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

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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 (N265TAA), 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 N265TAA 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 phypathogenetic and symbiotic elements, members of the Rhizobiaceae may carry additional plasmids, usually cryptic (25, 40, 54, 59, 62).

In the genus Agrobacterium, conjugal 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 conjugal 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.

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 acquisition 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 tra genes and traCDG operons likely involved in conjugal 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 conjugal 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
TraA of pTiCS8 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 carboxy-terminal domain resembles helicases of IncN, IncW, and IncF plasmids. Furthermore, the results of pRetCFN42d relaxation-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-CaCl2) 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 electropod manipulator apparatus (BTX 680, San Diego, Calif.). Electropodent cells were prepared according to the instructions of the manufacturer and stored at −80°C. For electropodation, cells were thawed on ice, mixed with plasmid DNA (0.3 to 0.5 μg/ml of cell suspension), and transduced 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 600 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 (purified 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...
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 pRetCN42d TraA gene was cloned in the pET22c (+) vector. First, the DNA fragment corresponding to pRetCN42d bp 144991 to 145776 was PCR amplified with oligonucleotide primers TraANdeI-F and TraAXhhoI-R (Table 2). The resulting DNA was digested with endonucleases NdeI and XhoI and cloned into the corresponding sites of plasmid pJB3Tc19, resulting in plasmids pRetCFN42d and pJBSB, respectively. TraAXhhoI-R was used as a host. Cells containing the pRetCN42d plasmid were grown in 1 liter of LB broth containing Km (50 mg ml⁻¹). Protein expression was induced at an A₆₀₀ of 0.6 by adding 0.5 mM isopropyl-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), 100 mM NaCl, 20% glycerol, 500 mM imidazole. To obtain a higher level of N265TraA purification was carried out by Ni-nitrilotriacetic acid (NTA). The cell pellet was resuspended in 35 ml of buffer (100 mM NaH₂PO₄, 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 pre-equilibrated 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 overexpression and purification. For purification of the pRetCN42d TraA N-terminal fragment, the E. coli BL21 derivative strain C41-DE3 was used as a host. Cells containing the pTEN265TraA expressing plasmid were grown in 1 liter of LB broth containing Km (50 mg ml⁻¹). Protein expression was induced at an A₆₀₀ of 0.6 by adding 0.5 mM isopropyl-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 IQAGEN recommendations for Ni-nitrilotriacetic acid (NTA). The cell pellet was resuspended in 35 ml of buffer (100 mM NaH₂PO₄, 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 pre-equilibrated 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 mM NaCl, 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.

Oligonucleotides and labeling. Unlabeled oligonucleotides were purchased from MWG-BIOTECH. Oligonucleotides were labeled at the 5′ end using [γ-³²P]ATP (3,000 Ci mmol⁻¹) and polynucleotide kinase (New England Biolabs) (55). Unlabeled [γ-³²P]ATP was eluted from the mixture by Micro-Spin T M 25 column (Amersham) purification.

N265TraA overexpression and purification. For purification of the pRetCN42d TraA N-terminal fragment, the E. coli BL21 derivative strain C41-DE3 was used as a host. Cells containing the pTEN265TraA expressing plasmid were grown in 1 liter of LB broth containing Km (50 mg ml⁻¹). Protein expression was induced at an A₆₀₀ of 0.6 by adding 0.5 mM isopropyl-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 IQAGEN recommendations for Ni-nitrilotriacetic acid (NTA). The cell pellet was resuspended in 35 ml of buffer (100 mM NaH₂PO₄, 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 pre-equilibrated 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 mM NaCl, 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 [³²P]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 MgCl₂, 100 mM NaCl). After 3.5 h
of incubation at 28°C, reaction products were treated with protease 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 nM. 

Binding reaction mixtures contained 1 nM radiolabeled 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, 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, 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, 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, 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, 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, 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, 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, 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, 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, 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 ol...
pRetCFN42d TraA central fragment exhibits between 20% and 22% identity with helicase domains of MOBp 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 pRetCFN42d TraA 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
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
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 $K_D$s 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-ACGTATATTGGCG 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 (ACGTATATTGGCG) among rhizobial and agrobacterial megaplasmids (Fig. 6A). This sequence, with a consensus ACNNNTA(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 (Fig. 2). The phylogenetic tree showed that the nic site were used. The presence of similar nic sequences in pRetCFN42d-related plasmids (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
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 regions (75.4% and 74.5% identity to pRetCFN42d, respectively).

An R. etli pSym derivative (pRetCFN42d::Tn5) 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::Tn5.1. These donors were mated with the R. etli CFNX218Spce 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::Tn5.1.

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::Tn5.1 mobilized the oriT regions of S. meliloti symbiotic plasmids pSymA (pJBSA) and pSymB (pJBBB) 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 conjugal 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::Tn5.1, their transfer frequencies were between 100- and 1,000-fold lower than that of pRetCFN42d::Tn5.1 (Table 4). In order to examine the possibility that the oriT fragments (300 bp) were missing some important information, two new constructions

<table>
<thead>
<tr>
<th>Construct (plasmid oriT)</th>
<th>No. of transconjugants</th>
</tr>
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<tbody>
<tr>
<td>None</td>
<td>1.48 × 10^{-3}</td>
</tr>
<tr>
<td>pJB3Tc19 (empty vector)</td>
<td>7.25 × 10^{-5}</td>
</tr>
<tr>
<td>pJBRec (traA fragment, no oriT)</td>
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<td>pJB42d (R. etli pRetCFN42d)</td>
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</tr>
<tr>
<td>pJBBB (S. meliloti pSymB)</td>
<td>1.02 × 10^{-2}</td>
</tr>
<tr>
<td>pJBNG (pNGR234a)</td>
<td>9.29 × 10^{-3}</td>
</tr>
<tr>
<td>pJBTi (pTiC58)</td>
<td>7.11 × 10^{-3}</td>
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</table>

* A. tumefaciens GM0023 harboring pRetCFN42d::Tn5.1 and pJBTi9p1 derivatives were mated with R. etli CFNX218Spce. The frequencies of pRetCFN42d::Tn5.1 transfer (Km-resistant transconjugants) and mobilization of pJBTi9 derivatives (Tc-resistant transconjugants) are expressed as the number of transconjugants per input recipient cell. The values are the means from three independent experiments.
(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::Tn5.1 (Table 5). Likewise, a plasmid carrying the same HindIII fragment but cloned in the opposite orientation (pJBdp6) was efficiently mobilized by pRetCFN42d::Tn5.1. 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::Tn5.1 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 Stul 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::Tn5.1 was obtained (strain At Tn5.1ΔtraAGm) (see Materials and Methods), and its ability to mobilize traA-oriT or traA-oriT constructs was tested. When coexisting with pJBdp1, plasmid p42d::Tn5.1ΔTraA, 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+ Sym (Table 5). Finally, the presence of the two traA mutant plasmids in the same donor cell (strain At p42d::Tn5.1ΔTraA 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 sitea

<table>
<thead>
<tr>
<th>Donor strain</th>
<th>No. of transconjugants</th>
<th>Km’</th>
<th>Tc’</th>
</tr>
</thead>
<tbody>
<tr>
<td>At p42d::Tn5.1(pJB3Tc19)</td>
<td>7.25 × 10^-4</td>
<td>&lt;10^-8</td>
<td></td>
</tr>
<tr>
<td>At p42d::Tn5.1(pJB42d)</td>
<td>1.17 × 10^-3</td>
<td>6.09 × 10^-6</td>
<td></td>
</tr>
<tr>
<td>At p42d::Tn5.1(pJBdp4)</td>
<td>9.6 × 10^-4</td>
<td>6.9 × 10^-6</td>
<td></td>
</tr>
<tr>
<td>At p42d::Tn5.1(pJBdp1)</td>
<td>6.11 × 10^-4</td>
<td>6.41 × 10^-4</td>
<td></td>
</tr>
<tr>
<td>At p42d::Tn5.1(pJBdp6)</td>
<td>6.47 × 10^-4</td>
<td>1 × 10^-3</td>
<td></td>
</tr>
<tr>
<td>At p42d::Tn5.1ΔtraA</td>
<td>8.05 × 10^-4</td>
<td>7.31 × 10^-6</td>
<td></td>
</tr>
<tr>
<td>At p42d::Tn5.1ΔtraA(pJBdp1)</td>
<td>&lt;10^-8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>At p42d::Tn5.1ΔtraA(pJBdp4)</td>
<td>4.7 × 10^-6</td>
<td>4.6 × 10^-3</td>
<td></td>
</tr>
<tr>
<td>At p42d::Tn5.1ΔtraA(pJBdp6)</td>
<td>4.1 × 10^-6</td>
<td>2 × 10^-3</td>
<td></td>
</tr>
<tr>
<td>At p42d::Tn5.1ΔtraA(pJBdp5)</td>
<td>&lt;10^-8</td>
<td>&lt;10^-8</td>
<td></td>
</tr>
</tbody>
</table>

a *A. tumefaciens* GM19023 harboring pRetCFN42d::Tn5.1 (At p42d::Tn5.1) or pRetCFN42d::Tn5.1ΔtraA (At p42d::Tn5.1ΔtraA) and pJB3Tc19 derivatives were mated with *R. etli* CFNX2188sp. 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_RSFI100, indicating that rhizobial relaxases belong to the MOBO 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 RSFI100 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_RSFI100 (57, 71), a central helicase domain was identified for pRetCFN42d TraA in this work. Indeed, an in-frame traA deletion (tra AΔStuI of pJbD5), 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 ACCTATAA or -TTTGCG-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 pIP02 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::Tn5.1 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 conjugal plasmids (32, 43, 72). Nonetheless, the fact that pRetCFN42d::Tn5.1 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, IncL, 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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