Ti Plasmid Conjugation Is Independent of *vir*: Reconstitution of the *tra* Functions from pTiC58 as a Binary System

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Two regions of the nopaline-type Ti plasmid pTiC58 are important for conjugal transfer of this element to recipient bacteria. These two regions were cloned into two independent replicons to produce a binary transfer system. For one region, *oriT/tra*, we constructed two derivatives, pFRtra and pDCtra-5. Each contains the *oriT* site and the two flanking, divergently transcribed *tra* operons that encode the DNA processing functions associated with the relaxosome. These two plasmids also carry *traR*, which encodes the transcriptional activator necessary for expression of transfer genes. The two plasmids differ by the amounts of *traB* sequence or sequence downstream of *traG* present in the construct. The second replicon, pPLE2, carries the *traI/trb* region. The *traI* gene confers production of the *Agrobacterium tumefaciens* *N*-acyl homoserine lactone autoinducer, while the remaining genes in the *trb* operon encode components of the mating bridge. Donors harboring the two plasmids mobilized the transfer of the plasmid carrying the *oriT/tra* region to an *A. tumefaciens* recipient at frequencies similar to that at which the intact Ti plasmid transferred. Plasmid pFRtra, which encodes most of *traB*, was mobilized at a frequency almost 10-fold higher than was pDCtra-5, which lacks most of the gene. *A. tumefaciens* donors also mobilized pFRtra to *Escherichia coli* and *Pseudomonas fluorescens* recipients at frequencies similar to those observed with *A. tumefaciens* recipients. *Rhizobium meliloti* harboring the binary system also transferred the *oriT/tra* component to these recipients. However, *E. coli* or *P. fluorescens* donors harboring the binary system did not transfer pFRtra to any of the recipients. Furthermore, while the *A. tumefaciens* and *R. meliloti* donors produced high levels of the autoinducer, the *P. fluorescens* and *E. coli* donors produced only trace amounts of this signal molecule. These results indicate that the *tra* system of pTiC58 is fully contained within the characterized *tra* and *trb* regions of the Ti plasmid, that conjugation does not require functions encoded by the *vir* system for maximal activity, and that while the Ti plasmid *tra* system recognizes diverse gram-negative bacteria as recipients, of the hosts tested, it functions only in members of the family *Rhizobiaceae*.

Ti plasmids of *Agrobacterium tumefaciens* encode two conjugal transfer systems. One, the *vir* system, transfers a specific portion of the Ti plasmid, called the T region, to plant cells (5, 54). Subsequent integration of the transferred DNA (T-DNA) into the plant cell nuclear genome and expression of gene sets encoded on this DNA fragment result in the conversion of the plant cells to crown gall tumor cells. The second system is responsible for the transmission of the entire Ti plasmid from a donor to a suitable bacterial recipient (13). Both conjugation systems are strongly regulated. Expression of the *vir* genes requires specific factors produced by wounded plant cells, as well as certain environmental conditions (5, 24). Similarly, conjugal transfer occurs only when the bacteria sense the presence of specific metabolites, called opines, produced by the crown gall tumors (13). In addition, expression of genes involved in conjugal transfer is regulated by an autoinducer-dependent quorum-sensing system (17, 41).

Our previous work defined three regions of the nopaline-

type Ti plasmid pTiC58 as being involved in conjugal transfer (3). One region, designated *tra*, maps near *acc*, which is necessary for uptake and utilization of agrocinosines A and B, the opines that induce transfer of this Ti plasmid (3, 20) (Fig. 1). This 6.6-kb region contains six identifiable genes arranged in two divergently transcribed units, the *traAFB* and *traCDG* operons (14). A 258-bp intergenic region separates *traC* and *traA* and contains the origin of transfer (*oriT*), as well as the promoters and other *cis*-acting elements required for regulated expression of the two *tra* operons (8, 9). Predicted products from three of the six *tra* genes are related to proteins required for transfer of other conjugal and mobilizable plasmids (14).

The second region, *trb*, maps between the nopaline catabolism locus, *noc*, and the plasmid replication region, *oriV/rep*, and is separated from *tra* by about 100 kb (Fig. 1). The locus encodes 12 open reading frames, the products of 11 of which share significant relatedness to the *trb* proteins of RP4 (33, 34). *trb* of RP4 is thought to encode proteins that form the membrane-associated macromolecular complex involved in mating-pair formation and in facilitating passage of the DNA molecule from donors to recipients (33, 34, 40). The 12th gene, *traI*, codes for the production of the *A. tumefaciens* autoinducer *N*-(3-oxo-octanoyl)-L-homoserine lactone (30).

The third region, originally called *TraI*, maps between *acc* and the *traAFB* operon. This region encodes *traR*, the product of which is required for activation of transcription of the *tra* and *trb* operons (41). *traR* is the only gene in the *TraI* region required for conjugation; the four remaining open reading frames at this locus appear to be associated with sensing and

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FIG. 1. Physical and genetic organization of pTiC58 and pTi15955. The maps are based on those from references 10 and 23. Regions of the Ti plasmids that were used for construction of the binary system are shown outside of the circular maps. Two of the four regions of pTiC58 and pTi15955 that are homologous based on heteroduplex analysis are shown as the shaded arcs labeled B and C. Loci on pTiC58 and pTi15955 include the following: *noc*, nopaline catabolism; *traI/trb*, autoinducer synthesis and mating bridge functions; *oriV/rep*, replication region; *oriT/tra*, origin of transfer and the *trans*-acting *tra* functions; *traR/traM*, transcriptional activator gene required for expression of *tra* genes and an anti-activator gene (27); *accR/acc*, master opine-conjugal transfer repressor (2) and agrocinopine catabolism; *vir*, virulence locus; *T_L*, T-region left; *T_R*, T-region right; *occ*, octopine catabolism; and *moc*, mannitol opine catabolism.

catabolism of some unknown carbon substrate (42). TraR is a member of the LuxR family of transcriptional activators and requires *Agrobacterium* autoinducer, also called AAI, as a coinducer (17, 41, 55). This factor is produced by the donor bacteria themselves and serves as a quorum-sensing signal (17, 30, 41). In turn, production of AAI requires *traI*, which is the first gene of the *trb* operon (17, 30).

Although all of the transfer-deficient mutants of pTiC58 isolated to date map to the *tra*, *trb*, and *traR* loci, it is possible

that other essential functions are encoded elsewhere on the Ti plasmid. In addition, our analysis of *lacZ* fusions in the regions surrounding *tra* suggests that the two divergent operons each encode additional downstream genes (14). Strains with mutations in these regions still conjugate at high frequencies, suggesting that the genes disrupted by these insertions are not involved in transfer. However, it is conceivable that other genes on the Ti plasmid suppress the phenotypes of these mutants. In this regard, two groups have reported that mutations in genes located in the *vir* region, which mediates T-DNA transfer to plants, influence conjugal transfer of the Ti plasmids (18, 48). It is possible that functions encoded by *vir* complement the mutations in these *tra*-linked regions. The fact that the *traAFB* operon of pTiC58 contains a span of DNA that is 80% identical to the *virF* region of pTi15955 suggests that this possibility cannot be excluded (14, 39).

Thus, it is important to determine whether all of the genetic information necessary for conjugation is contained within the *tra* and *trb* operons. To address this issue, we constructed a binary conjugal transfer system by cloning the known transfer regions of pTiC58 into two independent broad-host-range replicons. A similar strategy was used to define the essential transfer regions of the IncP1 α plasmid RP4 (33). In such a binary system, the plasmid that contains the *oriT* site is mobilized from the donor strain when provided with the necessary *trans*-acting functions encoded by the other genes essential for conjugation located on the second plasmid. Using this binary system we show that *traAF*, *traCDG*, *traR*, *traI*, and the *trb* operon provide all of the *trans*-acting functions required for high-frequency conjugal transfer of a vector containing the Ti plasmid *oriT*. *traB*, while necessary for maximal transfer frequencies, is not essential for conjugation. In addition, since the *oriT* region is contained on a broad-host-range replicon, we show by heterologous matings that the conjugal host range of the Ti plasmid extends to members of the families *Enterobacteriaceae* and *Pseudomonadaceae*. Finally, we demonstrate that the components of the conjugal transfer systems of the nopaline-type Ti plasmids and of the octopine-type Ti plasmids are functionally interchangeable.

MATERIALS AND METHODS

Bacterial strains and plasmids. Strains of *A. tumefaciens*, *Rhizobium meliloti*, *Escherichia coli*, and *Pseudomonas fluorescens* as well as the plasmids used in this study are listed in Table 1.

Media and growth conditions. Luria broth (Gibco-BRL) was used as the rich liquid medium. *A. tumefaciens* strains were grown on nutrient agar (NA; Difco) and in AB minimal medium (7) with mannitol (0.4%; ABM) as the carbon source. *R. meliloti* was grown in complete GTS medium (32). *P. fluorescens* was grown on L agar plates or in PM minimal medium (11) with glucose (0.4%) as the carbon source. Antibiotics were added to media at the following concentrations: for *A. tumefaciens*, rifampin (RIF) at 50 or 100 μ g/ml, streptomycin at 200 μ g/ml, kanamycin (KM) at 50 or 100 μ g/ml, carbenicillin at 100 μ g/ml, and tetracycline (TC) at 2.0 μ g/ml; for *R. meliloti*, neomycin at 100 μ g/ml, carbenicillin at 100 μ g/ml, and TC at 20 μ g/ml; for *E. coli*, RIF at 50 μ g/ml, streptomycin at 100 μ g/ml, KM at 50 μ g/ml, ampicillin at 100 μ g/ml, and TC at 20 μ g/ml; and for *P. fluorescens*, RIF at 100 μ g/ml, KM at 50 μ g/ml, and TC at 25 μ g/ml. Cultures of *A. tumefaciens* and *P. fluorescens* were grown at 28°C, while those of *E. coli* were grown at 28 or 37°C. All liquid cultures were grown with shaking to ensure adequate aeration.

Plasmid DNA isolations and recombinant DNA techniques. Plasmid DNA was isolated from *A. tumefaciens* and *E. coli* by an alkaline lysis procedure as previously described (21). Standard recombinant DNA techniques were used as described by Sambrook et al. (45). Plasmids were introduced into *E. coli* strains by transformation using CaCl_2 or by electroporation and into *A. tumefaciens*, *R. meliloti*, and *P. fluorescens* strains by electroporation or by biparental cross streak matings using *E. coli* S17-1 harboring the plasmid of interest as the conjugal donor (14, 46).

Construction of *tra* plasmids pFRtra and pDCtra-5. To construct pFRtra, a 2.7-kb *PstI-KpnI* fragment which contains the 3' end of the *traC* gene along with all of *traD* and *traG* was excised from pRK-KP2.8 (Table 1) and cloned into the multiple cloning site of pRKE33, resulting in plasmid pDCCKPE33 (Table 1). The

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype, phenotype, or characteristic ^a	Source or reference
Strains		
<i>E. coli</i>		
DH5 α	<i>supE44</i> Δ <i>lacU169</i> (ϕ 80 <i>lacZ</i> Δ M15) <i>hsdR17</i> <i>recA1</i> <i>endA1</i> <i>gyrA96</i> <i>thi-1</i> <i>relA1</i>	45
S17-1	Pro ⁻ Res ⁻ Mod ⁺ <i>recA</i> ; integrated RP4-Tet::Mu-Kan::Tn7, Mob ⁺	46
HB101Rif	<i>recA13</i> <i>leuB6</i> <i>proA2</i> Rif ^r	Our collection
<i>R. meliloti</i> AK631	Nod ⁺ Fix ⁺ 5-Fu ^r	32
<i>A. tumefaciens</i>		
NT1	C58 cured of pTiC58	50
C58C1RS	C58 cured of pTiC58; Rif ^r Str ^r	49
<i>P. fluorescens</i>		
1855.344	Wild-type isolate of <i>P. fluorescens</i>	11
1855.344RS	Rif ^r Str ^r	Our collection
Plasmids		
pRKE33	<i>Eco</i> RI fragment 33 from pTiC58 encoding the <i>traR</i> gene cloned into pRK415; Tc ^r	Our collection
pDCKPE33	2.7-kb <i>Kpn</i> I- <i>Pst</i> I fragment from the <i>tra</i> region of pTiC58 cloned into pRKE33; Tc ^r	This study
pDCII1	pTHB58 containing Tn3HoHo1 insertion III; Tc ^r Cb ^r	14
pFRtra	<i>tra</i> region from pTiC58 cloned into pDCKPE33; Tc ^r Cb ^r	This study
pMP5	<i>Hind</i> III fragment 3 containing Tn3HoHo1 insertion 5 cloned in pCP13/B; Tc ^r Cb ^r	27
pDCHSE33	3.8-kb <i>Hind</i> III- <i>Sst</i> I fragment from the <i>tra</i> region of pTiC58 cloned into pRKE33; Tc ^r	This study
pDCtra-5	<i>tra</i> region from pTiC58 cloned into pDCHSE33; Tc ^r	This study
pPLE2	<i>Eco</i> RI fragment 2 from pTiC58 cloned into pDSK519; Km ^r	36
pJS4159K1	<i>Kpn</i> I fragment 1 from pTi15955 cloned into pJS400; Km ^r MOA ⁺	15
pDCI41E33	<i>traG::lacZ traR</i> in pDSK519; Km ^r autoinducer reporter plasmid	8

^a Abbreviations: Rif^r, RIF resistance; Str^r, streptomycin resistance; Tc^r, TC resistance; Cb^r, carbenicillin resistance; Km^r, KM resistance; 5-Fu^r, 5-fluorouracil resistance; MOA⁺, mannopinic acid utilization; Mob⁺, mobilizing.

remaining sequences of the *tra* locus were excised from plasmid pDCII1 as an 11-kb *Kpn*I fragment. pDCII1 is the cosmid clone pTHB58 containing Tn3HoHo1 insertion III located at the 3' end of *traB* (Table 1; Fig. 2) (14). The *Kpn*I fragment which carries 240 bp of *traC*, the interoperonic *oriT* region, *traA*, *traF*, approximately 900 bp of the 1,266-bp *traB* open reading frame, and approximately 6 kb of DNA from Tn3HoHo1 insertion III including the *bla* gene was ligated into pDCKPE33. These cloning steps resulted in the reconstitution of the *tra* region of pTiC58 including the entire *traCDG* operon, the intergenic region, *traA*, *traF*, and all but the last 328 nucleotides of *traB* (Fig. 2; Table 1).

The second *tra* plasmid, pDCtra-5, was constructed in a manner similar to that of pFRtra. However, most of the *traB* open reading frame was eliminated, and the clone contains an additional 3.4 kb beyond the *Pst*I site that delimits the left end of the pFRtra clone (Fig. 2). A 3.8-kb *Hind*III-*Sst*I fragment was cloned from plasmid pMP5 into the multiple cloning site of pRKE33 to produce pDCHSE33 (Table 1). This fragment contains approximately 1.6 kb of *tra* region DNA including the 591-bp 3' portion of *traA*, all of *traF*, approximately 100 bp of *traB*, and approximately 2.6 kb of Tn3HoHo1 DNA from insertion 5 (Fig. 2). The remaining elements needed to reconstitute the entire *tra* region were added by cloning *Hind*III fragment 4 from pTiC58 in the proper orientation into pDCHSE33 to produce pDCtra-5 (Fig. 2; Table 1). This construct lacks the *bla* gene of Tn3HoHo1.

Matings. Matings to assess conjugal transfer were performed on nitrocellulose filters essentially as described by Cook and Farrand (9). Donors and recipients, at an input ratio of approximately 1:1, were cocultured on the filters at 28°C for 2 or 24 h, depending upon the experiment. At the time that the filters were prepared, dilutions of the donor culture also were prepared and plated onto NA, L agar, or complete GTS agar medium to determine input donor titers. Filters containing only the donor and only the recipient were prepared and incubated in parallel as controls. Following recovery from the filters in a solution of 0.9% NaCl, the cell suspensions were diluted, and volumes of each dilution were plated onto media selective for the transconjugants. Conjugation frequencies are expressed as transconjugants recovered per input donor. In some cases transfer was assayed by the spot plate mating technique directly on selective media (3).

Assay for production of autoinducer. An overnight culture of the *A. tumefaciens* indicator strain, NT1(pDCI41E33), was used to inoculate 5 ml of ABM containing melted 0.7% agar and 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal; 40 μ g/ml) to a density of about 10⁷ CFU/ml. The soft agar suspension was overlaid onto a petri dish containing 20 ml of ABM solidified with 1.5% agar. After the overlay had gelled, colonies to be tested for production of an active acyl-homoserine lactone autoinducer were patched onto the surface,

and the plate was incubated at 28°C overnight. The indicator strain contains a copy of *traR* and a *lacZ* fusion to *traG* carried on the IncQ cloning vector pDSK519 (31). Because it lacks *traI*, the strain does not produce an active autoinducer. Consequently, the strain does not express the reporter fusion unless provided with an exogenous source of active autoinducer. In the assay, production of an active autoinducer by the strain being tested results in a diffuse blue zone from the lawn of the indicator bacteria surrounding the colony.

RESULTS AND DISCUSSION

Construction of *tra* region plasmids pFRtra and pDCtra-5.

Genetic analysis has indicated that two regions of pTiC58, now called *tra* and *trb*, encode functions essential for conjugation (Fig. 1) (3). Recent sequence analysis confirms that these two regions encode genes involved in conjugal transfer (14, 36). Our goal was to determine if these two regions comprise the minimum amount of genetic information from pTiC58 that is required for conjugal transfer. We used a strategy by which we divided these two regions among two compatible cloning vectors, thereby constructing a binary system. That component containing the *oriT* region should be transmissible from a strain harboring both elements. We chose pRK415 (IncP1 α) and pDSK519 (IncQ) as vectors for the binary constructs because the two are compatible with each other but neither is mobilized by the conjugal transfer system of pTiC58 (9).

Because the *oriT* region of the Ti plasmid is flanked by two divergent sets of genes encoding the *tra* functions (14), we opted to clone this as a single unit on one component of this system. pRKE33, which is pRK415 containing *Eco*RI fragment 33 from pTiC58 (Table 1), was used as the vector for the *tra* clones. The *Eco*RI fragment encodes the transcriptional activator, TraR, which is necessary for expression of the *tra* and *trb* operons (14, 30).

FIG. 2. Organization of the *oriT/tra* region of pTiC58 and of the binary *tra* plasmids pFRtra and pDCtra-5. A restriction map of the *tra* region of pTiC58 (D), as well as the organization of the two *tra* operons (B), is shown. The filled box (A) denotes the major part of homology region C that is common between the nopaline- and the octopine-type Ti plasmids (also see Fig. 1). The vertical lines mark the positions of Tn3HoH1 insertions mapping to the region as described in reference 14. The horizontal crossbars indicate the direction of transcription reported by the *lacZ* gene of the transposon. The phenotypes with respect to conjugal transfer (Tra) and expression of β -galactosidase (β -Gal) are presented for each insertion mutant (C). (E) The organization of the two *tra* plasmids. The extent of the *tra* region contained within each clone is aligned with the Ti plasmid locus shown in the restriction map. That portion of each clone corresponding to Tn3HoH1 sequences is illustrated as a thick solid line labeled with a number corresponding to the insertion mutant from which it was derived. The dashed thick line represents transposon DNA from the restriction site within the transposon used for cloning that is not part of the *tra* clone. *EcoRI* fragment 33 from pTiC58 (dark shaded box) carries *traR* (41).

Two recombinant clones containing the *tra* region were constructed as described in Materials and Methods (Fig. 2). Plasmid pFRtra contains about 72% of *traB*, while pDCtra-5 contains only about 100 bp of the 5' end of this gene (Fig. 2). At the other end, pDCtra-5 contains about 3.5 kb of DNA immediately to the left and downstream of the *traCDG* operon. Plasmid pFRtra lacks this DNA (Fig. 2). Insertion mutations in this region of the full-size Ti plasmid do not affect conjugal transfer. However, our analysis of *lacZ* fusions in this region suggested that it contains genes that are cotranscribed with the *tra* operon (14).

The second component of the binary system is pPLE2. This plasmid contains a 14-kb insert of DNA from pTiC58 cloned into the IncQ vector, pDSK519. The properties of pPLE2, as well as the complete nucleotide sequence of the Ti plasmid insert, will be presented in a future publication (36). Briefly, the insert in this plasmid contains the entire *trb* region, *traI* (30), *nocR*, and most of the *repA* gene of the Ti plasmid replication system (36). These determinants account for the entire coding capacity of the insert. The *traI* gene, which is the first gene of the *trb* operon, is required for production of the *Agrobacterium* autoinducer *N*-(3-oxo-octanoyl)-L-homoserine lactone (30). This signal molecule is required by TraR as a coinducer of the *tra* and *trb* operons (17, 30, 42).

Mobilization of pFRtra and pDCtra-5 to recipient bacteria. Each of the *tra* plasmids, pFRtra and pDCtra-5, was introduced into *A. tumefaciens* NT1 and NT1(pPLE2) (Table 1). In matings with *A. tumefaciens* C58C1RS, both *tra* plasmids were

mobilized at high frequencies (Table 2). Mobilization of the *tra* binary clones depended on the presence of pPLE2; donors lacking this plasmid showed no detectable mobilization activity (Table 2). Moreover, donors harboring only pPLE2 also failed to yield transconjugants. Both of these controls are consistent with the fact that the *tra* system of pTiC58 does not mobilize IncP1 α or IncQ plasmids (9). Plasmid DNA was isolated from a subset of transconjugants and subjected to restriction endonuclease analysis. In all cases the transconjugants harbored only the *oriT-tra* plasmid (results not shown). Moreover, we never observed any rearrangements in these plasmids.

These results confirm our previous genetic and sequence analyses which showed that, at a minimum, the essential *tra* locus spans about 6.6 kb and includes *traA*, *traF*, *traC*, *traD*, and *traG* (14). *traB*, while required for maximum transfer frequency, is not essential for the process. pFRtra, which contains most of *traB*, was mobilized at a frequency similar to the transfer frequency of the intact Ti plasmid (Table 2). However, pDCtra-5, which lacks most of the gene, was mobilized to an *Agrobacterium* recipient at a frequency 7.5-fold lower than that of pFRtra (Table 2). These effects of 3' deletions in *traB* on transfer frequencies are consistent with our observation that transposon insertions near the 5' end of the gene diminish transfer of the Ti plasmid to a greater extent than do those near the 3' end of the gene (14). That pFRtra is mobilized at a high frequency also indicates that the genes located downstream from and cotranscribed with the *traCDG* operon are not required for conjugation. This is consistent with our ob-

servations that insertion mutations in this region on the intact Ti plasmid have no detectable effect on conjugal transfer (14). Moreover, since the binary donors lack a Ti plasmid, the results argue against a role for *vir* functions suppressing these mutations on the full-size Ti plasmid.

These results show that *tra* and *trb* encode all of the components of the Ti plasmid required for interbacterial conjugation. This is interesting in light of two studies indicating that mutations in the *vir* system, which mediates T-DNA transfer from *A. tumefaciens* to plants (54), affect conjugal transfer of the Ti plasmid (18, 48). If functions encoded by *vir* genes are involved in conjugal transfer of the Ti plasmid, then eliminating the entire *vir* regulon should affect conjugation. That both *tra* plasmids are mobilized at high frequencies is not consistent with a role for any of the *vir* proteins in *tra*-mediated conjugal transfer. However, it is clear that certain transposon insertion mutations in *vir* genes can drastically reduce conjugal transfer frequencies, even when the regulon is not induced (18, 38, 48). Based on these results, others have proposed that the conjugal transfer system is dependent upon certain Vir proteins for maximum activity (18, 48). As an alternative, we suggest that the mutant Vir proteins gratuitously interfere with the conjugal transfer system. It also is possible that certain transposon insertions in upstream genes in a given *vir* operon promote expression of the downstream genes of that operon and that such expression interferes with the *tra* system. Both possibilities are consistent with the observations that the effects of these mutations in *vir* on conjugation are allele specific; some but not all insertion mutations in a given *vir* gene affect *tra*-mediated transfer (18, 48). In either case, we would predict that these insertions result in the expression of Vir proteins that interfere with proper assembly or functioning of the conjugation apparatus encoded by *tra*.

The *trb* region from pTiC58 is contained within approximately 10.5 kb of the 14-kb insert present in pPLE2 (36). Together with the 6.6-kb *tra* region and the 0.7-kb *traR* gene, the total amount of DNA from pTiC58 necessary for conjugal transfer of the plasmid is approximately 18 kb. The regions coding for conjugal transfer of pTiC58 and of other plasmids, such as RP4 (IncP1 α , 18 kb) (40), pKM101 (IncN, 15 kb) (43, 51), and R388 (IncW, 15 kb) (6, 35), are approximately the same size. In contrast, IncFI plasmids such as F and the Ti plasmid *vir* system commit substantially more DNA to conjugative processes. The coding regions for conjugal transfer of F span about 33 kb, representing approximately 30% of the plasmid (16). The *vir* regulons comprising the T-DNA transfer systems of Ti plasmids span approximately 44 and 29 kb of pTi15955 and pTiC58, respectively (24). However, several of the *vir* genes are not essential for transfer of T-DNA to plants. Certain genes, such as *virF*, increase host range (39), while others, such as *virB1* (4), *virE2* (53), *virD3* (37), and those of the *virC* operon (26), are not essential but are required for maximum tumorigenicity. Based on nucleotide sequence data for the *vir* regions of nopaline- and octopine-type Ti plasmids, we estimate that the essential components of the T-DNA transfer system are encoded within approximately 20 kb of DNA from the Ti plasmid.

Host range of the binary system. Ti plasmids have a narrow replicative host range, being stably maintained only within species of *Agrobacterium* and *Rhizobium* (25). However, using a phage rescue strategy, Sprinzl and Geider (47) showed conjugal transmission of pTiC58 to *E. coli*. We examined the host range of the Ti plasmid conjugal transfer apparatus using our broad-host-range-based binary system. Both pFRtra and pDCtra-5 were mobilized at high frequencies from the binary *A. tumefaciens* donor to an *E. coli* recipient (Table 2). Addi-

TABLE 2. *trans* mobilization of pFRtra and pDCtra-5 from binary strains to *A. tumefaciens*, *E. coli*, and *P. fluorescens* recipients

Donor	Frequency of mobilization to ^a :		
	C58C1RS	HB101Rif	1855.344RS
NT1(pTiC58 Δ accR)	1.8×10^{-3}	NT	NT
NT1(pPLE2)	$<1 \times 10^{-7}$	$<1 \times 10^{-7}$	$<1 \times 10^{-7}$
NT1(pFRtra)	$<1 \times 10^{-7}$	$<1 \times 10^{-7}$	$<1 \times 10^{-7}$
NT1(pFRtra, pPLE2)	2.4×10^{-3}	1.0×10^{-2}	5.1×10^{-3}
NT1(pFRtra, pJS4159K1)	7.0×10^{-3}	2.2×10^{-2}	2.7×10^{-2}
NT1(pDCtra-5)	$<1 \times 10^{-8}$	$<1 \times 10^{-8}$	NT
NT1(pDCtra-5, pPLE2)	3.2×10^{-4}	1.9×10^{-3}	NT

^a The number of transconjugants recovered per input donor cell. Values represent the averages from at least two independent experiments. In the mating between NT1(pTiC58 Δ accR) and C58C1RS, transconjugants were selected on AB minimal medium containing arginine and nopaline as the sole carbon sources (2). In all other matings, with C58C1RS and 1855.344RS as recipients, transconjugants were selected for on NA containing RIF, streptomycin, and carbenicillin [NT1(pFRtra) donor] or RIF, streptomycin, and TC [NT1(pDCtra-5) donor]; with HB101Rif as the recipient, transconjugants were selected for on L agar containing RIF and ampicillin [NT1(pFRtra) donor] or ampicillin and TC [NT1(pDCtra-5) donor]. NT, not tested; C58C1RS, *A. tumefaciens* recipient; HB101Rif, *E. coli* recipient; 1855.344RS, *P. fluorescens* recipient (Table 1).

tionally, pFRtra was mobilized to *P. fluorescens* at frequencies similar to its transfer to an *Agrobacterium* recipient (Table 2). These findings extend the host range of the conjugal transfer system of the Ti plasmid to a member of the genus *Pseudomonas*. The results also indicate that the core *tra* and *trb* regions are sufficient for high-frequency conjugal transfer to recipients other than members of the family *Rhizobiaceae*. Whatever role *traB* plays in conjugation, it is not required for high-frequency transfer to either of these heterologous recipients. Nor are the genes downstream from the *traCDG* operon required for the extended conjugal host range of the system; pFRtra, which lacks this region (Fig. 2), is transmissible to all three recipients at high frequency (Table 2). Our results are consistent with the current notion that bacterial conjugation systems are indiscriminant with respect to recipients. At the extreme, *tra* systems are capable of mediating transkingdom gene transfer. In addition to the *vir*-mediated transfer of DNA from *A. tumefaciens* to plants, several plasmids, including F, can mediate gene transfer from a bacterial donor to fungal recipients (19, 22).

Taken as a whole, these studies indicate that in nature Ti plasmid transfer might occur between *A. tumefaciens* cells and a wide range of bacterial recipients. Although the Ti plasmid cannot replicate within hosts outside of the family *Rhizobiaceae*, it does remain within a nonpermissive host for a period of time sufficient to detect transfer by expression of markers carried in by the Ti plasmid (47). Moreover, transposons can be delivered to *P. fluorescens* by Ti plasmid-mediated conjugation (28). Transient existence of the Ti plasmid within a nonpermissive host may be important in gene flux among bacterial populations. This is an especially interesting possibility considering that the *iaaH* and *iaaM* genes of *Pseudomonas syringae* pv. savastanoi, which code for the synthesis of indol-3-acetic acid by this bacterium, are related at the nucleotide sequence level to those encoded on the T region of octopine- and nopaline-type Ti plasmids (52). That the Ti plasmid may be transmissible to *P. syringae* pv. savastanoi by conjugation provides a mechanism by which this plant-pathogenic pseudomonad could have acquired these genes from *Agrobacterium*.

Expression of *tra* and *trb* in other hosts. Most Ti plasmid genes do not confer the expected phenotypes when present in *E. coli*. In certain cases, it appears that these genes are not transcriptionally expressed in the enteric host. In other cases,

TABLE 3. Expression of the Ti plasmid conjugal transfer system in other gram-negative bacteria

Donor	Transfer of pFRtra to ^a :			Production of autoinducer (mm) ^b
	C58CIRS	HB101Rif	1855.344RS	
<i>A. tumefaciens</i> NT1(pFRtra, pPLE2)	5.6×10^{-3}	5.1×10^{-3}	5.0×10^{-3}	42
<i>R. meliloti</i> AK631(pFRtra, pPLE2)	2.6×10^{-3}	5.4×10^{-5}	2.0×10^{-3}	39
<i>P. fluorescens</i> 1855.344(pFRtra, pPLE2)	$<1 \times 10^{-8}$	$<1 \times 10^{-8}$	$<1 \times 10^{-8}$	10
<i>E. coli</i> HB101(pFRtra, pPLE2)	$<1 \times 10^{-8}$	$<1 \times 10^{-8}$	$<1 \times 10^{-8}$	10

^a Transconjugants recovered per input donor.

^b Diameter of the blue zone surrounding the test colony. The color results from the β -galactosidase activity expressed from the *traR*-autoinducer-dependent *traG::lacZ* fusion in the reporter strain NT1(pDCI41E33) seeded in the overlay (see Materials and Methods). Expression of this fusion is dependent upon an exogenous source of the acyl-homoserine lactone signal. Each value includes the diameter of the test colony (about 8 mm).

the genes may be transcribed but traits still are not expressed (11). However, the genes encoding mannopine utilization from pTi15955 confer on *P. fluorescens* the ability to use this opine as the sole carbon source (11, 29). We examined the ability of the Ti plasmid conjugation system to function in alternative hosts by constructing derivatives of *R. meliloti* AK631, *E. coli* HB101, and *P. fluorescens* 1855.344 each harboring pFRtra and pPLE2. The *R. meliloti* donor harboring the complete binary system transferred pFRtra when mated with *A. tumefaciens*, *E. coli*, and *P. fluorescens* recipients (Table 3). Frequencies of transfer were somewhat lower than those observed with the *A. tumefaciens* donor. As with the *A. tumefaciens* donors, transfer from *R. meliloti* was dependent upon the presence of both members of the binary complex (data not shown). The *R. meliloti* donor also produced a large amount of autoinducer (Table 3). In comparison, neither the *E. coli* nor the *P. fluorescens* strain acted as a conjugal donor (Table 3). Furthermore, although each of these strains produced a detectable amount of AAI, neither produced nearly as much of the *N*-acyl homoserine lactone signal as did NT1(pFRtra, pPLE2) or AK631(pFRtra, pPLE2) (Table 3). Since *traR* is present in all of these strains, we suggest that the activator is unable to initiate transcription of the *tra* and *trb* operons in the enteric and pseudomonad hosts. This is consistent with our observations that transcriptional and translational fusions of *lacZ* to several *tra* genes do not express detectable levels of β -galactosidase activity in *E. coli* hosts, even when *traR* and AAI are provided in excess (14).

***trb* regions from nopaline- and octopine-type Ti plasmids are interchangeable.** The nopaline- and octopine-type Ti plasmids share four regions of significant DNA sequence homology as measured by electron microscopic heteroduplex analysis (12). Two of these regions, B and C (Fig. 1), include the *trb* and *tra* regions of pTiC58, respectively (14, 36). We replaced pPLE2, which contains *trb* of pTiC58, with pJS4159K1 (15), which contains the corresponding region from the octopine-type Ti plasmid, pTi15955 (Fig. 1; Table 1) (10, 12, 23). NT1(pFRtra, pJS4159K1) mobilized pFRtra to an *A. tumefaciens* recipient at a frequency that was similar to that for mobilization from strain NT1(pFRtra, pPLE2) (Table 2).

This finding is consistent with our observation that a transfer-constitutive derivative of pTi15955 mobilizes recombinant clones containing the minimum *cis*-acting *oriT* sequence of pTiC58 at frequencies similar to those observed for mobilization by pTiC58 (9). Thus, the mating bridges presumably encoded by the *trb* regions of the two Ti plasmids, and the components encoded by *tra* that act at the *oriT* to produce the DNA transfer intermediate, are functionally equivalent and interchangeable. Recent analyses have shown that the *tra* and *trb* regions of pTiC58 and pTiR10, an octopine-type Ti plasmid essentially identical to pTi15955, are greater than 80% identi-

cal at the nucleotide sequence level (1, 14, 36). Clearly, the transfer systems of these two Ti plasmid types share the same aboriginal source.

Our results demonstrate that the *tra* system functions independently from *vir*. The converse also is true. Rogowsky et al. (44), also using a binary system, showed that a clone containing only the *vir* regulon of pTiC58 transferred to host plants DNA flanked by T-region borders contained on a second plasmid. Thus, the *vir* transfer system is completely independent of *tra*. This supports our model that the two conjugal transfer systems derived from different ancestors and that Ti plasmids such as pTiC58 and pTi15955 may have arisen by cointegration between two plasmids, each encoding its own conjugal transfer system (1, 14).

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