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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

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We have determined the DNA sequences of two unlinked regions of octopine-type Ti plasmids that contain genes required for conjugal transfer. Both regions previously were shown to contain sequences that hybridize with *tra* genes of the nopaline-type Ti plasmid pTiC58. One gene cluster (designated *tra*) contains a functional *oriT* site and is probably required for conjugal DNA processing, while the other gene cluster (designated *trb*) probably directs the synthesis of a conjugal pilus and mating pore. Most predicted Tra and Trb proteins show relatively strong sequence similarity (30 to 50% identity) to the Tra and Trb proteins of the broad-host-range IncP plasmid RP4 and show significantly weaker sequence similarity to Vir proteins found elsewhere on the Ti plasmid. An exception is found in the Ti plasmid TraA protein, which is predicted to be a bifunctional nickase-helicase that has no counterpart in IncP plasmids or among Vir proteins but has homologs in at least six other self-transmissible and mobilizable plasmids. We conclude that this Ti plasmid *tra* system evolved by acquiring genes from two or three different sources. A similar analysis of the Ti plasmid *vir* region indicates that it also evolved by appropriating genes from at least two conjugal transfer systems. The widely studied plasmid pTiA6NC previously was found to be nonconjugal and to have a 12.65-kb deletion of DNA relative to other octopine-type Ti plasmids. We show that this deletion removes the promoter-distal gene of the *trb* region and probably accounts for the inability of this plasmid to conjugate.

It is widely appreciated that *Agrobacterium* strains are able to transfer segments of oncogenic DNA from their large Ti plasmids to the nuclei of infected plant cells, resulting in the formation of crown gall tumors. This conjugation-like process is mediated by the products of approximately 25 Ti plasmid-encoded *vir* genes (59). It may be less widely appreciated that the *vir* region contains only one of two DNA transfer systems found on Ti plasmids, since most Ti plasmids are able to undergo conjugal transfer between bacterial hosts (16). It was postulated some years ago that a single conjugation system might mediate both kinds of DNA transfer, but more recently it was shown that these processes are genetically distinct. Although two reports indicate that these two DNA transfer systems may interact (26, 51), other studies indicate that *tra* gene mutations do not affect T-DNA transfer to plants and, conversely, *vir* gene mutations do not abolish interbacterial conjugation (4, 8, 30). This suggests that, at least under most conditions, these transfer systems are functionally quite distinct. However, before the present study was initiated, it still remained possible that these two systems were the products of an evolutionarily recent gene duplication. We provide data that argue against such an origin and suggest a more complicated evolutionary lineage for *tra* and *vir* functions.

Several laboratories are studying how *Agrobacterium tumefaciens* perceives and responds to chemical signals that originate from plant hosts. While most of these studies focus on wound-released signals (29), attention recently has been focused on signals released from crown gall tumors (3, 54). These tumors release a variety of compounds called opines which provide nutrients for agrobacteria (10). Many or perhaps all opines induce the expression of genes that direct their uptake and catabolism. Particular opines, designated conjugal opines, also stimulate Ti plasmid conjugation by enhancing expression of the Ti plasmid-encoded *traR* gene (3, 16, 22). The TraR protein, together with a 3-oxo-octanoyl homoserine lactone called AAI, then activates multiple *tra* promoters in a quorum-dependent fashion (21, 22, 24, 25, 31, 32, 44). In addition, the octopine-type TraR protein regulates at least six genes that have no direct role in conjugation (24). To begin understanding the functions of conjugation genes and to find other genes that may be regulated by TraR, we have determined the sequences of two clusters of genes required for conjugation, as well as flanking DNA probably encoding functions distinct from conjugal transfer.

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MATERIALS AND METHODS

DNA sequencing. DNA between coordinates 13.2 and 18.85 (as defined in reference 13) was subcloned from cosmid pVK236 (34) as an *Eco*RI-*Bam*HI fragment into pDELTA 2 (Bethesda Research Laboratories), and in vivo deletions of the resulting plasmid, pCF329, were created by using the Deletion Factory System 2.0 (Bethesda Research Laboratories). The resulting plasmids were purified by using SpinBind columns (FMC Bioproducts), and the inserts

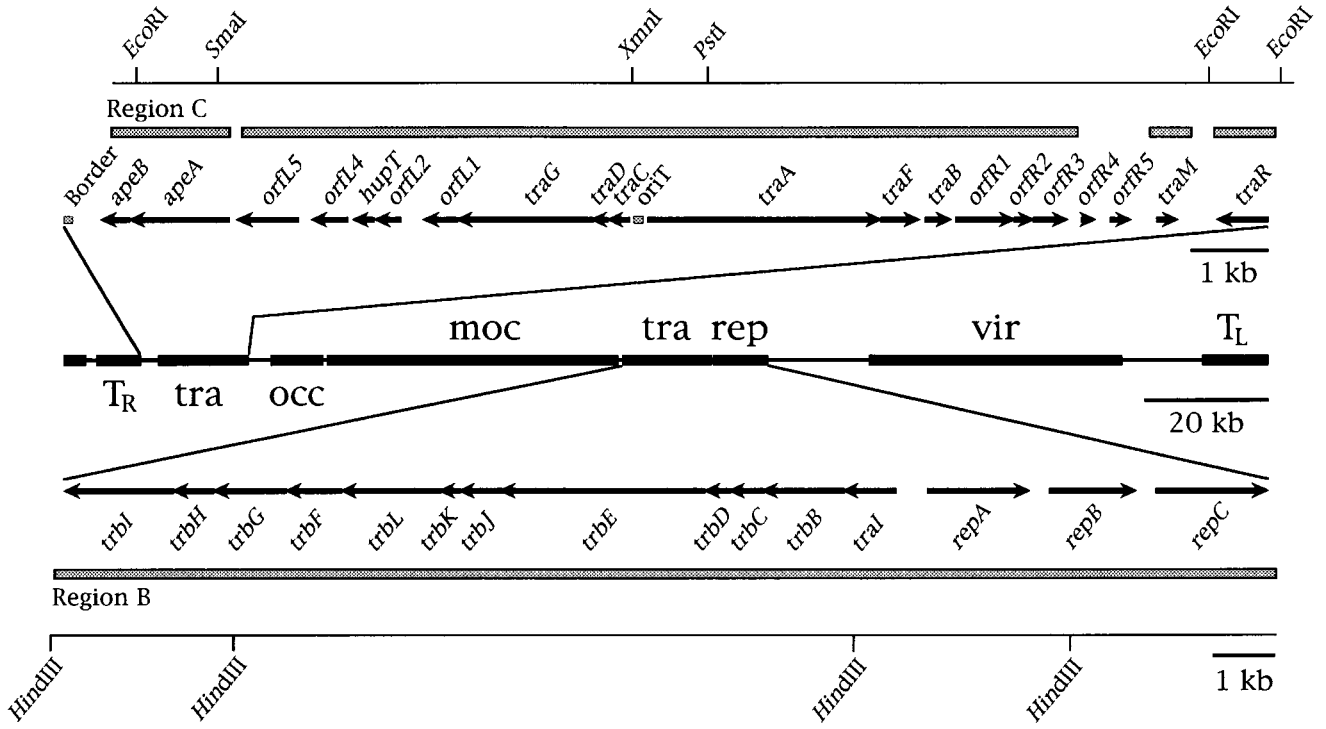

FIG. 1. Genetic map of the *tra* and *trb* clusters of octopine-type Ti plasmids. *traR* and *traI* encode quorum-sensing positive transcriptional regulators, while *traM* encodes a TraR antagonist. The other *tra* genes are thought to be required for conjugal DNA processing, and the *trb* genes are thought to encode a conjugal pore. *apeA* and/or *apeB* confers exclusion of bacteriophage AP-1. Other Ti plasmid loci include T_L and T_R (DNA that is transferred to plant nuclei), *occ* (octopine catabolism), *moc* (mannityl opine catabolism), *rep* (vegetative replication), and *vir* (required for T_L DNA and T_R DNA transfer). Arrows indicate the direction of transcription.

were sequenced with an ABI-373A Stretch automated DNA sequencer (ABI) and primers that hybridize to vector sequences. Custom-made primers that hybridize to Ti plasmid DNA were used as needed to complete the sequences. Sequencing reactions were carried out with *Taq* DNA polymerase and DyeDeoxy Terminator Sequencing kits (ABI). Data were analyzed by using ABI DNA analysis software (version 2.0.1). *Hin*dIII fragment 20 (coordinates 85.8 to 88.1) was subcloned from pYDH902 (11) into pBluescript SK(+) and subjected to automated sequencing with vector-specific and custom-made primers.

DNA segments between coordinates 18.85 and 26.45 and between 88.1 and 95.85 were subcloned from pYDH49E and pYDH902, respectively (11), into pBluescript SK(+) and subcloned again by using restriction sites within these fragments. The resulting plasmids were purified by using Qiagen-20 columns (Qiagen) and sequenced with an ALF automated DNA sequencer (Pharmacia). Sequencing reactions were carried out with 5′-fluorescein primers and either Auto-Read sequencing kits (Pharmacia) or Sequitherm cycle sequencing kits (Epicentre Technologies). Custom-made primers were used as needed to complete the sequences. The data were analyzed by using ALF Manager, version 2.5 (Pharmacia). All DNA sequences were determined on both strands. Final analysis of all sequences was carried out by using Lasergene (DNASTAR), Genepro (Riverside Scientific), BLAST (1), PILEUP, and PRETTYPLOT (12).

Localization of *oriT*. *Bam*HI fragments to be tested for *oriT* function were subcloned into the broad-host-range cloning vector pJA401.2 (a Km^r derivative of pCOS2EMBL [46]). A 1.1-kb *Xmn*I-*Pst*I fragment that lies within *Bam*HI-20 was subcloned between the *Sma*I and *Pst*I sites of pTZ19R (60), excised as an *Eco*RI-*Hin*dIII fragment, and inserted between the *Eco*RI and *Hin*dIII sites of pINIIA2 (42). The fragment was excised from the resulting plasmid as an *Xba*I-*Bam*HI fragment and introduced between the *Xba*I and *Bam*HI sites of pJA401.2, creating pJA431.1.

Plasmid pJA401.2 and its derivatives were introduced into strain C58C1(pGV2260) (7) and tested for conjugal transfer of Km^r in filter matings with *Escherichia coli* HB101 carrying the Tc^r plasmid pJA23.20 as described by Simon et al. (50). Donors and recipients were incubated overnight on nitrocellulose filters placed on AT medium containing 1 mg of octopine per ml, serially diluted, and plated on solid AT medium containing 15 μg of tetracycline and 12.5 μg of kanamycin per ml.

Visualization of the TraA protein. Codons 1 to 1046 of *traA* were subcloned downstream of an inducible *tac* promoter by digesting the plasmid pMS470Δ8 (2) with *Nde*I and *Hin*dIII and introducing *traA* sequences as a 3.4-kb *Bal*I-*Hin*dIII fragment in the presence of an oligonucleotide adapter. The fusion junction of the resulting plasmid (pJA480.32) contains the sequence AAGGAGATATA CATAGTGGCCATC, where the underlined bases represent adapter DNA. pJA480.32 was introduced into *E. coli* DS410, and minicells derived from the resulting strain were incubated with [^{35}S]methionine according to a published protocol (41).

Nucleotide sequence accession numbers. The nucleotide sequences determined in this study have been deposited in the GenBank DNA sequence database (accession numbers U43674 and U43675).

RESULTS

DNA sequences of two regions of pTiR10 required for conjugal transfer. Four regions of octopine-type Ti plasmids form heteroduplexes with the nopaline-type Ti plasmid pTiC58 that are visible with an electron microscope (15), and two of the these regions of pTiC58 (B and C) contain genes required for Ti plasmid conjugal transfer (4). The sequences of regions B and C of octopine-type Ti plasmids were partially determined in previous studies. *traR* and *traM* lie at the right end of region C (21, 22), while *traI*, *repA*, *repB*, and *repC* lie at the right end of region B (22, 52) (Fig. 1). In this study, we have completed the DNA sequencing of region B (10 kb of new sequence) and region C (13.25 kb of new sequence). Sequences of region B were derived by using pTi15955. Sequences of region C were derived by using two different Ti plasmids: coordinates 13.20 to 18.85 (13) were sequenced by using pTiA6NC DNA, while coordinates 18.85 to 26.45 were sequenced by using pTi15955 DNA. However, the physical maps of these plasmids are virtually identical (except for a large deletion in pTiA6NC; see below), strongly suggesting that their DNA sequences are extremely well conserved (13). From this analysis, we have identified 14 new open reading frames (ORFs) whose predicted products are homologous to known Tra proteins of other plas-

mids (see below) as well as 15 ORFs encoding proteins with other functions, some of which are unknown.

Eighteen previously unidentified ORFs were found to lie between the left end of region C and *traM* (Fig. 1), all of which would be transcribed divergently from the *oriT* site (described below). It is not known which of these ORFs are functional genes, but sequence similarities between several of their products and proteins found in other plasmids (see below) strongly suggest that at least these ORFs encode functional proteins. All ORFs in this region except *orfR4* and *orfR5* are located within regions that are conserved in pTiC58 (see Fig. 1, shaded bars at top).

A. tumefaciens strains containing either an octopine-type or a nopaline-type Ti plasmid are resistant to infection by bacteriophage AP-1. Five transposon insertions in an octopine-type Ti plasmid abolished resistance to this bacteriophage but did not affect conjugation (8). All insertions were localized within *Eco*RI fragment 1 and within *Sma*I fragment 3b and therefore were located within a 1.3-kb region near the left end of region C. This region was designated *ape* (for AP-1 exclusion). This 1.3-kb interval lies wholly within a 468-codon ORF, which we therefore tentatively designate *apeA*. It remains possible that the Ape phenotype requires the next ORF (designated *apeB*) in addition to (or even instead of) the *apeA* gene and that the five transposon insertions were Ape^- by virtue of polar effects upon *apeB*.

Eleven ORFs were identified between the left end of region B and *traI* (Fig. 1). All were oriented in the same direction as *traI* and could be transcribed as a single operon beginning with *traI*. Previous genetic studies indicate that *traI* is the first gene in an operon required for conjugation (22, 32). All of these genes are located within regions that are conserved in pTiC58 (see Fig. 1, shaded bar at bottom).

Each of these ORFs was used to search for homologous proteins in the dynamically translated GenBank-EMBL DNA sequence database using BLAST (1). The BESTFIT algorithm of the GCG DNA sequence analysis software (Genetics Computer Group, Madison, Wis.) (12) was used to determine the degree of similarity (Table 1), while the PILEUP and PRETTYPLOT algorithms (Genetics Computer Group) were used to align homologous pairs of proteins (Fig. 2). Many predicted proteins in these regions are homologous to proteins implicated in conjugal transfer of other plasmids. In particular, the closest homologs of TraG, TraF, and all Trb proteins are encoded by the IncP plasmid RP4 (43), and the corresponding Ti plasmid genes were therefore given names corresponding to their homologous genes in RP4. In several cases, the only known homolog is found in RP4. Furthermore, the order of Ti plasmid *trb* genes is the same as that of their RP4 counterparts except for one apparent translocation, since the *trbJ*, *trbK*, and *trbL* genes are the last three genes in the RP4 *trb* operon but lie further upstream in the octopine-type Ti plasmid (Fig. 1). Some of these predicted proteins are more weakly related to the Tra proteins of the IncN plasmid pKM101 (45) and the F plasmid (20), to Vir proteins encoded elsewhere on the Ti plasmid (55), and to the Ptl proteins of *Bordetella pertussis* (57), which secrete pertussis toxin. We also found that an ORF designated *hupT* could encode a protein that has a strong sequence similarity to the HU family of histone-like proteins (Fig. 3A). The ORFs *traB*, *traC*, and *traD* are not homologous to any known *tra* genes and were designated *tra* genes for correspondence with homologous genes from pTiC58 (17).

Although most of the predicted Tra and Trb proteins are most closely related to their IncP homologs, this is not true of TraA. TraA is not related to any Tra protein in RP4 but is related to Tra proteins in other plasmids. Surprisingly, the amino terminus of TraA is similar to a subset of these proteins while the central region of TraA is similar to a different subset (Table 1 and Fig. 2). The amino-terminal 370 amino acids of TraA show strong similarity to known or putative conjugal nickases of three mobilizable plasmids (pTF1, RSF1010, and pSC101) and to the nickase of the self-transmissible plasmid pIP501. This region shows far weaker similarity to the TrwC protein of R388. Figure 2 shows an alignment with the relatively well-characterized MobA protein of RSF1010. Within this region, the amino-terminal 200 amino acids are particularly well conserved.

The central 500 amino acids of TraA are most similar to the TraI protein of the F plasmid (20), to the TrwC protein of R388 (38), and to the TraHI protein of pKM101 (unpublished data). Figure 2 shows an alignment between this portion of TraA and the F plasmid TraI protein. TraI of the F plasmid is a bifunctional protein with nickase and helicase activities (58), and the region that is homologous to the Ti plasmid TraA is thought to contain the helicase activity (38). These alignments suggest that the Ti plasmid TraA protein has nickase and helicase activities and that it may have evolved by a fusion between a gene encoding a nickase and another gene encoding a helicase. The carboxyl-terminal 250 amino acids of TraA are not detectably related to any other known protein.

Visualization of a truncated *traA* product. TraA is an exceptionally large protein (1,100 amino acids; predicted molecular mass, 123.7 kDa). Interestingly, *traA* contains a possible internal translation start site at codon 847, which is preceded by a sequence (AAGGAG-N_9) whose sequence and position closely agree with those of the consensus *E. coli* ribosome binding site (28). If translation were to start here, a 265-amino-acid (29.7-kDa) protein would be produced. This region of TraA is located adjacent to its helicase domain, although the two regions have little if any overlap. To provide evidence that TraA translation extends well past codon 847 and to determine whether this gene has an internal translation start site, we attempted to visualize the product or products of this locus by using the plasmid pJA480.32. This plasmid contains codons 1 to 1046 of TraA (followed closely by a translation stop site within the vector sequences) and would therefore direct the synthesis of 117.4- and 23.4-kDa truncated proteins. Figure 4 shows that upon transcriptional induction, this plasmid directs synthesis of a 103-kDa protein and a 19-kDa protein. Both of these molecular masses correspond reasonably well with the predicted masses. We conclude that full-length TraA is at least this large and that translation probably starts at or near codon 847.

Localization of the *oriT* site of pTi15955. The *oriT* sequence of pTiC58 was previously localized to a 65-nucleotide fragment (6) (Fig. 3B). To determine whether the *oriT* of octopine-type plasmids is located at a similar site, we subcloned all *Bam*HI fragments of region C into the broad-host-range plasmid pCOS2EMBL and introduced the resulting plasmids into strain C58C1(pGV2260). pGV2260 is a self-transmissible deletion derivative of pTi15955 (7). Only *Bam*HI fragment 20 conferred *oriT* function (data not shown). A 1.1-kb *Xmn*I-*Pst*I fragment subcloned from *Bam*HI fragment 20 (creating pJA431.1) also was found to have a functional *oriT*. C58C1(pGV2260)(pJA431.1) transferred kanamycin resistance to an *E. coli* strain at an efficiency of 10^{-3} per donor after overnight matings.

The *Xmn*I site of this fragment (GAATCCCTTC) lies within the *traC* promoter (23) (Fig. 3B), while the *Pst*I site lies well within the *traA* structural gene. This fragment contains a region that is 72% identical to the minimal *oriT* of pTiC58 (Fig. 3B). Within this region, the left half is far more conserved than

TABLE 1. Similarities between Tra or Vir proteins of octopine-type Ti plasmids and other proteins

Protein	Homolog	Accession number	% Identity	% Similarity	No. of gaps	Alignment score[a]
TraA (aa 1–370)[b]	pIP501 ORF	L39769	30	57	16	188 (19.0)
	pTF1 MobL	X52699	31	49	9	186 (16.9)
	RSF1010 MobA	X04830	29	52	15	151 (6.7)
	pSC101 ORF	X01654	25	45	12	141.2 (6.6)
	R388 TrwC	X63150	20	47	11	141.9 (4.2)
	pTiA6NC VirD2	M14762	16	44	12	127.5 (2.9)
	RecD	X04582	17	41	10	134.3 (1.2)
	pKM101 TraHI	U43676	20	45	13	133.9 (0.9)
	F TraI	M54796	26	47	18	134.8 (0.2)
TraA (aa 371–875)[b]	pKM101 TraHI	U43676	27	48	16	230.1 (14.8)
	F TraI	U01159	28	54	24	229.7 (13.6)
	R388 TrwC	X63150	30	52	16	223.5 (11.9)
	RecD	X04582	29	54	18	183.7 (3.8)
	pSC101 ORF	X01654	23	48	19	133.4 (2.6)
	RSF1010 MobA	X04830	22	46	22	180.9 (1.0)
	pIP501 ORF	L39769	16	41	18	165.0 (0.1)
	pTiA6NC VirD2	M14762	18	44	16	139.3 (0.0)
	pTF1 MobL	X52699	23	48	14	131.4 (−0.3)
TraF	RP4 TraF	L27758	35	61	4	114.5 (18.3)
TraG	RP4 TraG	L27758	23	46	19	257.0 (13.0)
	R388 TrwB	X63150	24	51	22	209.4 (9.0)
	pTiA6NC VirD4	X06045	23	47	17	236.2 (7.0)
	pSK41 TraK	L19570	24	47	14	201.4 (6.0)
	F TraD	U01159	20	48	21	212.6 (1.8)
TrbB	RP4 TrbB	L27758	48	65	1	274.4 (54.9)
	PtlH	L10720	32	55	11	170.2 (18.6)
	pTiA6NC VirB11	J03216	32	57	12	174.7 (17.2)
	pKM101 TraG	U09868	32	56	11	162.1 (15.8)
	OutE	X70049	27	54	14	148.1 (7.5)
TrbC	RP4 TrbC	L27758	34	59	3	89.7 (12.6)
TrbD	RP4 TrbD	L27758	47	66	0	80.9 (17.9)
TrbE	RP4 TrbE	L27758	52	70	5	757.8 (87.6)
	pTiA6NC VirB4	J03216	23	48	23	354.5 (17.8)
	pKM101 TraB	U09868	22	45	19	339.4 (16.0)
	PtlC	L10720	23	49	25	347.4 (15.5)
	F TraC	U01159	21	46	35	293.6 (7.2)
TrbJ	RP4 TrbJ	L27758	23	47	6	125 (10.3)
TrbK	RP4 TrbK	L27758	21	36	0	30.3 (4.1)
TrbL	RP4 TrbL	L27758	27	55	8	231.4 (18.0)
	pKM101 TraD	U09868	22	53	12	152.2 (4.8)
TrbF	RP4 TrbF	L27758	38	58	3	158.2 (22.1)
TrbG	RP4 TrbG	L27758	44	65	4	223.9 (36.4)
TrbH	RP4 TrbH	L27758	33	65	7	65.2 (4.6)
TrbI	RP4 TrbI	L27758	36	55	8	280.8 (34.1)
	PtlG	L10720	27	49	11	167.8 (14.2)
	pKM101 TraF	U09868	27	51	12	184.9 (13.3)
	pTiA6NC VirB10	J03216	23	49	13	165.7 (9.3)
pTiA6 VirD2 (aa 1–200)[b]	RP4 TraI (aa 1–200)	L27758	25	44	5	70.3 (3.4)
pTiA6 VirD4	RP4 TraG	L27758	27	52	22	288.1 (17.8)
	pSK41 TraK	L19570	22	46	16	229.5 (12.0)
	F TraD	U01159	20	46	29	234.6 (6.2)
	R388 TrwB	X63150	19	44	17	190.2 (4.8)

[a] The BESTFIT algorithm (12) was used with a gap weight of 3.0 and a gap length weight of 0.1. Numbers in parentheses are *z* values (37), calculated by shuffling the second sequence in each line 50 times, aligning each shuffled sequence with the first sequence, subtracting the average of these scores from the original alignment score, and dividing by the standard deviation of the 50 alignment scores. *z* values of ≥10.0 are generally interpreted as compelling evidence of common ancestry, while *z* values of ≥5.0 are interpreted as good evidence of common ancestry, particularly when additional information supports this conclusion (14).

[b] aa, amino acids.

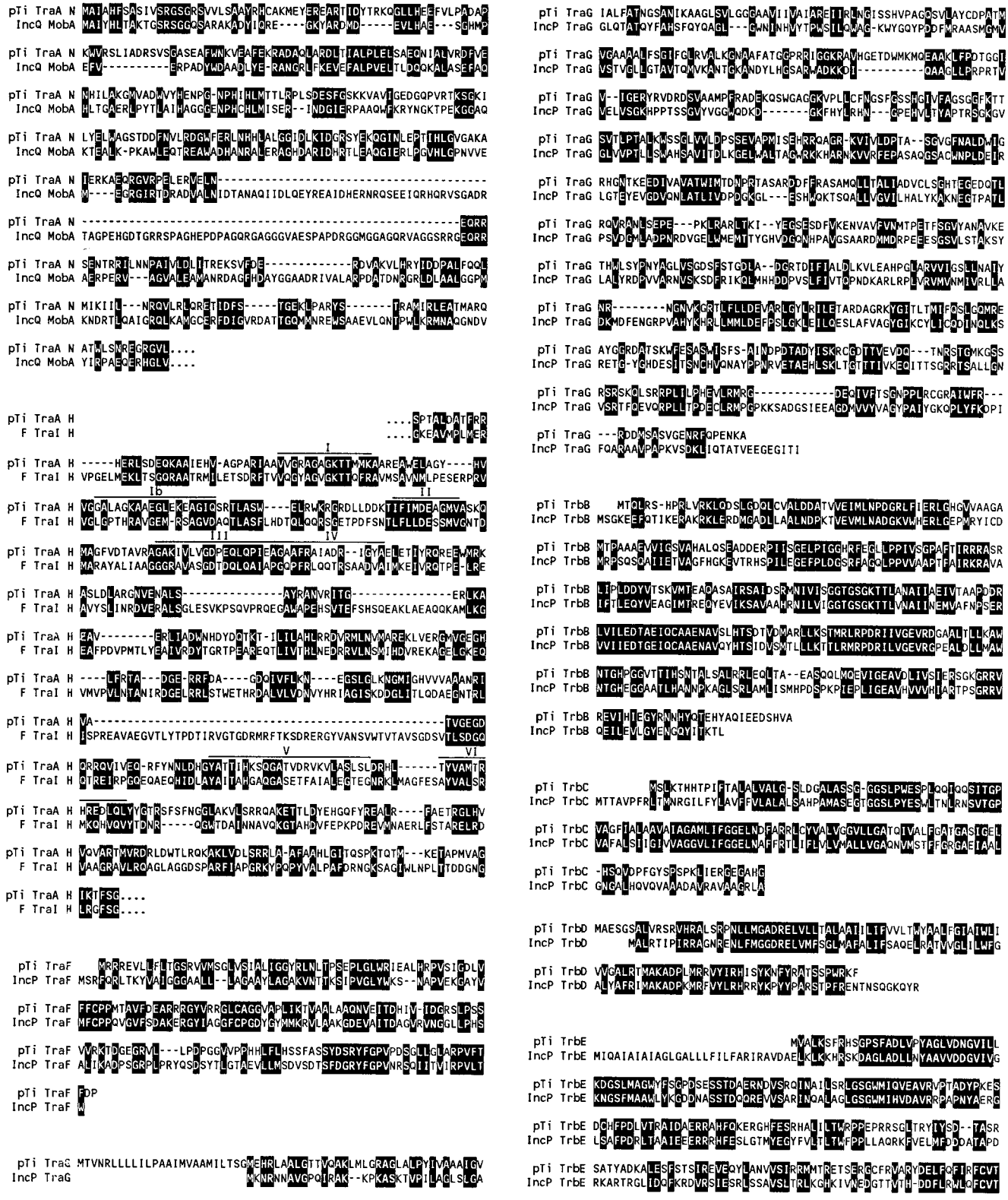

FIG. 2. Alignments of predicted proteins encoded by the *tra* and *trb* regions of octopine-type Ti plasmids with those of *tra* systems of other plasmids. The PILEUP and PRETTYPLOT algorithms were used with default settings, except that the threshold was 0.45. pTi TraA N refers to the putative nickase domain of TraA, while pTi TraA H refers to the putative helicase domain of TraA. Amino acids that are identical or conserved are shown as white letters on a black background. Dashes were introduced to optimize alignments, and bars over sequences indicate motifs conserved among a family of bacterial helicases.

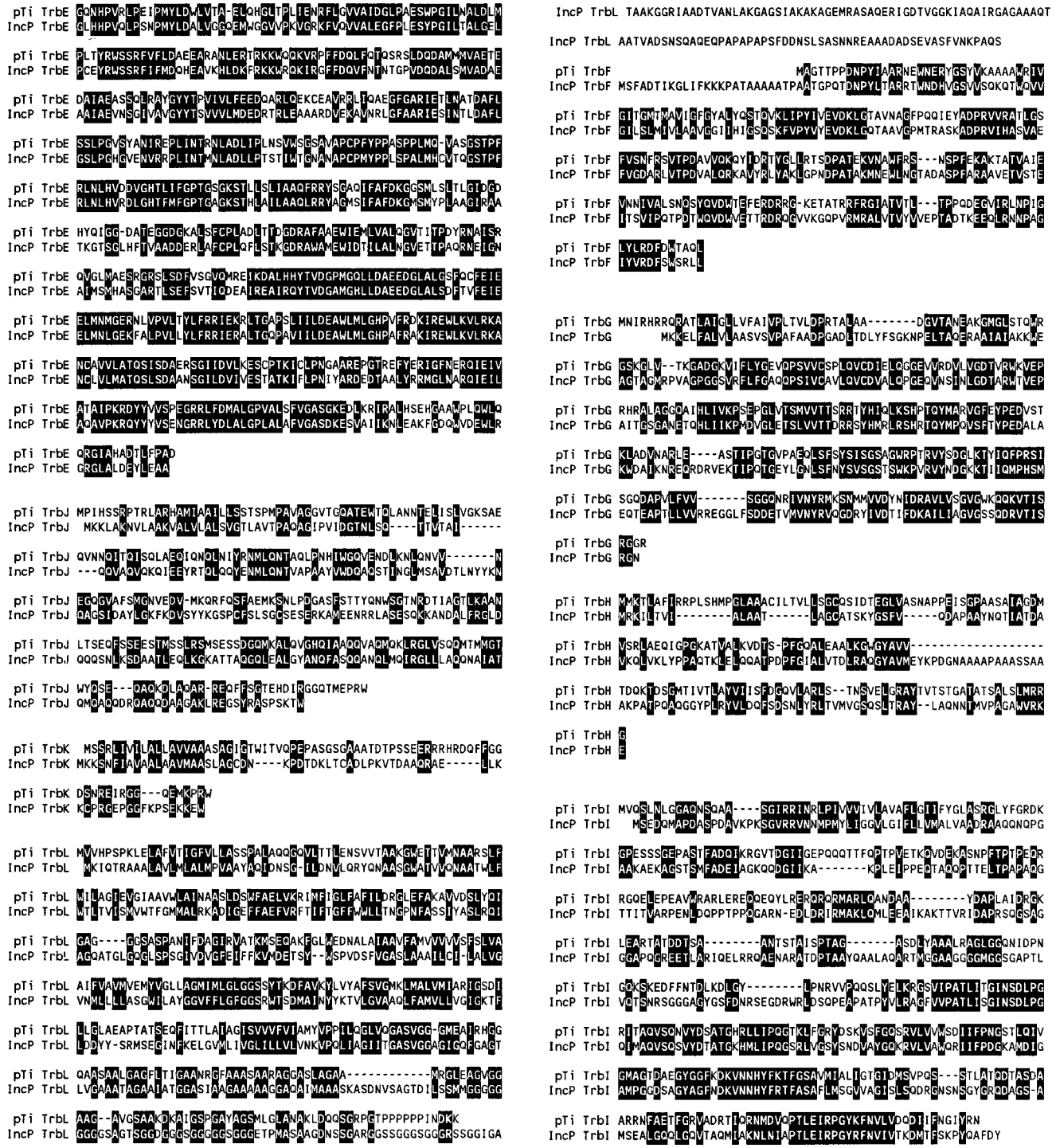

FIG. 2—*Continued.*

the right half and also is similar to the *oriT* sites of other plasmids (6) (Fig. 3B, shaded box), suggesting that it might provide a binding site for the conjugal nickase. This region lies immediately downstream of the *traA* transcription start site (23).

pTiA6NC contains a deletion of the *trbI* gene. While most Ti plasmids are able to conjugate, the widely studied plasmid pTiA6NC is conjugation defective (unpublished observations). This plasmid was previously reported to have a 12.65-kb deletion relative to most other octopine-type Ti plasmids (34, 49). Another wild-type octopine-type Ti plasmid, pTi89.10, has a very similar (or possibly identical) deletion (9). Although this deletion lies mostly within the *mac* region, which is required for catabolism of mannopinic acid, its right end appeared to extend into the *trb* region. We cloned a 3.1-kb *Bam*HI fragment from pTiA6NC that contains both endpoints of this de-

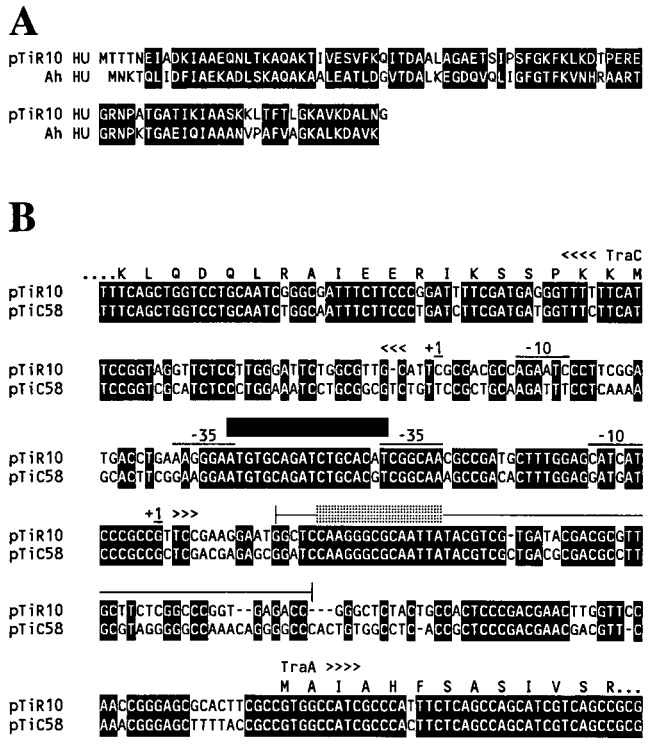

FIG. 3. Alignment of DNA sequences of pTiR10 or proteins encoded by pTiR10 and homologous sequences. (A) Sequence similarity (43% identity) between the predicted protein encoded by the *hupT* ORF and the Hu protein of *Aeromonas proteolytica* (27). (B) Alignment of DNA sequences of octopine-type and nopaline-type Ti plasmids in the region containing the *traA* and *traC* promoters and the pTiC58 *oriT*. The solid bar represents a probable binding site for the TraR transcriptional activator (23), the single horizontal line represents the minimal *oriT* site of pTiC58, the shaded bar represents sequence similar to the *oriT* sites of other plasmids (6), and the arrowheads indicate the direction of translation. (C) Sequence similarity (47% identity) between the predicted product of an ORF in the *moa* region of pTiR10 and the HyuA protein of *Pseudomonas* sp. strain NS671 (35), which is one subunit of an enzyme that converts 5′-substituted hydantoins to the corresponding *N*-carbamyl amino acids. In all three panels, amino acid or nucleotide sequences that are identical (or, for amino acids, conserved) are indicated by white letters on a black background.

letion. By sequencing the left end of this fragment, we precisely identified the endpoints of this deletion. The right endpoint of this deletion lies at codon 46 of *trbI*, indicating that this deletion removed 387 codons of this gene. Although we have not proven that *trbI* is required for conjugation, the deletion of this gene probably accounts for the conjugation defect of this Ti plasmid.

To determine whether pTiA6NC is capable of detectable conjugation, we assayed conjugal transfer of the Occ phenotype by A348 (which carries this Ti plasmid) after extended mating in the presence of high levels of octopine. Conjugation was not detected (fewer than 10^{-8} transconjugants resulted per donor; data not shown). Conjugation of other Ti plasmids is strongly elevated by overexpression of the Ti plasmid TraR

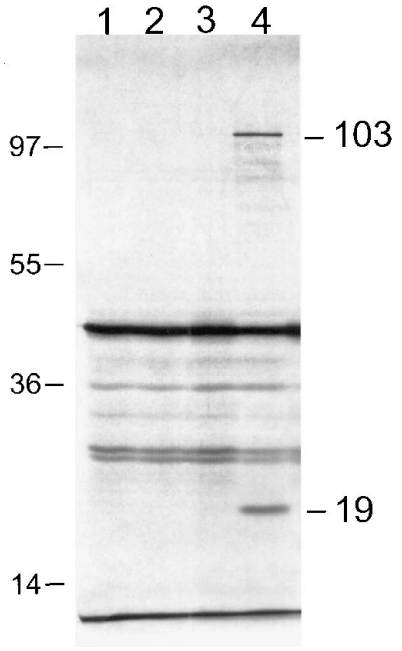

FIG. 4. Visualization of the TraA protein. Minicells obtained from DS410(pMS470Δ8) (lanes 1 and 2) or DS410(pJA480.32) (lanes 3 and 4) were incubated in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 1 mM isopropyl-β-D-thiogalactopyranoside prior to labeling with [^{35}S]methionine. Radiolabeled proteins were size fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The numbers on the left are molecular mass standard positions (in kilodaltons).

protein (22). To determine whether overexpression of TraR could allow detectable conjugation of pTiA6NC, we introduced a multicopy plasmid (pCF218) containing TraR fused to a foreign promoter (22). A348(pCF218) was able to conjugally transfer Occ, although the efficiencies were about two orders of magnitude lower than those for R10(pCF218) (Table 2). This finding indicates that *trbI* is not absolutely essential for conjugation but is required for wild-type conjugal efficiencies. Residual levels of conjugation might be due to some other protein that can functionally substitute for TrbI. One candidate would be VirB10, which is homologous to TrbI, although the *virB10*

TABLE 2. Partial restoration of conjugation of pTiA6NC by overexpression of the *traR* gene of pTiR10

Strain	Genotype[a]	Conjugation efficiency[b] determined on	
		ATGN[c]	ATO[d]
A348(pSW172)[e]	pTiA6NC, vector	$<1 \times 10^{-8}$	$<5 \times 10^{-8}$
A348(pCF218)[f]	pTiA6NC, *traR*^{mc}	1.1×10^{-4}	3.0×10^{-4}
R10(pSW172)	pTiR10, vector	$<1 \times 10^{-8}$	5×10^{-3}
R10(pCF218)	pTiR10, *traR*^{mc}	6.5×10^{-2}	5×10^{-2}

[a] mc, multicopy.
[b] Occ$^+$, rifampicin-resistant, streptomycin-resistant transconjugants per input donor after overnight mating with *A. tumefaciens* C58C1RS (see reference 21 for experimental details).
[c] Overnight mating on solid medium containing AT salts and buffer amended with 0.5% glucose and 15 mM $(NH_4)_2SO_4$.
[d] Overnight mating on solid medium containing AT salts and buffer amended with 2 mg of octopine per ml.
[e] pSW172 is an IncP broad-host-range vector (5).
[f] pCF218 is a derivative of pSW172 that overexpresses TraR.

gene was probably expressed at extremely low levels during these experiments.

In the course of localizing the 12.65-kb deletion in pTiA6NC, we obtained the sequence of 351 nucleotides that lie just to the left of this deletion and are localized in the *moa* region (kilobase coordinates 74.7 to 75.0 [13, 17]). This fragment could encode a protein that shows strong sequence similarity (47% identity at the amino acid level) (Fig. 3C) to an internal region of the *hyuA* gene of *Pseudomonas* sp. strain NS671. *hyuA* encodes one subunit of the enzyme hydantoinase, which directs the conversion of 5′-substituted hydantoins to the corresponding *N*-carbamyl-L-amino acids (35).

DISCUSSION

The primary goal of this study was to identify the predicted *tra* and *trb* genes of octopine-type Ti plasmids and to compare them with homologous genes of other plasmids. We have not determined which of these genes are essential for conjugation or whether these genes are sufficient for conjugation. However, we have identified a homolog for virtually every RP4 *tra* or *trb* gene that is essential for RP4 conjugation (43). The only exceptions are the RP4 *traI*, *traJ*, and *traK* genes. *traI* encodes the *oriT*-specific nickase, while the *traJ* product helps TraI recognize *oriT*; the *traK* product also binds to *oriT* (61). In place of *traI*, we found a member of a different family of nickases, while the Ti plasmid *traB*, *traC*, and/or *traD* could encode products functionally analogous to the RP4 *traJ* and *traK* products. These data provide suggestive evidence that we have identified all essential *tra* genes. It also remains unclear whether each of the *tra* genes identified here is essential for conjugation. Nonpolar mutations in each gene and extensive complementation analysis will be required to address this question, and this remains for future studies.

From the *tra* sequences determined here, we can make hypotheses about the evolution of these genes and the evolution of Ti plasmids in general. Most of the predicted Tra proteins and all of the predicted Trb proteins resemble RP4 Tra proteins, while TraA has no counterpart in RP4 but has homologs in several other transfer systems. Of the proteins homologous to TraA, the bifunctional 180-kDa TraI protein of the F plasmid is the only one studied at the biochemical level. TraI is an *oriT*-specific nickase and remains covalently bound to the 5′ terminus of the transferred strand. TraI also has DNA helicase activity (this protein also is known as helicase I [40, 47]). Sequence analysis suggests that the amino-terminal half of TraI contains the nickase activity while the carboxyl-terminal half contains the helicase activity (38). It is interesting that most characterized *tra* systems are predicted to encode related helicases (including IncW, IncQ, IncN, and the Ti plasmid *tra* region), and it is possible that those systems that lack helicases (IncP and Ti plasmid *vir*) may rely on host-encoded proteins for this activity. The *traI* gene of the F plasmid also encodes a 94-kDa protein (designated TraI*) which starts at an in-phase internal initiation codon (53) and is predicted to contain helicase activity. We do not find a plausible internal translation start site at or near the corresponding site of TraA, suggesting that if TraA contains both nickase and helicase activities, these functions must be tethered together in a single protein. As described above, we have provided evidence that TraA does have an internal translation start site localized much closer to the carboxyl terminus.

As described above, TraF, TraG, and all Trb proteins are most closely related to Tra proteins in RP4. TraF of RP4, although genetically linked to genes required for conjugal DNA processing, is itself required for the mating bridge synthesis or function, since mutations in it block sensitivity to bacteriophages that bind to the pilus (19). TraG is highly conserved in all known conjugation systems, and all TraG homologs have hydropathy profiles that suggest an integral membrane topology. The homologous protein of F (TraD) binds single-stranded DNA in vitro and is required for efficient DNA replacement synthesis in donor cells (33). TrbB and TrbD, like their IncP counterparts, have nucleotide binding motifs (36) and may provide energy for the export of DNA or the export of other Trb proteins.

While most of these proteins are rather strongly conserved with RP4 proteins, TrbK and TrbH are only weakly conserved. TrbK has been implicated in preventing conjugal entry of other IncP plasmids (39). TrbK is sufficient for this phenotype when overexpressed but requires TrbJ when expressed at low levels. TrbJ also is essential for conjugation. TrbK of RP4 has a lipid attachment motif and has been postulated to be a lipoprotein (36). In contrast, TrbK of the Ti plasmid lacks this lipid attachment motif and in particular lacks the cysteine that is the putative site of lipid attachment. These striking differences between the TrbK proteins of the two systems suggest that the entry exclusion system of the octopine Ti plasmid might function poorly or not at all. In preliminary assays in which these genes were expressed in a conjugal recipient, we were unable to detect entry exclusion (data not shown). Lack of entry exclusion could play a significant role in Ti plasmid biology, since Ti plasmid conjugation is stimulated by high densities of conjugal donors (23). This suggests that donors might conjugate with other donors; such matings would be prevented by an entry exclusion system.

We have seen that the Ti plasmid *tra* region appears to have evolved by appropriating *tra* genes from at least three sources. Remarkably, the *vir* regulon appears to have had a similar evolutionary history. The *virB*-encoded putative mating bridge is closely related to the mating bridge of the IncN plasmid pKM101 and to the Ptl protein secretion system of *B. pertussis*, while it is much more weakly related to the RP4-encoded Trb mating bridge (45). In contrast, the VirD2 and VirD4 genes show the closest similarity to their counterparts in IncP plasmids (Table 1), and the T-DNA borders, which are nicked by VirD1 and VirD2, are similar to the *oriT* of IncP plasmids but not to other *oriT* sequences (56). The similarity between VirD2 and TraI of RP4 is weak but is probably significant, since the amino termini and active-site tyrosine residues are correctly aligned. Furthermore, unlike most other nickases of conjugal plasmids, both VirD2 and TraI of RP4 lack helicase domains.

We propose that an ancestor of the Ti plasmid may have arisen by the fusion of two conjugal plasmids and that one *tra* system in this fusion retained its original function while the other could have evolved to transfer DNA to plant nuclei (Fig. 5). According to this model, a plasmid with an IncN-type *tra* region may have fused with a plasmid that had an IncP-type *tra* system. The IncN-type mating bridge would then have evolved to transfer DNA to plant cells, while the IncP-type mating bridge would have retained its original function. As described above, the mobilization proteins of the Vir region resemble IncP-type mobilization functions. Conversely, the nickase domain of TraA resembles the MobA protein of the IncQ plasmid RSF1010, while the helicase domain of TraA resembles the helicases of IncN, IncW, and IncF plasmids. It is apparent that several DNA translocations would be required to create these chimeric transfer systems. Part, but not all, of this genetic reassortment could have occurred by a simple exchange of nickase functions between the two transfer systems.

Our finding that a sequence fragment found between kilobase coordinates 74.7 and 75.0 is similar to an internal frag-

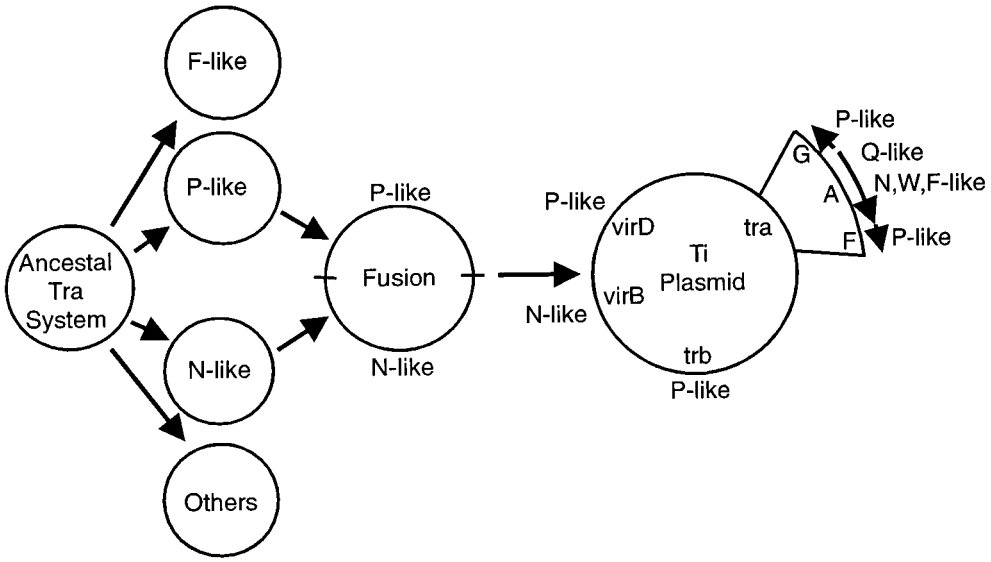

FIG. 5. A possible scheme for the evolution of Ti plasmids, in which divergent evolution of an ancestral conjugal transfer system led to several homologous systems. All or part of a plasmid containing an IncN-like (N-like) *tra* system is postulated to have become fused to all or part of a plasmid having an IncP-like (P-like) *tra* system. Subsequently, these systems diverged in function such that the mating bridge of the IncN-like system evolved into the *virB* operon while the mating bridge of the IncP-like system retained its original function in conjugation. At some point in evolution, the *vir* system appears to have acquired an IncP-like nickase while the *tra* system appears to have acquired a dual-function protein with nickase and helicase activities. The helicase domain could have been obtained from an IncN-like, IncW-like (W-like), or IncF-like (F-like) plasmid, while the nickase domain is related to a third group of nickases that includes MobA of the IncQ plasmid RSF1010.

ment of a hydantoinase gene could be interpreted as evidence of catabolism of a similar substrate. Evidence of such genes or enzymatic activities in *A. tumefaciens* has been reported (35, 48). However, this region of the Ti plasmid is known to direct the catabolism of mannityl opines (Fig. 1). A fragment spanning kilobase coordinates 70.2 to 88.1 is sufficient for catabolism of mannopinic acid, and two subclones of this region (spanning kilobases 70.2 to 75.25 and 75.25 to 88.1) are both essential (18). As might be expected, pTi89.10, which has a deletion similar or identical to that of pTiA6NC, does not direct catabolism of this opine (9). Hydantoinases hydrolyze a cyclic CO-N (amide) bond, while mannopinic acid is catabolized by the hydrolysis of an H_2C-N bond (9). Although these reactions seem dissimilar, it nevertheless is plausible that the Ti plasmid-encoded hydantoinase homolog could be required for mannopinic acid catabolism.

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