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Characterization of Conjugal Transfer Functions of *Agrobacterium tumefaciens* Ti Plasmid pTiC58

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Physical characterization of 13 transposon Tn*5* insertions within the agrocinopine-independent, transfer-constitutive Ti plasmid pTiC58Tra^c identified three separate loci essential for conjugation of this nopaline/agrocinopine A + B-type Ti plasmid. Complementation analysis with relevant subcloned DNAs indicated that the three physically separated blocks of conjugal genes constitute distinct complementation groups. Two independent Tn*5* insertions within the wild-type, agrocinopine-dependent, repressed pTiC58 plasmid resulted in constitutive expression of conjugal transfer. These two insertions were physically indistinguishable and could not be complemented in *trans*. However, the Tra^c phenotype resulted when the Tn*5*-mutated fragment cointegrated into the wild-type Ti plasmid. While the spontaneous Tra^c mutant Ti plasmids were also derepressed for agrocinopine catabolism, those generated by Tn*5* insertions remained inducible, indicating that this apparent *cis*-acting site is different from that affected in the spontaneous mutants. No chromosomal Tn*5* insertion mutations were obtained that affected conjugal transfer. An octopine-type Ti plasmid, resident in different *Agrobacterium tumefaciens chvB* mutants, transferred at normal frequencies, demonstrating that this virulence locus affecting plant cell binding is not required for Ti plasmid conjugation. None of our conjugal mutants limited tumor development on *Kalanchoe diagremontiana*. Known lesions in pTiC58 *vir* loci had no effect on conjugal transfer of this Ti plasmid. These results show that pTiC58 Ti plasmid conjugal transfer occurs by functions independent of those required for transfer of DNA to plant cells.

Ti plasmids (58) enable *Agrobacterium tumefaciens* to induce the crown gall disease on a wide range of plants (reviewed in references 42, 47, 53, and 55). Characteristic neoplasias ensue upon expression of a set of Ti plasmid-specific transfer DNA (T-DNA) genes that are transferred from the bacterium and integrated into the plant host nuclear genome (reviewed in references 4, 9, 41, 54, and 59). Expression of additional T-DNA-encoded functions dictates the plant-specific synthesis and secretion of novel sugar and amino acid conjugates, collectively termed opines (43). These opines can serve as unique growth substrates for tumor-associated agrobacteria, with specific opine catabolic functions being encoded by the Ti plasmid (7, 14, 38). One can envision that this potential nutrient source provides a competitive advantage for plant-associated agrobacteria. Consequently, these additional Ti plasmid-encoded functions may have a significant bearing on overall *Agrobacterium* ecology. A population of *Agrobacterium* cells can take full advantage of this ability to catabolize opines if efficient Ti plasmid conjugal transfer ensures that the majority of colonizing agrobacteria contain a copy of the appropriate Ti plasmid. Indeed, the normally repressed Ti plasmid conjugative mechanisms are induced by specific, so-called conjugal, opines (18, 21, 26, 33–35, 44).

Tumors resulting from plant transformation by pTiC58-specific T-DNA synthesize the opines nopaline (25), nopalinic acid (19), and agrocinopines A and B (20). Each of these opines can serve as a sole source of carbon for strains of *Agrobacterium* that harbor pTiC58 (42, 43; G. T. Hayman and S. K. Farrand, manuscript in preparation). In addition, agrocinopines A and B, but not nopaline, induce the expression of pTiC58 conjugal transfer genes (18, 21) and also of the agrocinopine catabolic functions (21, 27).

We wished to characterize the conjugative mechanism of the nopaline-type Ti plasmid pTiC58 (59). This characterization can identify features of the conjugal mechanism that may play a role in plant transformation (30, 52). More importantly, this characterization can aid our understanding of how plant-specific signals communicate nutritional opportunities to plant-associated agrobacteria and how this communication contributes to a successful microbe-plant interaction.

This report describes the identification of three distinct regions of pTiC58 that are essential for conjugal plasmid transfer. Analysis of transposon insertions into these regions yielded detailed maps of the respective transfer (*tra*) regions of pTiC58 and thus provides a framework for future characterizations. The data described here also demonstrate that conjugation and oncogenicity are functionally independent, pTiC58-specific phenotypes.

(A portion of this work was presented at the 1988 American Phytopathological Society annual meeting [S. Beck von Bodman and S. K. Farrand, Phytopathology **78**:1587, abstr. no. 599, 1988].)

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and plasmids used in this study are described in Table 1. Plasmids pJK107, pJK190, pJK195, pJK502, and pJK710 (46) were transformed into strain NT1 prior to conjugal analysis.

Media and growth conditions. *Escherichia coli* and *Agrobacterium* strains were grown in L broth (GIBCO Laboratories) or on nutrient agar (Difco Laboratories). *Agrobacterium* strains were also cultured in AB minimal medium (10), AT minimal medium containing 0.15% $(NH_4)_2SO_4$ (52), or Stonier medium (51). Solid medium contained 1.5% agar (Difco). Minimal medium was supplemented with either glucose or mannitol to 0.5% or 2 mg of L-arginine hydrochloride (Sigma Chemical Co.) per ml plus inducing levels (0.2 mg/ml) of nopaline (22) (Sigma) as sources of carbon. Selec-

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TABLE 1. Bacterial strains and plasmids used in this work[a]

Strain or plasmid	Relevant plasmid	Relevant phenotype or genotype	Source or reference(s)
E. coli			
DH1		*recA1 thi-1* λ^-	39
1830	pJB4JI	*met-63 pro-22* Km^r Nm^r	3
2013(pRK2013)	pRK2013	Tra^+ *mob*$^+$	15
MC1061	pBR322::Tn*5*	Km^r Ap^r	2
A. tumefaciens			
NT1		Agr^r	55
C58C1RS		Rm^r Sm^r	21
C58	pTiC58	Agr^s Tra^i Noc^+	59
LBA4011(pTiC58Tra^c)	pTiC58Tra^c	Agr^{ss} Tra^c Noc^+ Rm^r	11, 32
NT1(pTiC58Tra^c)	pTiC58Tra^c	Agr^{ss} Tra^c Noc^+	This study
NT1(*tra2-17*)	pTiC58Tra^c::Tn*52*-17	Agr^{ss} Tra^- Noc^+	This study
NT1(*tra12-8*)	pTiC58Tra^c::Tn*512*-8	Agr^{ss} Tra^- Noc^+	This study
NT1(*tra17-59*)	pTiC58Tra^c::Tn*517*-59	Agr^{ss} Tra^- Noc^+	This study
NT1(*tra4-82*)	pTiC58Tra^c::Tn*54*-82	Agr^{ss} Tra^- Noc^+	This study
NT1(*tra3-16*)	pTiC58Tra^c::Tn*53*-16	Agr^{ss} Tra^- Noc^+	This study
NT1(*tra16-24*)	pTiC58Tra^c::Tn*516*-24	Agr^{ss} Tra^- Noc^+	This study
NT1(*tra15-26*)	pTiC58Tra^c::Tn*515*-26	Agr^{ss} Tra^- Noc^+	This study
NT1(*tra15-62*)	pTiC58Tra^c::Tn*515*-62	Agr^{ss} Tra^- Noc^+	This study
NT1(*tra9-74*)	pTiC58Tra^c::Tn*59*-74	Agr^{ss} Tra^- Noc^+	This study
NT1(*tra12-33*)	pTiC58Tra^c::Tn*512*-33	Agr^{ss} Tra^- Noc^+	This study
NT1(*tra2-16*)	pTiC58Tra^c::Tn*52*-16	Agr^{ss} Tra^- Noc^+	This study
NT1(*tra6-52*)	pTiC58Tra^c::Tn*56*-52	Agr^{ss} Tra^- Noc^+	This study
NT1(*tra19-22*)	pTiC58Tra^c::Tn*519*-22	Agr^{ss} Tra^- Noc^+	This study
NT1(*trc10-23*)	pTiC58::Tn*510*-23	Agr^s Tra^c Noc^+	This study
NT1(*trc42-87*)	pTiC58::Tn*542*-87	Agr^s Tra^c Noc^+	This study
NT1(*noc13-57*)	pTiC58::Tn*513*-57	Agr^{ss} Tra^c Noc^-	This study
NT1(*noc17-52*)	pTiC58::Tn*517*-52	Agr^{ss} Tra^c Noc^-	This study
NT1(pJK107)	pJK107	*virA* Tra^c Noc^+	46
NT1(pJK190)	pJK190	*virB* Tra^c Noc^+	46
NT1(pJK195)	pJK195	*virD* Tra^c Noc^+	46
NT1(pJK502)	pJK502	*virB* Tra^c Noc^+	46
NT1(pJK710)	pJK710	*virG* Tra^c Noc^+	46
A1020	pTiB$_6$806	*chvB* Tra^+ Occ^+	17
A1038	pTiB$_6$806	*chvB* Tra^+ Occ^+	17
A1045	pTiB$_6$806	*chvB* Tra^+ Occ^+	17
B$_6$806	pTiB$_6$806	Tra^+ Occ^+	17
Plasmids			
pCP13/B		Tc^r	14
pVK101		Tc^r Km^r	36
pTHB55	pCP13::*Bam*HI-partial(pTiC58)	Agr^r Tc^r	27
pTHB58	pCP13::*Bam*HI-partial(pTiC58)	Agr^r Tc^r	27
pTHB14	pCP13::*Bam*HI-14(pTiC58)	Tc^r Km^r	This study
pTHH6	pVK102::*Hin*dIII-partial(pTiC58)	Tc^r	27
pSVB20	pCP13::*Bam*HI-partial(pTiC58Tra^c)	Tc^r	This study
pTSVE33	pVK101::*Eco*RI-33(pTiC58)	Tc^r Km^r	This study
pBR322		Tc^r Ap^r	6
pBR322/10-23	pBR322::*Eco*RI-partial(pTiC58Tra^c::Tn5 of NT1trc10-23)	Km^r Ap^r	This study

[a] Abreviations: Agr^r, agrocin 84 resistant; Agr^s, agrocin 84 sensitive; Agr^{ss}, agrocin 84 supersensitive; Tra^+, conjugally active; Tra^i, agrocinopine-dependent conjugation; Tra^-, conjugally inactive; Tra^c, constitutive conjugal transfer; Noc^+, nopaline catabolism; Noc^-, deficient nopaline catabolism; Occ^+, octopine catabolism; Km^r, kanamycin resistance; Tc^r, tetracycline resistance; Ap^r, ampicillin resistance; Rm^r, rifampin resistance.

tive medium for *E. coli* contained either tetracycline (10 μg/ml), kanamycin (50 μg/ml), or ampicillin (100 μg/ml). Selective medium for *A. tumefaciens* contained tetracycline (2 μg/ml) or kanamycin (150 μg/ml) or both; rifampin (50 μg/ml) and streptomycin (500 μg/ml); or carbenicillin (50 μg/ml). *E. coli* cells were incubated at 37°C, and *A. tumefaciens* cells were incubated at 28°C.

Isolation of Tn5 transpositions. The suicide plasmid pJB4JI (3) in *E. coli* 1830 was used to introduce transposon Tn5 insertions into the genomes of *A. tumefaciens* C58 and NT1(pTiC58Tra^c) by the protocol described by Garfinkel and Nester (24), except that bacterial suspensions were spread onto AB minimal mannitol plates containing kanamycin (150 μg/ml).

Conjugal transfer assays. Kanamycin-resistant prototrophic *A. tumefaciens* progeny, randomly chosen from multiple transposon mutagenesis experiments, were tested for conjugal ability by direct spot matings on agar surfaces. The mating strategy exploited the transfer of Ti plasmid-encoded nopaline and arginine catabolic functions to compensate for a naturally occurring arginine-catabolic deficiency of the recipient strain C58C1RS (21, 44). Kanamycin-resistant donor cells were grown at 28°C on AB minimal mannitol plates containing kanamycin (150 μg/ml). These

colonies, when no older than 3 days, were transferred with sterile toothpicks as small patches to mating plates which were prepared in the following manner. Recipient cells, grown to late exponential phase in AT minimal mannitol medium containing rifampin and streptomycin, were harvested and washed twice with supplement-free AT minimal salt solution. The final cell pellet was suspended in 1/10 the culture volume, and 200 μl of this cell suspension (ca. 10^9 CFU/ml) was spread confluently over the surfaces of AT minimal plates containing arginine hydrochloride (2 mg/ml), nopaline (0.2 mg/ml), 0.15% $(NH_4)_2SO_4$, and counterselection antibiotics, usually rifampin and streptomycin. The plates were then incubated overnight at 28°C before inoculation with the donor strains. Ti plasmid transfer yielded growth of colonies at the spot-mating sites within 2 or 3 days. Transfer frequencies were determined essentially by the same protocol except that donor strains were grown to the late exponential phase in liquid AT minimal mannitol in the presence of antibiotics when applicable. The cells were washed twice in AT and diluted in 10-fold increments. The number of donor cells was determined by spotting 10 μl of appropriate dilutions on NA plates. The number of transconjugants was established by spotting 10 μl of the culture dilutions directly onto recipient plates. Transfer frequencies are expressed as number of transconjugants per input donor cell. The frequencies reported are averages of assays performed in triplicate. Conjugal transfer assays of *chvB* mutants, harboring the octopine-type plasmid $pTiB_6806$, were performed as detailed above, except that the donors were grown in AT minimal medium containing 0.15% $(NH_4)_2SO_4$ and octopine (Sigma) (1 mg/ml) and the mating plates contained octopine (1 mg/ml) and appropriate antibiotics.

Plasmid isolation and characterization. Plasmid DNA was isolated by the alkaline lysis method of Maniatis et al. (39) or as described by Slota and Farrand (48). Restriction endonuclease digestions were performed with the reaction conditions recommended by the supplier. DNA fragments were separated by agarose gel electrophoresis on 0.8% agarose in Tris-borate-EDTA buffer (39). Restriction fragments were transferred to membrane filters by the method of Southern (49) and hybridized with ^{32}P-labeled pBR322::Tn*5* plasmid DNA under standard conditions (39). Probes were radiolabeled with [α-^{32}P]dCTP (ICN) by nick translation with a commercial kit (Bethesda Research Laboratories).

Bacterial transformations. *E. coli* DH1 was transformed by standard procedures (39). *A. tumefaciens* NT1 was transformed by the protocol of Holsters et al. (29).

Cloning and subcloning. pTiC58Tra^c DNA was purified by centrifugation to equilibrium in CsCl-ethidium bromide gradients. Partial *Bam*HI restriction endonuclease digests of this DNA were cloned into the cosmid vector pCP13/B (14) by standard methods (39). Ligated DNA was packaged in bacteriophage λ with reagents supplied in a kit by Promega Biotech. Recombinant cosmid clones were isolated by transduction into *E. coli* DH1 as described by Hohn (28). Subclonings into pBR322 and pVK101 vectors were performed by standard techniques (39).

Construction of merodiploid strains. Recombinant plasmids were mobilized into *Agrobacterium* strains by triparental matings as described by Ditta et al. (15). Transconjugants were selected on AB minimal glucose plates containing antibiotics appropriate for the selection of the plasmids used. Merodiploidy was confirmed by plasmid DNA analysis.

Agrocin 84 sensitivity assays. Sensitivity to agrocin 84 was determined as described by Hayman and Farrand (27). Induction of supersensitivity to agrocin 84 by agrocinopines A and B was assessed by laying filter paper strips impregnated with the opines onto the surfaces of agrocin 84 assay plates (27).

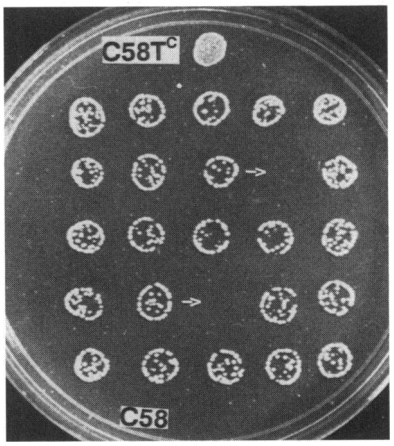

FIG. 1. Conjugal transfer assay. The plate shows a conjugal transfer assay and contains AT minimal nopaline-arginine medium plus rifampin and streptomycin. The recipient strain, C58C1RS, grown in liquid culture, was spread confluently over the agar surface and incubated at 28°C overnight prior to spot matings. Cells from randomly chosen kanamycin-resistant colonies derived from transposon mutagenesis matings were then transferred to this plate. Growth of transconjugants at spot-mating sites occurred after 3 days of incubation. Tn*5*-induced Tra$^-$ donors are identified by lack of transconjugant growth at spot-mating sites (arrows). Wild-type strain C58 produces no transconjugants because it requires agrocinopine A or B to induce conjugal transfer.

Virulence test. *Agrobacterium* strains were tested for tumorigenicity on *Kalanchoe diagremontiana* as described by Garfinkel and Nester (24).

RESULTS

Insertional mutagenesis to identify regions of pTiC58 required for conjugal transfer. We followed two transposon mutagenesis strategies to isolate mutants altered in conjugal transfer functions.

First, we used a spontaneous transfer-constitutive mutant, pTiC58Tra^c (Table 1), as a target plasmid to isolate Tn*5*-specific Tra$^-$ mutants. Three thousand kanamycin-resistant prototrophic *Agrobacterium* colonies from 10 separate crosses with *E. coli* 1830 were tested for Ti plasmid conjugal transfer. Transfer-deficient mutants were identified by their inability to generate arginine/nopaline-utilizing transconjugants when spot-mated with the recipient strain C58C1RS (Fig. 1). All apparent transfer-deficient mutants were tested for utilization of nopaline because mutants with insertions inactivating nopaline-catabolic (*noc*) genes would appear Tra$^-$ by this assay. This approach yielded 13 transfer-deficient and 3 nopaline-catabolism-deficient mutants (Tables 1 and 2).

Second, we isolated transfer-constitutive mutants of wild-type pTiC58 by screening Tn*5*-mutagenized, kanamycin-resistant progeny for donors that no longer required agrocinopines for induction of conjugal transfer. This second approach is identical to the first strategy with the exception that one looks for rare transfer-constitutive events among a pool of conjugally repressed strains. Two independent mutants out of 5,000 colonies tested were isolated that were able to transfer the Ti plasmid in the absence of agrocinopines (Tables 1 and 2).

TABLE 2. Conjugal transfer frequencies of *A. tumefaciens* strains harboring wild-type and mutant Ti plasmids[a]

Mutant locus	Strain	Plasmid	Transfer frequency (transconjugants/donor cell)
Wild type	C58	pTiC58	$<10^{-8}$
	NT1(pTiC58Tra^c)	pTiC58Tra^c	1×10^{-3}
	$B_6$806	pTi$B_6$806	$\sim 10^{-2}$
traI	NT1(*tra2-17*)	pTiC58Tra^c::Tn*52*-17	4×10^{-6}
	NT1(*tra12-8*)	pTiC58Tra^c::Tn512-8	$<10^{-8}$
	NT1(*tra17-59*)	pTiC58Tra^c::Tn517-59	$<10^{-8}$
	NT1(*tra4-82*)	pTiC58Tra^c::Tn54-82	$<10^{-8}$
	NT1(*trc10-23*)	pTiC58::Tn510-23	1×10^{-4}
	NT1(*trc42-87*)	pTiC58::Tn542-87	1×10^{-4}
traII	NT1(*tra3-16*)	pTiC58Tra^c::Tn53-16	$<10^{-8}$
	NT1(*tra16-24*)	pTiC58Tra^c::Tn516-24	$<10^{-8}$
	NT1(*tra15-26*)	pTiC58Tra^c::Tn515-26	1×10^{-6}
traIII	NT1(*tra15-62*)	pTiC58Tra^c::Tn515-62	$<10^{-8}$
	NT1(*tra9-74*)	pTiC58Tra^c::Tn59-74	$<10^{-8}$
	NT1(*tra12-33*)	pTiC58Tra^c::Tn512-33	$<10^{-8}$
	NT1(*tra2-16*)	pTiC58Tra^c::Tn52-16	$<10^{-8}$
	NT1(*tra6-52*)	pTiC58Tra^c::Tn56-52	$<10^{-8}$
	NT1(*tra19-22*)	pTiC58Tra^c::Tn519-22	$<10^{-8}$
vir	NT1(pJK107) (*virA*)	pJK107	6×10^{-3}
	NT1(pJK190) (*virB*)	pJK190	5×10^{-3}
	NT1(pJK195) (*virD*)	pJK195	2×10^{-4}
	NT1(pJK502) (*virB*)	pJK502	5×10^{-3}
	NT1(pJK710) (*virG*)	pJK710	4×10^{-3}
chvB	A1020	pTi$B_6$806	$\sim 10^{-3}$
	A1038	pTi$B_6$806	$\sim 10^{-3}$
	A1045	pTi$B_6$806	$\sim 10^{-3}$

[a] Abbreviations are as described in Table 1, footnote *a*.

Ti plasmids from each mutant were partially purified and transformed into strain NT1, selecting for growth on nopaline as the sole carbon source. In each case, the opine-utilizing transformants coinherited resistance to kanamycin, indicating that the Tn*5* transposon insertions are Ti plasmid specific. These transformants, when tested for conjugal transfer, display the Tra$^-$ or Tra^c phenotype of the original mutant from which the respective Ti plasmid DNA was isolated. These NT1 transformants were used in all subsequent experiments to ensure a common chromosomal background.

The frequencies of conjugal plasmid transfer were determined in triplicate assays for each mutant plasmid (Table 2). Eleven mutants failed to transfer their Ti plasmids at detectable frequencies. Two mutants, NT1(*tra2-17*) and NT1(*tra15-26*), exhibited detectable but greatly reduced transfer frequencies (1×10^{-6} to 4×10^{-6} transconjugants per donor cell) relative to the constitutive transfer frequency of the parent plasmid (1×10^{-3} transconjugants per donor cell). The two independent Tn*5*-induced constitutive mutants NT1(*trc10-23*) and NT1(*trc42-87*) transferred their Ti plasmids at frequencies of 1×10^{-4} per donor cell (Table 2).

Mapping the Tn*5* insertion sites. Ti plasmids purified from each of the Tn*5*-specific mutants listed in Table 2 were digested with five different restriction endonucleases to locate the sites of Tn*5* insertion (data not shown). Some restriction endonuclease digests were also probed with ^{32}P-labeled pBR322::Tn*5* plasmid DNA to confirm the altered banding patterns predicted from the restriction endonuclease analyses (data not shown). These analyses showed that the Tn*5* insertions are clustered in three regions of the plasmid (Fig. 2). Insertions between Ti plasmid coordinates 123 and 127 kilobases (kb) defined *tra* region I. This region included Tra$^-$ mutations *tra12-8* and *tra2-17*, which mapped in a previously unidentified small *Hin*dIII restriction fragment located between *Hin*dIII restriction fragments 3 and 31a of the map published by Depicker et al. (13). The insertions of Tra$^-$ mutants NT1(*tra17-59*) and NT1(*tra4-82*) mapped in the contiguous *Hin*dIII restriction fragment 3, as did the Tn*5*-specific insertions of the two Tra^c mutants NT1(*trc10-23*) and NT1(*trc42-87*). The two independently isolated Tra^c mutants contained Tn*5* insertions within pTiC58 that yielded indistinguishable banding patterns when analyzed with the five restriction endonucleases. *tra* region II, between coordinates 114 and 116, includes the mutations in mutants NT1(*tra16-24*), NT1(*tra3-16*), and NT1(*tra15-26*). The Tn*5* insertions mapped in *Hin*dIII restriction fragment 4. A cluster of five Tn*5* insertions identifies *tra* region III within coordinates 20 and 24. Mutants NT1(*tra15-62*) and NT1(*tra9-74*) contain insertions that mapped in *Hin*dIII restriction fragment 8; the insertions in NT1(*tra12-33*), NT1(*tra2-16*), and NT1(*tra6-52*) mapped in *Hin*dIII restriction fragment 35, while that in NT1(*tra19-22*) mapped to *Hin*dIII restriction fragment 26a.

Complementation analysis of Tn*5*-specific Tra$^-$ mutants. The cosmid pSVB20 (Fig. 2), with DNA from the spontaneous transfer-constitutive mutant plasmid pTiC58Tra^c, complemented the Tra-deficient phenotypes of mutants carrying mutations in *tra* region I (Fig. 3). Transfer frequencies were restored to between 10^{-4} and 10^{-5} transconjugants per input donor cell. In contrast, the cosmid pTHB55, which contains a comparable region from wild-type pTiC58, did not complement the *traI* mutants. Cosmid clone pTHB58 (27) complemented the three Tra$^-$ strains with mutations in *tra* region II. The merodiploids gave transfer frequencies of between 6×10^{-4} and 2×10^{-5} transconjugants per input donor cell. Five of the six *traIII* mutants were complemented by pTHH6, showing transfer frequencies between 2×10^{-4} and 5×10^{-5} transconjugants per input donor cell. The sixth mutant, NT1(*tra15-62*), was not complemented by pTHH6. Phenotypic and physical analyses of this merodiploid strain demonstrated stable coexistence of the mutagenized Ti plasmid and the recombinant plasmid pTHH6 (data not shown).

Complementation analysis of the Tn*5*-specific Tra^c mutants. Complementation of the Tn*5*-induced transfer-constitutive phenotype was tested only for strain NT1(*trc10-23*) because the transposon insertions in the two independent Tra^c mutations were indistinguishable by our mapping methods. Four different recombinant plasmids, pTHVB14, pTHB55, pSVE33, and pSVB20, containing inserts from either wild-type pTiC58 or pTiC58Tra^c, failed to repress the Tra^c phenotype of mutant NT1(*trc10-23*). The complemented strains yielded transfer frequencies between 3×10^{-4} and 2×10^{-5} transconjugants per input donor cell, values not significantly different from that for the Tn*5*-specific mutant itself.

In the absence of complementability, homogenotization of Tn*5*-specific *trc10-23* plasmid DNA into wild-type pTiC58 and concomitant conversion of the wild-type repressed

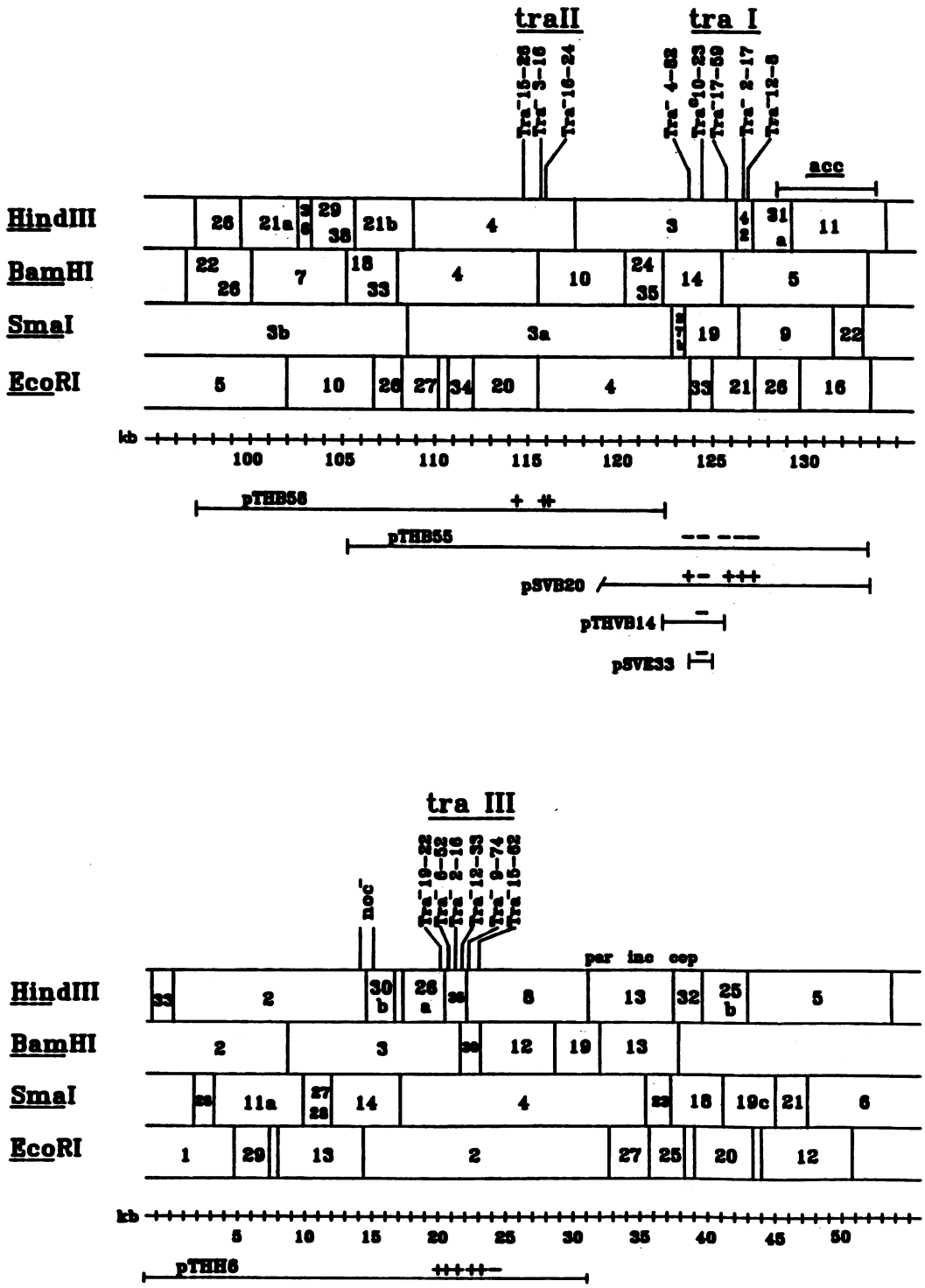

FIG. 2. Restriction map of Tn*5* insertions affecting conjugal transfer of pTiC58. The top panel shows a detailed map of *tra* region I defined within pTiC58 coordinates 123 and 127 and *tra* region II within coordinates 114 and 116. This map also shows the location of a previously unidentified *Hin*dIII fragment, *Hin*dIII-42, between *Hin*dIII-3 and *Hin*dIII-31a on the map published by Depicker et al. (13). The bottom panel depicts the mutations defining *tra* region III between coordinates 20 and 24 of the pTiC58 plasmid. Cosmid clones and subclones tested for complementation are represented as solid lines below the maps; the ability (+) or inability (−) to complement the corresponding mutant phenotypes is indicated. Genotypic designations are as follows: *noc*, nopaline catabolism; *acc*, agrocinopine catabolism; *par*, stable plasmid inheritance; *inc*, incompatibility with other *inc*Rh-1 members; *cop*, copy number control.

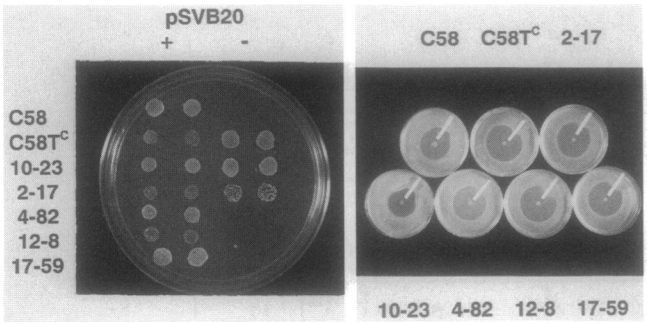

FIG. 3. Conjugation and agrocin 84 phenotypes of complemented and uncomplemented *traI* mutants. The left panel shows conjugal transfer assays. Donor strains, designated on the left, either with or without complementing cosmid pSVB20, were spotted onto the mating plate in duplicate. Inability to transfer the respective Ti plasmids is characterized by lack of growth at spot-mating sites. The right panel shows agrocin 84 assays. The wild-type strain C58 and the Tn*5*-induced Tra^c mutant, NT1(*trc10-23*), demonstrate an agrocinopine-inducible, agrocin 84-sensitive (Agr^s) phenotype (27), with a characteristic extension in the zone of growth inhibition next to a paper strip impregnated with agrocinopines A and B (27). The spontaneously constitutive mutant strain NT1(pTiC58Tra^c) (designated C58T^c) and the Tra$^-$ mutants derived from this strain demonstrate an agrocin-supersensitive phenotype characterized by a larger zone of growth inhibition and showing no response to agrocinopines (27).

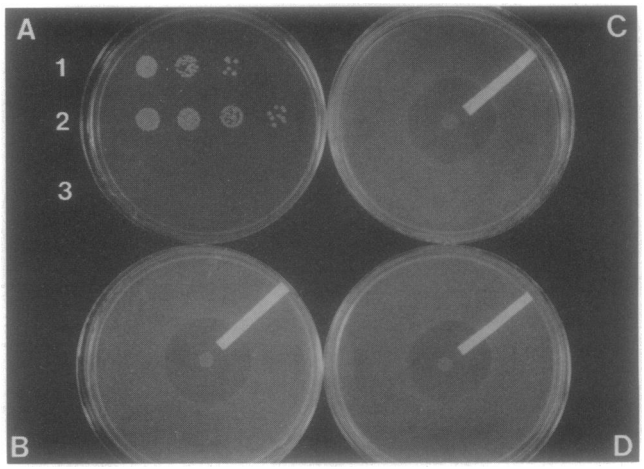

FIG. 4. Conjugation and agrocin 84 phenotypes of pTiC58 and its transfer-constitutive mutants. Plate A shows the conjugation phenotypes of NT1(pTiC58::Tn*5* *trc10-23*) (row 1), NT1(pTiC58Tra^c) (row 2), and C58 (row 3). Spots from left to right represent 10-fold dilutions of the donor strain. An approximately equal number of cells was plated for each donor at each dilution. The remaining plates show the agrocin 84 sensitivity phenotypes in response to agrocinopines A and B for (B) NT1(pTiC58Tra^c), (C) NT1(pTiC58::Tn*5* *trc10-23*), and (D) C58.

conjugal phenotype to the mutant phenotype would ensure that the observed constitutive conjugal transfer was caused by the transposon insertion. We were unable to isolate true homogenotes by using a pBR322 recombinant plasmid with *trc10-23* Tn*5*-specific DNA (including *Eco*RI fragments 34, 20, 4, 33::Tn*5*, and 21) by the procedure of Comai et al. (12). However, a number of plasmid cointegrates derived from single cross-over events were isolated. These hybrid plasmids carried a wild-type copy of *Eco*RI-33 in addition to a copy of *Eco*RI-33::Tn*5* from the transfer-constitutive mutant. All strains containing such a hybrid plasmid displayed the Tra^c phenotype of mutant NT1(*trc10-23*). Physical analysis using ^{32}P-labeled pBR322::Tn*5* DNA to probe restriction endonuclease digests of two such cointegrate plasmid DNAs showed that single recombinations occurred between the Ti plasmid and the clone within *Eco*RI fragment 4 (data not shown).

Agrocin 84 sensitivity assays as an indirect measure of agrocinopine uptake and catabolism. Conjugal transfer of pTiC58 is induced by agrocinopines A and B (18). Furthermore, virtually all spontaneous Tra^c mutants are also derepressed for catabolism of these opines (21; our unpublished observations). Accordingly, the Tn*5*-specific conjugal mutants were screened for opine-inducible agrocin 84 sensitivity to assess whether the respective mutations altered the regulatory status of the agrocinopine-catabolic (*acc*) locus (27). The spontaneous Tra^c parent strain NT1(pTiC58Tra^c) and the Tra$^-$ mutants derived from this strain all displayed a supersensitive phenotype (Agr^{ss}) (Fig. 3 and 4). In contrast, the Tn*5*-induced transfer-constitutive mutant, NT1(*trc10-23*), showed wild-type agrocinopine-dependent induction of the *acc* locus, characterized by an opine-induced extension in the zone of growth inhibition (Fig. 3 and 4).

Conjugal analysis of pTiC58-specific *vir* mutants. Mutations in octopine-type *vir* loci may play a role in conjugal transfer (S. Gelvin, personal communication). However, none of our pTiC58 Tra$^-$ mutants contained Tn*5* insertions in the *vir* region. Accordingly, we examined the conjugal transfer of a set of pTiC58Tra^c mutants with separate insertions in *virA*, *virB*, *virD*, and *virG* (32, 46) to determine whether known *vir* mutations limit conjugal transfer. As summarized in Table 2, the *vir* mutations tested showed conjugal transfer frequencies similar to that of the parent plasmid.

Effect of *tra* mutations on tumorigenicity. Schell and colleagues found during a general functional analysis of pTiC58 that one insertion mutant and those carrying several DNA deletions which give a Tra$^-$ phenotype were also altered in their ability to induce tumors (22, 30). Similarly, Tempé et al. (52) showed that tumorigenicity and Ti plasmid conjugal transfer have a common temperature-sensitive component. Accordingly, we tested our conjugal mutants for their ability to induce tumors on *K. diagremontiana*. Our results showed no appreciable difference in tumorigenesis between the Tra mutants and their parent strain NT1(pTiC58Tra^c) or wild-type strain C58 (data not shown).

Effect of chromosomal *chvB* mutations on conjugal transfer. There is evidence that mutations in two chromosomal loci, *chvA* and *chvB*, specifying *A. tumefaciens* attachment to plants (16), interfere with conjugal transfer of the *Agrobacterium* plasmid pAgK84 (5). We measured transfer frequencies of the octopine-type Ti plasmid pTiB$_6$806, resident in three *chvB* mutants of the C58 chromosomal background (16, 17). The results (Table 2) demonstrate that mutations in *chvB* do not interfere with conjugal transfer of pTiB$_6$806.

DISCUSSION

In this report we describe the isolation and characterization of Tn*5*-induced conjugal transfer mutants of the Ti plasmid pTiC58. Coinheritance of the Tra$^-$ character and Tn*5*-specific kanamycin resistance following transformation with the mutagenized plasmid DNAs suggests that the observed phenotypes result from the transposon insertions. This is strengthened by experiments showing that all mutations causing a Tra$^-$ phenotype, except *tra15-62*, could be

complemented in *trans* by recombinant clones containing inserts that overlapped the mutated regions. We predicted that our selection strategy should also identify mutations in *noc*, the operon encoding catabolism of nopaline (30). In fact, three such mutants were identified, and for the two analyzed, the transposon insertions mapped to the *noc* region (Fig. 2).

Mutagenesis and complementation analyses defined three separate regions of pTiC58 that are essential for conjugal transfer (Fig. 2). Based on the location of transposon insertions, *tra* region I extends a minimum of 4 kb, while *tra* region II is at least 2 kb in size. The two are separate loci, since both could be complemented in *trans* with cosmid clones containing nonoverlapping segments of the Ti plasmid (Fig. 2). *tra* region III is at least 3 kb in size, as defined by six independent Tn*5* insertions. The sizes for all three regions are minimum estimates, and site-directed mutagenesis will be required to fully define each locus. We cannot explain why the Tn*5* insertion of the Tra^- mutant carrying *tra15-62* is not complementable in *trans*. However, a more detailed characterization of *tra* region III can differentiate between a structural or regulatory mutation caused by the *tra15-62* transposon insertion.

The physical separation of Ti plasmid *tra* genes into three regions differs from the organization in the F plasmid, where the *tra* genes are grouped into a single 33-kb operon (56). However, it is similar to that of other conjugal plasmids, such as RP4 (1), pKM101 (57), and ColIB-P9 (45), in which Tra functions are organized into two or three groups of genes.

Mutations in *tra* region I could not be complemented by pTHB55, a recombinant clone derived from wild-type pTiC58 (27). However, pSVB20, a similar clone containing an insert from pTiC58Tra^c, restored conjugal transfer of the mutants whose mutations mapped to this region (Fig. 2 and 3). The spontaneous Tra^c Ti plasmid from which pSVB20 is derived also confers supersensitivity to agrocin 84, a phenotype associated with constitutive expression of *acc*, the agrocinopine-catabolic locus (Fig. 3) (27). This pleiotropic phenotype led Ellis et al. (21) to suggest that such spontaneously constitutive mutants are deficient in a common repressor that regulates both Tra functions and *acc*. Consistent with this hypothesis is the fact that agrocinopines A and B coinduce pTiC58-specific conjugal transfer and agrocinopine catabolism (18, 21, 27). We predict that the gene encoding this putative repressor is contained on both recombinant cosmid clones. If so, pTHB55, which is wild type with respect to this trait, is not expected to restore the Tra functions. Although it should complement these mutations, it should also complement the repressor mutation, making the Tra^c plasmid functionally Tra^- in the absence of the inducing opine. In contrast, pSVB20 is derived from the Tra^c Ti plasmid and presumably contains the mutant repressor gene. The work of Hayman and Farrand (27), suggesting that a repressor regulating *acc* is located within *Hin*dIII fragment 31a, is consistent with this interpretation, particularly since this *Hin*dIII fragment is common to both recombinant clones (Fig. 2).

The Tn*5*-induced Tra^c phenotype could not be complemented by any of a number of recombinant clones, including pSVB20 (Fig. 2 and 3). This raised the possibility that the mutation is not associated with the transposon insertion. Three observations argue against this interpretation. First, the two independent Tra^c mutants contained Tn*5* insertions in their Ti plasmids that are indistinguishable in location and polarity. Second, the constitutive phenotype was coinherited with the transposon-containing Ti plasmid. Third, the mutant phenotype was regenerated when the Tn*5*-containing region of the Tra^c Ti plasmid was inserted into wild-type pTiC58. The simplest interpretation of these results is that the transposon inserted into a *cis*-acting site. Constitutive expression of conjugal transfer genes could result from either of at least two mechanisms. First, the transposon may physically interfere with the binding of some negative regulatory component, allowing expression of the conjugal transfer genes in the absence of opine induction. Alternatively, the constitutive expression of *tra* genes may originate from Tn*5* transposon-specific promoters (2). The fact that both mutants contained the transposon inserted in the same polarity is consistent with this possibility. Regardless of which possibility is correct, our data establish that a transposon-specific mutation at this one locus is sufficient to overcome the wild-type repressed conjugal state and can lead to dominant constitutive expression of the conjugal phenotype.

The Tn*5*-generated Tra^c mutants differed from the spontaneous transfer-constitutive mutant Ti plasmid in two respects (Fig. 4 and Table 2). First, the Tra^c Tn*5* insertion mutants conjugally transferred at frequencies at least 10-fold lower than that of the spontaneous Tra^c mutant. Second, the spontaneous mutant is constitutive for both conjugal transfer and expression of *acc*. In contrast, the Tn*5*-induced mutants were only constitutive for conjugal transfer.

Our mutagenesis experiments failed to yield Tn*5* insertions in the chromosome that influenced conjugal transfer of pTiC58. However, a report in the literature suggests the lesions within *chvA* and *chvB*, two loci involved in *Agrobacterium* binding to plant cells, interfere with conjugal transfer of another *Agrobacterium* plasmid (5). Our results from conjugal transfer tests of the octopine-type Ti plasmid $pTiB_6806$ in the *chvB* mutant chromosomal background of C58 demonstrate that at least this chromosomal virulence gene is not required for octopine-type Ti plasmid conjugal transfer.

It was recently proposed that Ti plasmid-mediated transfer of T-DNA to plant cells is an adaptation of conjugal plasmid transfer (8, 50). Reports describing insertion and deletion mutations (22, 30) and temperature sensitivity properties (52) suggested that pTiC58-mediated conjugal transfer to bacterial recipients and T-DNA transfer to plants may have common elements. However, none of our Tra mutants harbored insertions mapping to known virulence determinants. Furthermore, all of the Tra mutants were tumorigenic on *K. diagremontiana* (data not shown). Finally, Ti plasmids with well-characterized mutations in selected *vir* operons were fully conjugal (Table 2). These combined observations indicate that conjugal transfer and virulence are separately specified phenotypes on pTiC58, but they do not rule out a model of T-DNA transfer with features mechanistically similar to those of bacterial conjugation.

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