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Identification of the *rctA* Gene, Which Is Required for Repression of Conjugative Transfer of Rhizobial Symbiotic Megaplasmids†

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An analysis of the conjugative transfer of pRetCFN42d, the symbiotic plasmid (pSym) of *Rhizobium etli*, has revealed a novel gene, *rctA*, as an essential element of a regulatory system for silencing the conjugative transfer of *R. etli* pSym by repressing the transcription of conjugal transfer genes in standard laboratory media. The *rctA* gene product lacks sequence conservation with other proteins of known function but may belong to the winged-helix DNA-binding subfamily of transcriptional regulators. Similar to that of many transcriptional repressors, *rctA* transcription seems to be positively autoregulated. *rctA* expression is greatly reduced upon overexpression of another gene, *rctB*, previously identified as a putative activator of *R. etli* pSym conjugal transfer. Thus, *rctB* seems to counteract the repressive action of *rctA*. *rctA* homologs are present in at least three other bacterial genomes within the order *Rhizobiales*, where they are invariably located adjacent to and divergently transcribed from putative *virB*-like operons. We show that similar to that of *R. etli* pSym, conjugative transfer of the 1.35-Mb symbiotic megaplasmid A of *Sinorhizobium meliloti* is also subjected to the inhibitory action of *rctA*. Our data provide strong evidence that the *R. etli* and *S. meliloti* pSym plasmids are indeed self-conjugative plasmids and that this property would only be expressed under optimal, as yet unknown conditions that entail inactivation of the *rctA* function. The *rctA* gene seems to represent novel but probably widespread regulatory systems controlling the transfer of conjugative elements within the order *Rhizobiales*.

Rhizobia are gram-negative soil bacteria that are able to establish nitrogen-fixing symbiotic associations with leguminous plants. Besides the chromosome, their genomes are usually constituted of large plasmids which carry genetic material relevant for diverse functions. In some species, the plasmid contribution to the total genome size is certainly astonishing (from 25 to 50% of the genome size [20, 42]). Most of the genes for nodulation and nitrogen fixation are usually located in one of these large replicons, known as symbiotic plasmids (pSyms), or clustered in "symbiosis islands" within the chromosome. In other cases, the participation of another plasmid in symbiosis is considered important enough to justify the denomination of pSym. For example, *Sinorhizobium meliloti* carries two megaplasmids: pSymA (1.35 Mb), the "true" pSym, contains most of the genes needed for nodulation and nitrogen fixation, whereas pSymB (1.68 Mb) harbors exopolysaccharide biosynthetic genes (19), which are also required for the establishment of symbiosis.

Advances in the knowledge of the transfer systems of these pSyms have been hindered by some particular characteristics of these replicons, such as their large size and the difficulty of detecting their transfer under laboratory conditions. However, there is abundant evidence of symbiotic plasmid transfer among these bacteria in soil (11, 26, 50, 56, 58). In addition, genome sequencing has revealed that many rhizobial pSyms carry genes potentially involved in conjugal transfer (19, 22, 46). For example, putative *D*NA *t*ransfer and *r*eplication genes (*dtr*) involved in the processing of plasmid DNA during conjugative transfer have been identified in several rhizobial pSyms, including pSymA of *S. meliloti* (2), pRetCFN42d (pSym of *Rhizobium etli* [22]), and pNGR234a (pSym of the broad-host-range strain *Rhizobium* sp. strain NGR234 [46]). These Dtr functions are commonly encoded by two divergently transcribed operons, *traA* and *traCDG*, with a hypothetical origin of conjugative transfer (*oriT*) in the intergenic regions (reviewed in reference 52).

Other genes likely involved in the conjugal transfer of pSym plasmids have also been identified, such as complete *trb*-like systems (in pNGR234a [46] and pRL1JI, the pSym of *Rhizobium leguminosarum* bv. viciae [59]) or *virB*-like (2, 22) type IV secretion systems (T4SS), which potentially code for *m*ating *p*air *f*ormation (Mpf) systems required for the transfer of DNA across the membranes of donor and recipient cells during conjugation (52).

Despite the evidence indicating the potential of these plasmids for conjugation, their transfer under laboratory conditions is usually undetectable or occurs at negligible frequencies. Uncovering the regulatory networks that likely control the conjugative transfer of most of these plasmids is one of the

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keys to identifying the environmental conditions promoting the transfer of these symbiotic megaplasmids and to better understanding the role of pSym lateral spread in rhizobial ecology.

In *Agrobacterium*, a genus of the *Rhizobiaceae* family, conjugal transfer of the tumor-inducing plasmid (pTi) is a quorum sensing-dependent highly regulated process (5, 31). Opines produced by the plant during infection control transcription from at least five promoters in gene operons involved in plasmid transfer (38, 47). Some rhizobial symbiotic and nonsymbiotic plasmids with quorum-sensing conjugal transfer regulation systems have been reported as well (15, 25, 27, 28, 40, 57). However, other rhizobial pSyms, such as pRetCFN42d of *R. etli* CFN42 and pSymA of *S. meliloti* 1021, seem to have no quorum sensing-like genes carried in their genomes (2, 22). This suggests that these plasmids either have different regulatory systems controlling their transfer or, as suggested previously (22), may have lost the capacity for efficient lateral spread.

The presence of *S. meliloti* pSymA has been described as an ancient event of lateral transfer mediated by import of pSymA from an unknown bacterium, as indicated by its distinctive G+C content and codon usage (19), but this plasmid has never been described as self-transmissible under laboratory conditions.

In *R. etli*, the ability of the pSym pRetCFN42d to cointegrate with the accompanying conjugative plasmid pRetCFN42a provides an alternative means for lateral spread (10, 12, 57). However, Pérez-Mendoza and coworkers have recently identified a gene (yp028) involved in the promotion of *R. etli* pSym transfer at relatively high frequencies in standard media in the absence of pRetCFN42a (45). The identification of this gene opened the possibility that conjugal transfer functions encoded by the *R. etli* pSym might be silent under laboratory conditions and that transfer could be activated with the participation of yp028 under as yet unknown conditions.

Here we report the identification of the *rctA* gene present in the symbiotic plasmids of *R. etli* and *S. meliloti*. *rctA* prevents the transfer of these two megaplasmids in standard media and is required for the repression of conjugal transfer genes under these conditions. The existence of this novel regulatory system to actively limit pSym transfer under certain conditions further supports our interpretation that conjugative transfer of these megaplasmids may be a highly regulated process responding to as yet unknown signals.

MATERIALS AND METHODS

Bacterial strains and plasmids. All bacteria and plasmids used for this work are listed in Table 1. *R. etli* and *S. meliloti* strains were grown at 30°C on TY (tryptone-yeast extract-$CaCl_2$ [6]). *Escherichia coli* and *Agrobacterium tumefaciens* were grown on Luria-Bertani (LB) medium (54). When required, antibiotics were added at the following concentrations: nalidixic acid (Nx), 20 μg/ml; spectinomycin (Spc), 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 (Gm), 10 μg/ml; rifampin, 50 μg/ml; streptomycin (Sm), 100 μg/ml for *R. etli* and *A. tumefaciens*, 200 μg/ml for *S. meliloti*, and 25 μg/ml for *E. coli*; and tetracycline (Tc), 2 μg/ml for *Rhizobium* and 10 μg/ml for *E. coli*.

Bacterial matings. Donor strains, grown to an approximate optical density at 600 nm (OD_{600}) of 0.2, and recipient strains, grown to late exponential phase, were washed and mixed at a 1:1 ratio of donor to recipient. Mating mixtures were resuspended in 50 μl of TY medium and loaded onto a sterile nitrocellulose filter with a 0.45-μm pore size. Filter mating mixtures were deposited on TY-agar plates and incubated overnight at 30°C. Cells were resuspended by vigorous vortexing and diluted in liquid medium. To calculate transfer frequencies, donor, recipient, and transconjugant CFU were counted after mating disruption and plating of serial dilutions. Transconjugants were selected on plates supplemented with appropriate antibiotics. The transfer frequency was expressed as the number of transconjugants per output recipient. Donor and recipient levels of spontaneous resistance to selective antibiotics were also determined.

Cell transformations. Bacterial transformation was carried out by electroporation using an electrocell manipulator apparatus (BTX 600). Electrocompetent cells were prepared according to the instructions of the manufacturer and then 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 then transferred to a 0.2-cm-electrode-gap chilled cuvette. A pulse with a 2.5-kV/cm field strength, a 6.8-ms length, and a 129-Ω set resistance was applied, and cells were immediately suspended in 1 ml of TY or LB medium and then 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 profiles. Plasmid profiles were visualized by the Eckhardt procedure (16), as modified by Hynes and McGregor (32).

DNA hybridizations. Total genomic DNAs were isolated by standard procedures (41), digested with the endonucleases EcoRI and BamHI, electrophoresed in 0.8% agarose gels, and then transferred to positively charged nylon membranes by the method of Southern (54). DNA hybridization probes were labeled with digoxigenin according to the manufacturer's instructions (Roche, Barcelona, Spain). Hybridization and membrane washes were carried out under high-stringency conditions. Membranes were prepared for chemiluminescence detection (Roche) and exposed to Kodak X-Omat film (Sigma).

PCR, cloning, and sequencing. Standard DNA techniques were used as described previously by Sambrook et al. (54). The Tn*5* insertions of the different mutants were subcloned into pUC18 as EcoRI fragments. Transposon arms were subcloned, and insertion sites were mapped by DNA sequencing using an IS*50*-specific primer. For each insertion, sequence stretches of between 600 nucleotides (nt) and >1,000 nt were obtained. Sequencing was performed with an ABI 373 automated sequencer. DNA sequence editing, translation, and analysis were performed with the Vector NTI 5.5 software package (Oxford Molecular) and the program BLAST from the network service at NCBI (1). The RctA winged-helix DNA-binding domain was identified using the program Superfamily 1.65 (23; http://supfam.mrc-lmb.cam.ac.uk/SUPERFAMILY/). Three-dimensional prediction structures of proteins were prepared using the 3D-JIGSAW program (version 2.0) (3, 4, 14; http://www.bmm.icnet.uk/servers/3djigsaw/).

All oligonucleotides used for constructions are listed in Table S1 in the supplemental material. Different gene fusions to the β-glucuronidase reporter gene *gusA* were constructed by cloning the pRetCFN42d *traA-traC* and yp037-*rctA* DNA regions into the vector p53gus. Primers UptraAE and LwtraCE were used to amplify a 1,394-bp DNA fragment containing the *traC* promoter, and primers UptraAXb and LwtraCK were used to amplify a 1,363-bp DNA fragment containing the *traA* promoter. These two PCR fragments were cloned into a dephosphorylated blunt-ended pMOS*Blue* vector. Among the two possible orientations, the correct one was identified by PCR using UptraA− (E or Xb) and the vector-specific primer T7 and then cloned as a SpeI/KpnI (in the p53R*etraC*::*gus* construct) or XbaI/KpnI (in the p53R*etraA*::*gus* construct) fragment into p53gus, which was previously digested with the corresponding restriction enzymes. p53R*eyp037*::*gus* and p53R*eyp038*::*gus* were constructed by cloning the PCR fragments containing the yp037 promoter (amplified with yp037up and yp037lw) and the yp038 promoter (amplified with yp038up and yp038lw) into p53gus, which was previously digested with XhoI/XbaI (restriction enzyme sites are underlined in the primer sequences).

S. meliloti gene fusion plasmids were constructed by cloning a *traA1-traC* fragment of 480 bp, previously amplified with primers SmtraACup and SmtraAClw, into the pGEM-T Easy cloning vector. Using the XbaI, EcoRI, and SalI restriction enzyme sites at the 5′ ends of the primers, different fragment orientations were obtained with respect to the *gusA* reading frame in p53gus. All p53gus derivatives were introduced into rhizobial strains by conjugation using the S17-1 mobilizing strain.

For complementation experiments, different plasmids derived from pTE3 were constructed. The different *rctA* alleles were amplified using specific primers with restriction sites at their 5′ ends to facilitate subsequent cloning in front of the *trp* promoter in pTE3. pTEYp038 contains the *rctA* gene of pRetCFN42d as a 750-bp fragment previously amplified with primers RerctA-F and RetrctA-R. pTEAtu5160 contains the *rctA* gene of pAtC58 as a 450-bp fragment that was amplified with the primers AtrctA-F and AtrctA-R. pTESMa1323 contains the *rctA* gene of pSymA as a 430-bp fragment that was obtained after amplification with primers SmrctA-F and SmrctA-R.

Construction of an *S. meliloti* pSymA SMa1323 mutant. The oligonucleotides used to construct the pSymA SMa1323 mutant are listed in Table S1 in the supplemental material. Two pairs of primers, SmrctA1-SmrctA2 and SmrctA3-SmrctA4,

TABLE 1. Bacterial strains and plasmids used for this study

Strain or plasmid	Relevant features	Reference or source
Strains		
S. meliloti		
1021	Wild-type strain, Sm^r	41
1021 RctA$^-$	1021 derivative (SMa1323::Sm/Spc)	This work
10OTSS	1021 derivative (Δ*otsA*::Sm/Spc)	A. Dominguez (EEZ)
R. etli		
CE3	Sm^r derivative of wild-type strain CFN42	49
CFNX182	CE3 derivative cured of p42a	9
CFNX218Spc	CE3 derivative (p42a$^-$ p42b$^-$ p42c$^-$ p42d$^-$ p42eΔ p42f) Spc^r	This work
CFNX218Spc Tn5.1	CFNX218Spc derivative with pRetCFN42d::Tn5.1	This work
CFNX218Spc Tn5.2	CFNX218Spc derivative with pRetCFN42d::Tn5.2	This work
CFNX218Spc Tn5.6	CFNX218Spc derivative with pRetCFN42d::Tn5.6	This work
CFNX218Spc Tn5.8	CFNX218Spc derivative with pRetCFN42d::Tn5.8	This work
CFNX218Spc Tn5.13	CFNX218Spc derivative with pRetCFN42d::Tn5.13	This work
A. tumefaciens		
C58	Wild-type, nopaline-resistant strain	63
GMI9023	Plasmidless C58 derivative	53
At Tn5.1	GMI9023 derivative with pRetCFN42d::Tn5.1	This work
At Tn5.2	GMI9023 derivative with pRetCFN42d::Tn5.2	This work
At Tn5.6	GMI9023 derivative with pRetCFN42d::Tn5.6	This work
At Tn5.8	GMI9023 derivative with pRetCFN42d::Tn5.8	This work
At Tn5.13	GMI9023 derivative with pRetCFN42d::Tn5.13	This work
E. coli		
DH5α	*supE44 Dlac U169 f80 lacZDM 5hsdR171 recA1 endA1 gyrA96 thi-1 relA1*	24
S17-1	*thi pro recA hsdR hsdM* RP4-2-Tc::Mu-Km::Tn7	55
Plasmids		
pTE3	IncP cloning vector carrying *Salmonella enterica* serovar Typhimurium *trp* promoter, Tc^r	17
pMOS *Blue*	PCR cloning vector, Ap^r	Amersham
pGem-T Easy	PCR cloning vector, Ap^r	Promega
pSUP202	Mobilizable Tc^r Cm^r Ap^r suicide vector	55
pHP45Ω	pBR322 derivative with the Sm/Spc cassette; Sm^r Spc^r Ap^r	48
pUC18	2,690-bp cloning vector, Ap^r	61
p53Gus	pBBR1MCS5 derivative with a *gus* gene of pWM5 (pBBR1MCS5::*uidA*)	L. Girard (CCG, México)
p53R*etraA*::*gus*	pRetCFN42d *traA*::*gus* fusion in p53Gus	This work
p53R*etraC*::*gus*	pRetCFN42d *traC*::*gus* fusion in p53Gus	This work
p53*yp037*::*gus*	pRetCFN42d *yp037*::*gus* fusion in p53Gus	This work
p53*yp038*::*gus*	pRetCFN42d yp038::*gus* fusion in p53Gus	This work
p53SmtraA1::*gus*	*S. meliloti* 1021 *traA1*::*gus* fusion in p53Gus	This work
p53SmtraC::*gus*	*S. meliloti* 1021 *traC*::*gus* fusion in p53Gus	This work
pTEYp028	pTE3 with yp028 cloned in front of *trp* promoter, yp028 (Con)	45
pTEYp038	pTE3 with yp038 cloned in front of *trp* promoter, yp038 (Con)	This work
pTEAtu5160	pTE3 with Atu5160 cloned in front of *trp* promoter	This work
pTESMa1323	pTE3 with SMa1323 cloned in front of *trp* promoter	This work

were used to amplify two DNA fragments which flanked the *S. meliloti* 1021 SMa1323 gene. Each PCR product was cloned into pUC18, the R fragment was cloned as a SmaI/XbaI fragment, generating pUC18-R, and the L fragment was cloned as a BamHI/HindIII fragment, generating pUC18-L. A triple ligation reaction included an Sm/Spc resistance gene cassette (a BamHI fragment purified from the pHP45Ω vector), the R fragment (as a BamHI/HindIII fragment purified from pUC18-R), and pUC18-L (previously linearized with BamHI/HindIII). The resulting construct (Ap, Sm, and Spc resistant; pUC18LCR) was restricted with SmaI/EcoRV, and the corresponding LCR fragment (containing the fragment L plus the resistance Cassette plus R) was cloned into suicide plasmid pSUP202 and introduced by conjugation into strain 1021 of *S. meliloti*. Allele replacement events were selected as described previously (55), and *rctA* mutants were verified after hybridization with a labeled plasmid, pUC18LCR, as a probe.

Gene expression assays. Rhizobial strains containing reporter gene fusion plasmids were grown in TY selective medium to an approximate OD_{600} of 0.8. Cultures were diluted (1/200 to 1/500) in TY medium containing 20 μg/ml Gm to an approximate OD_{600} of 0.4 to 0.6. Cells in 1.5 ml of medium were pelleted, washed three times with sterile distilled water to remove any traces of TY, and finally resuspended in 1.5 ml of assay buffer (dithiothreitol, 5 mM; EDTA, 1 mM; Na_2HPO_4-NaH_2PO_4, 50 mM). Three hundred microliters of this sample was stored at −20°C for the Bradford Bio-Rad protein assay. Another 200 μl was used to determine the β-glucuronidase activity by mixing with 740 μl of assay buffer, 50 μl of 0.1% sodium dodecyl sulfate, and 100 μl of chloroform and vortexing twice for 15 seconds each time. Samples were incubated at 37°C for 10 min, and then 10 μl of 100 mM NPG (4-nitrophenyl-β-D-glucuronide), preheated at 37°C, was added. Samples were incubated at 37°C until they turned yellow. The reactions were ended by adding 200 μl of 1 M Na_2CO_3. Samples were centrifuged for 5 min (12,000 rpm), and the absorbance of the upper phase of each sample was measured at 405 nm with a spectrophotometer. Values of β-glucuronidase activity were expressed as specific β-glucuronidase activities (nmol/min/mg of protein). Mean values and standard deviations were calculated from at least three independent experiments.

RESULTS

Isolation and genetic characterization of conjugative transfer regulatory elements in the *R. etli* symbiotic plasmid. The

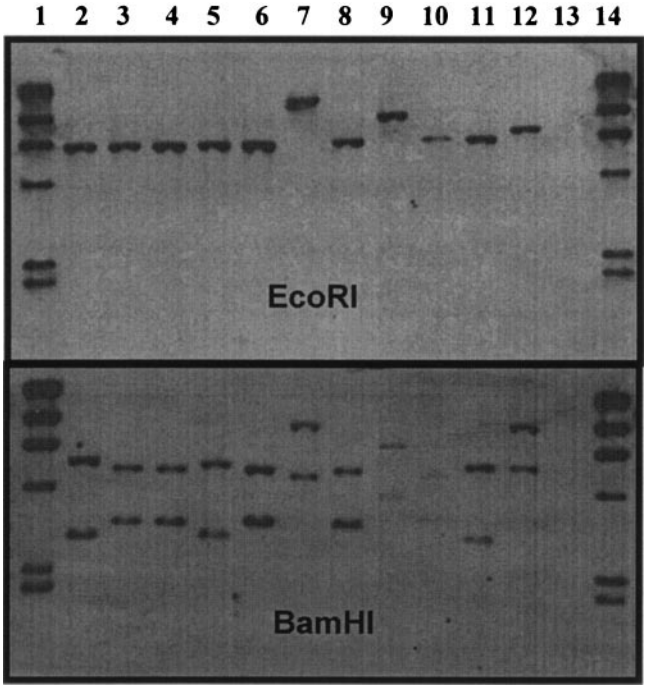

FIG. 1. Southern blots of EcoRI- and BamHI-digested genomic DNAs of 11 *A. tumefaciens* GMI9023 Km^r transconjugants, with digoxigenin-labeled Tn*5* as a probe. Lanes: 1 and 14, digoxigenin-labeled DNA molecular weight marker; 2 to 12, transconjugants harboring a Tn*5* insertion; 13, *A. tumefaciens* GMI9023.

symbiotic plasmid of *R. etli* CFN42, pRetCFN42d, has never been shown to be self-transmissible. However, data from a previous work indicated that conjugative transfer of this pSym may be silent under laboratory conditions, suggesting the possibility of an active repression system in this plasmid (45). In order to identify putative genes participating in the repression of *R. etli* pSym conjugal transfer, strain CFNX182 of *R. etli* (pRetCFN42a$^-$) was subjected to random Tn*5* mutagenesis using the suicide vector pSUP2021 (55). About 2.2×10^6 transposants were obtained from 10 independent matings, where each CFNX182 Km^r transconjugant should harbor a single Tn*5* insertion in its genome. The pool of CFNX182 transposants was used as a donor en masse in conjugation with the plasmidless *A. tumefaciens* GMI9023 strain as the recipient. Transposon insertions causing a loss of functionality of a hypothetical repressor of conjugative plasmid functions would result in a relief of repression and therefore would allow plasmid transfer. Tn*5* insertions able to promote the transfer of any plasmid in strain CFNX182 could be easily identified among *A. tumefaciens* GMI9023 Km^r transconjugants. Some 400 GMI9023 transconjugants were obtained from such mating. Plasmid profiles of 11 randomly chosen transconjugants showed that they all carried a plasmid with a similar size to that of the *R. etli* symbiotic plasmid pRetCFN42d. Southern hybridization analysis of digested genomic DNAs revealed that each transconjugant harbored a single copy of Tn*5* in its genome (Fig. 1). Transposon insertions could be grouped into five different types, which were named Tn*5*.1, Tn*5*.2, Tn*5*.6, Tn*5*.8, and Tn*5*.13. These results suggested that different Tn*5* insertions were producing the hypothetical derepression of plasmid transfer and that most, if not all, such insertions seemed to have occurred on the pSym plasmid pRetCFN42d but not on any other of the four additional plasmids carried by *R. etli* CFNX182.

TABLE 2. Transfer frequencies of different pRetCFN42d Tn*5* mutant derivatives from a plasmidless *A. tumefaciens* strain to *R. etli* CFNX218Spc

Mutant plasmid	Frequency of transfer[a] to *A. tumefaciens* GMI9023
pRetCFN42d::Tn*5*Mob	ND
pRetCFN42d::Tn*5*.1	1.48×10^{-3}
pRetCFN42d::Tn*5*.2	1.94×10^{-3}
pRetCFN42d::Tn*5*.6	3.14×10^{-4}
pRetCFN42d::Tn*5*.8	3.81×10^{-7}
pRetCFN42d::Tn*5*.13	2.13×10^{-7}

[a] Expressed as the number of transconjugants per input receptor cell. Frequencies are the averages of at least three independent experiments. pRetCFN42d::Tn*5*Mob is a pRetCFN42d Km^r derivative used as a control. ND, no transconjugants detected.

To verify that the mutant pRetCFN42d plasmids had indeed gained self-transmissibility, the five different types of *A. tumefaciens* GMI9023 derivatives were then individually used as donors for conjugation with *R. etli* CFNX218Spc. All the Tn*5* insertions promoted transfer of the Tn*5*-encoded Km resistance from *A. tumefaciens* to *R. etli*, but at three different frequencies (Table 2). Insertions Tn*5*.1 and Tn*5*.2 promoted transfer at relative high frequencies under laboratory conditions (about 10^{-3} transconjugants per recipient cell), whereas the Tn*5*.6 mutant plasmid was transferred at a ninefold lower frequency than the Tn*5*.1 or Tn*5*.2 insertion (about 10^{-4}); insertions Tn*5*.8 and Tn*5*.13 also promoted the transfer of pRetCFN42d, but at much lower frequencies (Table 2). Plasmid profiles of the various transconjugant types confirmed the transfer of the Tn*5*-tagged pRetCFN42d mutants in all cases. Individual *R. etli* transconjugants arising from each of the later matings were again used as donors in new matings with *A. tumefaciens* GMI9023 as the receptor strain. The conjugal transfer frequencies from the individual *R. etli* CFNX218Spc donors were similar to those previously obtained from the *A. tumefaciens* GMI9023 donors (data no shown). These results confirmed that Tn*5* insertions harbored by pRetCFN42d and selected with our strategy promoted conjugative transfer of this plasmid in standard laboratory media without the help of any other *R. etli* plasmid, in contrast to the wild-type plasmid, which shows no detectable self-transfer under these conditions (10, 45).

The EcoRI fragments from the mutant plasmids containing the transposon insertions promoting high-frequency transfer of pRetCFN42d, i.e., Tn*5*.1, Tn*5*.2, and Tn*5*.6, were cloned, and the Tn*5* flanking DNAs were sequenced. Sequence comparison with the pRetCFN42d genome sequence allowed the identification of the DNA regions interrupted by Tn*5*. Insertions Tn*5*.1 and Tn*5*.2 were located within the same EcoRI fragment but were 67 bp apart. In both mutants, the transposon had interrupted the coding sequence of the yp038 gene (Fig. 2), located adjacent to a hypothetical *virB*-like operon present in pRetCFN42d. In contrast, insertion Tn*5*.6 was mapped upstream of the yp028 open reading frame (Fig. 2). yp028 is located downstream of *traA*, the hypothetical plasmid relaxase

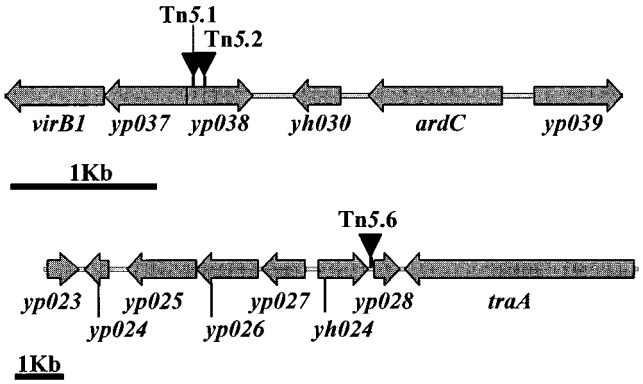

FIG. 2. Locations of different Tn*5* insertions in the pRetCFN42d genome. Transposon positions are shown as black triangles.

gene. Recently, yp028 was described as a gene which may act in the activation of conjugal transfer of the *R. etli* symbiotic plasmid (45).

yp038 represses conjugal transfer of the *R. etli* symbiotic plasmid. yp038 encodes a hypothetical protein of 149 amino acids that lacks significant similarity to any protein sequence of known function described in the databases. Nevertheless, this gene displays sequence conservation with several open reading frames of unknown function present in genomes of different bacteria within the order *Rhizobiales* (see below; Fig. 3A). Using the software program Superfamily 1.65 (23, 39), a "winged-helix" DNA-binding domain was identified in the hypothetical products of yp038 and its homologues (Fig. 3A). The winged-helix DNA-binding proteins constitute a subfamily within the large ensemble of helix-turn-helix proteins (18). The three-dimensional structure predicted by the 3D-JIGSAW (version 2.0) comparative modeling program (3, 4, 14) showed for Yp038 a similar α/β topology (Fig. 3B) to that of the winged-helix domains of several crystallized transcriptional regulators (35, 62).

The high pRetCFN42d transfer frequencies observed upon interruption of yp038 (insertions Tn*5*.1 and Tn*5*.2) suggested that this gene could act by repressing conjugal transfer of the *R. etli* symbiotic plasmid. To confirm this hypothesis, *R. etli* cells harboring Tn*5*.1 and Tn*5*.2 pSym mutants were complemented in *trans* with a yp038 gene under the control of the *trp* promoter (pTEyp038). Conjugal transfer of the mutant plasmids was then tested in matings with *A. tumefaciens* GMI9023 as the recipient. In all cases, the presence of the cloned yp038

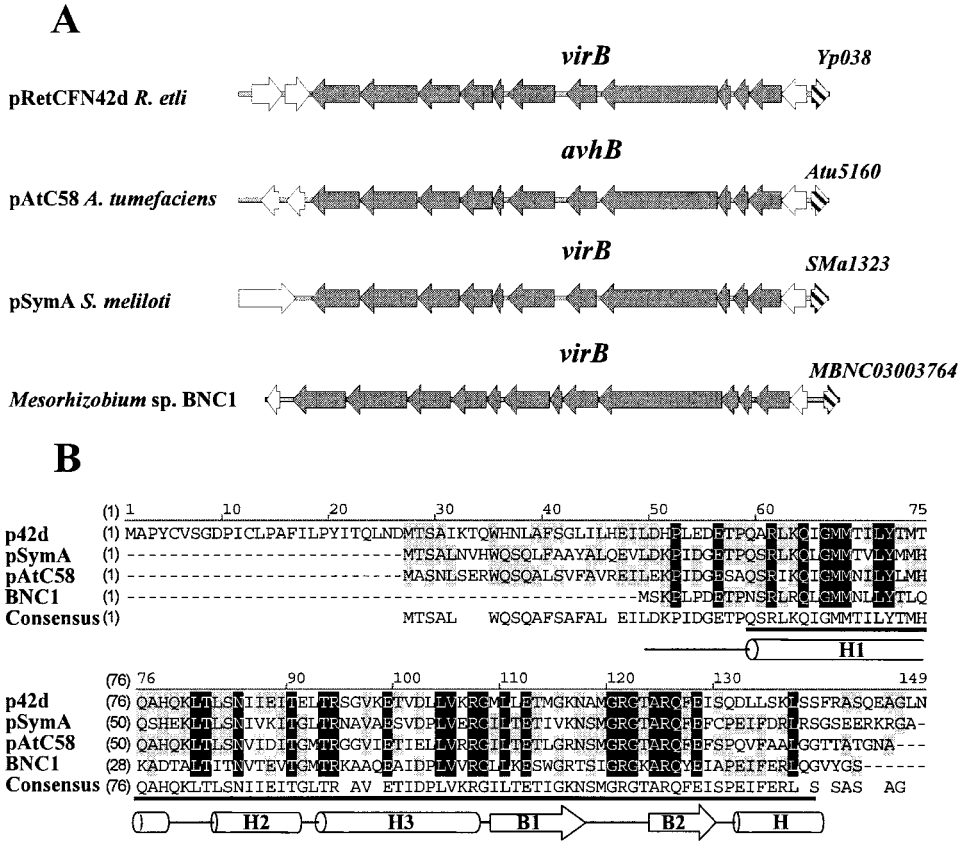

FIG. 3. (A) Genomic locations of *rctA* copies in pRetCFN42d from *R. etli*, pSymA from *S. meliloti*, pAtC58 from *A. tumefaciens*, and the chromosome of *Mesorhizobium* sp. strain BNC1. RctA open reading frames are shown as striped arrows, and *virB*-like genes are shown as gray arrows. (B) Sequence alignment of RctA homologs. Black-shaded residues are conserved in all four sequences, and gray-shaded amino acids are conserved in at least two of the four sequences. The consensus sequence is depicted below, and the putative winged-helix DNA-binding region is underlined. The α-helix and β-sheet regions of the putative winged-helix DNA-binding region of these four *rctA* copies, predicted by the 3D-JIGSAW program, are indicated with cylinders and arrows, respectively.

TABLE 3. Influence of *rctA* genotype on expression of *R. etli* pSym conjugal transfer genes

Transcriptional fusion	CFNX218 background[a]	Relevant genotype[b]	β-Glucuronidase activity[c]	Relative expression[d]
traA::*gus*	p42d::Tn*5*Mob	*rctA*$^+$	10.41 ± 1.82	1
	p42d::Tn*5*.1	*rctA*	61.89 ± 5.90	5.94
	p42d::Tn*5*.1 + pTEReyp038	*rctA*(Con)	5.46 ± 1.25	0.52
traC::*gus*	p42d::Tn*5*Mob	*rctA*$^+$	9.34 ± 2.04	1
	p42d::Tn*5*.1	*rctA*	62.08 ± 7.66	6.65
	p42d::Tn*5*.1 + pTEReyp038	*rctA*(Con)	5.30 ± 0.89	0.56
yp037::*gus*	p42d::Tn*5*Mob	*rctA*$^+$	60.2 ± 8.1	1
	p42d::Tn*5*.1	*rctA*	282 ± 44.73	4.68
	p42d::Tn*5*.1 + pTEReyp038	*rctA*(Con)	87.2 ± 22.4	1.44
yp038::*gus*	p42d::Tn*5*Mob	*rctA*$^+$	332.3 ± 36.6	1
	p42d::Tn*5*.1	*rctA*	53.4 ± 4	0.16
	p42d::Tn*5*.1 + pTEReyp038	*rctA*(Con)	354.1 ± 44.73	1.07

[a] Expression was assayed in *R. etli* strain CFNX218Spc containing pRetCFN42d::Tn*5*Mob (a pRetCFN42d Km^r derivative) or the pRetCFN42d::Tn*5*.1 mutant.
[b] *rctA*$^+$, wild-type *rctA*; *rctA*, no active *rctA; rctA*(Con), *rctA* expressed from *trp* promoter in pTE3 vector.
[c] Specific β-glucuronidase activity is expressed as nmol/min/mg of protein. Standard deviations were calculated from at least three independent experiments.
[d] Ratio of specific β-glucuronidase activity in a given background with respect to the corresponding wild-type background (p42d::Tn*5*Mob).

gene resulted in a complete inhibition of transfer of the mutant pSyms, confirming the important role of yp038 in silencing the conjugative transfer of *R. etli* pSym. In contrast, complementation of the pRetCFN42d Tn*5*.1 mutant derivative with a yp038 gene under the control of the *trp* promoter in the *A. tumefaciens* GMI9023 background resulted in a reduction of only 2 to 3 log of the transfer frequency of the mutant plasmid but not in the complete inhibition of pSym transfer (data not shown). This result suggested that the product of the yp038 gene expressed in *trans* was not able to totally repress plasmid conjugation in the *A. tumefaciens* genetic background.

We also analyzed the effect of a yp038 mutation on the expression of pRetCFN42d conjugal transfer genes by using transcriptional reporter gene fusions. The expression of the putative Dtr (p53Re*traA*::*gus* and p53Re*traC*::*gus*) and Mpf (p53*yp037*::*gus*) genes was elevated (between 4.7- and 6.7-fold) in a yp038 mutant (pRetCFN42d::Tn*5*.1) with regard to the wild-type plasmid (Table 3). Furthermore, the expression of all of these genes returned to the wild-type levels when the yp038 mutation was complemented in *trans* with a cloned yp038 gene (pTEyp038) (Table 3). In contrast to the case for conjugal transfer genes, we observed that *rctA* expression (yp038::*gus* fusion) was greatly reduced in a RctA$^-$ background and returned to wild-type levels when a cloned *rctA* gene was provided in *trans* (Table 3). These data suggest that *rctA* expression is positively autoregulated.

These results clearly show that yp038 actively participates in keeping low expression levels of genes likely involved in the conjugal transfer of pRetCFN42d. In view of the role of yp038 in the regulation of conjugal transfer of pRetCFN42d, we have renamed this gene *rctA* (*r*egulation of *c*onjugal *t*ransfer).

Overexpression of yp028 activates the expression of pRetCFN42d conjugal transfer genes. In a previous work, we demonstrated that the overexpression of yp028 promotes the conjugal transfer of pRetCFN42d (45). Here we have found that the Tn*5*.6 insertion, located upstream of the yp028 coding sequence (Fig. 2), promotes transfer of the *R. etli* symbiotic plasmid at similar frequencies to those observed when yp028 is overexpressed (Table 2) (45).

To determine whether the overexpression of yp028 or the Tn*5*.6 transposon insertion could affect the transcription of pRetCFN42d conjugal transfer genes, we analyzed the expression of the *traA* and *traC* genes, as well as that of the yp037 gene, the hypothetical first gene of the *virB*-like operon coding for the putative Mpf of pRetCFN42d. The expression of yp028 from the *trp* promoter led to enhanced expression of hypothetical transfer genes (between 4.1- and 6.1-fold) (Table 4). Likewise, the Tn*5*.6 insertion increased the expression of the hypothetical transfer genes between four- and fivefold (data not shown). Thus, this mutation had a similar effect to that observed when the yp028 gene was overexpressed.

The location of the Tn*5* insertion upstream of the yp028 gene coding region, the observed plasmid pSym transfer frequencies, and the effect on the expression of conjugal transfer genes from pRetCFN42d allow us to propose that the Tn*5*.6 insertion may have the effect of deregulating the yp028 gene. Altogether, these results support the idea that yp028 acts as a positive element in the regulation of pRetCFN42d conjugal transfer. Therefore, we have renamed this gene *rctB*.

***rctB* has a role in reducing *rctA* expression.** As shown above, the conjugal transfer of the *R. etli* symbiotic plasmid seems to be promoted by the action of the *rctB* gene and repressed by

TABLE 4. Influence of cloned yp028 gene on expression of pRetCFN42d conjugal transfer genes

Background[a]	β-Glucuronidase activity[b]	Relative expression[c]
p53*gus* (empty vector)	7.15 ± 1.49	
p53*traA*::*gus*	10.41 ± 1.82	1
p53*traA*::*gus* + pTEYp028	42.70 ± 4.4	4.1
p53*traC*::*gus*	9.51 ± 1.88	1
p53*traC*::*gus* + pTEYp028	50.06 ± 3.23	5.26
p53*yp037*::*gus*	60.20 ± 8.1	1
p53*yp037*::*gus* + pTEYp028	369 ± 28.4	6.13
p53yp038::*gus*	332.3 ± 36.6	1
p53*yp038*::*gus* + pTEYp028	176.7 ± 28.4	0.53

[a] Expression was assayed in *R. etli* CFNX218Spc containing pRetCFN42d::Tn*5*Mob (a pRetCFN42d Km^r derivative).
[b] Specific β-glucuronidase activity is expressed as nmol/min/mg of protein. Standard deviations were calculated from at least three independent experiments.
[c] Ratio of specific β-glucuronidase activity in a given background with respect to the corresponding wild-type background (absence of pTEYp028).

the *rctA* gene. Therefore, we wondered whether the functions of these conjugal transfer regulators were somewhat related. Using a transcriptional fusion of the *rctA* gene to the *gus* reporter gene (p53*yp038*::*gus*), we analyzed the effect of *rctB* on the expression of *rctA*. As shown in Table 4, overexpression of the *rctB* gene cloned under the control of the *trp* promoter led to a significant reduction of *rctA* expression (Table 4), indicating that the *rctB* product interferes with *rctA* expression. A similar effect on *rctA* expression was observed in the pRetCFN42d::Tn*5*.6 mutant (data not shown). These results suggest that the role of *rctB* in enhancing the expression of conjugal transfer genes might be an indirect phenomenon due to a negative effect of the *rctB* product on *rctA* expression or activity.

The *rctA* gene is present in several bacteria within *Rhizobiales*. Although the predicted *rctA* gene product has no significant sequence conservation with proteins of known function, it displays sequence conservation (between 45% and 57% sequence identity; Fig. 3B) with at least three other open reading frames of unknown function present in the sequenced genomes of different bacterial species within the *Rhizobiales* order. The hypothetical products of SMa1323 located in pSymA of *S. meliloti* 1021 and of Atu5160 (from pAtC58, the cryptic plasmid of *A. tumefaciens* C58) not only show very significant sequence conservation with RctA but are also located in similar genomic contexts, divergently transcribed from *virB*-like operons carrying the hypothetical plasmid T4SS (Fig. 3A). Indeed, for *R. etli* and *S. meliloti* these genes were annotated *virB1* to *virB11* (19, 22).

A third *rctA* homolog has been identified within the unfinished genome sequence of a gram-negative bacterium that was isolated by its ability to degrade chelating compounds such as EDTA, nitrilotriacetate, and diethylenetriaminepentaacetate (34, 43, 44). This strain was initially classified as *Agrobacterium* sp. strain BNC1 (7) but was recently renamed *Mesorhizobium* sp. strain BNC1, and its genome is being sequenced by the DOE Joint Genome Institute (NZ_AAED00000000). The shotgun sequence shows that the genome of this bacterium is composed of a unique 4,922,255-bp replicon. Similar to the rhizobium and *A. tumefaciens* counterparts, the *rctA* gene homolog from this bacterium seems to be located adjacent to a hypothetical *virB*-like operon (Fig. 3A).

Recently, Chen and coworkers (13) have described the functionality of the pAtC58 T4SS, named AvhB. This system is essential for pAtC58 plasmid transfer, suggesting that AvhB products comprise an Mpf system (13). Although pAtC58 carries a *rctA* homolog (60), this plasmid displays high-frequency conjugative transfer under laboratory conditions, in contrast to the *R. etli* and *S. meliloti* pSyms (13, 53). This suggests that either the pAtC58 *rctA* copy is not functional or the genetic background may influence the activity of *rctA*.

To test whether the *rctA* homologs from *S. meliloti* pSymA and pAtC58 are functional, the putative *S. meliloti* and *A. tumefaciens rctA* homologs were placed under the control of the heterologous *trp* promoter (pTESMa1323 and pTEAtu5160) and then introduced into a pRetCFN42d RctA$^-$ mutant (pRetCFN42d::Tn*5*.1). Using *A. tumefaciens* GMI9023 as the recipient, the transfer of pRetCFN42d::Tn*5*.1 from *R. etli* CFNX218Spc became undetectable in the presence of either the pSymA or pAtC58 *rctA* homolog, showing that both of these genes were able to replace the *R. etli rctA* gene in silencing the conjugal transfer of the pRetCFN42d plasmid.

TABLE 5. Effect of *rctA* mutation on expression of *S. meliloti* pSymA conjugal transfer genes

Transcriptional fusion	Background[a]	β-Glucuronidase activity[b]	Relative activity[c]
p53Sm*traA*1::*gus*	1021	5.03 ± 0.65	1
	1021 RctA$^-$	11.38 ± 0.59	2.26
p53Sm*traC*::*gus*	1021	70.62 ± 6.05	1
	1021 RctA$^-$	284.59 ± 6.31	4.03

[a] Expression levels were measured in *S. meliloti* strain 1021 and a 1021 RctA$^-$ mutant.
[b] Activity is indicated as specific β-glucuronidase activity (nmol/min/mg of protein). Standard deviations were calculated from at least three independent experiments.
[c] Relative activities are expressed as ratios of specific β-glucuronidase activity in the given backgrounds with respect to the corresponding 1021 wild-type background.

***S. meliloti rctA* gene represses conjugal transfer of the symbiotic plasmid pSymA.** As shown above, pSymA of *S. meliloti* also seems to contain a functional *rctA* copy, the SMa1323 gene. To test whether this gene has any influence in the repression of conjugal transfer of *S. meliloti* pSymA, an SMa1323 mutant derivative of strain 1021 was constructed, and pSymA transfer was tested in a mating with *A. tumefaciens* GMI9023 as the recipient. An *S. meliloti* 1021 derivative carrying pSymA tagged with an Sm/Spc cassette in the *otsA* gene was used as the donor in a control mating. The *rctA* mutation generated increments of *S. meliloti* pSymA transfer frequencies under laboratory conditions from undetectable to 1×10^{-5}, suggesting strong similarities in the regulation of pSym conjugal transfer between *S. meliloti* and *R. etli*. Furthermore, we also tested whether the *rctA* mutation in pSymA affects the expression of the conjugal transfer genes *traA1* and *traC*. Similar to the case for *R. etli*, a *rctA* mutation in *S. meliloti* resulted in enhanced expression of both Dtr genes (Table 5), which also correlates with the effect of this mutation on pSym transfer. Thus, the symbiotic megaplasmids from both species, *R. etli* and *S. meliloti*, share not only similar conjugal transfer genetic organizations but also similar genetic regulatory systems that limit their transfer under standard laboratory conditions.

DISCUSSION

The ability to nodulate leguminous plants provides rhizobia with the capacity to exploit an exclusive ecological niche and therefore with significant advantages over a strictly saprophytic lifestyle. Thus, it seems reasonable to think that the gain of the genetic information necessary to nodulate a specific host should be a very important event in the evolution of these soil bacteria. Genetic and ecological studies have shown that symbiotic plasmids do transfer in soil, as evidenced by the presence of similar pSyms in different genomic backgrounds (21, 36). These studies suggest the occurrence of horizontal transfer during the diversification of natural populations of rhizobia. Indeed, typical conjugal transfer genes have been identified in most pSym genomes sequenced so far.

Several symbiotic plasmids of *R. leguminosarum*, such as pRL1JI (33) and pRL5JI (8) of *R. leguminosarum* bv. viciae, pSym5 of *R. leguminosarum* bv. trifolii (29), and pRP2JI (37) of

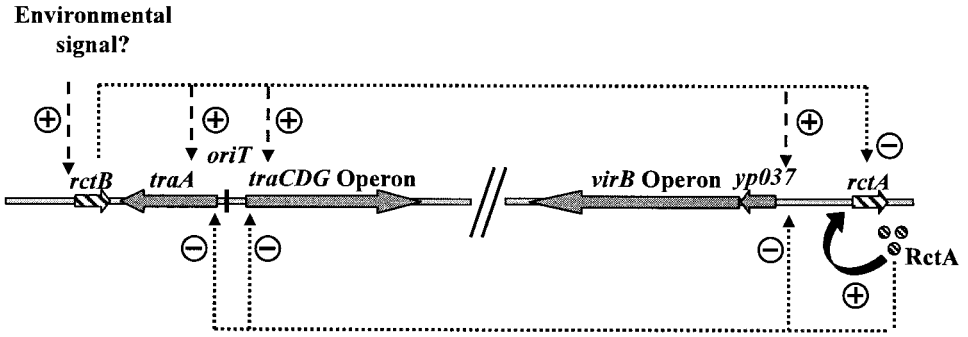

FIG. 4. Proposed model for conjugal transfer regulation of the *R. etli* symbiotic plasmid. +, activation; −, repression or inhibition. The lower part shows the normal status, i.e., the status under unfavorable conditions for conjugation (laboratory media), with high levels of RctA expression maintaining the silencing of conjugal transfer genes. When optimal environmental conditions for plasmid conjugation are found (upper part), the expression of *rctB* is enhanced; the *rctB* gene product interferes with *rctA* gene expression or RctA activity, relieving the expression of conjugal transfer genes so that plasmid conjugation can proceed.

R. leguminosarum bv. phaseoli, have been shown to be self-transmissible. However, for most rhizobial symbiotic plasmids, significant conjugal transfer does not occur under laboratory conditions, with transfer frequencies ranging from very low to undetectable.

In the particular case of *R. etli*, the transfer of pRetCFN42d has always been regarded as a cointegration phenomenon with the resident self-transmissible plasmid pRetCFN42a (10, 12, 57). Based on this, some authors suggested that the putative *oriT* and *tra* genes identified in the pRetCFN42d genome could be nonfunctional and viewed as evolutionary relics (22).

The demonstration of the functionality of the pRetCFN42d *mob* region and the identification of the yp028 gene as a possible activator of pRetCFN42d conjugative transfer provided the first strong evidence that this plasmid is self-transmissible (45). These findings led us to hypothesize the existence of a transfer regulation system that might repress the conjugal transfer functions under laboratory conditions. Here we have identified the *rctA* gene as an essential element of a regulatory system silencing the conjugative transfer of the *R. etli* symbiotic plasmid in standard laboratory media. Correlating with this, *rctA* is also required to maintain low expression levels of conjugal transfer genes in this plasmid. In contrast to that of transfer genes, the expression of *rctA* under laboratory conditions is high and seems to be positively autoregulated, as deduced from the low *rctA* expression levels observed in a *rctA* mutant. The putative *rctA* gene product shows no sequence homology to other proteins of known function. However, an analysis of the secondary and tertiary structures indicated that the predicted *rctA* gene product belongs to the winged-helix DNA-binding protein subfamily, which includes a number of both activators and repressors of gene transcription (18, 51). Altogether, the data suggest that the *rctA* gene product directly represses the transcription of transfer genes to prevent plasmid conjugation under nonfavorable conditions. The fact that the pRetCFN42d *rctA* mutant derivatives show self-transmissibility at high frequencies indicates that pSym conjugation can only be achieved under conditions leading to inactivation of the *rctA* gene product or to a reduction of *rctA* gene expression. This seems to be a requirement to obtain enhanced expression of the conjugal transfer genes *traA* and *traCDG* and the putative *virB*-like genes encoding a likely Mpf system. The *rctB* gene plays an important role in this regulatory process, as *rctA* expression is greatly reduced under conditions where *rctB* expression is enhanced, thereby leading to enhanced expression of transfer genes. Since *rctA* seems to autoregulate its own expression, it is not possible to anticipate whether the *rctB* product directly represses *rctA* transcription or interferes with the activity of the *rctA* gene product. There are two plausible mechanisms to explain the operation of this system. One of these entails binding of the RctA protein to its respective operators; this binding might be antagonized by protein-protein interactions with RctB. Alternatively, RctB might also bind to DNA, hindering the repression mediated by RctA. Resolution between these alternatives must await the purification of these proteins for use in binding assays in vitro.

Nonetheless, the data accumulated allow us to propose a working model for the regulation of *R. etli* pSym conjugative transfer (Fig. 4). We hypothesize that under unfavorable conditions for conjugation, like those present in the laboratory, the high expression of *rctA* allows the cell to maintain low expression levels of conjugal transfer genes so that efficient plasmid conjugation cannot proceed. Under favorable yet unknown conditions, the expression of *rctB* would be enhanced through an unknown mechanism, thereby leading to a reduction of *rctA* expression and/or activity which would result in the relief of expression of conjugal transfer functions and the transfer of pSym.

The regulatory network controlling pRetCFN42d conjugal transfer is probably very different from those described for other symbiotic and nonsymbiotic rhizobial plasmids, where plasmid transfer seems to be a quorum sensing-dependent phenomenon (15, 30, 57). This assumption is supported by the novel and important roles of the *rctA* and *rctB* genes in the control of pRetCFN42d conjugation, as described in this work, together with the absence of putative quorum-sensing gene homologs in pRetCFN42d (22).

We have also shown that conjugative transfer of the *S. meliloti* 1.35-Mbp pSymA is subject to *rctA*-dependent repression under laboratory conditions, by a mechanism likely similar to that found for pRetCFN42d. As for the *R. etli* pSym plasmid, our data provide strong evidence that the *S. meliloti* megaplasmid pSymA is self-conjugative but that this property is only expressed under certain conditions.

Since *rctA* is functional in both plasmids, it seems reasonable to think that both pSyms may share other elements of the conjugation regulatory network. However, we have been unable to identify an *rctB* homolog within the pSymA sequence, which suggests that the derepression of conjugal transfer genes in both plasmids may be achieved through different regulatory cascades or in response to different environmental conditions.

The *rctA* gene does not seem to be exclusive to rhizobial Sym plasmids. The *A. tumefaciens* pAtC58 plasmid also contains an *rctA* gene, and we have shown that this gene, when expressed from a heterologous promoter, is able to replace the *R. etli* allele in inhibiting pRetCFN42d conjugative transfer. However, unlike the *R. etli* and *S. meliloti* pSyms, pAtC58 was reported to conjugate under laboratory conditions (13, 53), in spite of harboring a *rctA* gene. It is possible that *rctA* is not expressed in *A. tumefaciens*, or more likely, the RctA proteins are not able to cause a complete repression of plasmid conjugation in the *Agrobacterium* background. Evidence favoring the latter possibility comes from the facts that *R. etli* RctA was not fully able to repress the conjugation of the pRetCFN42d Tn*5*.1 derivative in the GMI9023 background, whereas the product of the *A. tumefaciens rctA* allele was able to totally repress the conjugation of pRetCFN42d *rctA* mutants in *R. etli* cells.

We have also identified a homolog of *rctA* within the genome of the biodegradative bacterium *Mesorhizobium* sp. strain BNC1, where it is also located divergently transcribed from a hypothetical *virB*-like operon. According to the unfinished genome sequence, this bacterium carries a single replicon and no extrachromosomal elements. Thus, it is possible that the *rctA* and *virB*-like genes found in this genome belong to a conjugative element integrated in the chromosome.

The presence of a system for the repression of conjugal transfer genes might represent a widespread regulatory system for conjugative elements within the *Rhizobiales* order as an alternative to the better-known quorum sensing-dependent regulation described for several rhizobial and agrobacterial plasmids. The existence of an active system to silence the expression of plasmid conjugal transfer genes suggests the importance of achieving lateral spread only under the most favorable conditions. Uncovering additional elements in this regulatory network will probably be the best way to identify the optimal conditions for pSym transfer in nature, a key aspect to fully understand the ecology and evolution of this group of symbiotic bacteria.

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