Site-Directed Mutations in the Relaxase Operon of RP4

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Mutations were constructed by site-directed mutagenesis in the relaxase operon of the broad-host-range plasmid RP4. The mutations were constructed in smaller plasmids, recombined into the 60-kb RP4 plasmid, and tested for their ability to transfer. The relaxase operon contains the transfer genes *traJ*, *traH*, and *traI*, which are involved in nicking at the transfer origin to generate the single strand destined to be transferred to the recipient cell. In the first mutant, the C terminus of TraI was truncated, leaving TraH intact. This mutant decreased transfer by approximately 500-fold in *Escherichia coli*, and the *traI* mutation could be complemented by a wild-type copy of *traI* in *trans* in the donor. The *traI* mutation similarly decreased transfer between a variety of gram-negative bacteria. A site-specific mutation was made by the polymerase chain reaction-based unique-site mutagenesis procedure to alter the start site of *traH*. This mutation had no effect on intraspecific *E. coli* transfer but reduced transfer by up to sevenfold for some gram-negative bacteria. The *traH* mutation had no effect on plasmid stability. Thus, neither TraH nor the C terminus of TraI is required for conjugative transfer, but both increase mating efficiency in some hosts.

Plasmid RP4 (essentially identical to RK2) is a broad-host-range conjugative plasmid carrying multiple antibiotic-resistance genes (12). Although RP4 was discovered clinically in gram-negative organisms, it can mediate gene transfer to virtually any other bacterium, including gram-positive bacteria (for a recent review, see reference 10). Several features of the mechanism of transfer of this plasmid are analogous to infection of plant cells by the T-DNA of *Agrobacterium* Ti virulence plasmids. For both of these systems, the double-stranded plasmid is nicked (1, 9, 30; for a recent review, see reference 33) at homologous sites to initiate transfer. An *in vitro* assay was developed to identify which gene products from the TraI region of RP4 were required for specific nicking at the transfer origin, *oriT* (19). With this assay, it was determined that one of the proteins required for open circular DNA formation was TraJ, which recognized and bound to the *oriT* DNA sequence of RP4. TraI then bound to the TraJ-*oriT* complex, and the DNA was nicked at the *oriT* site. In addition, TraI bound covalently to the 5' end of the nick site (23). A third protein, TraH, associated *in vitro* with the TraIJ-containing relaxosome but produced only a minimal increase in the nicking activity. These proteins are encoded within the relaxase operon (Fig. 1). TraH is encoded by a different reading frame contained within the C-terminal portion of *traI*.

TraJ and TraI have functional analogs encoded by the VirD operon of the *Agrobacterium* Ti plasmid system, proteins VirD1 and VirD2, respectively. The VirD1-VirD2 complex nicks the T-DNA border sequences of Ti plasmid DNA, and then VirD2 binds tightly (probably covalently) to the 5' end of the nicked DNA (6, 13, 15, 31, 36). A part of the border sequence is identical to the IncP nick region (32). VirD2 also contains nuclear targeting sequences that guide the T-DNA into the nucleus of the plant recipient (16, 29). The overall arrangement of the VirD operon of the Ti plasmid resembles that of the RP4 TraI region (37) containing the relaxase operon and the downstream *traG* locus.

VirD2 has functional homology and limited structural homology to the N terminus of TraI (20, 21). The nicking and covalent binding domains of TraI occur within the N-terminal half of this protein (8, 22, 23), as in VirD2 (5, 35). However, since TraI is nearly twice the size of VirD2, whether the C terminus of the protein has any function in the transfer of RP4 is an important issue.

Also within both the VirD and TraI operons, a small overlapping open reading frame occurs out of frame, but in the same orientation as that of *virD2* and *traI*, respectively (37). This open reading frame (*virD3* in the Ti plasmid; *traH* in RP4) is located at the 3' termini of both genes. Different *agrobacterium* Ti and Ri plasmids encode *virD3* genes of different sizes, depending on the presence or absence of a putative insertion sequence-like element (16). This gene has been deleted from *Agrobacterium tumefaciens* nopaline pTiT37 and *Agrobacterium rhizogenes* pRiA4 and found to have no effect on virulence (16). A mutation was made in *virD3* that was nonpolar over *virD4* and was as efficient as the wild-type gene for tumorigenesis on *Kalanchoe* leaves and the stems of tomato, pea, and sunflower plants (16). VirD3 thus has no effect on host range. Production of the VirD3 protein has never been demonstrated *in vivo* (16), implying that the encoding DNA may be a remnant of evolution. The corresponding TraH protein is expressed in bacteria containing RP4, and antibodies have been made to TraH (37). Since TraH is made *in vivo* and was observed to associate with the relaxosome *in vitro*, its effect on transfer of RP4 was investigated in this study.

The strategy employed in this work was to create two specific mutants that lead to (i) a truncated TraI protein, leaving TraH intact, and (ii) a normal TraI protein and no TraH produced. The mutation that eliminates the TraI C terminus and the TraH knockout mutation were recombined into the 60-kb RP4 plasmid and compared with the native plasmid for conjugative transfer, stability, and host range. The results suggest that the C terminus of TraI increases transfer efficiencies but that TraH has a minimal effect by any of the criteria tested.

Construction of *traI* and *traH* mutants. Mutations in *traI*

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FIG. 1. Organization of the relaxase operon. The *oriT* *nic* site is indicated, as are the relaxase operon promoter (P_{L1}) and primase operon promoter (P_{L2}). The open triangle represents the 38-bp deletion in RP4-14, which was 1,414 kb from the start of *traI*. The small vertical arrow indicates the site-specific *traH* knockout mutation in RP4-21, 1,542 bp from the *traI* start site.

and *traH* in RP4 (Fig. 1) were initially constructed on plasmid pSC3, containing the 3-kb *AccI* fragment of RP4 (including *oriT*, *traJ*, *traI*, and *traH*) cloned into the *HincII* site of pUC9Cm. The smaller mutant plasmids were then recombined into RP4, and the cointegrate was resolved such that the mutant alleles replaced the wild-type alleles.

To construct plasmid RP4-14 (with a *TraI* C-terminal deletion), pSC3 was digested with *HincII* and *NotI*, the sticky *NotI* ends were filled in with the large fragment of DNA polymerase I (Klenow), and the plasmid was self-ligated to give a 38-bp deletion (Table 1). These procedures placed a translational stop codon in *traI* 11 codons downstream from the *NotI* site but did not alter the *traH* gene. The resulting *TraI* protein was truncated from 731 to 481 amino acids. Since recombination of the mutant allele into RP4 required two crossover events on each side of the mutation, an equal amount of homologous DNA on either side of the mutation gave optimal results. Thus, the *oriT* and *traJ* genes were omitted from the *AccI* fragment by digestion with *AflIII* and a site within the multiple cloning site of the vector, followed by blunt-end filling and self-ligation as described above. The resulting pSC14 plasmid contained approximately 0.8 to 0.9 kb on either side of the *NotI*-*HincII* deletion. This deletion was verified by restriction enzyme analysis.

In plasmid RP4-21 (*traH* knockout), the ATG initiation codon in *traH* was changed to ACG by polymerase chain

reaction (PCR)-based unique-site mutagenesis (24). The PCR primer used to construct the *TraH* start codon mutation also included a second base change to create a (new) *DdeI* restriction site. Neither of these two base changes altered the amino acids in *TraI* because of degeneracy at the third codon position. This 23-bp primer plus a second primer which contained a mutation in the vector *EcoRI* unique restriction site were used to generate a PCR fragment. The fragment obtained by PCR was annealed to denatured pSC3, and the rest of the second strand was synthesized with the large fragment of DNA polymerase I. The resulting heteroduplex plasmid was electroporated into *Escherichia coli* BMH71-18 (*mutS*; kindly provided by Jac Nickoloff), a strain defective in DNA repair, and DNA was isolated from the pool of transformants. Following digestion with *EcoRI*, the DNA was transformed into *E. coli* DH5 α , and clones were screened for loss of the *EcoRI* site and acquisition of a new *DdeI* site. The new plasmid (pSC21) was sequenced to confirm the *traH* start site alteration.

Recombination of mutations into RP4. The *traI* and *traH* mutations were recombined into RP4 by a modified version of the previously described procedure (2, 7). Plasmids pSC14 and pSC21 were transformed into *E. coli* C2110N x^r (*recA*⁺ *polA*) (nalidixic acid resistant) containing RP4. The resulting Cm^r clones (containing RP4 cointegrates with pSC14 or pSC21) were grown overnight with shaking (200 rpm, 37°C) in Luria broth (LB) (26), and then a 1% inoculum was made in LB. Resolution of the cointegrates was selected by cycloserine treatment in the presence of chloramphenicol. After 15 min of growth at 37°C with shaking at 200 rpm, chloramphenicol was added to 20 μ g/ml. Following an additional 45 min of incubation with shaking at 37°C, cycloserine (Sigma Chemical Co., St. Louis, Mo.) was added to a final concentration of 100 μ g/ml, and the culture was incubated for another 45 min. Cells were pelleted and plated onto LB agar, and individual colonies were tested for sensitivity to chloramphenicol. Twelve Cm^s clones were screened by clone analysis (14) for loss of the *NotI* site in RP4-14 or presence of a new *DdeI* site in RP4-21. Half of the clones contained the desired mutations in RP4.

Phenotype and complementation of the *TraI* C-terminal deletion mutant. For all subsequent mating experiments,

TABLE 1. Bacterial plasmids

Plasmid	Description	Marker(s) ^a	Reference(s)
pUC9Cm	Cm ^r derivative of pUC9	Cm ^r	32
pUC18	Multicopy replicon	Ap ^r	34
RP4	60-kb native plasmid	Km ^r Tc ^r Ap ^r	
RK231	49.5-kb <i>PstI</i> deletion derivative of RK2	Km ^r Tc ^r	18
pSC3	3-kb <i>AccI</i> fragment of RP4 in <i>HincII</i> site of pUC9Cm	Cm ^r	This work
pSC14	<i>AflIII</i> - <i>AccI</i> fragment containing 38-bp deletion in <i>traI</i> in pUC9Cm	Cm ^r	This work
RP4-14	RP4 with 38-bp deletion in <i>traI</i>	Km ^r Tc ^r Ap ^r	This work
pSC21	pSC3 with <i>traH</i> ATG-to-ACG site-specific mutation	Cm ^r	This work
RP4-21	RP4 with <i>traH</i> ATG-to-ACG site-specific mutation	Km ^r Tc ^r Ap ^r	This work
pVWDG23110	22.1-kb <i>TraI</i> region-containing <i>PstI</i> - <i>BamHI</i> fragment cloned into pBR329 (4)	Km ^r	17
pDB173	Expression vector with <i>traI</i> fused to <i>tac</i> promoter	Ap ^r	19
pSC28	pDB173 with <i>traI</i> truncation at the <i>NotI</i> site and <i>XbaI</i> linker (stop codons in 3 frames)	Ap ^r	This work
pACYC184	P15A replicon	Cm ^r Tc ^r	3, 25
pSC16	3-kb <i>AccI</i> fragment from pSC3 cloned in pACYC184	Cm ^r	This work
pAZ505 ^b	1.5-kb aminoglycoside resistance gene in pBR322	Tb ^r	28
pSC31	Tb ^r gene from pAZ505 ^b cloned in pACYC184	Tb ^r Cm ^r	This work

^a Cm^r, resistant to chloramphenicol; Ap^r, resistant to ampicillin; Tc^r, resistant to tetracycline; Km^r, resistant to kanamycin; Tb^r, resistant to tobramycin; Cb^r, resistant to carbenicillin.

^b Kindly provided by Karen Shaw.

TABLE 2. Media used in mating experiments

Recipient	Medium ^a used in mating expts to select donor, recipient, or transconjugant					
	<i>E. coli</i> JA221 ^b as donor			<i>E. coli</i> JA221 as recipient		
	Donor	Recipient	Transconjugant	Donor	Recipient	Transconjugant
<i>E. coli</i> JA221Nx ^r	LB-KAN	LB-NAL	LB-KAN-NAL			
<i>K. pneumoniae</i>	LB-KAN	LB-STR	LB-KAN-STR	LB-KAN-STR	LB-NAL	LB-KAN-NAL
<i>S. flexneri</i>	LB-KAN	LB-STR	LB-KAN-STR	LB-KAN-STR	LB-NAL	LB-KAN-NAL
<i>P. stutzeri</i>	LB-KAN	LB-STR	LB-KAN-STR	LB-KAN-STR	LB-NAL	LB-KAN-NAL
<i>P. putida</i>	LB-TOB	M9	M9-KAN	M9-KAN	LB-TOB	LB-TOB-KAN
<i>P. maltophilia</i>	LB-KAN	M9	M9-KAN	M9-KAN	LB-NAL	LB-KAN-NAL
<i>P. aeruginosa</i>	LB-CAR	M9	M9-CAR	M9-KAN	LB-TOB	LB-TOB-KAN

^a Medium abbreviations: LB, Luria broth agar; M9, minimal medium agar. Antibiotic abbreviations and concentrations used: NAL, nalidixic acid (30 µg/ml); KAN, kanamycin (50 µg/ml); STR, streptomycin (50 µg/ml); TOB, tobramycin (10 µg/ml); CAR, carbenicillin (500 µg/ml).

^b Genotype of JA221, *leuB trpE5 lacY recA hsdR hsdM⁺* (11).

late-log-phase cultures of bacteria were centrifuged and adjusted to 100 Klett units with 10% saline, mixed in a 1:4 donor/recipient ratio, and mated on nitrocellulose filters for 35 min. The titers of donors and recipients were determined for each experiment. Mating efficiencies were determined as the number of transconjugants divided by the number of recipients. The recipient was used as the denominator in this calculation in order to be consistent with our previous work. The media used for selection of donors, recipients, and transconjugants are indicated in Table 2. All matings in this work were performed two or three times in independent experiments, and the results were averaged.

Table 3 shows the transfer efficiencies of the TraI C terminus inactivation mutant (RP4-14) compared with wild-type RP4. For intraspecific mating experiments of *E. coli* using strain DH5α as the donor and strain HB101 as the recipient, truncation of TraI decreased transfer efficiency consistently by approximately 100- to 1,000-fold. The mutation in *traI* could be complemented in *trans* by the addition of a wild-type copy of *traI* in pACYC184 (pSC16 [Table 3]). Complementation in *trans* occurred only if pSC16 was provided in the donor. No complementation resulted if pSC16 was present only in the recipient.

To ensure that the TraI protein was not simply degraded when the C terminus was deleted, the truncated protein was visualized on immunoblots. For this experiment, a *traI* expression plasmid (pDB173) was truncated at the *NotI* site. An *XbaI* linker containing stop codons in all three frames was inserted into the *NotI* site to produce plasmid pSC28. A Western blot of whole-cell lysates of pSC28 induced with isopropyl-β-D-thiogalactopyranoside (IPTG) was reacted

with antiserum to TraI (23). The immunoblot showed that this clone produced a truncated product of TraI (data not shown); thus, this protein is expressed as a stable product. This expression system was used to construct the truncated form of TraI, since pDB173 contains *traI* fused to a translational initiation signal from phage T7 gene 10 and the GTG native start codon of *traI* has been replaced with a more efficient ATG start site (19). TraI is not detectable on immunoblots if expressed from its native promoter and start site. *NotI* was chosen to check for stability of the truncated TraI product, since pDB173 contains several *HincII* sites and could not be readily truncated with this enzyme. We have made several deletion derivatives of *traI* in pDB173, both upstream and downstream of the *NotI* site in pDB173, and observed them on immunoblots compared with the wild-type plasmid pDB173. In each case, the truncated product was expressed the same as the wild-type protein (data not shown).

Phenotype of *traH* site-specific mutant. The results of intraspecific *E. coli* mating experiments using RP4-21 show that inactivation of the start codon in *traH* has no effect on transfer (Table 3). The amino acid sequence of TraI was not altered by this single base pair change or by the introduction of a new *DdeI* restriction site. The sequence of the mutation in RP4-21 was confirmed by the following procedure. A PCR fragment was made from RP4-21 encompassing *traH* plus 107 bp of DNA upstream of the start site of *traH*. This fragment was subcloned into pUC18, and the sequence determined with Sequenase version 2 (U.S. Biochemical Corp., Cleveland, Ohio). No bases had been altered downstream of this mutation in RP4-21 through the *traH* coding region.

In order to prove that TraH is not expressed from an alternate start site, the *traH* mutation on plasmid pSC21 was compared with wild-type *traH* on plasmid pSC3 in immunoblots (Fig. 2). Western blots of whole-cell lysates of *E. coli* DH5α containing pSC21 or pSC3 were reacted with antibody made to TraI and reacted separately to TraH (37). These blots indicated that TraH was not produced by the clone containing pSC21 (Fig. 2), while full-length TraI was made (data not shown). The control plasmid, pSC3, expressed both TraI and TraH.

Host-range effects of the TraI C-terminus truncation and TraH inactivation. Gram-negative bacterial strains other than *E. coli* used in broad-host-range studies (Table 4) were a gift from the laboratory of Don Helinski. Mating efficiencies for the C-terminus deletion of TraI were consistently lower in intra- or interspecific matings with *E. coli* JA221 as

TABLE 3. Transfer efficiencies of wild-type and mutant RP4 plasmids

Mating and plasmid(s)	Mating frequency ^a
<i>E. coli</i> DH5α (donor) × HB101 (recipient)	
RP4	1 × 10 ⁻¹
RP4-14	2 × 10 ⁻⁴
RP4-21	2 × 10 ⁻¹
<i>E. coli</i> JA221 (donor) × JA221Nx ^r (recipient)	
RP4 + pACYC184	2 × 10 ⁻²
RP4-14 + pACYC184	6 × 10 ⁻⁵
RP4 + pSC16	7 × 10 ⁻²
RP4-14 + pSC16	2 × 10 ⁻²

^a Mating frequency = number of transconjugants/number of recipients.

FIG. 2. Solid-phase immunoassay of a *traH*⁻ mutant. Cell extracts of strains harboring pDB173, pSC3, or pSC21 were electrophoresed on a sodium dodecyl sulfate–15% polyacrylamide gel. Extracts were prepared as described previously (17). Aliquots corresponding to 0.15 A_{600} of the bacterial culture were applied. Proteins were transferred electrophoretically from the gel to a nitrocellulose membrane (27). Immunological reactions were visualized as described previously (17). Anti-TraH serum was diluted 750-fold. Molecular mass standards are fluorescein isothiocyanate-labeled bovine serum albumin (BSA) and lysozyme (LYS). Extracts of IPTG-induced (+) and uninduced (–) cells were used.

the donor. *Pseudomonas putida* had the greatest difference (over 1,000-fold) in transfer efficiency between RP4 and RP4-14. An exception to this occurred when *Pseudomonas maltophilia* was the recipient, which resulted in a transfer efficiency for RP4-14 that was indistinguishable from that for RP4. Mating RP4-14 from these species to *E. coli* JA221 also resulted in decreased transfer, except for *P. putida* as the donor.

For the *traH* mutant, RP4-21, transfer efficiencies decreased slightly for the recipients *Pseudomonas stutzeri*, *P.*

TABLE 4. Broad-host-range mating frequencies

Species used in mating expt	Mating frequency ^a with plasmid:		
	RP4 (wild type)	RP4-14 (Δ TraI)	RP4-21 (Δ TraH)
<i>Escherichia coli</i> as donor			
<i>Escherichia coli</i>	8×10^{-2}	2×10^{-3}	9×10^{-2}
<i>K. pneumoniae</i>	1×10^{-1}	7×10^{-3}	1×10^{-1}
<i>Shigella flexneri</i>	2×10^{-1}	9×10^{-3}	2×10^{-1}
<i>Pseudomonas stutzeri</i>	8×10^{-3}	2×10^{-4}	5×10^{-3}
<i>Pseudomonas putida</i>	1×10^{-1}	8×10^{-5}	6×10^{-2}
<i>Pseudomonas maltophilia</i>	2×10^{-1}	3×10^{-1}	3×10^{-1}
<i>Pseudomonas aeruginosa</i>	1×10^{-2}	3×10^{-4}	4×10^{-3}
<i>Escherichia coli</i> as recipient			
<i>Klebsiella pneumoniae</i>	2×10^{-1}	3×10^{-4}	3×10^{-2}
<i>Shigella flexneri</i>	1×10^{-1}	3×10^{-2}	1×10^{-1}
<i>Pseudomonas stutzeri</i>	1×10^{-2}	7×10^{-6}	4×10^{-3}
<i>Pseudomonas putida</i>	9×10^{-2}	8×10^{-2}	8×10^{-2}
<i>Pseudomonas maltophilia</i>	7×10^{-2}	5×10^{-3}	2×10^{-1}
<i>Pseudomonas aeruginosa</i>	6×10^{-2}	1×10^{-3}	5×10^{-2}

^a Mating frequency = number of transconjugants/number of recipients.

TABLE 5. Plasmid stabilities^a

Bacterial species	No. of cells with plasmid:	
	RP4 (wild type)	RP4-21 (Δ TraH)
<i>E. coli</i>	100 100	100 100
<i>S. flexneri</i>	100 99	100 100
<i>P. stutzeri</i>	100 83	97 100
<i>P. maltophilia</i>	99 100	100 100
<i>P. aeruginosa</i>	99 90	89 98

^a Results from duplicate experiments of 7 days of passage without antibiotic selection. A total of 100 cells was tested.

putida, and *Pseudomonas aeruginosa* when *E. coli* was the donor. Transfer of RP4-21 from *E. coli* to the other gram-negative bacteria did not result in a change from wild-type efficiencies, except for *Pseudomonas maltophilia*, which had a slight increase. When the recipient was *E. coli*, a decrease of seven- and threefold was noted for the donors *Klebsiella pneumoniae* and *P. stutzeri*, respectively, while *P. maltophilia* increased in efficiency by threefold.

Stability studies with the TraH-defective RP4 (RP4-21). Since the knockout mutation in *traH* had only a minimal effect on transfer and previous studies showed that TraH was expressed from RP4, we wondered whether TraH had a role in the stabilization of RP4. Plasmid stabilities of RP4 and RP4-21 in some of the strains used in the broad-host-range mating experiments were tested. Bacteria were grown to stationary phase and then subcultured for 7 days in LB without antibiotic selection for the IncP plasmid. Cells were then plated onto LB agar, and 100 individual colonies were streaked onto LB agar with and without kanamycin at 50 μ g/ml (or carbenicillin at 500 μ g/ml for *P. aeruginosa*). Two independent sets of stability data were collected. Table 5 shows the stabilities of RP4 and RP4-21 in some gram-negative bacteria under these conditions. Plasmid stabilities for RP4 and RP4-21 were essentially identical in all the strains tested. Therefore, TraH does not play a role in plasmid maintenance.

Analogy to Agrobacterium Ti plasmid. The inactivation of the C terminus of TraI revealed that the last one-third of the molecule was required for the optimal transfer of RP4 in most gram-negative bacteria. Transfer from *E. coli* to *P. maltophilia* and from *P. putida* to *E. coli* resulted in similar efficiencies for RP4 and RP4-14, suggesting that these strains can correct for the TraI C-terminus defect. The C terminus of VirD2 in *A. tumefaciens* has a role in agroinfection and tumor formation (16). The important feature of the VirD2 C terminus in the Ti plasmid for all of these functions is the nuclear localization sequence (NLS), since deletion of this moiety significantly lowered the efficiency of transfer (16). Interestingly, the C-terminal deletion in VirD2 which eliminated the NLS did not abolish transfer but reduced it by 10- to 100-fold. A second NLS is located in the N terminus of VirD2, and other transfer proteins (such as VirE2) also contain these sequences. Thus, it is possible that targeting to

the nucleus and integration can be compensated to some extent in this VirD2 mutant by NLS motifs in other proteins.

The C terminus of TraI may be involved in targeting to the recipient cell wall, since TraI binds covalently to the single-stranded DNA upon nicking. Perhaps other proteins can compensate for the elimination of this targeting sequence in *P. maltophilia* and in *E. coli* when *P. putida* is the donor. This defect cannot be complemented when a wild-type copy of TraI is present only in the recipient, which suggests that the TraI C terminus functions only in the donor. However, it is also possible that the defect in the C terminus of TraI can only be complemented in *cis* and that TraI actually functions in the recipient on the same strand of DNA to which it has covalently bound.

The elimination of TraH had a minimal effect on transfer of RP4. However, recipients *P. stutzeri*, *P. putida*, and *P. aeruginosa* exhibited reduced transfer efficiencies of two- to threefold, respectively, when *traH* was inactivated. In addition, when *E. coli* was the recipient, the transfer efficiency of *K. pneumoniae* was reduced about sevenfold and that of *P. stutzeri* was decreased by threefold. These effects of a *traH* deletion can be put in perspective if it is taken into consideration that the formation of relaxed DNA in the *in vitro* assay increased insignificantly by the addition of purified TraH. These small improvements in transfer for some organisms may represent an evolutionary advantage for TraH over several generations. It remains to be determined whether TraH assists in transfer, although it is clear that it is not required for either nicking or conjugative transfer in any of the gram-negative bacteria tested.

The results obtained with TraH are consistent with those obtained for VirD3 in the *Agrobacterium* Ti plasmid. No significant difference in virulence or host range was noted for nonpolar *virD3* mutants expressing only the N-terminal third of the protein. However, its function in the natural infection process may not be readily measurable in the laboratory, and slight improvements in tumorigenicity or agroinfection may not be detected. The deletion of TraH and the C-terminal inactivation of TraI suggest that these two proteins are not critical components for conjugative transfer of RP4 but rather optimize the yield of transconjugants. These moieties may have survived evolutionary culling because they induce a small selective advantage manifested over many generations.

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