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Essential Components of the Ti Plasmid *trb* System, a Type IV Macromolecular Transporter

PEI-LI LI,[1] INGYU HWANG,[1]† HEATHER MIYAGI,[2] HEATHER TRUE,[2]‡ AND STEPHEN K. FARRAND[1,2]*

Departments of Crop Sciences[1] and Microbiology,[2] University of Illinois at Urbana-Champaign, Urbana, Illinois 61801

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The *trb* operon from pTiC58 is one of three loci that are required for conjugal transfer of this Ti plasmid. The operon, which probably codes for the mating bridge responsible for pair formation and DNA transfer, contains 12 genes, 11 of which are related to genes from other members of the type IV secretion system family. The 12th gene, *traI*, codes for production of *Agrobacterium* autoinducer (AAI). Insertion mutations were constructed in each of the 12 genes, contained on a full-length clone of the *trb* region, using antibiotic resistance cassettes or a newly constructed transposon. This transposon, called mini-Tn*5*P*trb*, was designed to express genes downstream of the insertion site from a promoter regulated by TraR and AAI. Each mutation could *trans* complement downstream Tn*3*HoHo1 insertions in the *trb* operon of full-sized Ti plasmids. When marker-exchanged into the transfer-constitutive Ti plasmid pTiC58Δ*accR* mutations in *trbB*, *-C*, *-D*, *-E*, *-L*, *-F*, *-G*, and *-H* abolished conjugal transfer from strain UIA5, which lacks the 450-kb catabolic plasmid pAtC58. However, these mutants retained residual conjugal transfer activity when tested in strain NT1, which contains this large plasmid. The *trbJ* mutant failed to transfer at a detectable frequency from either strain, while the *trbI* mutant transferred at very low but detectable levels from both donors. Only the *trbK* mutant was unaffected in conjugal transfer from either donor. Transfer of each of the marker-exchange mutants was restored by a clone expressing only the wild-type allele of the corresponding mutant *trb* gene. An insertion mutation in *traI* abolished the production of AAI and also conjugal transfer. This defect was restored by culturing the mutant donor in the presence of AAI. We conclude that all of the *trb* genes except *trbI* and *trbK* are essential for conjugal transfer of pTiC58. We also conclude that mutations in any one of the *trb* genes except *traI* and *trbJ* can be complemented by functions coded for by pAtC58.

The Ti plasmids of *Agrobacterium tumefaciens* code for two distinct conjugal transfer systems. One, mediated by the Vir system, transfers T-DNA into the plant cells but also can mobilize transfer of a suitable plasmid to recipient bacteria (for a recent review, see reference 17). The second, which constitutes the major pathway for Ti plasmid transfer, operates through a functionally and physically separated system called Tra. Expression of the Tra system on at least two Ti plasmids is tightly regulated at the transcriptional level through a complex signalling circuitry that involves opines produced by the crown gall tumors plus a LuxR-LuxI-type quorum-sensing mechanism (5, 22, 31, 37, 38, 46).

The Tra system of pTiC58 consists of two physically separated gene sets, *tra* and *trb*, which contain all of the genes essential for conjugal transfer (15). The *tra* region encodes the origin of conjugal transfer (*oriT*) and two sets of genes organized as divergently expressed operons (14, 24). Three of the six genes flanking the *oriT* region are related to essential *tra* genes from IncP and IncQ plasmids. The products of some of these genes comprise the DNA transfer and replication (Dtr) function of the Ti plasmid Tra system and most probably form the relaxosome complex at the *oriT* site. The second region, *trb*, is located at the 2 o'clock position on the plasmid and is flanked by *noc*, the locus conferring catabolism of nopaline, and *oriV*/*rep*, the locus for vegetative replication.

The *trb* genes are believed to encode the mating pair formation (Mpf) apparatus required for the physical translocation of DNA from donors to recipients. Sequence analysis and genetic studies have shown that this region contains 12 genes, *traI* and *trbB*, *-C*, *-D*, *-E*, *-J*, *-K*, *-L*, *-F*, *-G*, *-H*, and *-I*, organized in a single operon (42). Expression of this operon is controlled by the quorum-sensing activator, TraR, and the acyl-homoserine lactone signal, *Agrobacterium* autoinducer [AAI; *N*-(3-oxooctanoyl)-L-homoserine lactone], which is synthesized by the gene product of *traI* (38, 42, 43). The *trb* genes of pTiC58 are closely related in sequence and organization to the 11 *trb* genes from the *tra2* core region of the IncP plasmids RP4 and R751. Genes of the *trb* system also are related to those of several other bacterial conjugation or protein secretion systems (42), including the VirB system of *A. tumefaciens* (49), the Ptl system of *Bordetella pertussis* (21, 55), the Tra system of plasmid F (27), the *cag* system of *Helicobacter pylori* (10, 36, 50), and the Dot system of *Legionella pneumophila* (48, 53).

In RP4, all but one of the 11 core *trb* genes are required for conjugal transfer (34). Similarly, 10 of the 11 *virB* genes are essential for the transfer of T-DNA to plant cells (8). Although the *trb* system of the Ti plasmid is related to these two systems, which of the Ti plasmid *trb* genes are essential for conjugal transfer remains unknown. In this report we describe the construction of a minitransposon carrying the promoter region of

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

† Present address: Plant Protectants Research Unit, Korean Research Institute of Bioscience and Biotechnology, Yusung, Taejon, South Korea 305-600.

‡ Present address: Howard Hughes Medical Institute, Department of Molecular Genetics and Cell Biology, The University of Chicago, Chicago, IL 60637.

TABLE 1. Bacterial strains and plasmids used

Bacterial strain or plasmid	Relevant genotype, phenotype, or characteristic(s)[a]	Source or reference(s)
Escherichia coli		
DH5α	*supE44* Δ*lacU169*(ϕ80*lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	47
S17-1	*pro* Res$^-$ Mod$^+$ Mob$^+$ Amp^r Cm^r Tp^r Str^r	15
S17-1λ-*pir*	*pro* Res$^-$ Mod$^+$ Mob$^+$ Amp^r Cm^r Tp^r Str^r λ::*pir*	24
2174(pPH1JI)	*met pro* Gm^r Sp^r, used to isolate homogenotes	24
Agrobacterium tumefaciens		
C58	Wild-type pathogenic strain carrying pTiC58 and pAtC58	Our collection
NT1	Ti plasmid-cured C58	15
NT1(pTiC58Δ*accR*)	NT1 harboring a Tra^c mutant of pTiC58	6
UIA5	Ti plasmid- and cryptic plasmid-cured C58, Rif^r Str^r	14
C58C1RS	Ti plasmid-cured C58, Rif^r Str^r	15
C58C1EC	Ti plasmid-cured C58, Ery^r Cm^r	Our collection
Plasmids		
pCF1	3,680-bp *Bam*HI fragment 13 encoding *traI*, *trbB*, and *repA* from pTiC58 cloned in pTZ18U, Amp^r	38
pDCI41E33	*traG*::*lacZ traR* in pDSK519, Km^r, autoinducer reporter plasmid	15
pDEK-*m*[b]	pTiC58Δ*accR*::Tn*3*HoHo1 marker-exchanged mutants, Cb^r	42
pDSK519	Broad-host-range IncQ cloning vector, Km^r	41
pHM1	Delivery plasmid for mini-Tn*5*P*trb*, Amp^r Km^r	This study
pHM25	*Hin*dIII fragment 8 containing Tn*3*HoHo1 from pPLE2-25 cloned in pRK415, Tc^r	38
pHM25-70	pHM25::mini-Tn*5*P*trb*, Tc^r Km^r	This study
pJB3	Broad-host-range IncP cloning vector, Amp^r	9
pKK38	Broad-host-range IncP cloning vector, *tac* promoter, Tc^r	David Nunn
pKK38ASH	Derivative of pKK38 with extra cloning sites, Tc^r	This study
pPLE2-25	pPLE2 with Tn*3*HoHo1 insertion in *trbE*, Cb^r	38, 42
pPLKmPtrb	Km-P*trb* cassette from mini-Tn*5*P*trb* cloned in pBluescript SK(+), Amp^r Km^r	This study
pPLK-*n*[c]	pTiC58Δ*accR*::mini-Tn*5*P*trb* marker-exchanged mutants, Km^r	This study
pPLKΔC	pTiC58Δ*accR trbC*::Km-P*trb* marker-exchanged mutant, Km^r	This study
pPLKΔK	pTiC58Δ*accR trbK*::*nptI* marker-exchanged mutant, Km^r	This study
pPLtra	*tra* region and *traR* from pTiC58 cloned in pDSK519, Tc^r	This study
pPLtrb*X*[d]	PCR-generated individual *trb* gene ORFs cloned in pKK38ASH or pKK38, Tc^r	This study
pRK415	Broad-host-range IncP cloning vector, Tc^r	41
pRKtrb	11,003-bp *Bgl*II-*Xba*I fragment containing the *trb* region of pTiC58 cloned in pRK415, Tc^r	This study
pRKtrb-*n*	pRKtrb::mini-Tn*5*P*trb* mutants, Tc^r Km^r	This study
pRKtrbΔC	pRKtrb with a Km-P*trb* cassette in *trbC*, Tc^r Km^r	This study
pSB315	pUC4K derivative containing *nptI*, Km^r	32
pSVB33	1.8-kb *Eco*RI fragment encoding *traR* from pTiC58 cloned in pSa152, Km^r Gm^r	46
pTra17-52	pTiC58Δ*accR nocR*::Tn*5* 17-52, Noc$^-$ Tra^c Km^r	6
pUTmini-Tn5Km	Delivery plasmid for mini-Tn*5*Km, Amp^r Km^r	20

[a] Abbreviations: Rif^r, rifampin resistance; Str^r, streptomycin resistance; Tc^r, tetracycline resistance; Cb^r, carbenicillin resistance; Km^r, kanamycin resistance; Amp^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Gm^r, gentamicin resistance; Em^r, erythromycin resistance; Sp^r, spectinomycin resistance; Tp^r, trimethoprim resistance; Tra^c, transfer constitutive.

[b] *m* refers to the allele number of the Tn*3*HoHo1 insertion as shown in Fig. 3C.

[c] *n* refers to the allele number of the mini-Tn*5*P*trb* insertion as shown in Fig. 3B.

[d] trb*X* refers to *trb* genes. *X* can be *B*, *C*, *D*, *E*, *J*, *K*, *L*, *F*, *G*, *H*, or *I*.

the *traI-trb* operon and the use of this element to generate complementable mutations in the *trb* genes of pTiC58. Results from matings with these mutants indicate that unlike the case for the RP4 *trb* system, only 9 of the 11 Ti plasmid *trb* genes are required for conjugal transfer. Our results also indicate that the large catabolic plasmid pAtC58 harbored by *A. tumefaciens* C58 not only can mobilize the IncQ plasmid RSF1010 at low frequency (14) but also can complement the mutations in most of the *trb* genes of the Ti plasmid.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and plasmids. The strains of *A. tumefaciens* and *Escherichia coli* and the plasmids used in this study are listed in Table 1. *A. tumefaciens* strains were grown at 28°C in L broth (LB [47]) (Gibco-BRL, Gaithersburg, Md.), in AB minimal medium (11), or on nutrient agar plates (Difco Laboratories, Detroit, Mich.). Mannitol or glucose, at a final concentration of 0.2%, was used as the sole carbon source in the minimal medium. *E. coli* strains were grown at 37°C in LB or on L agar plates. Antibiotics were added at the following concentrations when required: for *A. tumefaciens*, carbenicillin at 100 or 200 μg/ml, gentamicin at 30 μg/ml, kanamycin at 100 μg/ml, rifampin at 50 μg/ml, streptomycin at 200 μg/ml, erythromycin at 100 μg/ml, chloramphenicol at 30 μg/ml, and tetracycline at 2 μg/ml; for *E. coli*, ampicillin at 100 μg/ml, kanamycin at 50 μg/ml, rifampin at 50 μg/ml, and tetracycline at 10 μg/ml. X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; Gibco-BRL) was included in media at 40 μg/ml to assess the production of β-galactosidase.

DNA manipulation and plasmid constructions. Ti plasmids were isolated as described by Hayman and Farrand (35). Other plasmids were isolated by an alkaline lysis method (47). Standard recombinant DNA techniques were used as described by Sambrook et al. (47). Digestions with restriction endonucleases were conducted according to the manufacturers' instructions, and digestion products were separated by electrophoresis in agarose gels, using Tris-borate-EDTA buffer.

PCR. The *traI-trb* promoter region was amplified from pCF1 by using AmpliTaq DNA polymerase (Perkin-Elmer, Foster City, Calif.) and oligomers 5′-GGGCGGCCGCCCGATTCTTCAAATGC-3′ and 5′-GAGCGGCCGCATCGTAATCTCCGC-3′. *Pfu* DNA polymerase (Stratagene, La Jolla, Calif.) was used to amplify each of the 11 *trb* genes, and the products were cloned into pKK38ASH or pKK38. In each case, the 5′ primer was designed to generate an *Nco*I, *Rca*I, *Stu*I, or *Afl*III site allowing for in-frame fusion of the second codon of the *trb* gene to an ATG initiation codon provided by the vector. The sequences of the primers used for these amplifications are available upon request.

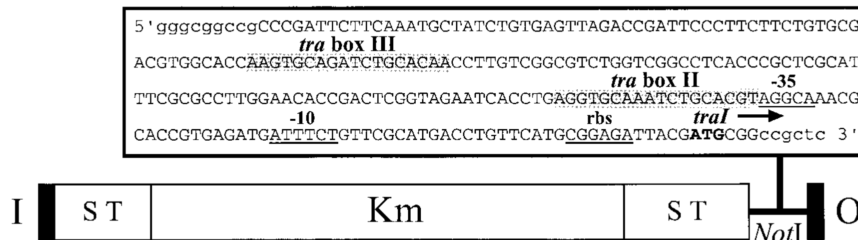

FIG. 1. Structure of mini-Tn*5*P*trb*. The 236-bp *traI-trb* promoter region was amplified from pCF1 by PCR, and the product was cloned into the *Not*I site of mini-Tn*5*Km (19, 20). *tra* box II and *tra* box III, the 18-bp almost perfect inverted repeat sequences that are conserved in LuxRI-type quorum sensing regulatory systems (29, 38); −10 and −35, the promoter elements of the *traI-trb* operon identified in pTiR10 (29); rbs, the putative ribosomal binding site of *traI*; S, translational stop; T, transcriptional terminator; Km, kanamycin resistance gene; I and O, the 19-bp I and O ends of Tn*5*.

Mini-Tn*5* mutagenesis and homogenotization. A method based on mutagenesis with a mini-Tn*5* transposon as described by de Lorenzo and Timmis (20) was used to transpose mini-Tn*5*P*trb* from pHM1 into the promoterless *trb* reporter clone pHM25 and into the full-length *trb* clone pRKtrb. The transposon delivery strain S17-1λ-*pir*(pHM1) was mated with the target strain, DH5α(pHM25) or DH5α(pRKtrb), on a 0.22-μm-pore-size filter, and the filter was incubated at 37°C for 6 h on the surface of an L agar plate. Following this incubation, the cells on the filter were suspended in 3 ml of LB, serial dilutions were prepared, and 0.1-ml volumes were spread on L agar plates containing kanamycin and tetracycline. The plates were incubated at 37°C, and colonies that appeared were combined. Plasmid DNA was extracted from the pool and used to transform *E. coli* DH5α with selection for resistance to kanamycin and tetracycline on L agar plates. Independent colonies were isolated and purified, and the locations and orientations of insertions of mini-Tn*5*P*trb* in the target plasmid were mapped by restriction endonuclease analysis. Insertion mutations of interest in pRKtrb were homogenotized into pTiC58Δ*accR* by using pPH1JI as the eviction plasmid as previously described (24). Proper marker exchanges in the Ti plasmids were confirmed by restriction endonuclease analysis. pPH1JI was cured from these strains by continuous growth of the strain in LB without gentamicin, the selection marker of pPH1JI. Alternatively, the marker-exchanged Ti plasmid was isolated and introduced into *A. tumefaciens* NT1 via electroporation. Transformants resistant to kanamycin but remaining susceptible to gentamicin were retained for further study.

β-Galactosidase assay. Quantitative assays for β-galactosidase activity were conducted as described previously (37). Each sample was analyzed in triplicate, and activity was expressed as units of β-galactosidase per 10^9 CFU.

Conjugation assays. Conjugal transfer of the Ti plasmid and of the *oriT-tra* plasmids, pFRtra and pPLtra, of the binary transfer system (15) to the *A. tumefaciens* recipient strains C58C1RS and C58C1EC was assayed by a filter mating method as described previously (14). Samples were plated in triplicate, and the values obtained were used to calculate the average number of transconjugants that arose for each mating. Transfer frequencies were expressed as numbers of transconjugants obtained per input donor cell. Each set of matings was repeated once or twice. Although absolute transfer frequencies usually differed, the patterns of transfer were similar from one experiment to the next. Thus, in each case we present data from a single experiment in which all of the matings shown were conducted in parallel.

Analysis of AAI production. AAI production was assayed by the semiquantitative plate method using *A. tumefaciens* NT1(pDCI41E33) as the indicator strain as previously described (15). A diffuse blue zone on the assay plate indicates the production of an active acyl-homoserine lactone by the strain being tested.

RESULTS

Construction and evaluation of mini-Tn*5*P*trb*. To construct a mini-Tn*5* transposon carrying the *traI-trb* promoter, a 236-bp fragment containing the promoter region of the *traI-trb* operon and the first two codons of *traI* was amplified by PCR using primers containing a *Not*I site as described in Materials and Methods. The amplified fragment was cloned into the unique *Not*I site in pUTmini-Tn*5*Km (20), and the fidelity of the sequence and orientation of the insert were confirmed by nucleotide sequencing. The resulting plasmid is designated pHM1, and the minitransposon is designated mini-Tn*5*P*trb* (Fig. 1).

We tested this transposon by mutagenizing a promoterless reporter plasmid, pHM25 (Fig. 2). This plasmid, which is a derivative of pPLE2-25, contains *Hin*dIII fragment 8 of pTiC58 with a Tn*3*HoHo1 insertion in *trbE* but lacks the 5′ end of *traI* and the entire upstream *traI-trb* promoter region (38). Although the *lacZ* of Tn*3*HoHo1 is oriented in the proper direction, the construct does not express β-galactosidase activity (38). Following mutagenesis of pHM25 with mini-Tn*5*P*trb*, we identified an insertion derivative, pHM25-70, in which the transposon is located just upstream of *traI* and is oriented such that P*trb* (the *trb* promoter) can drive expression of *trb*. Strains harboring this plasmid expressed β-galactosidase activity but only when both TraR and AAI were provided (Table 2). Thus, the cloned promoter in this newly constructed minitransposon, when inserted in the proper orientation, can express downstream genes, and this expression is dependent on the quorum-sensing regulators, TraR and AAI.

Phenotype of pRKtrb::mini-Tn*5*P*trb* mutants. pRKtrb, which contains the entire *trb* region, was mutagenized with mini-Tn*5*P*trb* as described in Materials and Methods. Using restriction endonuclease analysis, we identified 14 independent insertions, all oriented in the correct direction and representing at least one insertion in 9 of the 11 *trb* genes (Fig. 3B). To generate nonpolar mutations in the two remaining genes, *trbC* and *trbK*, the following cloning strategies were used (Fig. 3A). For *trbC*, a cassette containing P*trb* and the *nptII* gene was constructed from mini-Tn*5*P*trb*. This cassette, called Km-P*trb*, retains the kanamycin resistance gene and the *traI* promoter region but lacks some restriction sites and the insertion sequence elements of the transposon. The cassette was cloned between the internal *Bam*HI and *Nru*I sites in *trbC*, thus replacing 112 bp of the gene with the Km-P*trb* cassette. Subsequent cloning resulted in the replacement of wild-type *trbC* by the *trbC* deletion-insertion allele within the full-length *trb* clone, pRKtrb, to generate pRKtrbΔC. For *trbK*, an *nptI* cassette coding for resistance to kanamycin was excised from pSB315 and cloned between the internal *NruI* sites within the gene. This cassette lacks a transcriptional terminator, and the promoter of the *nptI* gene is known to express genes downstream of the insertion (32). This resulted in an allele of *trbK* deleted for 133 internal residues and containing the *nptI* cassette oriented such that the *trb* genes downstream from *trbK* will be expressed from the promoter of *nptI*.

Each mutation was assessed for any strong polar effects by testing its ability to complement a Ti plasmid derivative with a Tn*3*HoHo1 insertion in a downstream *trb* gene (Fig. 3C) (42). These Tn*3*HoHo1 insertion derivatives do not transfer at detectable frequencies, but transfer can be restored to wild-type levels ($\sim 10^{-2}$ transconjugant per input donor) by introducing a full-length *trb* clone such as pPLE2 (42) or pRKtrb (data not shown). Among the mini-Tn*5*P*trb* derivatives of pRKtrb tested, 13, including at least one in each *trb* gene, complemented the Tn*3*HoHo1 insertion mutations located downstream in the

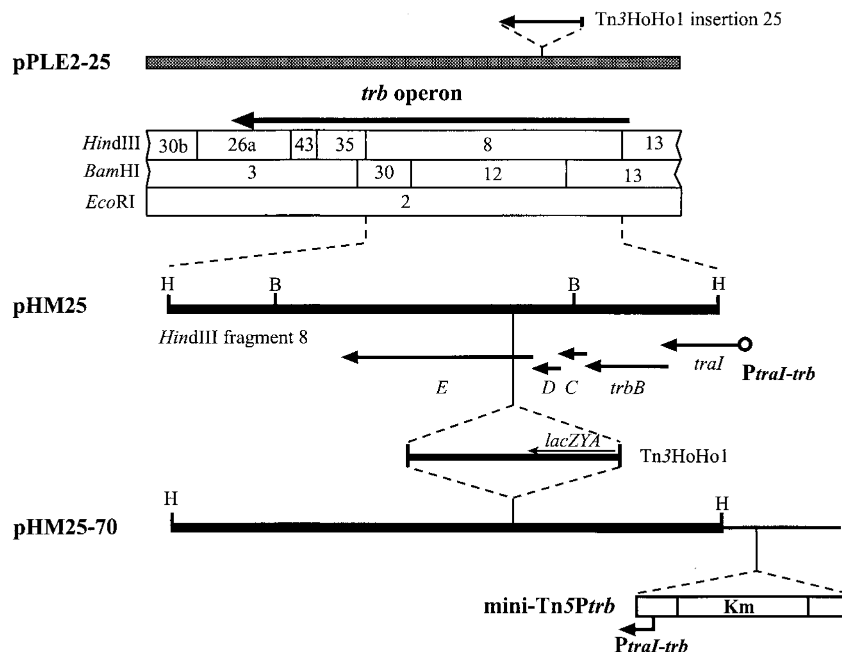

FIG. 2. Physico genetic organization of pPLE2-25 and construction of pHM25 and pHM25-70. The restriction map is according to the published sequence (42). pPLE2-25 contains the entire *trb* region of pTiC58 and a Tn*3*HoHo1 insertion in *trbE*. pHM25 is derived from pPLE2-25 by cloning the *Hin*dIII fragment 8 including the Tn*3*HoHo1 insertion into pRK415 (38). The open circle represents the *traI-trb* promoter region, which is just upstream of the *Hin*dIII site. pHM25-70 contains a mini-Tn*5*P*trb* insertion just upstream of the truncated *traI* gene. The transposons are not drawn to scale.

tester Ti plasmids (Table 3). pRKtrb-2 with a mini-Tn*5*P*trb* insertion in *trbE* failed to complement the test plasmid, suggesting that the insertion in this mutant exerts a strongly polar effect on expression of downstream *trb* genes. On the other hand, another *trbE* mutant, pRKtrb-5 restored transfer of the test plasmid to a reasonable level. These results indicate that in most cases the mini-Tn*5*P*trb* insertion and the *nptI* cassette mutants express *trb* genes located downstream of the insertion sites at levels allowing formation of a functional *trb* transporter.

The mini-Tn*5*P*trb*, Km-P*trb*, or *nptI* insertion allele of each *trb* gene was marker exchanged into pTiC58Δ*accR*, and each Ti plasmid was tested for its conjugal properties. With two exceptions, all such donors exhibited reduced but detectable transfer frequencies compared to that of the transfer-constitutive (Tra^c) Ti plasmid (Table 4). The mini-Tn*5*P*trb* insertion in *trbJ* completely abolished conjugal transfer, while the *nptI* cassette in *trbK* had virtually no effect on transfer frequencies. We considered the possibility that pPH1JI, the R751 derivative used as the eviction plasmid in the marker exchange, was complementing the *trb* mutations in the Ti plasmids. However, when tested in a donor lacking pPH1JI, each mutant Ti plasmid except the *trbJ* mutant continued to transfer at a low but detectable frequency (Table 4). Again, transfer of the *trbK* mutant occurred at near-wild-type frequencies. Strain NT1 harbors a 450-kb catabolic plasmid called pAtC58 (23). To determine whether this plasmid was contributing transfer functions, we introduced each of the mutant Ti plasmids into UIA5, a derivative of NT1 cured of pAtC58. When these strains were used as donors, all except those with a mutation in *trbK* or *trbI* failed to transfer at a detectable frequency (Table 4). The *trbI* mutant showed an approximately 3- to 4-orders-of-magnitude decrease in transfer frequency compared to the parent Ti plasmid, whereas the mutation in *trbK* had no effect on the transfer frequency. To confirm that the *trbI* mutation does not abolish conjugal transfer, we tested pDEK-9 and pDEK-64, two derivatives of pTiC58Δ*accR* with independent Tn*3*HoHo1 insertions in *trbI* (Fig. 3C). We previously reported that these Ti plasmids failed to transfer (42). However, when we increased the sensitivity of the assay by plating less diluted samples of the mating mix, we observed transfer of pDEK-9 and pDEK-64 at frequencies of 9.4×10^{-8} and 1.5×10^{-7} respectively.

TABLE 2. Mini-Tn*5*P*trb* restores TraR-AAI-dependent *lacZ* expression of a promoterless *trbE*::*lacZ* reporter fusion

Test strain[a]	β-Galactosidase activity (U/10^9 CFU)			
	−TraR		+TraR[b]	
	−AAI	+AAI[c]	−AAI	+AAI
pPLE2-25	3	4	146	NT[d]
pHM25	6	7	5	6
pHM25-70	5	5	4	85

[a] All plasmids are harbored in NT1.
[b] TraR was supplied by the *traR*-expressing clone pSVB33.
[c] Synthetic AAI was added to the culture at a concentration of 25 nM.
[d] NT, not tested. The strain itself produces AAI.

Complementation analysis. Each of the *trb* genes was amplified by PCR using primers containing *Nco*I, *Rca*I, *Stu*I, or *Afl*III sites, depending on the 5′-end sequence of the gene, and either *Hin*dIII, *Pst*I, or *Bam*HI sites immediately following the stop codon of the gene. These PCR products were cloned into pKK38ASH, which is a derivative of pKK38 into which we inserted extra cloning sites for *Hin*dIII, *Pst*I, and *Bam*HI (Fig. 4). Each of the *trb* open reading frame (ORF) clones was introduced in *trans* into UIA5 harboring a derivative of pTiC58Δ*accR* with a mini-Tn*5*P*trb* insertion in the corresponding *trb* gene. When mated with C58C1EC, all complemented donor strains transferred the mutant Ti plasmids, although the

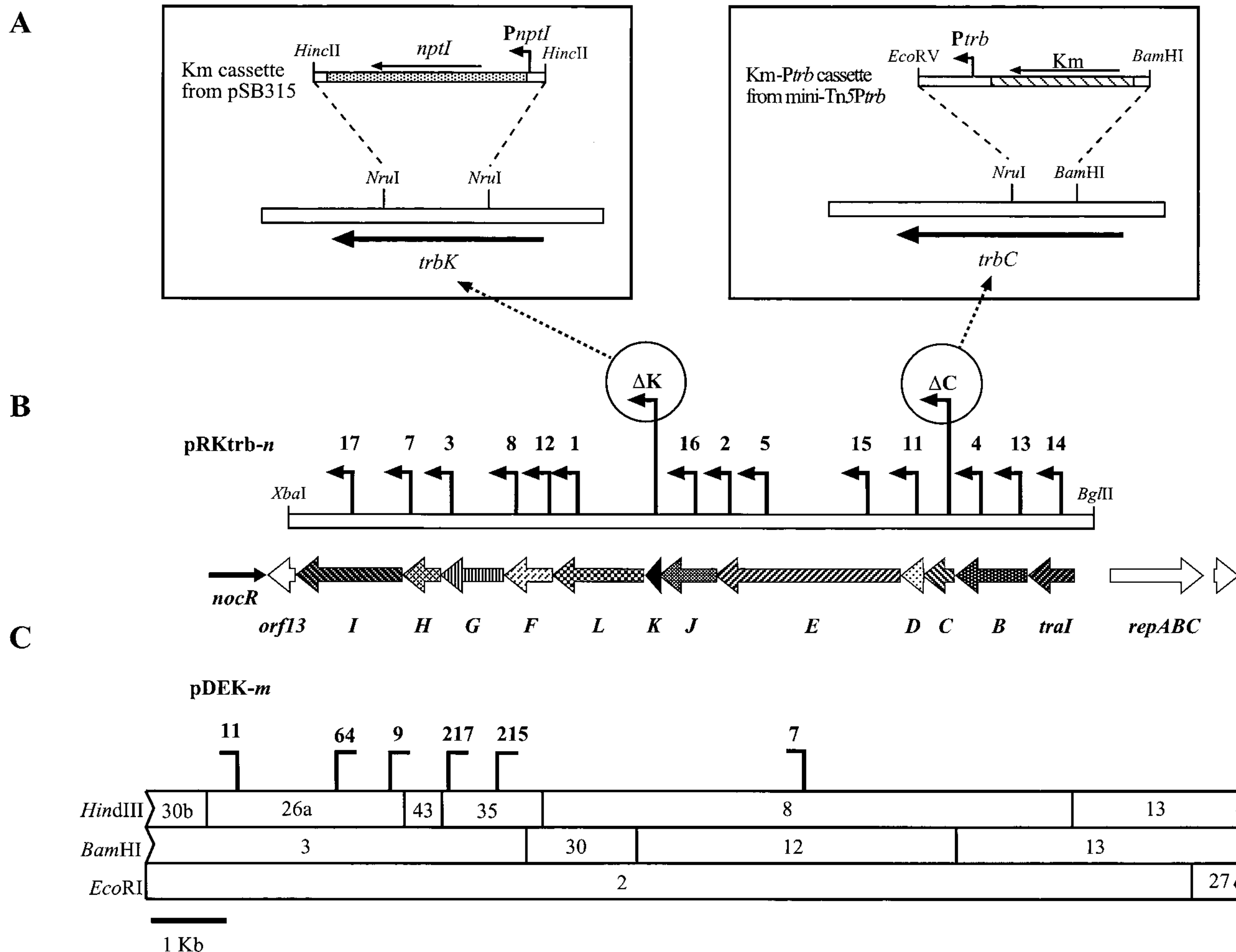

FIG. 3. Mutational analysis of the *trb* genes of pTiC58. (A) Cloning strategies for constructing nonpolar insertions in *trbC* and *trbK*. (B) Locations of mini-Tn*5*P*trb* insertions in pRKtrb. Each vertical bar represents an independent insertion, and the horizontal arrow indicates the orientation of the *traI-trb* promoter of the transposon. (C) Restriction map of the *trb* region and locations of the Tn*3*HoHo1 insertions in pTiC58Δ*accR*::Tn*3*HoHo1 which were used in complementation assays to test polarity of the mini-Tn*5*P*trb* insertion mutants of pRKtrb.

efficiency of complementation varied for different mutations (Table 4). Complemented donors with mutations in *trbC*, *trbD*, *trbE*, *trbL*, and *trbF* transferred the test plasmid at near-wild-type levels. On the other hand, the *trbB* and *trbJ* mutants were only poorly complemented, while mutants with insertions in *trbG*, *trbH*, and *trbI* transferred their Ti plasmids at intermediate frequencies (Table 4).

Conjugal transfer of a nonpolar *traI* mutant can be restored by adding AAI. We also identified a mini-Tn*5*P*trb* insertion in *traI*, the first gene of the *trb* operon (Fig. 3B). An *Agrobacterium* strain harboring this mutant plasmid, pRKtrb-14, does not produce AAI at detectable levels even in the presence of TraR (Table 5). Complementation assays using pTiC58Δ*accR*::Tn*3*HoHo1 indicated that this insertion mutation is not strongly polar (Table 3). Attempts to marker exchange this mutation in pTiC58Δ*accR* were not successful. Thus, we assessed the effect of the disruption of *traI* on conjugal transfer by testing the ability of pRKtrb-14 to mobilize pPLtra, which is a pDSK519 derivative containing the *tra* operons, the Ti plasmid *oriT*, and *traR* of the Ti plasmid, in a binary transfer system. NT1 harboring both pPLtra and pRKtrb-14 mobilizes the *tra* plasmid from the Ti plasmid *oriT* only at very low frequency (Table 5). This transfer rate is similar to that observed from strain NT1(pPLtra), which lacks the *trb* component, and is about 3 orders of magnitude lower than that observed from NT1(pPLtra, pRKtrb), which contains the wild-type Trb system of the Ti plasmid (Table 5). This basal level of mobilization of pPLtra is commonly observed when an RSF1010 derivative is harbored in strain NT1 (14). However, the mobilization frequency of the *tra* plasmid in NT1(pPLtra, pRKtrb-14) was restored to that of NT1(pPLtra, pRKtrb) by adding exogenous AAI (Table 5). Mobilization of the *oriT* plasmid from NT1(pPLtra) was not stimulated by addition of AAI.

DISCUSSION

***traI* and 9 of the 11 *trb* genes of pTiC58 are essential for conjugation.** Mutations in all but two *trb* genes resulted in complete loss of conjugal activity of the Ti plasmid (Table 4). The first gene of the *trb* operon is *traI*, the only known function of which is the synthesis of AAI, the essential signal for the quorum-sensing regulation of Ti plasmid conjugal transfer (30, 38, 43). Consistent with this, a mini-Tn*5*P*trb* mutation in *traI*

TABLE 3. Complementation analysis of mini-Tn*5*P*trb* mutants of pRKtrb

pRKtrb::mini-Tn*5*P*trb* mutant	Gene mutated	pTiC58Δ*accR*::Tn*3*HoHo1 mutant[a]	Transfer frequency of pTiC58Δ*accR*::Tn*3*HoHo1 derivative
None	None	pDEK-11[b]	9.0×10^{-2}
pRKtrb-14	*traI*	pDEK-7	3.1×10^{-2}
pRKtrb-4	*trbB*	pDEK-7	2.3×10^{-1}
pRKtrb-13	*trbB*	pDEK-7	2.6×10^{-1}
pRKtrbΔC[c]	*trbC*	pDEK-7	2.2×10^{-3}
pRKtrb-11	*trbD*	pDEK-7	8.8×10^{-2}
pRKtrb-2	*trbE*	pDEK-215	$<10^{-7}$
pRKtrb-5	*trbE*	pDEK-215	4.1×10^{-3}
pRKtrb-15	*trbE*	pDEK-215	1.6×10^{-4}
pRKtrb-16	*trbJ*	pDEK-215	9.4×10^{-5}
pRKtrb-1	*trbL*	pDEK-217	1.4×10^{-3}
pRKtrb-8	*trbF*	pDEK-217	4.1×10^{-4}
pRKtrb-12	*trbF*	pDEK-217	5.1×10^{-4}
pRKtrb-3	*trbG*	pDEK-9	3.9×10^{-3}
pRKtrb-7	*trbH*	pDEK-9	4.4×10^{-3}

[a] All derivatives of pTiC58Δ*accR*::Tn*3*HoHo1 except pDEK-11 failed to transfer at a detectable frequency in the absence of the complementing plasmids.
[b] pDEK-11 is a derivative of pTiC58Δ*accR* with a Tn*3*HoHo1 insertion in *nocR* (42).
[c] Contains a replacement mutation in *trbC* as described in the text.

abolished normal conjugation in a binary transfer assay but the wild-type phenotype could be restored by supplying exogenous AAI (Table 5). Thus, we conclude that the first gene in the *trb* operon, *traI*, is essential for Ti plasmid conjugal transfer but only because it is required for synthesis of the quorum-sensing signal. A nonpolar mutation in *trbK* has virtually no effect on conjugal transfer, suggesting that the product of the gene is not essential for the *trb*-encoded Mpf apparatus. However, *trbK* does play a role in conjugation; when present in a recipient, this gene confers entry exclusion against closely related Ti plasmids (44). This is consistent with studies of RP4 in which *trbK* is not required for conjugal transfer but is responsible for entry exclusion (33, 34). Hence the conservation between these two systems extends, at least in one case, to the function of the individual genes, even though the TrbK proteins from the two plasmids show considerable sequence divergence (42).

TABLE 4. Conjugal transfer frequency of pTiC58Δ*accR*::mini-Tn*5*P*trb* mutants and complementation of these mutants with *trb* gene clones

Mutant Ti plasmid	*trb* gene mutated	Transfer frequency when mated from:			
		NT1 (Ti plasmid only)	UIA5		
			Ti plasmid only	+pKK38ASH	+*trb* ORF clone
pTra17-52	None	1.6×10^{-1}	2.4×10^{-2}	NT[a]	NT
pPLK-4	*trbB*	7.6×10^{-4}	$<10^{-8}$	$<10^{-8}$	8.3×10^{-5}
pPLKΔC	*trbC*	6.4×10^{-4}	$<10^{-8}$	$<10^{-8}$	4.4×10^{-2}
pPLK-11	*trbD*	1.2×10^{-5}	$<10^{-8}$	$<10^{-8}$	5.6×10^{-3}
pPLK-5	*trbE*	2.1×10^{-6}	$<10^{-8}$	$<10^{-8}$	2.0×10^{-3}
pPLK-16	*trbJ*	$<10^{-7}$	$<10^{-8}$	$<10^{-8}$	3.8×10^{-6}
pPLKΔK	*trbK*	5.5×10^{-2}	2.9×10^{-3}	1.2×10^{-4}	1.2×10^{-3}
pPLK-1	*trbL*	3.3×10^{-6}	$<10^{-8}$	$<10^{-8}$	2.0×10^{-3}
pPLK-12	*trbF*	6.6×10^{-4}	$<10^{-8}$	$<10^{-8}$	1.8×10^{-2}
pPLK-3	*trbG*	4.7×10^{-6}	$<10^{-8}$	$<10^{-8}$	1.1×10^{-4}
pPLK-7	*trbH*	3.7×10^{-4}	$<10^{-8}$	$<10^{-8}$	2.0×10^{-4}
pPLK-17	*trbI*	8.7×10^{-5}	5.0×10^{-6}	2.8×10^{-6}	4.1×10^{-4}

[a] NT, not tested.

TABLE 5. Conjugal mobilization of pPLtra by the *traI*::mini-Tn*5*P*trb* mutant of pRKtrb can be restored by addition of AAI

Test strain	AAI production	Conjugal transfer frequency (transconjugants/input donor)	
		−AAI	+AAI[a]
NT1(pPLtra)	−	3.9×10^{-6}	4.0×10^{-7}
NT1(pPLtra, pRKtrb)	+++	4.7×10^{-3}	NT[b]
NT1(pPLtra, pRKtrb-14)	−	3.2×10^{-6}	4.8×10^{-3}

[a] Synthetic AAI was added at a concentration of 40 nM, and the culture was grown for an additional 6 h.
[b] NT, not tested.

Although members of the type IV secretion family share many characteristics, not all systems contain the same sets of genes. For example, the *virB* system of Ti plasmids and the *trb* system of RP4 have only six genes in common. Moreover, only *trbI*/*virB10* is present in every known type IV secretion system characterized to date, including the very distantly related systems such as *cag* of *H. pylori*, which contains only four *trb* homologs (10, 36, 50), and *dot* of *L. pneumophila*, which contains only two *virB* homologs (48, 53). *trbI* of RP4 has been reported to be essential for conjugal transfer (34). Similarly, *virB10*, the *trbI* homolog of the Ti plasmid Vir system, apparently is required for T-strand transfer to plants (8, 54) as well as for mobilization of RSF1010 to bacteria (28). Yet our results indicated that disruption of *trbI* of pTiC58, while severely reducing the frequency, did not abolish conjugal transfer. That transfer could be restored to near-normal levels when we supplied a copy of *trbI* in *trans* to the mutant Ti plasmid indicates that only the mutation in *trbI* is responsible for the decreased conjugal transfer activity. It is conceivable that the insertion of mini-Tn*5*P*trb* in *trbI* did not completely destroy the TrbI protein and that the remaining N- or C-terminal portion, or both parts of the protein, still retains partial function. However, our derivatives of pTiC58Δ*accR* with Tn*3*HoHo1 insertions in *trbI* also exhibit very low but detectable levels of transfer. Furthermore, our results are consistent with the observation that pTiA6NC, an octopine-type Ti plasmid containing a deletion that removes 90% of *trbI*, conjugally transfers at a very low but detectable frequency (2). Thus, we conclude that *trbI* of the Ti plasmid is not essential for conjugation but is required for transfer at wild-type efficiencies.

Although the function of TrbI is unknown, VirB10, the TrbI homolog of the Ti plasmid *virB* system, is believed to play a crucial role in assembling the mating pore complex. Several groups have proposed that VirB10 functions as an anchor by interacting with other VirB proteins, including VirB7 and VirB9, to form a high-molecular-weight complex (3, 4, 8, 13, 25, 26). However, the *trb* systems of the Ti plasmid and RP4 contain neither a VirB7 nor a VirB9 homolog. Thus, TrbI and VirB10 may play different roles in their two respective Mpf systems.

Mini-Tn*5*P*trb* as a tool to generate complementable mutations in the *trb* operon. Using transposable elements as mobile promoters to study polycistronic transcriptional units has proved to be useful (for a review, see reference 7). Transposons such as Tn*5virB* (16) and mini-Tn*5*-*lacI*^{q}/P*trc* (18) have been successfully applied in the genetic analyses of complex operons in *A. tumefaciens* and *Pseudomonas* spp. Our analyses indicate that when inserted in the proper location and correct orientation, mini-Tn*5*P*trb* can provide a promoter capable of expressing downstream genes, and that expression from this

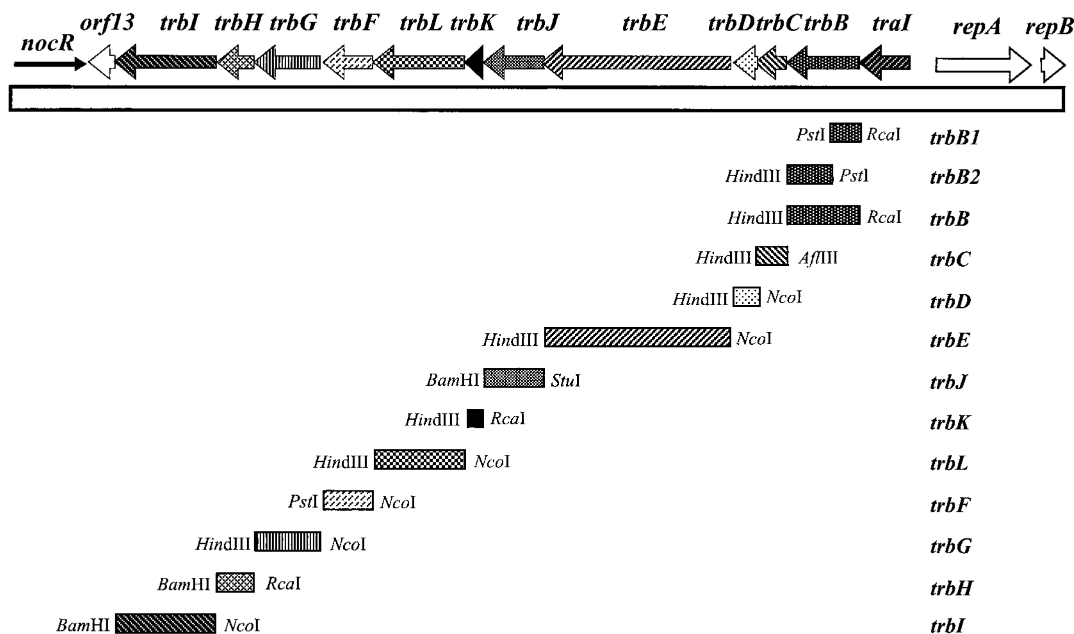

FIG. 4. Construction of individual *trb* gene ORF clones. PCR products corresponding to each of the *trb* genes were cloned into the expression vector as described in Materials and Methods. Due to the lack of suitable restriction sites, the *trbB* clone was obtained by first amplifying the 5′ and 3′ halves of the gene separately to generate *trbB1* and *trbB2* and then cloning *trbB2* into *trbB1*. All PCR products were cloned in pKK38ASH except for *trbK*, which was cloned in pKK38.

promoter is regulated by TraR and AAI. However, the transcriptional activity from the *traI-trb* promoter of mini-Tn*5*P*trb* in pHM25-70 was only about 60% of that observed when the *trbE*::*lacZ* fusion was expressed from the native *traI-trb* promoter in the original clone pPLE2-25 (Table 2). This difference in expression probably is due to the location of the insertion and the distance between the insertion and the immediate downstream gene. It also is possible that early termination of transcription occurs due to translational stops in the other reading frames. Therefore, for any given insertion some degree of polarity may be expected, and examination of more than one insertion in each gene may be necessary to obtain a suitable nonpolar mutation. Such factors may account for the difference in the ability of the two *trbE* mutants to *trans* complement a downstream mutation in the *trb* operon. Polar effects also may arise from the disruption of the preceding gene in a translationally coupled gene cluster as observed in other studies (8, 34). However, the promoter in mini-Tn*5*P*trb* contains a ribosomal binding site which may allow translational reinitiation of downstream, translationally coupled genes. With respect to our analysis, each of the Ti plasmid *trb* genes is preceded by a sequence that could serve as a ribosomal binding site (reference 42 and data not shown). Even so, that some of our *trb* mutants could not be complemented to wild-type levels of transfer suggests that mini-Tn*5*P*trb* insertions can induce some degree of polarity on the expression of downstream genes. Such effects may account for the relatively weak complementation of the *trbB* and *trbJ* mutations by the complementing cloned genes.

Mini-Tn*5*P*trb* provides several advantages for studying the *trb* genes. First, compared to MURFI linker insertion, which involves cloning and subcloning steps (34, 45), or DNA polymerase-directed site-specific deletion, which usually requires extensive in vivo and in vitro DNA manipulations, transposon mutagenesis is a relatively quick and easy way to generate acceptably nonpolar mutations in a large gene cluster. Second, using a cognate promoter such as P*trb* ensures that the same regulatory mechanism controls expression of both the downstream genes, which are transcribed from the transposon, and the upstream genes, which are transcribed from the native promoter of the operon. However, in common with all transposon mutagenesis schemes, insertions of mini-Tn*5*P*trb* in small genes can be difficult to obtain. Such was the case of *trbC* and *trbK* in this study even though the minitransposon insertions appeared to be evenly distributed throughout the *trb* region.

The role of the catabolic plasmid pAtC58 in conjugal transfer. In addition to the well-studied Ti plasmids, most isolates of *Agrobacterium* spp. harbor other extrachromosomal elements. These replicons usually are very large, but little is known concerning traits they confer on their bacterial hosts. *A. tumefaciens* C58 harbors at least one such plasmid, pAtC58, with a size variously estimated at 450 to 550 kb (1, 12, 39, 40). This plasmid, which codes for catabolism of a set of Amadori compounds produced by rotting vegetation and also by some crown gall tumors (52), is self-conjugal (51) and can mobilize an RSF1010 derivative at low but detectable frequency (14). Our results suggest that components of the transfer system of pAtC58 can substitute for certain of the *trb* functions of pTiC58. Thus, NT1, which harbors pAtC58, transfers most of our *trb* mutants at low frequency, while these same mutant plasmids fail to transfer from UIA5, a strain that lacks pAtC58 but otherwise is nearly isogenic to NT1 (Table 4). However, our *oriT-tra* plasmids such as pFRtra and pDCtra-5 are not mobilized from NT1 in the absence of a functional Ti plasmid *trb* system (15). Moreover, Ti plasmid *trb* mutants derived by insertion of Tn*3*HoHo1, which can exert strong polarity, fail to transfer from an NT1 donor (42). These observations suggest that the Mpf of pAtC58 is not itself able to substitute for that of the Ti plasmid. Rather, we propose that Mpf components of pAtC58 can replace some but not all of those coded for by the *trb* operon of pTiC58 to form a functional chimeric conjugal transporter. In this regard, only the *trbJ* mutant of pTiC58 failed to transfer at detectable frequencies from a donor har-

boring pAtC58 (Table 4). This observation suggests that the function coded for by this gene is an essential component of, and highly specific to, the Ti plasmid transporter and cannot be replaced by the corresponding Mpf component of pAtC58. Such a dependence on the Ti plasmid *trbJ* product for transfer from the Ti plasmid relaxosome might explain why the Mpf of pAtC58 cannot substitute for that of pTiC58. Interestingly, pPH1JI, which codes for a *trb* system closely related to that of pTiC58 (42), apparently does not complement any mutations in the Ti plasmid *trb* operon. Our nonpolar *trb* mutants do not transfer from a donor harboring pPH1JI at frequencies any higher than from donors lacking this IncP1β plasmid (data not shown). This is reminiscent of our observation that although the Dtr components of pTiC58 and RSF1010 are related, the Ti plasmid will not mobilize the IncQ plasmid (14). Determining the functional and phylogenetic interrelationships of these type IV systems and the points at which specificity is conferred should aid us in understanding how these transporters recognize and translocate their substrates.

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REFERENCES

1. **Allardet-Servent, A., S. Michaux-Charachon, E. Jumas-Bilak, L. Karayan, and M. Ramuz.** 1993. Presence of one linear and one circular chromosome in the *Agrobacterium tumefaciens* C58 genome. J. Bacteriol. **175:**7869–7874.
2. **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 an IncP plasmid and distantly related to Ti plasmid *vir* genes. J. Bacteriol. **178:**4248–4257.
3. **Banta, L. M., J. Bohne, S. D. Lovejoy, and K. Dostal.** 1998. Stability of the *Agrobacterium tumefaciens* VirB10 protein is modulated by growth temperature and periplasmic osmoadaption. J. Bacteriol. **180:**6597–6606.
4. **Beaupré, C. E., J. Bohne, E. M. Dale, and A. N. Binns.** 1997. Interactions between VirB9 and VirB10 membrane proteins involved in movement of DNA from *Agrobacterium tumefaciens* into plant cells. J. Bacteriol. **179:**78–89.
5. **Beck von Bodman, S., G. T. Hayman, and S. K. Farrand.** 1992. Opine catabolism and conjugal transfer of the nopaline Ti plasmid pTiC58 are coordinately regulated by a single repressor. Proc. Natl. Acad. Sci. USA **89:**643–647.
6. **Beck von Bodman, S., J. E. McCutchan, and S. K. Farrand.** 1989. Characterization of conjugal transfer functions of *Agrobacterium tumefaciens* Ti plasmid pTiC58. J. Bacteriol. **171:**5281–5289.
7. **Berg, C. M., and D. E. Berg.** 1996. Transposable element tools for microbial genetics, p. 2588–2612. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. ASM Press, Washington, D.C.
8. **Berger, B. R., and P. J. Christie.** 1994. Genetic complementation analysis of the *Agrobacterium tumefaciens virB* operon: *virB2* through *virB11* are essential virulence genes. J. Bacteriol. **176:**3646–3660.
9. **Blatny, J. M., T. Brautaset, H. C. Winther-Larsen, 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.
10. **Censini, S., C. Lange, Z. Xiang, J. E. Crabtree, P. Ghiara, M. Borodovsky, R. Rappuoli, and A. Covacci.** 1996. *cag*, a pathogenicity island of *Helicobacter pylori*, encodes type I-specific and disease-associated virulence factors. Proc. Natl. Acad. Sci. USA **93:**14648–14653.
11. **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.
12. **Cho, K., C. Fuqua, and S. C. Winans.** 1997. Transcriptional regulation and locations of *Agrobacterium tumefaciens* genes required for complete catabolism of octopine. J. Bacteriol. **179:**1–8.
13. **Christie, P. J.** 1997. *Agrobacterium tumefaciens* T-complex transport apparatus: a paradigm for a new family of multifunctional transporters in eubacteria. J. Bacteriol. **179:**3085–3094.
14. **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 T-region borders. J. Bacteriol. **174:**6238–6246.
15. **Cook, D. M., P.-L. Li, F. Ruchaud, S. Padden, and S. K. Farrand.** 1997. Ti plasmid conjugation is independent of *vir*: reconstitution of the *tra* functions from pTiC58 as a binary system. J. Bacteriol. **179:**1291–1297.
16. **Dale, E. M., A. N. Binns, and J. E. Ward, Jr.** 1993. Construction and characterization of Tn*5virB*, a transposon that generates nonpolar mutants, and its use to define *virB8* as an essential virulence gene in *Agrobacterium tumefaciens*. J. Bacteriol. **175:**887–891.
17. **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*. Kluwer Academic Publishing, Dordrecht, The Netherlands.
18. **de Lorenzo, V., L. Eltis, B. Kessler, and K. N. Timmis.** 1993. Analysis of *Pseudomonas* gene products using *lacIq*/P*trp-lac* plasmids and transposons that confer conditional phenotypes. Gene **123:**17–24.
19. **de Lorenzo, V., M. Herrero, U. Jakubzik, and K. N. Timmis.** 1990. Mini-Tn*5* transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in gram-negative eubacteria. J. Bacteriol. **172:**6568–6572.
20. **de Lorenzo, V., and K. N. Timmis.** 1994. Analysis and construction of stable phenotypes in Gram-negative bacteria with Tn*5* and Tn*10*-derived minitransposon. Methods Enzymol. **235:**386–405.
21. **Farizo, K. M., T. G. Cafarella, and D. L. Burns.** 1996. Evidence for a ninth gene, *ptlI*, in the locus encoding the pertussis toxin secretion system of *Bordetella pertussis* and formation of a PtlI-PtlF complex. J. Biol. Chem. **271:**31643–31649.
22. **Farrand, S. K.** 1993. Conjugation of *Agrobacterium* plasmids, p. 255–291. *In* D. Clewell (ed.), Bacterial conjugation. Plenum Press, New York, N.Y.
23. **Farrand, S. K.** 1998. Conjugal plasmids and their transfer, p. 199–233. *In* H. P. Spaink, A. Kondorosi, and P. J. J. Hooykaas (ed.), The *Rhizobiaceae*. Kluwer Academic Publishing, Dordrecht, The Netherlands.
24. **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.
25. **Fernandez, D., G. M. Spudich, X. R. Zhou, and P. J. Christie.** 1996. The *Agrobacterium tumefaciens* VirB7 lipoprotein is required for stabilization of VirB proteins during assembly of the T-complex transport apparatus. J. Bacteriol. **178:**3168–3176.
26. **Finberg, K. E., T. R. Muth, S. P. Young, J. B. Maken, S. M. Heitritter, A. N. Binns, and L. M. Banta.** 1995. Interactions of VirB9, −10, and −11 with the membrane fraction of *Agrobacterium tumefaciens*: solubility studies provide evidence for tight associations. J. Bacteriol. **177:**4881–4889.
27. **Frost, L. S., K. Ippen-Ihler, and R. A. Skurray.** 1994. An analysis of the sequence and gene products of the transfer region of the F sex factor. Microbiol. Rev. **58:**162–210.
28. **Fullner, K. J.** 1998. Role of *Agrobacterium virB* genes in transfer of T complexes and RSF1010. J. Bacteriol. **180:**430–434.
29. **Fuqua, C., and S. C. Winans.** 1996. Conserved *cis*-acting promoter elements are required for density-dependent transcription of *Agrobacterium tumefaciens* conjugal transfer genes. J. Bacteriol. **178:**435–440.
30. **Fuqua, W. C., and S. C. Winans.** 1994. A LuxR-LuxI type regulatory system activates *Agrobacterium* Ti plasmid conjugal transfer in the presence of a plant tumor metabolite. J. Bacteriol. **176:**2796–806.
31. **Fuqua, W. C., S. C. Winans, and E. P. Greenberg.** 1994. Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. J. Bacteriol. **176:**269–275.
32. **Galán, J. E., C. Ginocchio, and P. Costeas.** 1992. Molecular and functional characterization of the *Salmonella* invasion gene *invA*: homology of InvA to members of a new protein family. J. Bacteriol. **174:**4338–4349.
33. **Haase, J., M. Kalkum, and E. Lanka.** 1996. TrbK, a small cytoplasmic membrane lipoprotein, functions in entry exclusion of the IncPα plasmid RP4. J. Bacteriol. **178:**6720–6729.
34. **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.
35. **Hayman, G. T., and S. K. Farrand.** 1988. Characterization and mapping of the agrocinopine-agrocin 84 locus on the nopaline Ti plasmid pTiC58. J. Bacteriol. **170:**1759–1767.
36. **Hofreuter, D., S. Odenbreit, G. Henke, and R. Haas.** 1998. Natural competence for DNA transformation in *Helicobacter pylori*: identification and genetic characterization of the *comB* locus. Mol. Microbiol. **285:**1027–1038.
37. **Hwang, I., D. M. Cook, and S. K. Farrand.** 1995. A new regulatory element modulates homoserine lactone-mediated autoinduction of Ti plasmid conjugal transfer. J. Bacteriol. **177:**449–458.
38. **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.
39. **Jumas-Bilak, E., C. Maugard, S. Michaux-Charachon, A. Allardet-Servent, A. Perrin, D. O'Callaghan, and M. Ramuz.** 1995. Study of the organization of the genomes of *Escherichia coli*, *Brucella melitensis* and *Agrobacterium tumefaciens* by insertion of a unique restriction site. Microbiology **141:**2425–2432.
40. **Jumas-Bilak, E., S. Michaux-Charachon, G. Bourg, M. Ramuz, and A. Allardet-Servent.** 1998. Unconventional genomic organization in the alpha subgroup of the *Proteobacteria*. J. Bacteriol. **180:**2749–2755.
41. **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.
42. **Li, P.-L., D. M. Everhart, and S. K. Farrand.** 1998. Genetic and sequence analysis of the *trb* locus on pTiC58, a mating-pair formation system related to members of the type IV secretion family. J. Bacteriol. **180:**6164–6172.
43. **Moré, M. I., L. D. Finger, J. L. Stryker, C. Fuqua, A. Eberhard, and S. C. Winans.** 1996. Enzymatic synthesis of a quorum-sensing autoinducer through use of defined substrates. Science **272:**1655–1658.
44. **Nelson, C. D., C. E. Bratis, and S. K. Farrand.** Unpublished data.
45. **Perlman, D., and H. O. Halvorson.** 1986. The MURFI linker for multiple reading frame insertion of a sense or nonsense codon into DNA. Nucleic Acids Res. **14:**2139–2155.
46. **Piper, K. R., S. Beck von Bodman, and S. K. Farrand.** 1993. Conjugation factor of *Agrobacterium tumefaciens* regulates Ti plasmid transfer by autoinduction. Nature (London) **362:**448–450.
47. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Press, Cold Spring Harbor, N.Y.
48. **Segal, G., M. Purcell, and H. A. Shuman.** 1998. Host cell killing and bacterial conjugation require overlapping sets of genes within a 22-kb region of the *Legionella pneumophila* genome. Proc. Natl. Acad. Sci. USA **95:**1669–1674.
49. **Shirasu, K., P. Morel, and C. I. Kado.** 1990. Characterization of the *virB* operon of an *Agrobacterium tumefaciens* Ti plasmid: nucleotide sequence and protein analysis. Mol. Microbiol. **4:**1153–1163.
50. **Tummuru, M. K. R., S. A. Sharma, and M. J. Blaser.** 1995. *Helicobacter pylori picB*, a homologue of the *Bordetella pertussis* toxin secretion protein, is required for induction of IL-8 in gastric epithelial cells. Mol. Microbiol. **18:**867–876.
51. **van Montagu, M., and J. Schell.** 1979. The plasmids of *Agrobacterium tumefaciens*, p. 71–95. *In* K. N. Timmis and A. Pühler (ed.), Plasmids of medical, environmental and commercial importance. Elsevier/North Holland Biomedical Press, Amsterdam, The Netherlands.
52. **Vaudequin-Dransart, V., A. Petit, W. S. Chilton, and Y. Dessaux.** 1998. The cryptic plasmid of *Agrobacterium tumefaciens* cointegrates with the Ti plasmid and cooperates for opine degradation. Mol. Plant-Microbe Interact. **11:**583–591.
53. **Vogel, J. P., H. L. Andrews, S. K. Wong, and R. R. Isberg.** 1998. Conjugative transfer by the virulence system of *Legionella pneumophila*. Science **279:**873–876.
54. **Ward, J. E., Jr., E. M. Dale, P. J. Christie, E. W. Nester, and A. N. Binns.** 1990. Complementation analysis of *Agrobacterium tumefaciens* Ti plasmid *virB* genes by use of a *vir* promoter expression vector: *virB9*, *virB10*, and *virB11* are essential virulence genes. J. Bacteriol. **172:**5187–5199.
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.