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Essential Motifs of Relaxase (TraI) and TraG Proteins Involved in Conjugative Transfer of Plasmid RP4

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Two essential transfer genes of the conjugative plasmid RP4 were altered by site-directed mutagenesis: *traG* of the primase operon and *traI* of the relaxase operon. To evaluate effects on the transfer phenotype of the point mutations, we have reconstituted the RP4 transfer system by fusion of the transfer regions Tra1 and Tra2 to the small multicopy replicon ColD. Deletions in *traG* or *traI* served to determine the Tra phenotype of mutant plasmids by *trans* complementation. Two motifs of TraG which are highly conserved among TraG-like proteins in several other conjugative DNA transfer systems were found to be essential for TraG function. One of the motifs resembles that of a nucleotide binding fold of type B. The relaxase (TraI) catalyzes the specific cleaving-joining reaction at the transfer origin needed to initiate and terminate conjugative DNA transfer (W. Pansegrau, W. Schröder, and E. Lanka, Proc. Natl. Acad. Sci. USA 90:2925–2929, 1993). Phenotypes of mutations in three motifs that belong to the active center of the relaxase confirmed previously obtained biochemical evidence for the contributions of the motifs to the catalytic activity of TraI. Expression of the relaxase operon is greatly increased in the absence of an intact TraI protein. This finding suggests that the relaxosome which assembles only in the presence of TraI in addition to its enzymatic activity plays a role in gene regulation.

Plasmids of incompatibility group P (IncP) are conjugative broad-host-range plasmids carrying multiple antibiotic resistance genes. The prototype IncP plasmid RP4 (60,099 bp [42]) was discovered in a clinically notable *Pseudomonas* strain. RP4 can replicate in diverse gram-negative organisms, but the host range defined by its conjugative transfer ability is considerably greater than the range of vegetative replication proficiency. Suitable vector constructions carrying the IncP transfer system mediated transfer from gram-negative organisms to gram-positive bacteria and to yeasts. Evolutionary and functional relationships to other conjugative DNA transfer systems include Ti/Ri plasmids that direct T-DNA transfer from agrobacteria to plant cells (27, 29, 42, 43). These properties recommend RP4 as a model system for studying conjugative processes (for reviews, see references 15, 17, and 42).

Functions responsible for RP4 transfer are encoded by two distinct regions of the genome, designated Tra1 and Tra2. Genetic analyses recently identified the core of Tra1 and Tra2 needed for intraspecific conjugative DNA transfer in *Escherichia coli* (26, 28). Transfer functions are classified in two groups: the DNA transfer and replication system (Dtr) and the apparatus of mating pair formation (Mpf) responsible for the donor-recipient contact during conjugation. Mpf genes map in Tra2 and include the *traF* gene of Tra1. Tra1 codes for all of the Dtr genes, in particular for components of the relaxosome, the initiation complex of the transfer replication (39, 56).

Since the contribution of each of the Tra1 genes to the transfer phenotype has been evaluated recently, we are extending our studies to the more detailed understanding of the structure-function relationship of single essential Dtr components. Site-directed mutagenesis of *tra* genes resulting in single amino acid replacements serves as an experimental approach.

Here we describe the effect on the transfer phenotype of *traG* and *traI* point mutations. Both TraG and TraI fulfill key functions in conjugative DNA transfer. TraG is the only RP4-encoded component needed for mobilization of IncQ plasmid RSF1010 in addition to the Mpf system (28, 57). Furthermore, TraG is proposed to interact with both the relaxosome and the membrane components involved in the DNA transfer process (28, 30). TraI is needed for relaxosome formation and mediates site- and strand-specific cleavage at the transfer origin (*oriT*) by which TraI becomes covalently attached to the 5′ end of the DNA strand destined for transfer to recipient cells (44). The covalent TraI-DNA complex functions as an intermediate in the pathway for single-stranded DNA transmission proposed to occur through a rolling circle mechanism (44, 58).

For convenient quantitative analyses of the transfer phenotype by complementation, we reconstituted the RP4 transfer system by fusion to the ColD replicon. Mutant *tra* genes were supplied on plasmids in *trans*. The differential pattern of transfer phenotypes of 5 *traG* and 14 *traI* mutations is discussed. A quantitative immunological analysis of *traI* deletion derivatives demonstrated that TraI might also play a role in the regulation of expression of the relaxase operon.

MATERIALS AND METHODS

Nomenclature. RP4 Tra1 genes were designated *tra* (42, 62); Tra2 genes were designated *trb* (27, 42).

Bacteria, bacteriophages, and plasmids. *E. coli* strains used in this study were SCS1 [*endA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1 recA1 gyrA96 relA1*], a DH1 derivative (19); HB101 (*hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44*) (4); HB101 Nx^r, a spontaneous nalidixic acid-resistant derivative of HB101; and BMH71-18 {*thi supE* Δ(*lac-proAB*) *mutS*::Tn*10* [F′(*proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15*)} (61). Phages Pf3 (49), PRR1 (36), and PRD1 (35) were propagated as described previously (49). Plasmids are listed in Table 1.

Media. Cells were grown in yeast-tryptone medium (YT) (33) buffered with 25 mM 3-(*N*-morpholino)propanesulfonic

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TABLE 1. Plasmids used in this study

Plasmid	Selective marker(s)[a]	Relevant genotype	Replicon	Description[b]	Size (bp)	Reference or source
pBR329	Ap, Cm, Tc	*oriT*^{0}	pMB1	Cloning vector	4,151	7
pBS140	Ap	*traG*$^+$	pMB1	pJF119HEΩ[RP4 *Hin*cII-*Sph*I 48.710–46.495 kb[c]]	7,502	57
pBS141	Ap	*traG*$^+$	RSF1010	pMMB67HEΩ[RP4 *Hin*cII-*Sph*I 48.710–46.495 kb]	11,040	This work
pDB10	Ap, Cm	*traF-traM*[d] *oriT*	pMB1	pBR329Ω[*Bam*HI; RP4 *Bfa*I-*Bfa*I 45.893–53.462 kb]	11,744	This work
pDB10*traI:Afl*II	Ap, Cm	*traF-traM oriT traI*$^+$	pMB1	pDB10[RP4 *traI:Afl*II 50.660 kb]	11,744	This work
pDB11	Ap, Cm	*traF-traM oriT traG*^{0}	pMB1	pDB10Δ[RP4 *Sfi*I-*Ssp*I 48.374–46.670 kb]	10,037	This work
pDB12	Ap, Cm	*traF-traM oriT traI*^{0}	pMB1	pDB10*traI:Afl*II Δ[RP4 *Afl*II-*Afl*II 50.660–50.052 kb]	11,141	This work
pDB12m	Ap, Cm	*traF-traM oriT traI*^{0}	pMB1	pDB12Ω[*Afl*II$_i$ MURFI linker]	11,155	This work
pDB126	Cm	*traF-traM oriT trbB-trbM*[e]	ColD	pML123Ω[*Bam*HI; RP4 *Bfa*I 45.893–53.462 kb]	22,666	This work
pDB127	Cm	*traF-traM oriT trbB-trbM traG*^{0}	ColD	pDB126Δ[RP4 *Sfi*I-*Ssp*I 48.374–46.670 kb]	20,959	This work
pDB128	Cm	*traF-traM oriT trbB-trbM traI*^{0}	ColD	pDB126*traI:Afl*II Δ[RP4 *Afl*II-*Afl*II 50.660–50.052 kb]	22,063	This work
pDB128m	Cm	*traF-traM oriT trbB-trbM traI*^{0}	ColD	pDB128Ω[*Afl*II$_i$ MURFI linker]	22,077	This work
pDB173	Ap	*traI*$^+$	pMB1	pMS119HEΩ[T7 gene *10* SD, *Nde*I-*Ear*I adaptor, RP4 *Ear*I-*Ssp*I 50.656–48.374 kb]	7,653	39
pML123	Cm	*trbB-trbM*[e]	ColD	pGZ119EHΩ[*Eco*RI-*Bam*HI; *Eco*RI-*Xmn*I adaptor, RP4 *Xmn*I-*Not*I 18.841–30.042 kb]	15,073	28
pVWDG23110Δ0.1	Km	*traA-upf54.8*	pMB1	pBR329Δ[*Pst*I-*Bam*HI 2.755–0.606 kb]Ω[RP4 *Pst*I-*Apa*I 37.942–54.709 kb]	18,926	28

[a] Ap, ampicillin; Cm, chloramphenicol; Tc, tetracycline; Km, kanamycin.
[b] Cloning vectors pJF119HE (pMB1 replicon [12]), pMMB67HE (RSF1010 replicon [12]), pMS119HE (1), and pGZ119EH (ColD replicon [26]) were described elsewhere.
[c] RP4 coordinates of inserted fragments are given (42).
[d] Contains *traH*, *traL*, and *traM* and the Tra1 region essential for intraspecific *E. coli* matings, called the Tra1 core (*traF*, *traG*, *traI*, *traJ*, and *traK*).
[e] Tra2 region: *trbB*, *trbC*, *trbD*, *trbE*, *trbF*, *trbG*, *trbH*, *trbI*, *trbJ*, *trbK*, *trbL*, and *trbM*.

acid (sodium salt; pH 8.0) and supplemented with 0.1% glucose and 25 μg of thiamine hydrochloride per ml. Appropriate antibiotic supplements used for selection of bacteria were ampicillin (sodium salt; 100 μg/ml); chloramphenicol (10 μg/ml), kanamycin sulfate (30 μg/ml), and nalidixic acid (sodium salt; 30 μg/ml).

DNA methodology. Standard molecular cloning methods were used as described previously (47). For plasmid DNA sequencing, templates were purified by ethidium bromide-CsCl gradients and denatured by alkali (0.2 M NaOH). DNA sequencing was performed by using T7 Sequenase (Pharmacia) and specific synthetic oligonucleotides 18 to 25 nucleotides in length as primers.

Preparation of the substrate oligonucleotide for phosphoamino acid analysis of TraI oligonucleotide adducts. A 13-mer oligodeoxyribonucleotide (TTCACCTATCCTG, 1 nmol) was labeled at the 3′ end with subsaturating amounts of [α-^{32}P]dCTP (2.5 MBq, 110 TBq/mmol, 22.5 pmol), using terminal deoxynucleotidyltransferase (Amersham). Following incubation at 37°C for 90 min under the conditions recommended by the manufacturer, unlabeled dCTP (10 nmol) and a second aliquot of terminal deoxynucleotidyltransferase were added. Incubation was continued for 60 min, then the chase reaction was stopped by addition of EDTA (final concentration 50 mM), and the labeled oligonucleotide was purified by gel filtration.

Generation of mutations. *traI* and *traG* mutants used for complementation analyses were generated by site-specific oligonucleotide-directed mutagenesis as described by Sayers et al. (48). Oligonucleotide primers applied for *traI* mutagenesis were Y22L (GCTGGTGAAGCTCATCACCGA), Y22R (GCTGGTGAAGCGCATCACCGA), and Y22F+T24A (GCTGGTGAAAGTTCATCGCCGAACGAGC); those for *traG* mutagenesis were K187T (CGCTCCGGCACGGGCGTCGGC), K209T (CACCGACCTGACAGGCGAGTT), E211Q (CCTGAAAGGCCAGTTGTGGGC), D449N (GATGATGCTCAACGAGTTCCC), and K456T (GCCTGGGCACGCTCGAAATCCC). Deviations from the wild-type RP4 sequence (62) are underlined. Oligonucleotides used for generation of other *traI* mutations have been described previously (45). Following mutagenesis, base exchanges introduced both in *traI* and in *traG* were verified by nucleotide sequencing.

To introduce a new restriction site (*Afl*II) at the 5′ end of *traI*, the method of Deng and Nickoloff (9) was used. Two mutagenic oligonucleotide primers were annealed to a denatured plasmid target. One primer contained the *Afl*II site (GTCGCTTTCTTAAGGGAGCGCATGGGG); the second contained a mismatch changing the unique *Pst*I restriction site of the vector to a sequence not cleavable by *Pst*I (GTTGCCATTGCTCGAGGCATCGTGG). T4 DNA polymerase and T4 DNA ligase served to extend the primers and to ligate the ends of the polymerization products. The heteroduplex plasmids formed in this way cannot be linearized by *Pst*I. The mismatch repair-defective *E. coli* BMH71-18 was transformed

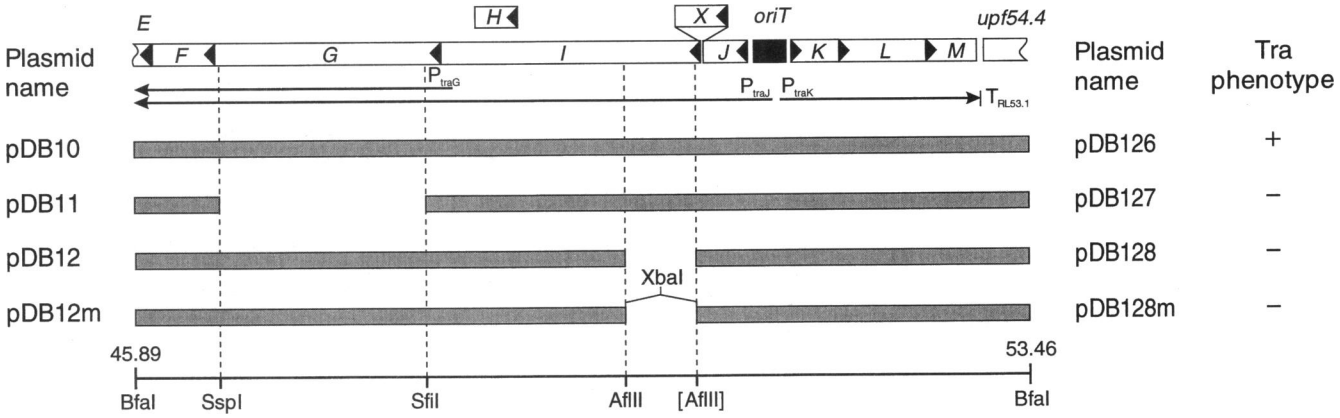

FIG. 1. Physical and genetic map of the RP4 Tra1 core region and notation of *traI* and *traG* deletion derivatives. The gene organization is shown at the top; genes are drawn as bars, and arrowheads mark the gene starts. *E* through *M*, *tra* genes; *upf54.4*, an unknown plasmid function (42); P, promoters (arrows indicate transcripts); T, a rho-independent terminator. To construct a plasmid carrying the RP4 Tra1 core region, RP4 DNA was digested with *Bfa*I. The *Bfa*I fragment B mapping between RP4 kilobase coordinates 45.893 and 53.462 was isolated and inserted into the *Bam*HI site of pBR329. To connect the *Bfa*I and *Bam*HI-created ends, a *Bam*HI-*Bfa*I adaptor which consists of two synthetic oligonucleotides (GATCCGGTACCCC and TGGGGTACCG) and contains a *Kpn*I site was used. The resulting plasmid, pDB10, served for generation of deletion derivatives. Isolation of pDB10 fragments *Ssp*I-*Sal*I and *Sal*I-*Sfi*I (*Sfi*I ends blunted by T4 DNA polymerase) led to pDB11 after ligation. pDB11 lacks 1,707 bp of *traG* between the *Sfi*I and *Ssp*I sites. Site-directed mutagenesis of pDB10 *traI* gene from CTTGAT to CTTAAG (*Afl*II) as described in Materials and Methods resulted in plasmid pDB10*traI*:*Afl*II. Excision of the 603-bp *Afl*II fragment of pDB10 *traI*:*Afl*II and religation yielded in the *traI* deletion derivative pDB12. Generation of blunt ends at the unique pDB12 *Afl*II site by T4 DNA polymerase followed by MURFI linker insertion (46) led to pDB12m. In the case of pDB12m, the unique *Xba*I site introduced by the MURFI linker is depicted. Shaded bars represent Tra1 parts present in pDB10 and its deletion derivatives pDB11 and pDB12/pDB12m. Their construction permitted convenient subcloning of the Tra1 core region by inserting the corresponding *Bam*HI fragments into pML123 (28). The resulting plasmid, pDB126, contains the core RP4 transfer system (Fig. 2), including all essential parts of regions Tra1 and Tra2 required for intraspecific *E. coli* matings. Derivatives pDB127 and pDB128/pDB128m are devoid only of *traG* and *traI*, respectively. At the right, plasmid names and corresponding transfer phenotypes are listed. +, transmission frequency (number of transconjugants per donor cell per hour) of approximately 5×10^{-1}; −, transmission frequency of $<1 \times 10^{-7}$. A physical map of plasmid RP4 between kilobase coordinates 45.893 and 53.462 with restriction sites relevant for deletion mutagenesis is shown at the bottom.

with the *Pst*I-treated DNA, allowing the nonlinearized heteroduplex plasmids to establish and to segregate. Recovered plasmid DNA was treated again with *Pst*I. Retained circular DNA containing the mutations in both strands finally served to transform SCS1.

A MURFI linker (46) was inserted into the *Afl*II site of *traI* deletion derivatives. To obtain blunt ends for linker insertion, cleaved DNA was treated with T4 DNA polymerase. Resulting derivatives were confirmed by nucleotide sequencing.

Conjugations. For quantitative filter matings, appropriate donor cells (0.5 ml, A_{600} of 0.3) and HB101 Nx^r recipient cells (4.5 ml, A_{600} of 0.3) were mixed and filtered on a Millipore nitrocellulose filter (0.45-μm pore size, 25-mm diameter). The filter was incubated for 1 h at 30°C on a YT agar plate without selection. Cells were suspended, and aliquots of serial dilutions were plated on appropriate selective medium (30 μg of nalidixic acid and 10 μg of chloramphenicol per ml to select for HB101 Nx^r containing pDB126, pDB127, or pDB128; 30 μg of nalidixic acid and 100 μg of ampicillin per ml to select for HB101 Nx^r containing pBS141 or derivatives). The transmission frequency is the number of transconjugants per donor cell.

Test for phage susceptibility. Bacteria were seeded onto the surface of selective medium with YT extract soft agar (0.7%) and bottom agar (1.5%). Dilutions of the phage stocks were spotted on these plates and incubated overnight at 37°C. Efficiency of plating was determined by comparing PFU of various strains with those of an RP4-containing strain.

Solid-phase immunoassay. Proteins were visualized by immunoblotting as described previously (26). Fluorescence signals were quantified with a FluorImager 575 (courtesy of Molecular Dynamics).

RESULTS

Reconstitution of the RP4 transfer system by fusion to a ColD replicon. For the convenient analysis of *tra* point mutations, we have reconstituted the RP4 transfer system by combining the two transfer regions and fusing them to a ColD replicon. The ColD replicon was chosen because the mutated *tra* genes were available on pMB1- or RSF1010-type plasmids, i.e., ColD-compatible replicons. Knowledge of previous experiments defining the minimal Tra regions (28) was exploited to construct plasmids carrying Tra1 and Tra2 core as cassettes. The RP4 *BfaI* fragment B served to generate the Tra1 core cassette because this fragment includes all functions of Tra1 needed for intraspecific *E. coli* matings; i.e., the loci *traF*, *traG*, *traH*, *traI*, *traJ*, *traK*, *traL*, and *traM* are present on pDB10 (Fig. 1; Table 1). Although *traL* and *traM* are nonessential *tra* genes, we decided to include them because lack of *traM* results in a 200-fold reduction of the transmission frequency. Plasmid pML123 carries all essential Tra2 functions and suitable restriction sites for introducing the Tra1 cassette (Table 1). The resulting plasmid, pDB126 (Fig. 2), offers several experimentally useful features. First, using an *E. coli* host as donor as well as recipient, pDB126 is a conjugative plasmid with the properties of the fully functional RP4 transfer system. Plasmid pDB126 is transmitted with high frequency, and the isolates from recipients are of identical size and have the same transfer properties when reused in a donor strain (data not shown), indicating that after transfer, the plasmid was installed properly in the recipient. Second, the multicloning sites on pDB126 allow the Tra cassettes to be easily exchanged. Third, the pDB126 system allows us to construct defined deletion deriv-

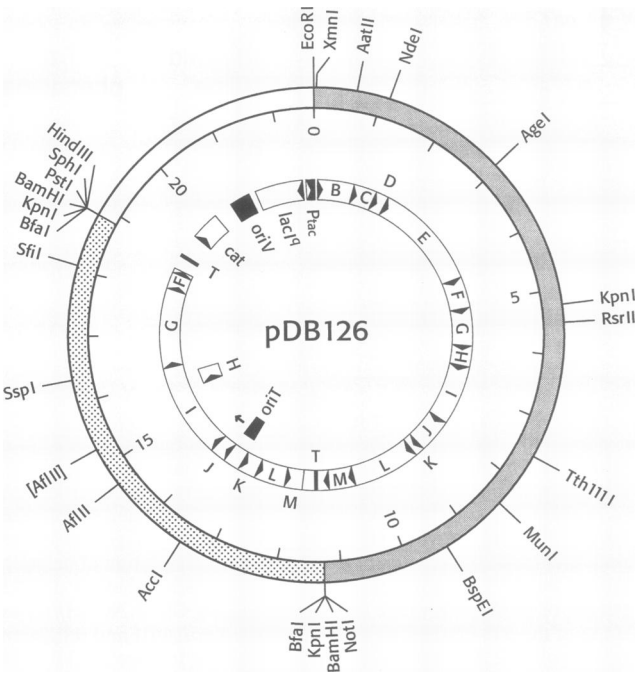

FIG. 2. Physical and genetic map of plasmid pDB126. To construct the reconstituted RP4 transfer system containing plasmid, the 7,593-bp *Bam*HI fragment of pDB10 (Fig. 1) encompassing the Tra1 core region was inserted into the *Bam*HI site of RP4 Tra2 plasmid pML123. pML123 lacks *trbA* and the intergenic portion of pDB1756Δ1.1.2 upstream of the *trbB* gene (28). The terminator sequence ($T_{RL53.1}$) (42) present in the inserted *Bam*HI fragment of the resulting plasmid pDB126 leads to termination of Tra1 transcription in both directions. Termination of Tra2 transcription takes also place at the Tra1 terminator. *E. coli rrnB* terminators are supplied in the vector portion. The leakiness of LacI-mediated *tac* promoter repression was sufficient to ensure expression of Tra2 functions. The portions of the vector pGZ119EH (Table 1; ▭) and regions Tra1 (▦) and Tra2 (■) are indicated on the outer circle. In the inner circle, gene starts are marked by black arrowheads, and Tra1 genes (*tra*) and Tra2 genes (*trb*) are designated with capital letters. Solid black segments represent terminators (T) as well as the origins of vegetative replication (oriV) and of transfer (oriT); the direction of transfer is indicated by an arrow. Vector loci depicted: cat, chloramphenicol acetyltransferase; oriV, origin of vegetative ColD replication; $lacI^q$, *lac* repressor; Ptac, *tac* promoter (marked by a white arrowhead). The restriction sites shown were important for analysis or the plasmid's construction.

atives of any *tra* gene for studying the influence of mutations on conjugative transfer by complementation in a binary plasmid system.

Generation of *traG* and *traI* transfer-deficient derivatives. For complementation studies, internal deletion derivatives of *traG* and *traI* were generated in pDB10 (Fig. 1; Table 2). To avoid polar effects on downstream genes, the constructions preserved the reading frames of *traG* and *traI* because at least for *traG* and *traF*, translational coupling has been shown (57). Almost the entire *traG* gene was deleted because the mutations to be tested are scattered within the first and second halves of *traG* (Fig. 1). *traI* mutations to be assayed map in the 5′-terminal third of its structural gene. Therefore, in a first approach, only 603 bp were removed (Fig. 1; Table 2). *BfaI* fragments of pDB11 (Δ*traG*) and pDB12 (Δ*traI*) were subcloned into the ColD/Tra2 plasmid pML123 to yield pDB127 and pDB128, respectively. However, subsequent attempts to isolate *E. coli* transformants containing both pDB173 (which carries the wild-type *traI* gene) and pDB128 (Δ*traI*) failed (see Table 4). No such effect was observed with the wild-type *traG* plasmids; transformation of HB101(pDB127 [Δ*traG*]) with pBS140 (pMB1 replicon, wild-type *traG*) or pBS141 (RSF1010 replicon, wild-type *traG*) was feasible. To circumvent the unexpected interference of the internally deleted Δ(I11-G211)TraI polypeptide with wild-type TraI protein a MURFI linker was inserted into the unique *Afl*II site (Fig. 1). Thus, the disruption of the *traI* reading frame prevented expression of *traI*, but it should not interfere with synthesis of TraH. The corresponding derivative pDB128m in HB101 gave normal transformation frequencies with the wild-type *traI* plasmid.

TABLE 2. *traG* or *traI* lesion in mutant plasmids

Plasmid	Mutation	Reading frame	No. of amino acid residues		
			TraG	TraI	Δ
pDB10	None	Untouched	635	732	
pDB11	Δ*traG*	Restored	66	732	570
pDB12	Δ*traI*	Restored	635	531	201
pDB12m[a]	*traI*m	Interrupted	635	10 + 2[b]	722

[a] m, 14-bp MURFI linker insertion CtagTCtagACtag containing an *Xba*I site (underlined) and amber codons (lowercase letters) in each reading frame.
[b] Two additional non-TraI amino acids result from the MURFI linker insertion.

The conjugative transfer properties of pDB127, pDB128, and pDB128m confirm previous data; strains harboring these plasmids are transfer deficient (13, 17, 57). Complementation of the transfer defects by corresponding *traI* and *traG* wild-type plasmids (pDB173, pBS140, and pBS141) demonstrates that conjugative transfer and mobilization could be fully restored (Fig. 3). Thus, the gene products TraG and TraI are required for RP4-mediated conjugation.

Two potential nucleotide binding motifs of TraG are essential for conjugative transfer. The TraG amino acid sequence

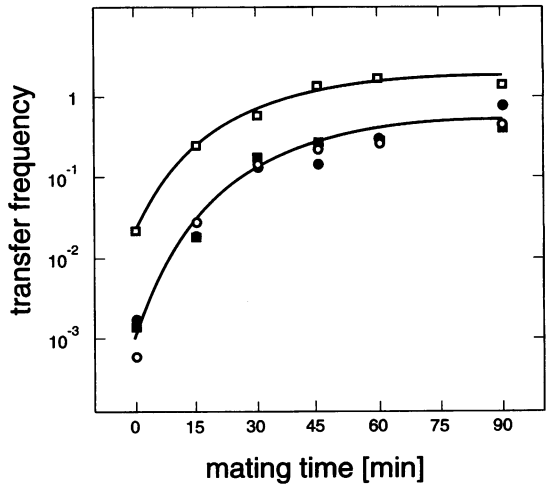

FIG. 3. Time course of transmission frequency dependent on different donor strains. Filter mating experiments were performed as described in Materials and Methods. To determine transmission frequencies of the plasmids (underlined) in HB101(pDB126) (○), HB101(pDB128m, pDB173) (●), and HB101(pDB127, pBS141) (■), HB101 Nx^r transconjugants were plated on chloramphenicol-nalidixic acid nutrient agar. Ampicillin nalidixic acid selection was used to measure the mobilization frequency of pBS141 in HB101(pDB127, pBS141) (□).

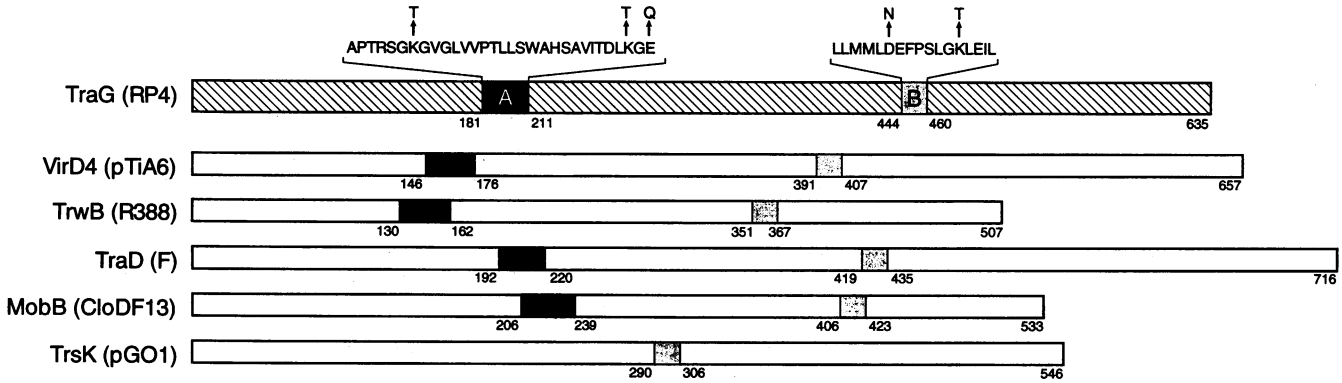

FIG. 4. Scheme of the domain structure of RP4 TraG and related proteins. The two TraG motifs (designated A and B) chosen for amino acid exchanges share common sequences with proteins from other transfer systems. Similarities between proteins RP4 TraG, Ti/Ri plasmid VirD4 (24, 60), and F plasmid TraD (23, 60) were described by Lessl et al. (30). Comparison was extended to MobB of CloDF13, a mobilizable plasmid from *Enterobacter cloacae* (53), and to TrsK of the conjugative plasmid pGO1 of *S. aureus* (34). Protein sequences are symbolized by open bars. The lengths (number of residues) are given at the ends of the bars. Conserved motifs are depicted with solid (A) or shaded (B) boxes, the boundaries are given by the corresponding number of amino acids. Amino acid sequences of the TraG motifs are shown at the top; amino acids exchanged by mutagenesis are indicated by arrows pointing at the mutant amino acid.

has three interesting features. At the N terminus, a signature for protein export has been found (30, 57, 62). A set of six different transfer systems encode TraG-like proteins: TraG (RP4/R751, IncP), VirD4 of the Ti and Ri plasmids, TraD of F and F-like plasmids, TrwB (R388, IncW), TrsK of the *Staphylococcus aureus* plasmid pGO1, and MobB of CloDF13, a nonconjugative but mobilizable plasmid (Fig. 4). With the exception of TrsK, in all of the TraG analogs, two nucleotide binding motifs, type A (54) and type B (14), have been located by computer search (30, 31, 41). TraG (RP4/R751) and VirD4 (pTi/pRi) contain a less conserved motif A, whereas conservation of motif B was high in all proteins considered. To understand why TraG is an important transfer function, we chose both potential nucleotide binding motifs as targets for site-directed mutagenesis. Rules for amino acid replacements were followed so that structural distortions in the protein would be minimal (3). For measuring conjugative plasmid transmission, the *traG* assay system consists of pDB127 (Δ*traG*) in the presence of pBS140 (carrying wild-type *traG* on a pMB1 replicon) or derivatives of pBS140 which carry specific mutations in *traG*. To assay the Tra system for the ability to mobilize RSF1010, pBS140 was replaced by pBS141, the corresponding RSF1010 derivative carrying *traG* (Fig. 3; Table 3). Mutations in both potential nucleotide binding motifs of *traG* affect the transfer and mobilization ability (K187T, K209T, E211Q, and D449N; Table 3). There was one mutation in each of the motifs (K187T in motif A and D449N in motif B) that inactivated the capacity to restore DNA transmission in the complementation assay. The mutations K209T and E211Q (both in motif A) resulted in 2,000- and 100-fold reduced transmission frequencies, respectively, compared with the wild type. Mutation K456T (motif B) did not affect transfer at all. The RSF1010 mobilization pattern of the five mutations follows that for transmission of pDB127.

The plating efficiency of donor-specific phages Pf3, PRD1, PRR1 on HB101(pDB126) was normal, and HB101(pDB127 [Δ*traG*]) could also function as a phage host without a decrease in plating efficiency, indicating that *traG* is not essential for phage growth. An influence of any of the *traG* mutations on phage reproduction was not observed (data not shown). This question was addressed because Fong and Stanisich (11) claimed that the *traG* locus is involved in propagation of phages, and therefore they named the gene *pilA*.

Active-site mutations of *traI* are transfer deficient. Purified relaxase mutant proteins have been characterized in vitro to evaluate their contributions to the catalytic activity of three motifs (Fig. 5) (45). Motifs I to III were found to be conserved among N-terminal portions of analogous proteins of several different DNA transfer systems, including VirD2 of Ti/Ri plasmids, NikB of R64, Rlx of *S. aureus* plasmids pS194, pC221, and pC223, and MobA of pTF-FC2 of *Thiobacillus ferrooxidans* (22, 40, 45). Residues in these motifs interact to exert the DNA cleaving-joining reaction of the relaxase which is needed for initiation and termination of the conjugative DNA metabolism during transfer. A defined role was assigned to each of the motifs by in vitro data: motif I contains the active-site tyrosine 22 that reacts during cleavage with the 5′ phosphate of the C at the nick site of *oriT* to form a phosphodiester with its aromatic hydroxyl group (44) (Fig. 5). Motif III probably is involved by facilitating the cleavage reaction through proton abstraction from the tyrosine 22 hydroxyl group by histidine 116. Motif II might be involved in

TABLE 3. Properties of *traG* point mutants

Motif	*traG* mutation[a]	Transmission frequency of pDB127[b]	Mobilization frequency of pBS141[c]
A	K187T	$<1 \times 10^{-7}$	$<1 \times 10^{-7}$
	K209T	2.5×10^{-5}	7.0×10^{-4}
	E211Q	5.7×10^{-4}	5.6×10^{-3}
B	D449N	$<1 \times 10^{-7}$	$<1 \times 10^{-7}$
	K456T	5.8×10^{-2}	6.5×10^{-1}
None	Wild type	4.3×10^{-2}	3.7×10^{-1}
	Vector[d]	$<1 \times 10^{-7}$[e]	$<1 \times 10^{-7}$[f]

[a] *traG* mutations on pBS140(pMB1 *oriT*^{0})/pBS141(RSF1010 *oriT*$^+$) are designated by the induced amino acid exchange of TraG protein: the first character stands for the wild-type amino acid, the number indicates its position, and the second character stands for the amino acid in the mutant.

[b] Transconjugants per donor cell [HB101(pDB127, pBS140)] in 60 min; to determine titers, cells were plated with nalidixic acid and chloramphenicol selection; each value represents an average of two independent experiments.

[c] Transconjugants per donor cell [HB101(pDB127, pBS141)] in 30 min. Titers were determined by plating of cells with nalidixic acid and ampicillin selection.

[d] Vectors carrying the *lac* repressor gene and *tac* promoter used for construction of pBS140 (pMS119HE) or pBS141 (pMMB67HE).

[e] pMS119HE.

[f] pMMB67HE.

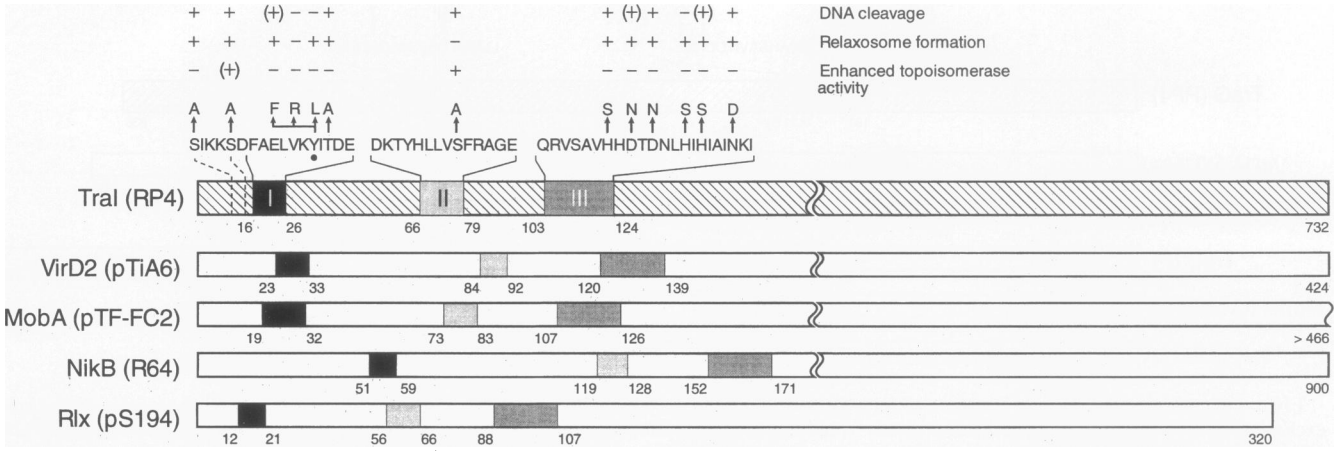

FIG. 5. Scheme of the domain structure of RP4 relaxase (TraI) and related proteins. A comparison of DNA relaxases from various origins shows the three conserved motifs as described elsewhere (22, 40, 45). Protein sequences are symbolized by open bars. The lengths (number of residues) are given at the ends of the bars. Gene designations of relaxases and plasmid sources (in parentheses) are listed at the left. Conserved motifs are represented by solid (I), lightly shaded (II), or darkly shaded (III) boxes. Motif boundaries are given by the corresponding number of amino acid positions. The amino acid sequence of TraI motifs is depicted, and TraI tyrosine 22 is marked by a dot. Amino acids exchanged by mutagenesis are indicated by arrows pointing at the mutant amino acid(s). In vitro properties of corresponding proteins are indicated at the top (45).

recognition and binding of the nick region at *oriT*. To study the in vivo properties of the *traI* mutations, we determined the Tra phenotype. The *traI* mutant assay system consisted of HB101(pDB128m) carrying in addition a pDB173 derivative with either the *traI* wild-type or a *traI* mutant gene (Table 4; Fig. 5).

Only 3 of the 14 *traI* mutant plasmids tested fully restored the Tra^+ phenotype of pDB128m: TraI S10A, S14A, and T24A. The remaining 11 *traI* mutant plasmids lead to a decrease of the pDB128m transmission frequency compared with the wild type (Table 4). Two mutants carrying an L or R substitution for Y22 rated below the detection level for DNA transmission. TraI Y22F restored the transmission frequency to about 10% of the wild-type level. This is an unexpected result, because tyrosine 22 is the catalytic site of the relaxase's active center (see above). A phenylalanine in place of tyrosine was expected to result in a complete transfer defect. How can the in vivo and in vitro results with the mutant plasmid and protein be explained?

Analysis of the in vitro cleavage reactions of nick region oligonucleotides unambiguously demonstrated that phosphotyrosine was obtained both with the wild-type protein and with

TABLE 4. Complementation of *traI* deletion mutants with *traI* point mutants

Motif	*traI* mutation in pDB173	Transformation with pDB173 derivative of:		Transmission to recipient[a] of pDB128 or pDB128m from:	
		HB101(pDB128)	HB101(pDB128m)	HB101(pDB128)	HB101(pDB128m)
I	S10A	−	+		2.6×10^{-1}
	S14A	−	+		2.4×10^{-1}
	Y22F	+	+	+	2.6×10^{-2}
	Y22L	+	+	ND[b]	$<1 \times 10^{-7}$
	Y22R	+	+	ND	$<1 \times 10^{-7}$
	T24A	−	+		3.1×10^{-1}
	Y22F, T24A	+	+		1.4×10^{-3}
II	S74A	−	+		6.3×10^{-2}
III	H109S	−	+		7.8×10^{-2}
	D111N	+	+	+	1.0×10^{-1}
	D113N	(+)[c]	+	+	9.2×10^{-2}
	H116S	+	+	−	8.5×10^{-5}
	H118S	(+)	+	+	2.5×10^{-2}
	N122D	−	+		8.6×10^{-2}
None	None (wild type)	−	+		3.5×10^{-1}
	pDB174[d]	−	+		ND
	pMS119HE[e]	+	+	−	$<1 \times 10^{-7}$

[a] Transconjugants per donor cell in 60 min; to determine titers, cells were plated with nalidixic acid-chloramphenicol selection; each value represents an average of two independent experiments.
[b] ND, not done.
[c] (+), small colonies, plasmid content extremely unstable.
[d] Antitranscriptive orientation of *traI* relative to the *tac* promoter.
[e] Vector carrying the *lac* repressor gene and *tac* promoter.

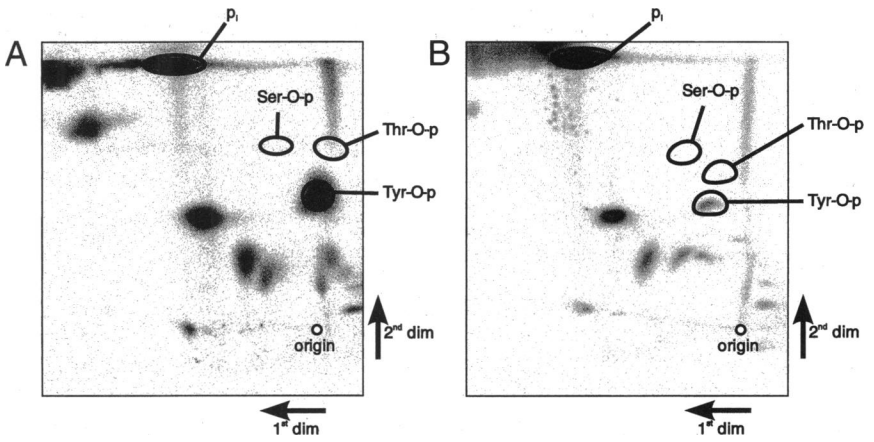

FIG. 6. Phosphoamino acid analysis of TraI oligonucleotide adducts. Internally labeled oligonucleotides containing the RP4 nick region (100 pmol) were incubated with TraI derivatives (100 pmol) as described previously (44), and cleavage products were captured by addition of EDTA (20 mM) and HCl (6 M). Samples were incubated at 110°C for 2 h and then lyophilized. *O*-phospho-L-serine (Ser-O-p), *O*-phospho-L-threonine (Thr-O-p), and *O*-phospho-L-tyrosine (Tyr-O-p) (1 μg of each) were added as internal references, and the samples were applied to cellulose thin-layer plates (200 by 200 mm; E. Merck). Electrophoresis was carried out in the first dimension at 1.5 kV in HCOOH, 2.2%–AcOH–7.8%–water (pH 1.9) and in the second dimension at 1.3 kV in 5% AcOH–0.5% pyridine–water (pH 3.5). Phosphoamino acids were visualized by reaction with ninhydrin, and radioactivity was detected by using the storage phosphor technology (Molecular Dynamics PhosphorImager 400B) (25). The spot labeled p_i was assigned to P_i. Direction of migration during two-dimensional electrophoresis is indicated by arrows. (A) Hydrolysis products of TraI-d(p*C)$_{5-10}$; (B) Like panel A except that a TraI Y22F preparation was used to treat internally labeled oligonucleotide.

the TraI Y22F preparation after acid hydrolysis (Fig. 6). N-terminal sequence analysis of purified TraI Y22F demonstrated that position 22 in the major fraction of the protein is occupied by phenylalanine, but traces of tyrosine were also found in this position. From these findings, we conclude that the preparation of overproduced TraI Y22F contains traces of wild-type TraI. This explains both the in vivo and in vitro results with this mutation, i.e., partial restoration of the transmission frequency and residual oligonucleotide cleavage activity, respectively.

Mutations in motifs II and III all gave reduced transmission frequencies. Some resulted in a 3- to 4-fold reduction (S74A, H109S, D111N, D113N, and N122D); H118S resulted in a 14-fold reduction, and H116S decreased the transmission frequency by about 4 orders of magnitude. The latter was found to exert the lowest specific activity in the cleavage reaction, indicating the importance of this histidine residue for the catalytic activity.

Another interesting finding of the complementation studies was that strain HB101(pDB128) could not be transformed with *traI* wild-type or mutant plasmids that were cleavage positive. In contrast, mutants strongly reduced or negative in cleavage activity resulted in nearly normal transformation frequencies of HB101(pDB128). When the latter were tested for plasmid transmission, the results were comparable to those obtained with the corresponding strains carrying pDB128m (Table 4). These mating experiments were not quantified because of lack of suitable controls. Plasmid pDB128 encodes an internally deleted TraI lacking the three motifs thought to contribute to the catalytic center (Fig. 1 and 5). Therefore, the observation is interesting, since it indicates that the internally deleted Δ(I11-G211)TraI protein interacts with a relaxosome assembled of wild-type components in a peculiar way.

Relaxase negatively regulates expression of the relaxase operon. To confirm the expected polypeptide pattern expressed from the mutant plasmids, solid-phase immunoassays of cell extracts of HB101 carrying pDB11, pDB12, or pDB12m were compared with those of HB101(pDB10) representing the wild-type situation. In addition to TraG and TraI proteins, TraF, TraH, TraJ, TraK, and TraL were monitored by using polyclonal antisera (Table 5). As expected, the HB101(pDB11) extract did not cross-react immunologically with the anti-TraG serum because the remaining 66-amino-acid polypeptide of TraG might escape detection because of its small size and/or rapid degradation. In contrast to the HB101(pDB12m) extract, internally deleted Δ(I11-G211)TraI protein was visible in an HB101(pDB12) extract.

The reaction of HB101(pDB11) with antisera against TraF, TraH, TraI, TraJ, TraK, and TraL showed a pattern similar to that of HB101(pDB10), indicating that TraG does not strongly influence the expression of these proteins (Table 5). However, the corresponding experiment with HB101(pDB12) or HB101(pDB12m) showed increased levels of TraF, TraG, TraH, TraJ, and TraK. The level of TraL did not change (Table 5). This finding suggests that TraI when assembled in the relaxosome is involved in regulation of relaxase operon expression.

DISCUSSION

Two separated regions on IncP plasmids, Tra1 and Tra2, are responsible for the plasmid's conjugation ability (26, 28, 37).

TABLE 5. Influence of TraI and TraG on expression of Tra1 genes

Plasmid in SCS1	Signal intensity[a] of given Tra1 gene product						
	F	G	H	I	J	K	L
pDB11 (Δ*traG*)	1.5	—[b]	1.1	0.3	0.7	1.0	1.0
pDB12 (Δ*traI*)	3.8	2.3	2.0	7.9[c]	7.8	1.7	0.85
pDB12m (*traI*m)	2.5	1.5	1.4	—	6.5	1.8	0.8

[a] Relative to the normalized value of 1.00, which corresponds to the signal in solid-phase immunoblots observed with SCS1(pDB10).
[b] —, no detectable cross-reaction.
[c] Internally deleted product.

Dissection and reconstitution of the DNA transfer system allowed us to define the core of the transfer genes, i.e., genes essential for mating to occur between *E. coli* cells. TraI and Tra2 function in *trans*, as demonstrated by subcloning onto two compatible replicons, and also in *cis*, shown by arranging the two Tra regions in a recombinant ColD vector plasmid. This plasmid (pDB126; 22,666 bp) contains approximately 19 kb of the IncPα Tra regions of RP4 encompassing 20 *tra* genes and the transfer origin. Three TraI genes, *traH*, *traL*, and *traM*, are not essential but may provide accessory transfer functions. *traH* has been shown to moderately enhance transfer in interspecific matings (6). Absence of *traM* causes a 200-fold reduction of the transmission frequency in *E. coli* matings, indicating a rather important role of the corresponding gene product (28). TraL and TraM share sequence similarity with VirC1 and VirC2 of the Ti plasmid complex (42). The *virC* genes are known to stimulate processing of T-DNA (51). A comparative biochemical analysis of the IncP and Ti components might give an answer about their roles. Ten of the eleven Tra2 genes (*trbB*, *trbC*, *trbE*, *trbG*, and *trbL* [28], *trbD* [50], and *trbF*, *trbH*, *trbI* and *trbJ* [17a, 32]) are known to encode essential transfer functions. *trbM* is nonessential in intraspecific *E. coli* matings and was kept only because of constructional reasons.

The presently available set of components of the IncPα Tra system allows a systematic analysis of the Tra functions. The plasmids might also be useful for the genetic manipulation of non-*E. coli* organisms. The complete system pVWDG 23110Δ0.1 (TraI)/pML123 (Tra2) (28), the core system pDB10 (TraI)/pML123 (Tra2), and the recombined core system pDB126 (TraI plus Tra2) offer a suitable variety of manipulations, as a result of presence of common restriction enzyme cleavage sites only in the vector portions, not in the Tra regions. The transfer properties in terms of frequency and plasmid installation in the recipient of the subcloned regions resemble those of RP4 except that the frequency of transmission is higher, probably because of the multicopy level providing a higher *tra* gene dosage. The same applies for RSF1010 (IncQ) mobilization by the reconstituted RP4 transfer system.

The transfer deficiency of a *traG* (pDB127) and a *traI* (pDB128/pDB128m) deletion and the complementation of these derivatives with the corresponding wild-type gene confirmed that both *traG* and *traI* encode essential Tra functions. The in vivo assay for defined point mutations in these genes could simply be done by *trans* complementation. The intriguing Tra property of the *traG* mutants, the mutations of which reside in potential nucleoside triphosphate (NTP) binding motifs of the protein demonstrates the importance of the corresponding residues. Although there is no experimental evidence for their involvement in nucleotide binding, it appears that intact consensus NTP binding motifs are required for the activity of TraG and TraG-like proteins. One type of enzyme that might have such a feature are DNA helicases. A rolling circle-type mode of the generation of the plasmid strand destined for transfer to the recipient cell would require a DNA-unwinding activity to displace the DNA strand transferred during conjugation (58). Interestingly, a DNA-unwinding enzyme has not been identified among the IncP Tra proteins, and DNA helicases presently known that are specified by other conjugative plasmids, F TraI and R388 (IncW) TrwC, are dispensable for ColE1 and ColE1/RSF1010 mobilization, respectively. RSF1010 encodes a replicative helicase (RepA) (21), but so far no evidence is available for an involvement of RepA in RP4-mediated mobilization of the IncQ plasmid (10). On the other hand, the Tra systems of the conjugative plasmids encode TraG-like proteins, TraD (F) and TrwB (R388), that are essential for the mobilization process and for plasmid self-transmission (5, 59). The nonconjugative plasmid CloDF13 is mobilized by F and by F with *traD* mutant alleles, indicating that TraD is not essential for CloDF13 transmission (59). The CloDF13-encoded MobB protein (Fig. 4) therefore might be a functional analog of TraD.

The RP4 TraI region encodes two proteins with potential nucleotide binding folds: TraG and TraL. The latter is a small (26-kDa) nonessential Tra protein. There are also two Tra2 proteins, TrbB and TrbE, with proposed A-type NTP binding motifs, both of which are indispensable for transfer. Only for TrbB is there preliminary evidence for DNA-independent interaction with ATP (unpublished data). Both proteins are likely to be involved in pilus and channel/pore formation. It is possible that a chromosomally specified helicase provides the activity required for DNA transfer; however, promising candidates for having a transfer-specific DNA unwinding activity appear to be also the TraG-like proteins.

Recently we proposed a model for the interplay of amino acid residues in three motifs located in the N-terminal portion of TraI that are involved in the cleaving-joining reaction of *nic* of *oriT* (45). We have demonstrated that tyrosine 22 in motif I of TraI is the active-center amino acid of the catalytic activity. Transesterification leads to intermediate formation of a phosphodiester between the phosphate of a cytidyl residue at *nic* and tyrosine 22 (44). At least two histidine residues, histidine 116 and histidine 118 of motif III, are thought to participate actively in the reaction. Residues of motif II are likely to play a role in recognition and binding of the nick region.

There was one drawback in our biochemical study of mutant TraI proteins. A TraI mutant protein thought to have a Tyr-to-Phe replacement at position 22 still resulted in residual cleaving-joining activity (45). Here we confirmed that TraI Y22F was also able to support transfer in vivo although at a reduced rate. That the in vitro reaction might result in a covalent bond with another amino acid (serine or threonine) or a tyrosine at another position was excluded (Fig. 6). The most likely explanation of this puzzling result is that our TraI mutant protein thought to be a homogeneous TraI Y22F was actually a mixture of TraI Y22F with a small amount of TraI wild-type protein. Because of the high sensitivity of our methods, we were able to draw this conclusion. An analogous result was recently documented for a Tyr-to-Phe mutation in the bacteriophage λ Int protein (18): a preparation of mutant protein Int Y342F was shown to possess 6 to 9% residual *attL* B-site cleavage activity. The tyrosine 342 residue serves as a nucleophile during strand cleavage and covalently attaches Int to the DNA through a phosphotyrosyl bond. In addition, the product of Int Y342F cleavage was still sensitive to proteinase K, indicative of a covalent protein-DNA complex (18). One explanation for these observations is misincorporation of amino acids during the course of overexpression of the *traI* and *int* mutant genes. Such mistakes could happen when the Phe codon UUC is translated into Tyr. Overexpressing cells may be depleted of loaded $tRNA^{Phe}$, so in a few cases $tRNA^{Tyr}$ is accepted as well (2, 8). Two additional mutations, TraI Y22L and Y22R, gave a negative transfer phenotype, which is in agreement with our original proposal. Applying these mutants for in vitro cleavage reactions in both cases revealed no detectable residual activity. Relaxosome formation was possible with TraI Y22L but not with TraI Y22R (Fig. 5), indicating that the Tyr-to-Leu mutation does not disturb the protein's secondary structure. The codon replacements in these cases

(CUC for Y22L and CGC for Y22R) were selected in such a way to avoid a too close relationship to the tyrosine codons (UAC and UAU).

It has been inferred from previous mutagenesis studies that the leader and relaxase operon are subject to a complex regulation (17, 42). It has also been shown that at least some of the genes of the relaxase operon are regulated at the translational level: *traF* by coupled translation to *traG* (57) and *traI* by an inefficient G-rich masked ribosome binding site possibly activated by translation of *traX* (38). The arrangement in the leader operon also suggests cotranslation between *traK/L* and *traL/M*. Evidence for regulation at the transcriptional level was deduced from our experiments analyzing the polypeptide expression patterns of *traG* and *traI* deletion mutants. We suggest that the complete relaxosome which only assembles in the presence of TraI acts as a transcriptional repressor. The internally deleted Δ(I11-G211)TraI protein (pDB12) most likely is unable to allow relaxosome formation because of the absence of the catalytic center (including motif II potentially involved in nick region recognition and binding). In the absence of wild-type TraI, the polypeptide level has significantly increased for both the relaxase operon components and TraK of the leader operon. This observation suggests that a functional relaxosome controls the accessibility of the promoters P_{traJ} and P_{traK} (42) by RNA polymerase, leading to a lower level of transcription. However, without direct determination of mRNA concentrations, other explanations for the increase of other *tra* gene products in the absence of TraI should be kept in mind: expression of *traI* from high-copy-number plasmids could exhaust certain resources required for the expression of the other Tra proteins. Alternatively, the wild-type TraI protein could be slightly deleterious for the cell so that in its absence expression of the other genes would be increased. However, at least the first possibility seems unlikely because a comparable increase of *tra* gene expression was observed independently of whether the internally deleted Δ*traI* (pDB12) or the *traI*m mutant (pDB12m) was applied for the analysis (Table 5).

The observation that pDB128-containing cells cannot be transformed with plasmids encoding TraI derivatives capable of DNA cleavage (Table 4) suggests an interaction between the latter and the internally deleted Δ(I11-G211)TraI protein encoded by pDB128. It is conceivable that the IncP relaxosome contains more than one copy of TraI, also because there is only one active-site tyrosine present in the protein. Rolling circle replication with generation of a unit-length copy of the DNA single strand to be transferred, however, requires a second cleavage event at the restored nick site (55, 58). In the case of the gene A protein of bacteriophage φX174, two active-site tyrosine residues exist that alternate in forming the phosphodiester with the plus-strand 5′-terminal nucleotide during successive rounds of reinitiation (20, 52). It has been shown that TraI protein in vitro can transfer a covalently bound oligonucleotide only to a preformed 3′ hydroxyl terminus at *nic* (44) but does not catalyze strand exchange of covalently bound oligonucleotides with oligonucleotides containing an intact nick site (41). Therefore, it is likely that at least two copies of TraI are involved in initiation and termination of IncP transfer DNA replication. If both the internally deleted version of TraI and a catalytically active form of TraI exist in the same cell, the presence of TraI heteromultimers in the relaxosomes could interfere with vital plasmid functions such as vegetative replication.

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