems. Nucleic Acids Res. **19:**3445.
38. **Petit, A., and J. Tempé.** 1978. Isolation of *Agrobacterium* Ti plasmid regulatory mutants. Mol. Gen. Genet. **167:**147–155.
39. **Pohlman, R. F., H. D. Genetti, and S. C. Winans.** 1994. Common ancestry between IncN conjugal transfer genes and macromolecular export systems of plant and animal pathogens. Mol. Microbiol. **14:**655–668.
40. **Porter, S. G., M. F. Yanofsky, and E. W. Nester.** 1987. Molecular characterization of the *virD* operon from *Agrobacterium tumefaciens*. Nucleic Acids Res. **15:**7503–7517.
41. **Rogowsky, P. M., B. S. Powell, K. Shirasu, T.-S. Lin, P. Morel, E. M. Zykprian, T. R. Steck, and C. I. Kado.** 1990. Molecular characterization of the *vir* regulon of *Agrobacterium tumefaciens*: complete nucleotide sequence and gene organization of the 28.63-kbp regulon cloned as a single unit. Plasmid **23:**85–106.
42. **Rosenberg, C., and T. Huguet.** 1984. The pAtC58 plasmid of *Agrobacterium tumefaciens* is not essential for tumor induction. Mol. Gen. Genet. **196:**533–536.
43. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
44. **Sastre, J. I., E. Cabezóne, and F. de la Cruz.** 1998. The carboxyl terminus of protein TraD adds specificity and efficiency to F-plasmid conjugative transfer. J. Bacteriol. **180:**6039–6042.
45. **Scherzinger, E., R. Lurz, S. Otto, and B. Dobrinski.** 1992. *In vitro* cleavage of double- and single-stranded DNA by plasmid RSF1010-encoded mobilization proteins. Nucleic Acids Res. **20:**41–48.
46. **Scholz, P., V. Haring, B. Wittmann-Liebold, K. Ashman, M. Bagdasarian, and E. Scherzinger.** 1989. Complete nucleotide sequence and gene organization of the broad-host range plasmid RSF1010. Gene **75:**271–288.
47. **Stachel, S. E., and E. W. Nester.** 1986. The genetic and transcriptional organization of the *vir* region of the A6 Ti plasmid of *Agrobacterium tumefaciens*. EMBO J. **5:**1445–1454.

48. **Toro, N., A. Datta, O. A. Carmi, C. Young, R. K. Prusti, and E. W. Nester.** 1989. The *Agrobacterium tumefaciens virC1* gene product binds to overdrive, a T-DNA transfer enhancer. J. Bacteriol. **171:**6845–6849.
49. **Van Larebeke, N., C. Genetello, J. Schell, R. A. Schilperoort, A. K. Hermans, J. P. Hernalsteens, and M. van Montagu.** 1975. Acquisition of tumor-inducing ability by non-oncogenic agrobacteria as a result of plasmid transfer. Nature (London) **255:**742–743.
50. **Vogel, A. M., and A. Das.** 1994. Mutational analysis of *Agrobacterium tumefaciens* pTiA6 *virD1*: identification of functionally important residues. Mol. Microbiol. **12:**811–817.
51. **Ward, J. E., E. M. Dale, and A. N. Binns.** 1991. Activity of the *Agrobacterium* T-DNA transfer machinery is affected by *virB* gene products. Proc. Natl. Acad. Sci. USA **88:**9350–9354.
52. **Waters, V. L., and D. G. Guiney.** 1993. Processes at the nick region link conjugation, T-DNA transfer and rolling circle replication. Mol. Microbiol. **9:**1123–1130.
53. **Waters, V. L., B. Strack, W. Pansegrau, E. Lanka, and D. G. Guiney.** 1992. Mutational analysis of essential IncPα plasmid transfer genes *traF* and *traG* and involvement of *traF* in phage sensitivity. J. Bacteriol. **174:**6666–6673.
54. **Watson, B., T. C. Currier, M. P. Gordon, M.-D. Chilton, and E. W. Nester.** 1975. Plasmid required for virulence of *Agrobacterium tumefaciens*. J. Bacteriol. **123:**255–264.
55. **Weiss, A. A., F. D. Johnson, and D. L. Burns.** 1993. Molecular characterization of an operon required for pertussis toxin secretion. Proc. Natl. Acad. Sci. USA **90:**2970–2974.
56. **Willetts, N. S., and C. Crowther.** 1981. Mobilization of nonconjugative IncQ plasmid RSF1010. Genet. Res. **37:**311–316.
57. **Winans, S. C., D. L. Burns, and P. J. Christie.** 1996. Adaptation of a conjugal transfer system for the export of pathogenic macromolecules. Trends Microbiol. **4:**64–68.
58. **Ziegelin, G., W. Pansegrau, B. Strack, D. Balzar, M. Kröger, V. Kruft, and E. Lanka.** 1991. Nucleotide sequence and organization of genes flanking the transfer origin of promiscuous plasmid RP4. J. DNA Seq. Map. **1:**303–327.