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The Relaxase of the *Rhizobium etli* Symbiotic Plasmid Shows *nic* Site *cis*-Acting Preference

Daniel Pérez-Mendoza,[1,2] María Lucas,[2] Socorro Muñoz,[1] José A. Herrera-Cervera,[1]† José Olivares, Fernando de la Cruz,[2] and Juan Sanjuán[1]*

Departamento de Microbiología del Suelo y Sistemas Simbióticos, Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas (CSIC), Granada, Spain,[1] and Departamento de Biología Molecular (Unidad asociada al CIB, CSIC), Universidad de Cantabria, C/Herrera Oria s/n, 39011 Santander, Spain[2]

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Genetic and biochemical characterization of TraA, the relaxase of symbiotic plasmid pRetCFN42d from *Rhizobium etli*, is described. After purifying the relaxase domain (N265TraA), we demonstrated *nic* binding and cleavage activity in vitro and thus characterized for the first time the nick site (*nic*) of a plasmid in the family *Rhizobiaceae*. We studied the range of N265TraA relaxase specificity in vitro by testing different oligonucleotides in binding and nicking assays. In addition, the ability of pRetCFN42d to mobilize different *Rhizobiaceae* plasmid origins of transfer (*oriT*) was examined. Data obtained with these approaches allowed us to establish functional and phylogenetic relationships between different plasmids of this family. Our results suggest novel characteristics of the *R. etli* pSym relaxase for previously described conjugative systems, with emphasis on the *oriT cis*-acting preference of this enzyme and its possible biological relevance.

Bacterial species belonging to the family *Rhizobiaceae* usually carry large plasmids essential for diverse functions that determine their lifestyles. For example, Ti plasmids enable *Agrobacterium tumefaciens* to induce crown gall disease in a wide range of plants (17, 29). Similarly, in many rhizobia, genes needed to establish nitrogen-fixing symbiotic associations with leguminous plants are located in plasmids, the so-called symbiotic plasmids (pSym). Besides phytopathogenic and symbiotic elements, members of the *Rhizobiaceae* may carry additional plasmids, usually cryptic (25, 40, 54, 59, 62).

In the genus *Agrobacterium*, conjugative plasmids have been described for *A. tumefaciens*, *A. radiobacter*, *A. rhizogenes*, and *A. vitis*, in which the virulent and opine catabolic plasmids have been well studied (10, 19). Tumor-inducing (Ti) plasmids of *A. tumefaciens* encode two transfer systems. The first system, called the *vir* system, mediates translocation of the transfer DNA from a bacterium to a plant cell. The second system is responsible for conjugative transfer of the Ti plasmid and is encoded by *tra* and *trb* genes. Ti plasmids can be transferred between bacterial populations that remain in the soil after plant infection. Opines produced by the plant after infection serve as growth substrates and are the signals that turn on expression of genes required for their utilization. Additionally, these signals regulate expression of the operons involved in plasmid transfer in a quorum-sensing (QS)-dependent manner. A population of *Agrobacterium* cells can take full advantage of the ability to catabolize opines if efficient Ti plasmid conjugal transfer ensures that the majority of colonizing agrobacteria contain a copy of the appropriate Ti plasmid.

* Corresponding author. Mailing address: Dpto. Microbiología del Suelo y Sistemas Simbióticos, Estación Experimental del Zaidín, Profesor Albareda 1, 18008 Granada, Spain. Phone: 34-958181600, ext. 259. Fax: 34-958129600. E-mail: juan.sanjuan@eez.csic.es.

† Present address: Dpto. Fisiología Vegetal, Facultad de Ciencias, Universidad de Granada, Campus Fuentenueva s/n, 18071 Granada, Spain.

Likewise, there is evidence for Sym plasmid exchange among rhizobia in soil (9, 31, 52, 67). Acquiring the ability to nodulate leguminous plants allows rhizobia to exploit an exclusive ecological niche, which has important advantages over a strictly saprophytic lifestyle. Therefore, the acquistion of the genetic information necessary to nodulate must have been an important event in the evolution of rhizobia, similar to what occurred in agrobacteria.

Compared to conjugal transfer of Ti plasmids, conjugal transfer of Sym plasmids has been studied less. Genome sequencing projects revealed the presence of potential conjugative systems in several of these plasmids. Typically, these systems consist of an *oriT* located between *traA* genes and *traCDG* operons likely involved in conjugative DNA processing and a *trb*-like (50, 69) or *virB*-like (4, 26) type IV secretion system (53). Conjugal transfer of *Rhizobium leguminosarum* bv. viciae symbiotic plasmid pRL1JI has been studied in great detail. The regulatory network that governs conjugative transfer of this plasmid depends on a QS regulatory relay (13). QS-dependent transfer has also been reported for other rhizobial nonsymbiotic plasmids (65).

Nevertheless, QS regulation of conjugal transfer is unlikely for other rhizobial plasmids, and recent studies have revealed the presence of novel regulation systems in the pSym plasmids of *Rhizobium etli* and *Sinorhizobium meliloti*, represented by the *rctA* gene encoding a repressor of plasmid conjugal transfer (48, 49).

Relaxases play a central role in DNA processing during bacterial conjugation. They initiate and end DNA transfer by catalyzing site- and strand-specific DNA cleavage reactions at *nic* of a given *oriT*. The sequence and structure of *nic* have been experimentally defined for different prototype plasmids (3, 21, 37, 45, 58, 66). However, little is known about the reactions involved in *oriT* processing by conjugative relaxases of phytopathogenic and symbiotic plasmids. Based on protein sequence conservation, Farrand and coworkers reported that

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant features	Reference or source
S. meliloti 1021	Wild-type strain, Sm^r	39
Rhizobium sp. strain NGR234	Wild-type New Guinea isolate	64
R. etli strains		
CE3	Sm^r derivative of strain CFN42	51
CFNX218Spc	CE3 derivative (p42a$^-$, p42b$^-$, p42c$^-$, p42d$^-$, p42eΔ, p42f$^-$) Spc^r	49
A. tumefaciens strains		
C58	Wild type, nopaline strain	73
GMI9023	Plasmidless C58 derivative	54
At Tn*5*.1	GMI9023 derivative with pRetCFN42d::Tn*5*.1	49
At Tn*5*.1Δ*traA*Gm	At Tn*5*.1 derivative (Δ*traA*::Gm^r)	This study
E. coli strains		
DH5α	*supE44* Δ*lacU169* ϕ80 *lacZΔM15 hsdR171 recA1 endA1 gyrA96 thi-1 relA1*	30
C41	BL21(DE3) derivative	42
Plasmids		
pET29C(+)	His tag expression vector, Km^r	Novagene
pETN265TraA	pET29C(+) with the first 795 bp of pRetCFN42d *traA*	This study
pGem-T Easy	PCR cloning vector, Ap^r	Promega
pJB3Tc19	IncP cloning vector; Tc^r Ap^r	8
pK18Δ*traA*Gm	pK18*mobSacB* derivative harboring pRetCFN42d *traA*-ΩGm	48
pJBRec	pJB3Tc19 with a 398-bp SacI/EcoRI fragment from pRetCFN42d *traA*	This study
pJB42d	pJB3Tc19 with 300 bp containing *nic* site of *R. etli* pRetCFN42d	This study
pJBSA	pJB3Tc19 with 300 bp containing *nic* site of *S. meliloti* pSymA	This study
pJBSB	pJB3Tc19 with 300 bp containing *nic* site of *S. meliloti* pSymB	This study
pJBNG	pJB3Tc19 with 300 bp containing *nic* site of *Rhizobium* sp. strain NGR234 pNGR234a	This study
pJBTi	pJB3Tc19 with 300 bp containing *nic* site of *A. tumefaciens* pTiC58	This study
pJBdp1	pJB3Tc19 carrying 7.7-kb HindIII fragment from pRe182R1a	48
pJBdp6	pJBdp1 derivative with the 7.7-kb HindIII fragment of pJBdp1 in reverse orientation	This study
pJBdp5	pJBdp1 derivative (pJBdp1 ΔStuI)	This study
pJBdp4	pJB3Tc19 carrying a 1,300-bp EcoRI fragment from pRe182R1a	48

[a] Sm^r, streptomycin resistant; Spc^r, spectinomycin resistant; Gm^r, gentamicin resistant; Km^r, kanamycin resistant; Ap^r, ampicillin resistant; Tc^r, tetracycline resistant.

TraA of pTiC58 is a chimeric protein consisting of 1,100 amino acids in which the amino-terminal domain resembles MobA, the relaxase of IncQ plasmid RSF1010, while the carboxyl-terminal domain resembles helicases of IncN, IncW, and IncF plasmids (1, 20). The smallest functional *oriT* of pTiC58 was defined as a 65-bp DNA fragment, and the location of *nic* was inferred from similarity with the well-defined sites of RSF1010 and pTF1 plasmids (7, 11, 58).

In this study we focused on purification of the TraA relaxase of the *R. etli* symbiotic plasmid. We determined for the first time the *nic* site of a plasmid in the family *Rhizobiaceae* and found some functional and phylogenetic relationships between rhizobial plasmids. Furthermore, the results of pRetCFN42d relaxase-*oriT* interaction experiments indicated that TraA of the *R. etli* symbiotic plasmid is a *cis*-acting protein, which has important ecological implications.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacteria and plasmids used are listed in Table 1. *R. etli*, *Rhizobium* sp. strain NGR234, and *S. meliloti* strains were grown at 30°C on TY (tryptone-yeast extract-$CaCl_2$) medium (6). *Escherichia coli* and *A. tumefaciens* were grown on Luria-Bertani (LB) medium (55). When required, antibiotics were added at the following concentrations: nalidixic acid, 20 μg/ml; spectinomycin, 100 μg/ml for *R. etli* and 200 μg/ml for *S. meliloti*; kanamycin (Km), 50 μg/ml for *R. etli* and *A. tumefaciens* and 200 μg/ml for *S. meliloti*; gentamicin, 10 μg/ml; rifampin, 50 μg/ml; streptomycin, 100 μg/ml for *R. etli* and *A. tumefaciens*, 200 μg/ml for *S. meliloti*, and 25 μg/ml for *E. coli*; tetracycline (Tc), 2 μg/ml for *Rhizobium* and 10 μg/ml for *E. coli*; and carbenicillin, 100 μg/ml for *A. tumefaciens*.

Bacterial transformation. Bacterial transformation was carried out by electroporation using an electrocell manipulator apparatus (BTX 600, San Diego, Calif.). Electrocompetent cells were prepared according to the instructions of the manufacturer and stored at −80°C. For electroporation, cells were thawed on ice, mixed with plasmid DNA (0.3 to 0.5 μg/ml of cell suspension), and transferred to a 0.2-cm-electrode-gap chilled cuvette. A pulse with a 2.5-kV/cm field strength, 6.8-ms length, and 129-Ω set resistance was applied, and cells were immediately suspended in 1 ml of TY or LB medium and incubated at 30°C for 15 h (*R. etli*) or at 37°C for 1 h (*E. coli*). Appropriate dilutions were plated on selective media.

Plasmid methodology, enzymes, and oligonucleotides. Plasmid DNA was purified as described by Sambrook et al. (55). DNA fragments were purified from agarose gels with silica using a GenElute gel extraction kit (Sigma). PCR amplification of DNA fragments up to 400 bp long was carried out with *Taq* DNA polymerase (Promega). For PCR amplification of larger fragments, High Fidelity Vent DNA polymerase (New England Biolabs) was used. Cloning techniques were carried out by using a standard methodology (55). Phage T4 polynucleotide kinase and T4 DNA ligase were obtained from New England Biolabs. Restriction endonucleases were purchased from Amersham. The oligonucleotides used (purchased from MWG-BIOTECH) are listed in Table 2.

Sequence treatment and phylogenetic analysis. A DNA sequence similarity search was carried out with the BLAST program from NCBI (2). Alignment was performed with CLUSTALW (63). Phylogenetic and molecular evolutionary analyses were conducted using MEGA, version 3.1 (34).

Conjugation experiments. Donor strains, grown to an optical density at 600 nm of 0.2, and recipient strains, grown to late exponential phase, were washed and mixed at a 1:1 ratio. Mating mixtures were resuspended in 50 μl TY medium and deposited onto sterile 0.45-μm-pore-size nitrocellulose filters. Filter mating mixtures were placed on TY agar plates and incubated overnight at 30°C. Cells were

TABLE 2. Oligonucleotides

Oligonucleotide	Sequence[a]	Location or fragment amplified
TraANdeI-F	TCACTCATATGGCGATCATGTTCGTCAGAG	Positions 144991 to 145776 of pRetCFN42d sequence (NC_004041)
TraAXhoI-R	TTTATCTCGAGGCGGCGGGCGAGAT	
OriTsdAB-F	AATGAATTCGCCTGCTCGTCGATCATCC	
OriTp42d-R	TCGAAAGCTTATCGATGATTTTGCAGCCA	With OriTsdAB-F, positions 145673 to 145972 of pRetCFN42d sequence (NC_004041)
OriTpSymA-R	TTGAAAGCTTGGATTTCGCGGCCATTC	With OriTsdAB-F, positions 515500 to 515799 of pSymA sequence (NC_003037)
OriTpSymB-R	AAGCAAGCTTTACCGTCACGGGCAAATAC	With OriTsdAB-F, positions 725786 to 726085 of pSymB sequence (NC_003078)
OriTpNGR234a-F	CAGCACGGCGCTGCGGCC	Positions 80004 to 80320 of pNGR234a sequence (NC_000914)
OriTpNGR234a-R	ATTCCGTCCGTCTCCGTGGCTG	
OriTpTiC58-F	CGGCTGACGATGCTGGCT	Positions 143729 to 144024 of pTiC58 sequence (NC_004972)
OriTpTiC58-R	TCCGGTCGCATCTCCCTG	
p42d-20mer[b]	ACGTATATTGCG/CCCTCAAA	pRetCFN42d
p42d-C-[b]	TTTGAGGGCGCAATATACGT	pRetCFN42d
p42d-28mer[b]	CGACAGCGACGTATATTGCG/CCCTCAAA	pRetCFN42d
p42d-34mer[b]	CGTCGCGACAGCGACGTATATTGCG/CCCTCAAAC	pRetCFN42d
p42d-32mer[b]	CAGCGACGTATATTGCG/CCCTCAAACAGATCG	pRetCFN42d
pTN-21mer[b]	ACGTATAATTGCG/CCCTTGGA	pTiC58 and pNGR234a
pSB-20mer[b]	TAGCCTATCCTG/CAATAGAC	pSB102
pRS-20mer[b]	CCGGTAAGTGCG/CCCTCCCC	RSF1010
PXF-20mer[b]	AAACCTATCCTG/CCCTAGAT	pXF51

[a] Restriction sites used in cloning are underlined.
[b] Oligonucleotide used in EMSA and nicking assays.

resuspended by vigorous vortexing and diluted in liquid medium. Transconjugants were selected on plates supplemented with appropriate antibiotics. The transfer frequency was expressed as the number of transconjugants per output recipient.

Plasmid construction. The truncated pRetCFN42d TraA gene was cloned in the pET29c(+) vector. First, the DNA fragment corresponding to pRetCFN42d bp 144991 to 145776 was PCR amplified with oligonucleotide primers TraANdeI-F and TraAXhoI-R (Table 2). The resulting DNA was digested with endonucleases NdeI and XhoI and cloned into the same sites of plasmid pET29c(+), resulting in plasmid pETN265TraA, the vector used for N265TraA overexpression and purification.

For construction of plasmids harboring the *oriT* regions of symbiotic plasmids pRetCFN42d of *R. etli* and pSymA and pSymB of *S. meliloti*, the corresponding 300-bp DNA fragments around the *nic* site (150 bp to each side) were PCR amplified using oligonucleotide OriTsdAB-F and either OriTp42d-R, OriTpSymA-R, or OriTpSymB-R. The resulting DNAs were digested with endonucleases EcoRI and HindIII and cloned into the same sites of plasmid pJB3Tc19, resulting in plasmids pJB42d, pJBSA, and pJBSB, respectively. *oriT* plasmids of symbiotic plasmid pNGR234a of *Rhizobium* sp. strain NGR234 and tumorigenic plasmid pTiC58 of *A. tumefaciens* were generated by a two-step procedure. First, the hypothetical *oriT* regions were amplified using oligonucleotide primers OriTpNGR234a-F and OriTpNGR234a-R and oligonucleotide primers OriTpTiC58-F and OriTpTiC58-R, respectively. The DNA fragments generated were independently cloned into the pGem-T Easy PCR cloning vector (Promega). Second, the two 300-bp EcoRI *oriT* fragments were independently cloned into the same sites of plasmid pJB3Tc19, resulting in plasmids pJBNG and pJBTi, respectively.

Two additional constructs were generated from the pJBdp1 plasmid (48): (i) pJBdp6 was obtained by digesting pJBdp1 plasmid DNA with HindIII, rejoining, and selecting a clone with the HindIII fragment in the inverse orientation; and (ii) pJBdp5 was obtained by digesting pJBdp1DNA with StuI and rejoining the ends, thus deleting two of the three StuI fragments present in pJBdp1.

Construction of a *traA* mutant derivative of pRetCFN42d::Tn*5*.1. Plasmid pK18Δ*traA*Gm was introduced by conjugation into *A. tumefaciens* strain GMI9023 harboring pRetCFN42d::Tn*5*.1, a Sym plasmid derivative of *R. etli*. Allele replacement was selected as described previously (56), and *traA* mutants were verified after hybridization with labeled plasmid pK18Δ*traA*Gm that was BamHI digested as a probe.

Oligonucleotides and labeling. Unlabeled oligonucleotides were purchased from MWG-BIOTECH. Oligonucleotides were labeled at the 5′ end using [γ-^{32}P]ATP (3,000 Ci mmol^{-1}) and polynucleotide kinase (New England Biolabs) (55). Unbound [γ-^{32}P]ATP was eliminated from the mixture by MicroSpinTM G-25 column (Amersham) purification.

N265TraA overexpression and purification. For purification of the pRetCFN42d TraA N-terminal fragment, the *E. coli* BL21 derivative strain C41-DE3 was used as a host. Cells containing the pTEN265TraA overexpressing plasmid were grown in 1 liter of LB broth containing Km (50 mg ml^{-1}). Protein expression was induced at an A_{600} of 0.6 by adding 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and incubation was continued for an additional 4 h. Cells were harvested and resuspended in 12 ml of 50 mM Tris-HCl (pH 7.6), 10 mM EDTA, 10% (wt/vol) sucrose. N265TraA purification was carried out by following QIAGEN recommendations for Ni-nitrilotriacetic acid (NTA). The cell pellet was resuspended in 35 ml of lysis buffer (100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea; pH 8). The lysate was stirred for 90 min at room temperature and then centrifuged at 10,000 × *g* for 30 min to pellet the cellular debris. Eight milliliters of Ni-NTA resin preequilibrated with lysis buffer was added to the supernatant (cleared lysate) and mixed gently by shaking for 60 min at room temperature. The lysate-resin mixture was loaded onto an empty column with the bottom cap still attached. Washing, refolding, and elution of N265TraA were performed with the Automated Econo System (Bio-Rad). The refolding of N265TraA was carried out by immobilizing one end of the protein linked to the Ni column to prevent intermolecular interactions, which lead to aggregate formation. Renaturation was performed using a linear 6 M to 0 M urea gradient (120 steps in 120 min) in 20 mM Tris (pH 7.6), 200 mM NaCl, 20% glycerol. N265TraA was eluted by adding 10 ml of elution buffer (20 mM Tris [pH 7.6], 200 NaCl mM, 20% glycerol, 500 mM imidazole). To obtain a higher level of N265TraA purity, a second round of Ni-NTA purification was carried out in the same conditions. Imidazole was removed by dialysis using cellulose tubing (Sigma) in 20 mM Tris (pH 7.6), 200 mM NaCl, 20% glycerol for 15 to 20 h at 4°C. The purified protein was stored at −20°C. No loss of activity was observed after 6 months of storage under these conditions.

Oligonucleotide cleavage and strand transfer reactions using oligonucleotides labeled at the 5′ end with [^{32}P]ATP. For oligonucleotide cleavage reactions, 12.5 μM N265TraA was incubated with 5′-labeled oligonucleotides (10 nM) in cleavage buffer (10 mM Tris-HCl [pH 7.6], 5 mM $MgCl_2$, 100 mM NaCl). After 3.5 h

TABLE 3. Homologs of pRetCFN42d TraA[a]

Organism	Protein	Protein size (amino acids)	Gene	Plasmid or chromosome	No. of amino acids covered in the alignment	% Similarity	% Identity
S. meliloti	TraA2	1,539	*traA2*	pSymB	1,552	86	75
S. meliloti	TraA1	1,539	*traA1*	pSymA	1,550	85	75
A. tumefaciens	TraA	1,609	TraA	pAtC58	1,552	83	71
Mesorhizobium sp. strain BNC1	MBNC03003747	1,557	MBNC03003747	Chromosome	1,564	76	64
A. tumefaciens	TraA	1,194	tiorf109	pTi-Sakura	1,278	54	39
R. etli	TraA	1,103	TraA	pRetCFN42a	788	62	46
A. rhizogenes	TraA	1,108	riorf112	pRi1724	796	62	45
A. tumefaciens	TraA	1,100	TraA	pTiC58	786	61	46
A. tumefaciens	TraA	1,108	TraA	pRiA4b	796	61	45
Rhizobium sp.	TraA	1,102	TraA	pNGR234a	786	61	45
O. carboxidovorans	TraA	1,173	TraA	pHCG3	979	50	37
M. loti	TraA	1,015	mll5729	Chromosome	811	52	37
M. loti	TraA	1,015	mll0964	Chromosome	819	52	36

[a] The highest values (more than 50% similarity) obtained with BLASTP (2) are shown. Amino acid sequence conservation is expressed as percent similarity or identity.

of incubation at 28°C, reaction products were treated with proteinase K (13.6 mg/ml) and 1% (wt/vol) sodium dodecyl sulfate as described by Grandoso et al. (27) and were analyzed by polyacrylamide gel electrophoresis, followed by autoradiography (55). In order to obtain a marker ladder, a mixture of dATP and ddATP (10:1) at a final concentration of 500 μM and 1 U of terminal deoxynucleotidyl transferase were added to the p42d-20mer *nic* cleavage reaction mixture (see Fig. 5, lane 5). Oligonucleotide strand transfer reactions were carried out and analyzed like the cleavage reactions, except that the reaction samples contained an additional unlabeled oligonucleotide (p42d-32mer) at a final concentration of 100 nM.

EMSA. Binding reaction mixtures contained 1 nM radiolabeled oligonucleotide, 1 μM competitor oligonucleotide, and concentrations of N265TraA ranging from 0 to 1 μM in electrophoresis mobility shift assay (EMSA) buffer (10 mM Tris-HCl [pH 7.6], 100 mM NaCl, 0.02 mM EDTA). The competitor oligonucleotide was a mixture of three unlabeled oligonucleotides. The reaction mixtures were incubated for 20 min at 20°C. Samples were loaded onto a 12% nondenaturing polyacrylamide Bio-Rad minigel (0.75 mm by 6.5 cm) and electrophoresed for 60 min at 100 V in 90 mM Tris-borate buffer (pH 8.2). The gels were dried and scanned with a Molecular Imager FX system (Bio-Rad). The results were analyzed using the Quantity One software (Bio-Rad). Binding constants were calculated by nonlinear fitting of the data to the binding equation using the Prism 3.0 program (GraphPad Software).

RESULTS

Phylogenetic position of pRetFCN42d TraA and other rhizobial relaxases. TraA is one of the largest proteins (1,553 amino acids) encoded in the *R. etli* symbiotic plasmid and was annotated as a putative relaxase (26). The highest BLASTP scores occurred with hypothetical relaxases of members of the rhizobial order (Table 3). Among the TraA homologues, the TraA1 and TraA2 proteins of *S. meliloti* Sym plasmids, TraA of the pAtC58 cryptic plasmid of *A. tumefaciens*, and MBNC03003747 of *Mesorhizobium* sp. strain BNC1 showed more than 60% sequence identity. All of these proteins were also large (around 1,555 amino acids) (Table 3). A second group of proteins with significant sequence conservation (35% to 46% sequence identity) included putative relaxases of various agrobacterial and rhizobial plasmids, as well as putative relaxases encoded by the *Mesorhizobium loti* chromosome, although all these proteins were significantly smaller (around 1,100 amino acids) than the *R. etli* TraA protein (Table 3).

Most conjugative relaxases belong to a single large protein superfamily (22), suggesting that there is a unique, shared DNA processing mechanism. Three common motifs were defined based on protein similarities and the known atomic structure of the TrwC relaxase of plasmid R388 (22). Motif I contains a catalytic Tyr residue involved in DNA cleavage-joining activity. Motif II contains an invariant acidic residue (Asp or Glu) homologous to Asp85 in TrwC that likely activates the hydroxyl group of the catalytic Tyr by proton abstraction (28). Motif III contains three conserved His residues constituting a metal ion binding site, known as the 3H motif. The three motifs contribute to the catalytic center of relaxases. A CLUSTALW alignment of pRetCFN42d TraA and its relatives (Table 3) showed that there was conservation of the three relaxase motifs (Fig. 1B). The invariant residues include the catalytic Tyr in motif I, a Glu in motif II, and three His residues in motif III. The phylogenetic tree shown in Fig. 2 was obtained from alignment of the relaxase domains (300 N-terminal amino acids). A few conspicuous representatives of the main relaxase families, MOB_Q, MOB_P, and MOB_F (22), as well as three relaxases of recently described environmental broad-host-range plasmids present in plant-associated bacteria (59, 61, 62), were also included. The tree shows that relaxases of agrobacterium and rhizobium megaplasmids and symbiotic islands form a well-separated monophyletic group, well supported by a boostrap value of 99%, having a common ancestor with MOB_Q relaxases (Fig. 2). Neighbor-joining analysis showed that relaxases of the plant-associated bacterial plasmids form a different clade and that the distance to the common ancestor with TraA-like relaxases is relatively great (Fig. 2).

MOB_Q relaxases have a domain structure consisting of an N-terminal relaxase domain and a C-terminal primase domain, as reported previously for RSF1010/R1162 (57, 71). On the other hand, Farrand and coworkers described a putative C-terminal helicase domain for TraA of pTiC58 in addition to the N-terminal relaxase (20). The central fragment of pRetCFN42d TraA (residues 390 to 815) (Fig. 1A) has five DNA helicase motifs. Two of them are the Walker A and B nucleoside triphosphate (NTP)-binding motifs, which are typically found in nucleoside triphosphatases (Fig. 1C) (68). In addition, this

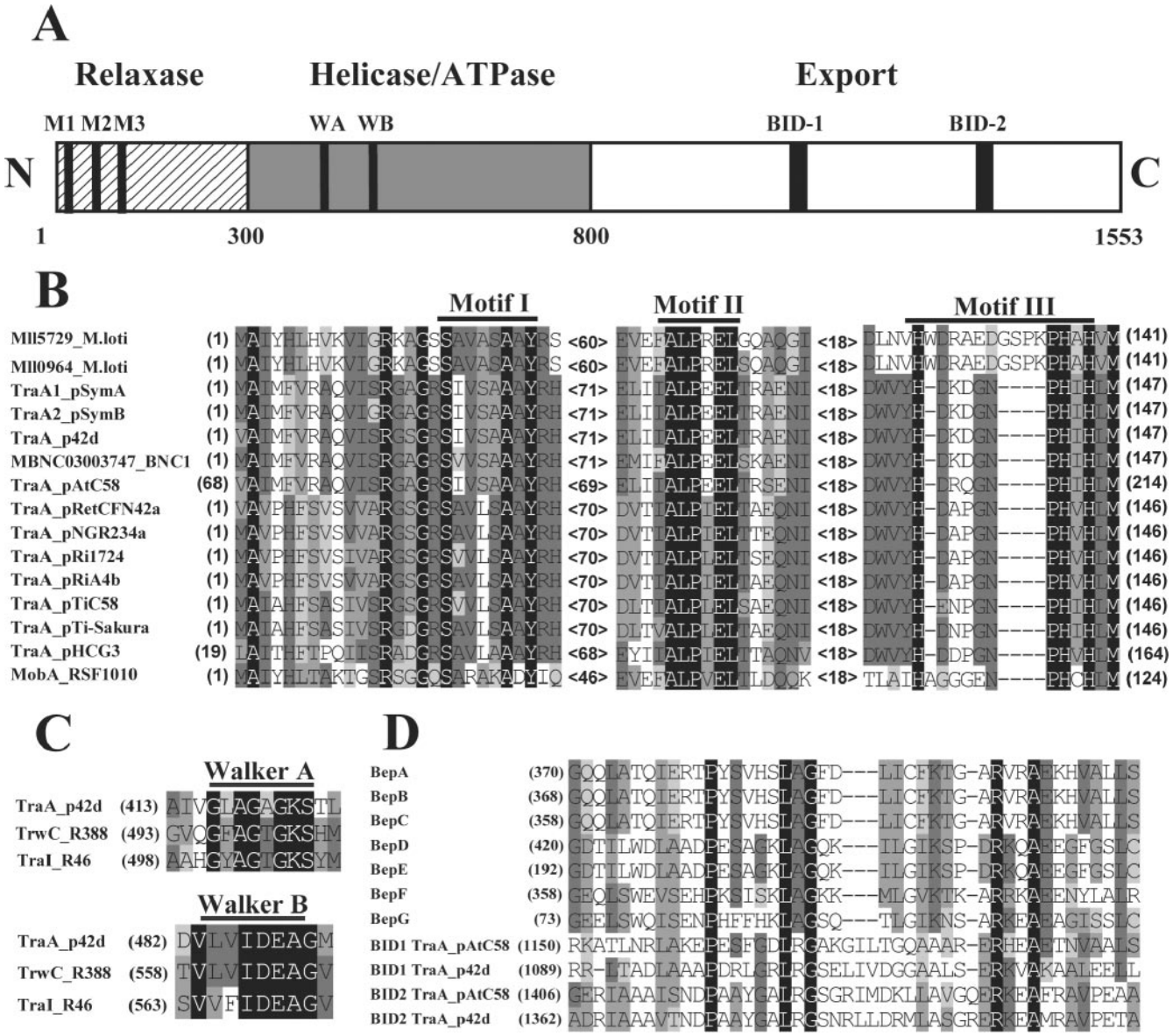

FIG. 1. (A) Schematic diagram of the domain organization of pRetCFN42d TraA. The N-terminal relaxase domain is indicated by a striped box, the helicase domain is indicated by a gray box, and the C-terminal domain containing intracellular delivery signatures (BID) is indicated by an open box. (B) Conserved motifs in the relaxase domain of pRetCFN42d TraA and related proteins. (C) Conserved ATPase Walker boxes in the helicase domain. (D) Conserved sequences in two hypothetical BID domains similar to BepA-BepG from *B. henselae* and TraA from *A. tumefaciens* pAtC58. White type with a black background indicates invariant residues; black type with a dark gray background indicates strongly conserved residues; black type with an intermediate gray background indicates similar residues; black type with a pale gray background indicates weakly conserved residues; and black type with a white background indicates nonconserved residues. Accession numbers are as follows: Mll5729 from *M. loti*, BAB52121; Mll0964 from *M. loti*, BAB48437; TraA1 from *S. meliloti* pSymA, A95325; TraA2 from *S. meliloti* pSymB, CAC49066; TraA from *R. etli* pRetCFN42d, NP_659868; MBNC03003747 from *Mesorhizobium* sp. strain BNC1, ZP_00193296; TraA from *A. tumefaciens* pAtC58, AC3173; TraA from *R. etli* pRetCFN42a, AAO43541; TraA from *Rhizobium* sp. strain NGR234(pNGR234a), T02782; TraA from *A. rhizogenes* pRi1724, NP_066693; TraA from *A. tumefaciens* pRiA4b, BAB47249; TraA from *A. tumefaciens* pTiC58, AAC17212; TraA from *A. tumefaciens* pTi-Sakura, NP_053349; TraA from *Oligotropha carboxidovorans* pHCG3, YP_015676; MobA of RSF1010, CAA28520; TrwC of R388, CAA44853; TraI of R46, NP_511201; and BepA, BepB, BepC, BepD, BepE, BepF, and BepG from *B. henselae*, CAD89506, CAD89508, CAD89509, CAD895010, CAD895011, CAD895012, and CAD895013, respectively.

pRetCFN42d TraA central fragment exhibits between 20% and 22% identity with helicase domains of MOB_F family relaxases TraI_F (amino acids 974 to 1417), TrwC_R388 (amino acids 471 to 945), and TraI_pKM101(amino acids 490 to 938). Together, these observations suggest that TraA_pRetCFN42d had a chimeric origin similar to that proposed for TraA_pTiC58 (1, 20).

It has recently been reported (60) that the *Bartonella* VirB/VirD4 system can translocate at least seven effector proteins (BepA to BepG) to target eukaryotic cells. The Bep proteins

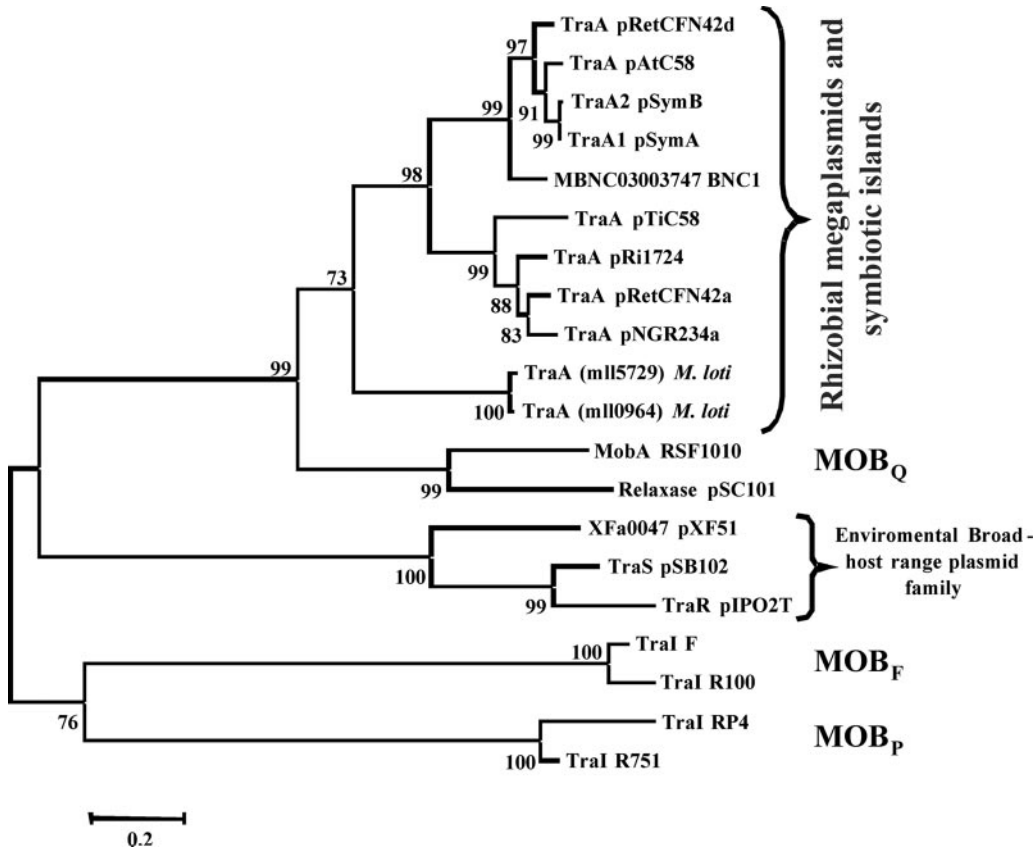

FIG. 2. Neighbor-joining phylogenetic analysis with bootstrap values (2,000 replicates) of TraA and related relaxase domains (300 N-terminal amino acids). Pairwise matrices of genetic distances were calculated using the Poisson correction parameter. The designations of relaxases and the corresponding plasmids are shown. Bootstrap values are indicated at the relevant branches. Accession numbers not given in the legend to Fig. 1 are as follows: relaxase of pSC101, CAA25820; XFa0047 from *Xylella fastidiosa* pXF51, Q9PHG1; TraS of pSB102, CAC79161; TraR of pIPO2T, CAC82755; and TraI of F, BAA97974.

have at least one copy of the Bep intracellular delivery (BID) domain and a short positively charged tail at the C terminus. This bipartite structure is sufficient to mediate VirB/VirD4-dependent intracellular delivery. Using the Cre recombinase reporter assay for translocation, Schulein and coworkers demonstrated that the pAtC58 relaxase TraA has a BID domain and a positively charged tail sequence at its C terminus, which efficiently direct its transfer from *Bartonella henselae* into human endothelial cells (60). Alignment of the pRetCFN42d TraA C terminus with BepA to BepG and TraA_pAtC58 revealed the presence of two hypothetical BID domains (from amino acid 1026 to amino acid 1165 and from amino acid 1301 to amino acid 1442) (Fig. 1A and D) and a positively charged tail (last 191 amino acids, with a net charge of 12). Conservation of the bipartite signal suggests that the pRetCFN42d TraA C-terminal domain has an important role.

***nic* site of pRetFCN42d and related plasmids.** The pRetCFN42d TraA protein N-terminal domain (N265TraA) was purified (Fig. 3) by affinity chromatography (Ni-NTA) (see Materials and Methods), as full-length TraA could not be overproduced. N265TraA binding to oligonucleotides containing its potential *nic* site was assayed by EMSA, using 5′-end-labeled oligonucleotides that were different sizes (p42d-20mer, p42d-28mer, and p42d-34mer) (Table 2). An oligonucleotide containing the

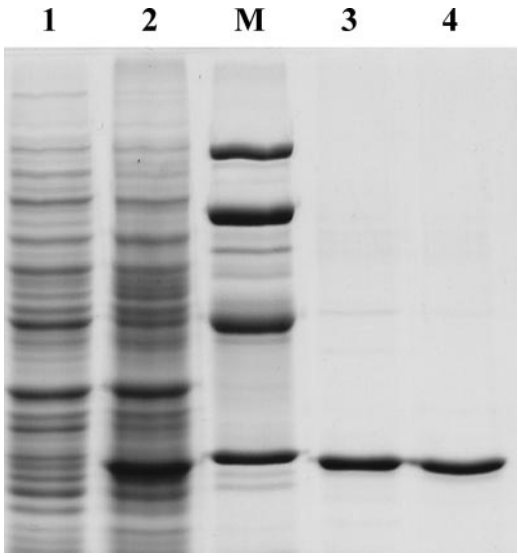

FIG. 3. Purification of protein N265TraA: results of 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis of 15-μl samples from pooled fractions from the different purification steps. Lane 1, crude extract before induction; lane 2, crude extract after 4 h of induction; lane 3, 0.6 μg of N265TraA eluted from Ni-NTA column; lane 4, 0.6 μg of N265TraA eluted from Ni-NTA column after a second round of protein purification; lane M, molecular weight marker (low-range prestained sodium dodecyl sulfate-polyacrylamide gel electrophoresis standards; Bio-Rad).

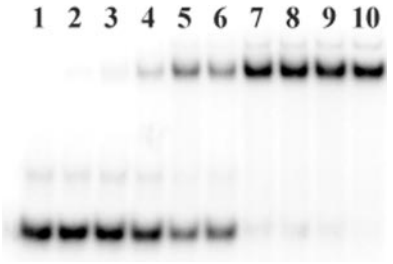

FIG. 4. Electrophoretic mobility shift assay of oligonucleotide p42d-20mer with protein N265TraA. Lanes 1 to 10 contained different concentrations of the N265TraA protein (0, 3, 10, 30, 100, 150, 300, 450, 800, 500, and 1000 nM, respectively) incubated with 1 nM p42d-20mer oligonucleotide, as described in Materials and Methods.

reverse complementary sequence of *nic* was included as a control (p42d-C-).

N265TraA shifted oligonucleotides p42d-20mer, p42d-28mer, and p42d-34mer even in the presence of a large excess of an unlabeled nonspecific mixture of oligonucleotides, showing similar dissociation constants (K_D) for the three oligonucleotides (the K_D were between 49 and 68 nM). The results of a gel retardation assay using 5′-end-labeled oligonucleotide p42d-20mer are shown in Fig. 4 as an example of a positive interaction with N265TraA. However, even a 1,000-fold molar excess of N265TraA did not shift oligonucleotide p42d-C-. These results showed that oligonucleotide binding was sequence specific and that the smallest oligonucleotide assayed (p42d-20mer) contained enough sequence information to be accurately recognized by N265TraA.

N265TraA *nic*-specific cleavage and strand transfer activities were analyzed using oligonucleotide p42d-20mer that was labeled with ^{32}P at its 5′ end. The protein cleaved the oligonucleotide, as judged from the smaller size of the resulting labeled product (Fig. 5, lanes 1 and 2). When the cleavage reaction mixture also contained an excess of unlabeled oligonucleotide p42d-32mer, a new larger labeled oligonucleotide (27-mer) was produced by DNA strand transfer (Fig. 4, lanes 6 and 7). Analogous products were obtained from the in vitro activities of other relaxases (36, 45, 66). By using a marker ladder, the exact nucleotide at which strand interruption occurred could be inferred (Fig. 5, lane 5). As shown in Fig. 5, N265TraA cleaved the p42d-20mer oligonucleotide, generating 5′-^{32}P-ACGTATATTGCG plus CCCTCAAA-3′, thus showing that the *nic* site of the *R. etli* symbiotic plasmid is at position 145830 of the pRetCFN42d sequence (accession no. NC_004041).

The alignment of pRetCFN42d-related *nic* sites showed that there was conservation of a 15-bp invariant sequence (ACGT ATA[A or -]TGCGCCCT) among rhizobial and agrobacterial megaplasmids (Fig. 6A). This sequence, with a consensus AC NNNTA(N_{1-2})TGCGCCCT sequence, was also conserved when other plasmids belonging to the MOB_Q family were included. The topology of the *nic* site phylogenetic tree obtained from this alignment was similar to that of the corresponding relaxase phylogenetic tree (compare Fig. 2 with Fig. 6B). If we judge the evolution of plasmids by the phylogeny of their relaxases or *nic* sites, we can conclude that agrobacterium and rhizobium TraA-containing megaplasmids form an isolated monophyletic group within MOB_Q that is closer to IncQ plasmids than to the environmental broad-host-range plasmids of plant-associated bacteria.

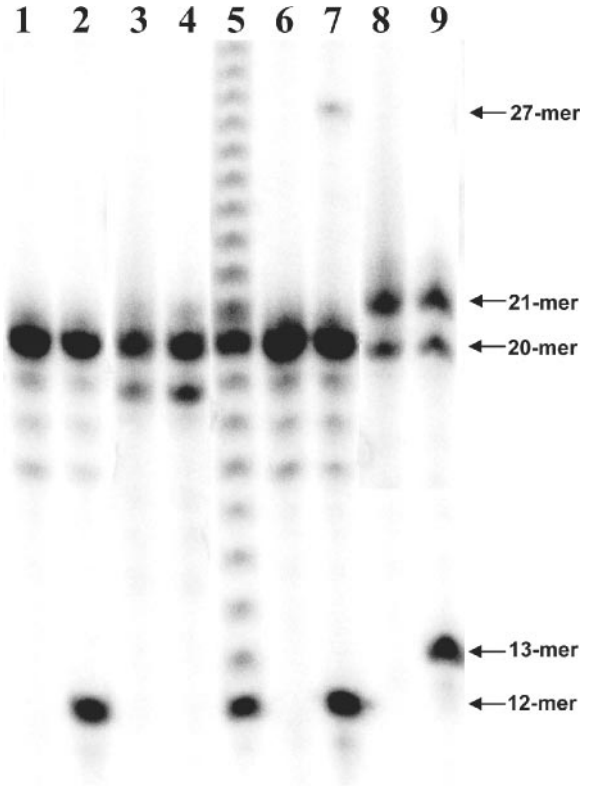

FIG. 5. In vitro cleavage and strand transfer reactions catalyzed by N265TraA. Reaction mixtures (10 μl) contained different 5′-labeled oligonucleotides (10 nM) incubated with or without N265TraA (12.5 μM). Reactions were carried out at 28°C for 3.5 h as described in Material and Methods. Lane 1, p42d-20mer; lane 2, p42d-20mer plus N265TraA; lane 3, p42d-C-; lane 4, p42d-C- plus N265TraA; lane 5, marker (p42d-20mer, N265TraA, dATP, ddATP, and terminal deoxynucleotidyl transferase); lane 6, p42d-20mer plus p42d-32mer; lane 7, p42d-20mer plus p42d-32mer plus N265TraA; lane 8, pTN-21mer; lane 9, pTN-21mer plus N265TraA. Product sizes are indicated by arrows.

Specificity of *oriT* recognition by pRetCFN42d conjugal transfer system. The N265TraA protein catalyzed DNA cleavage and strand transfer reactions when oligonucleotides encompassing its cognate *nic* site were used. The presence of similar *nic* sequences in pRetCFN42d-related plasmids (Fig. 6A) led us to compare the binding affinities of N265TraA for oligonucleotides containing different *nic* sites (Table 2). Thus, the N265TraA protein was incubated with oligonucleotides containing the *nic* sites of plasmids pTiC58/pNGR234a (pTN-21mer), RSF1010 (pRS-20mer), pSB102 (pSB-20mer), and pXF51 (pXF-20mer). N265TraA bound oligonucleotide pTN-21mer with an affinity similar to the affinity for p42d-20mer (K_D, 59 nM), whereas it did not bind the remaining oligonucleotides, even in the presence of a 1,000-fold molar excess of N265TraA.

N265TraA *nic*-specific cleavage was tested with oligonucleotide pTN-21mer. N265TraA cleaved the 21-mer oligonucleotide, producing a 13-mer labeled product (Fig. 5, lanes 8 and 9). This result shows that the TraA relaxase domain could bind

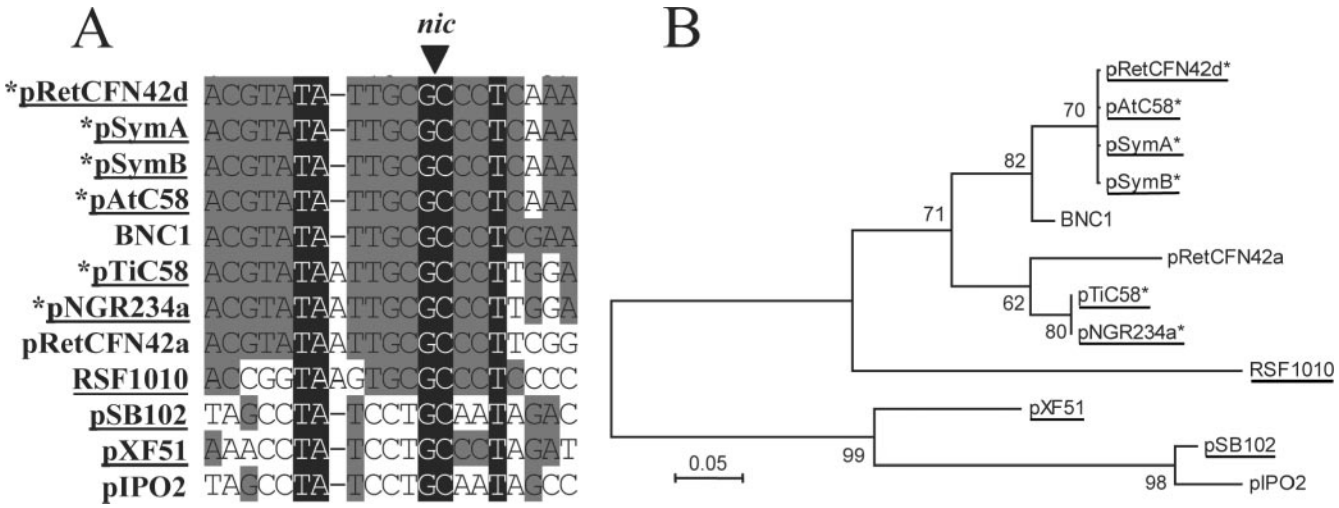

FIG. 6. *nic* sites of pRetCFN42d and related plasmids. Sites that were tested experimentally for N265TraA binding and *nic* cleavage are underlined. Asterisks indicate the sites that were bound and cleaved by N265TraA. (A) Alignment of *nic* site DNA sequences, showing invariant (black background) or conserved (gray background) nucleotides. The cleavage position is indicated by a solid triangle. (B) Neighbor-joining dendrogram with bootstrap values (2,000 replicates) constructed from the alignment. Pairwise matrices of genetic distances were calculated using the p-distance parameter.

and cleave only oligonucleotides containing the *nic* sites of closely related plasmids.

Besides the relaxase, other accessory proteins are also crucial for *oriT* recognition, as shown previously for other transfer systems (32, 43, 72). Mobilization assays were carried out to determine the specificity of the pRetCFN42d conjugal transfer system in vivo. Three-hundred-base-pair sequences around the hypothetical *nic* sites of pRetCFN42d and related plasmids were cloned in the vector pJB3Tc19. The *oriT* regions of the *S. meliloti* symbiotic plasmids pSymA and pSymB were selected since they contain very similar *oriT* regions (79.5% and 76.8% identity to the pRetCFN42d region, respectively). pTiC58 and pNGR234a were used as plasmids with less similar *oriT* regions (47.6% and 47.5% identity to pRetCFN42d, respectively).

An *R. etli* pSym derivative (pRetCFN42d::Tn*5*.1) which carries a mutation in the conjugal transfer repressor gene *rctA* provided *trans* mobilization functions, since wild-type pRetCFN42d does not transfer under laboratory conditions (49). Plasmid constructs were independently electroporated into an *A. tumefaciens* plasmidless strain containing pRetCFN42d::Tn*5*.1. These donors were mated with the *R. etli* CFNX218Spc recipient strain. A negative control plasmid (pJBRec) was constructed by cloning a 398-bp fragment outside pRetCFN42d *oriT*. Since mobilization experiments involved $RecA^+$ strains, pJBRec was used to evaluate transfer of the *oriT* constructs via homologous recombination with pRetCFN42d::Tn*5*.1.

Transconjugants for the symbiotic plasmid (Km-resistant colonies) and for the pJB3Tc19 derivatives (Tc-resistant colonies) were independently selected, and transfer and mobilization frequencies were determined (Table 4). The results showed that pNGR234a (pJBNG) and pTiC58 (pJBTi) *oriT* regions were not recognized by the pRetCFN42d conjugal transfer machinery. Transconjugants were not detected in the negative controls pJB3Tc19 and pJBRec, indicating that general recombination did not interfere with mobilization. In contrast, pRetCFN42d::Tn*5*.1 mobilized the *oriT* regions of *S. meliloti* symbiotic plasmids pSymA (pJBSA) and pSymB (pJBSB) at frequencies similar to the frequency for its own *oriT* (pJB42d) (Table 4). Thus, the evolutionarily related symbiotic plasmids of *R. etli* and *S. meliloti* have enough sequence similarity to be recognized by each other's conjugal transfer machinery. However, other megaplasmids that are included in this family but are more distantly related to pRetCFN42d (such as the symbiotic plasmid pNGR234a and the tumor-inducing plasmid pTiC58) have enough differences in their conjugative transfer systems to not be functionally interchangeable (Fig. 2 and 6).

pRetCFN42d TraA has a *nic* site *cis*-acting preference. Although the cloned *oriT* regions from *R. etli* and *S. meliloti* symbiotic plasmids could be mobilized by pRetCFN42d::Tn*5*.1, their transfer frequencies were between 100- and 1,000-fold lower than that of pRetCFN42d::Tn*5*.1 (Table 4). In order to examine the possibility that the *oriT* fragments (300 bp) were missing some important information, two new constructions

TABLE 4. Mobilization of different plasmid *oriT* regions in *trans* by plasmid pRetCFN42d::Tn*5*.1[a]

Construct (plasmid *oriT*)	No. of transconjugants	
	Km^r	Tc^r
None	1.48×10^{-3}	
pJB3Tc19 (empty vector)	7.25×10^{-4}	$<10^{-8}$
pJBRec (*traA* fragment, no *oriT*)	8.6×10^{-4}	$<10^{-8}$
pJB42d (*R. etli* pRetCFN42d)	1.17×10^{-3}	6.09×10^{-6}
pJBSA (*S. meliloti* pSymA)	1×10^{-3}	5.43×10^{-6}
pJBSB (*S. meliloti* pSymB)	1.02×10^{-3}	5.11×10^{-6}
pJBNG (pNGR234a)	9.29×10^{-4}	$<10^{-8}$
pJBTi (pTiC58)	7.11×10^{-4}	$<10^{-8}$

[a] *A. tumefaciens* GMI9023 harboring pRetCFN42d::Tn*5*.1 and pJB3Tc19 derivatives were mated with *R. etli* CFNX218Spc. The frequencies of pRetCFN42d::Tn*5*.1 transfer (Km-resistant transconjugants) and mobilization of pJB3Tc19 derivatives (Tc-resistant transconjugants) are expressed as the number of transconjugants per input recipient cell. The values are the means from three independent experiments.

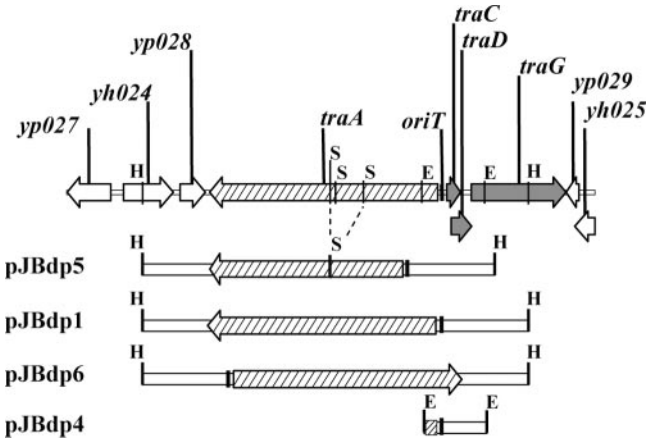

FIG. 7. Plasmid constructs containing pRetCFN42d *oriT*. The genetic organization of the *R. etli* symbiotic plasmid around *nic*, including relevant genes and restriction sites (E, EcoRI; H, HindIII; S, StuI), is shown. Schematic diagrams of the regions cloned in pJB3 derivatives used for mobilization experiments are shown at the bottom.

(pJBdp4 and pJBdp1) were used in mobilization experiments (Fig. 7). Even though the *oriT* DNA fragment contained in pJBdp4 was 1.3 kb long (406 bp upstream of *nic* and 923 bp downstream of *nic*), its mobilization frequency was similar to that of pJB42d (Table 5). This result indicated that the size of the pRetCFN42d *oriT* fragment in pJB42d was not a crucial factor for achieving efficient mobilization. However, pJBdp1 (with a 7.7-kb HindIII fragment containing the pRetCFN42d *nic* site) was mobilized at a frequency similar to that of the self-transmissible plasmid pRetCFN42d::Tn*5*.1 (Table 5). Likewise, a plasmid carrying the same HindIII fragment but cloned in the opposite orientation (pJBdp6) was efficiently mobilized by pRetCFN42d::Tn*5*.1. Therefore, this 7.7-kb HindIII fragment contained enough information to be mobilized at a high frequency.

Another possible explanation for the different mobilization frequencies observed for the small and large *oriT* fragments is the presence of an element (present in the 7.7-kb HindIII DNA fragment) that might be required in *cis* with the *nic* site to achieve efficient mobilization. The absence of this hypothetical *cis*-acting element in pJBdp4 and in the rest of the 300-bp *oriT* plasmid constructs would explain their inefficient mobilization.

There are a number of DNA-binding proteins whose ability to act in *trans* is severely compromised, and they have been termed *cis*-acting proteins (38). However, a *cis*-acting protein involved in conjugation has never been described. The presence of the *traA* gene in *cis* with the pRetCFN42d *nic* site only in *oriT* constructs exhibiting efficient mobilization (pJBdp1 and pJBdp6, as well as pRetCFN42d::Tn*5*.1 itself) prompted us to examine whether *traA* was a *cis*-acting element. For this purpose, two TraA$^-$ mutants were generated. Plasmid pJBdp5 was constructed by introducing an in-frame StuI deletion into pJBdp1 (Fig. 7) that knocked out the *traA* gene by removing the essential NTP-binding motifs of the TraA helicase domain. As shown in Table 5, mobilization of pJBdp5 was 2 logs lower than mobilization of pJBdp1. Thus, pJBdp5 lacking a functional *traA* gene was inefficiently mobilized, like other *oriT* constructs that lacked a *traA* gene in *cis* (Table 5). This suggested that the inefficient mobilization of pJBdp1, pJBdp4, and pJBdp5 was due to the absence of a *traA* gene encoding a functional relaxase in *cis* with the pRetCFN42d *nic* site.

In order to confirm the TraA *cis*-acting preference, a *traA* mutant derivative of pRetCFN42d::Tn*5*.1 was obtained (strain At Tn*5*.1Δ*traA*Gm) (see Materials and Methods), and its ability to mobilize *traA*$^+$-*oriT* or *traA*-*oriT* constructs was tested. When coexisting with pJBdp1, plasmid p42d::Tn*5*.1ΔTraA, which lacked a functional *traA* gene and therefore needed the *trans* activity of the pJBdp1-encoded TraA relaxase, was transferred at a frequency that was more than 200-fold lower than that of a *traA*$^+$ Sym plasmid, whereas plasmid pJBdp1 (*traA*$^+$-*oriT*$^+$) was transferred at a frequency as high as that of *traA*$^+$ pSym (Table 5). Finally, the presence of the two *traA* mutant plasmids in the same donor cell (strain At p42d::Tn*5*.1ΔTraA harboring pJBdp5) resulted in no detectable transfer of either plasmid, showing that mobilization required the *traA* function.

DISCUSSION

Relaxases are responsible for plasmid *oriT* recognition during conjugative transfer (for a review, see reference 70). The specificity for DNA binding and nicking activities resides in the N-terminal domain of these proteins (36, 58). In this work, the truncated yet functional N265TraA protein was purified. N265TraA, comprising the N-terminal 265 amino acids of TraA from *R. etli* plasmid pRetCFN42d, catalyzed DNA cleavage and strand transfer reactions in vitro on oligonucleotides encompassing its *nic* site. These experiments demonstrated for the first time the relaxase nicking activity of a protein required for conjugative transfer of a bacterial megaplasmid belonging to a member of the order *Rhizobiales* and allowed us to experimentally determine the pRetCFN42d *nic* site. The relaxase activity is associated with the 300-amino-acid N-terminal fragment of pRetCFN42d TraA, which exhibits high sequence conservation with many other putative rhizobial relaxases encoded in tumor-inducing and symbiotic megaplasmids and also in symbiotic islands (Table 3 and Fig. 2). Indeed, within the 300 N-terminal

TABLE 5. *cis*-acting preference of the *R. etli* symbiotic plasmid relaxase over its cognate *oriT* site[a]

Donor strain	No. of transconjugants	
	Km^r	Tc^r
At p42d::Tn*5*.1(pJB3Tc19)	7.25×10^{-4}	$<10^{-8}$
At p42d::Tn*5*.1(pJB42d)	1.17×10^{-3}	6.09×10^{-6}
At p42d::Tn*5*.1(pJBdp4)	9.6×10^{-4}	6.9×10^{-6}
At p42d::Tn*5*.1(pJBdp1)	6.11×10^{-4}	6.41×10^{-4}
At p42d::Tn*5*.1(pJBdp6)	6.47×10^{-4}	1×10^{-3}
At p42d::Tn*5*.1(pJBdp5)	8.05×10^{-4}	7.31×10^{-6}
At p42d::Tn*5*.1Δ*traA*	$<10^{-8}$	
At p42d::Tn*5*.1Δ*traA*(pJBdp1)	4.7×10^{-6}	4.6×10^{-3}
At p42d::Tn*5*.1Δ*traA*(pJBdp6)	4.1×10^{-6}	2×10^{-3}
At p42d::Tn*5*.1Δ*traA*(pJBdp5)	$<10^{-8}$	$<10^{-8}$

[a] *A. tumefaciens* GMI9023 harboring pRetCFN42d::Tn*5*.1 (At p42d::Tn*5*.1) or pRetCFN42d::Tn*5*.1Δ*traA* (At p42d::Tn*5*.1Δ*traA*) and pJB3Tc19 derivatives were mated with *R. etli* CFNX218Spc. The frequencies of Sym plasmid transfer (Km-resistant transconjugants) and mobilization of the pJB3Tc19 derivatives (Tc-resistant transconjugants) are expressed as the number of transconjugants per input receptor cell. The values are the means from three independent experiments.

residues of all these TraA-like relaxases we identified the three common motifs described for relaxase domains (22, 44, 70). The relaxase domain of pRetCFN42d TraA also exhibits sequence conservation (Fig. 1B) and a phylogenetic relationship (Fig. 2) with MobA_RSF1010, indicating that rhizobial relaxases belong to the MOB_Q family (22). The DNA targets for relaxases, the *nic* regions, showed phylogenies similar to those of TraA-like relaxase domains (compare Fig. 2 and 6B). Indeed, sequence conservation between *nic* regions of *Rhizobiales* megaplasmids and the *nic* region of RSF1010 could be observed (Fig. 6), underscoring the coevolution of relaxases and *nic* regions (46).

TraA-like relaxases are multidomain proteins. However, instead of a C-terminal primase domain, as reported previously for MobA_RSF1010 (57, 71), a central helicase domain was identified for pRetCFN42d TraA in this work. Indeed, an in-frame *traA* deletion (*traA*ΔStuI of pJBdp5), removing the known helicase NTP-binding motifs (Fig. 1C) (68), resulted in a nonfunctional protein (Table 5). The relaxase-helicase chimeric scheme was suggested previously for *A. tumefaciens* pTiC58 TraA (1, 20). Also, an additional C-terminal domain was identified in pRetCFN42d TraA, and this domain contains a bipartite signal (two BID signatures plus a short positively charged tail) probably involved in relaxase conjugative transport.

N265TraA bound and cleaved in vitro oligonucleotides encompassing the *nic* regions of several megaplasmids phylogenetically related to pRetCFN42d. It shifted oligonucleotides containing the sequence ACGTATA(A or -)TTGCG/CCCT, which is present in the *nic* region of conjugative elements grouped as rhizobial megaplasmids and symbiotic islands in Fig. 2. However, it failed to bind oligonucleotides encompassing *nic* regions of other plasmids from plant-associated bacteria, such as pSB102, which was isolated from a microbial population residing in the rhizosphere of alfalfa (59). pSB102, pXF51, and pIPO2 constitute a novel group of plasmids prevalent in hosts that associate with plants. The phylogenetic and in vitro EMSA analysis carried out with N265TraA showed that DNA relaxases of these broad-host-range plasmids are distantly related to TraA relaxases of symbiotic and tumor-inducing plasmids.

The in vivo *oriT* recognition experiments involving mobilization of 300-bp DNA fragments containing the *nic* sites of various agrobacterial and rhizobial plasmids were more restrictive than the in vitro assays. Thus, although plasmids pNGR234a and pTiC58 and *S. meliloti* pSymA and pSymB all harbor *nic* sites that could be recognized by N265TraA in vitro, pRetCFN42d::Tn*5*.1 mobilized the *oriT* regions from both *S. meliloti* pSym plasmids but not from pNGR234a and pTiC58. These results suggest the important role that other accessory conjugal transfer proteins must have in determining the specificity of *oriT* recognition, as determined previously for other conjugative plasmids (32, 43, 72). Nonetheless, the fact that pRetCFN42d::Tn*5*.1 mobilized in *trans* the *oriT* regions of *S. meliloti* megaplasmids pSymA and pSymB at frequencies similar to that of its cognate *oriT* (despite the fact that there is less than 80% sequence conservation) suggests that the *oriT* recognition specificity of the pRetCFN42d conjugal transfer machinery is relatively relaxed with respect to other systems (70).

A distinctive feature of the pRetCFN42d TraA relaxase is its *cis*-acting preference. Mobilization of pRetCFN42d *oriT* was 100- to 1,000-fold less efficient when *traA* was in *trans* with respect to the *nic* site. To our knowledge, this is the first report of a *cis*-acting relaxase. No *cis* effect was observed with conjugation of IncQ, IncF, IncP, IncW, IncI, IncN, and ColE1 plasmids (5, 12, 15, 18, 24, 33, 35) or with other *Rhizobiales* plasmids, such as pTi (11). The following three groups of DNA-binding proteins have been reported to act preferentially in *cis* (38): (i) bacterial transposases encoded by many insertion elements and transposing bacteriophage Mu (14, 47), (ii) proteins involved in replication of certain single-stranded phage and plasmids (23, 41), and (iii) some regulatory proteins (16). Inefficient translation and instability of the proteins have been reported to be important mechanisms for preferential *cis* action (14, 38). Future work will be directed toward identifying the underlying mechanisms for the pRetCFN42d TraA relaxase *cis*-acting preference.

The TraA relaxase *cis*-acting preference could be biologically relevant. The coexistence of several plasmids in rhizobia is very common. Indeed, *R. etli* type strain CFN42 harbors six different plasmids. We propose that a preference for *cis*-acting relaxases could compensate for relatively low specificity of *oriT* recognition by the conjugative transfer machinery. According to this scheme, activation of the relaxase from a given plasmid carrying a coupled *traA-nic* site would ensure preferential transfer of this plasmid even if other coexisting elements carry *oriT* regions that could be recognized by the relaxase. Thus, accidental mobilization of a plasmid would be prevented by ensuring efficient transfer of only the DNA molecules containing a coupled *traA-nic* site. This may be relevant for complex genomes like those of the rhizobia, where the coexistence of several plasmids and genome reorganization is frequent, as a way to make sure that conjugal transfer of large plasmids carrying important genetic information (i.e., symbiosis) takes place only under the most suitable conditions.

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