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Identification of Functional *mob* Regions in *Rhizobium etli*: Evidence for Self-Transmissibility of the Symbiotic Plasmid pRetCFN42d

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An approach originally designed to identify functional origins of conjugative transfer (*oriT* or *mob*) in a bacterial genome (J. A. Herrera-Cervera, J. M. Sanjuán-Pinilla, J. Olivares, and J. Sanjuán, J. Bacteriol. 180:4583-4590, 1998) was modified to improve its reliability and prevent selection of undesired false *mob* clones. By following this modified approach, we were able to identify two functional *mob* regions in the genome of *Rhizobium etli* CFN42. One corresponds to the recently characterized transfer region of the nonsymbiotic, self-transmissible plasmid pRetCFN42a (C. Tun-Garrido, P. Bustos, V. González, and S. Brom, J. Bacteriol. 185:1681-1692, 2003), whereas the second *mob* region belongs to the symbiotic plasmid pRetCFN42d. The new transfer region identified contains a putative *oriT* and a typical conjugative (*tra*) gene cluster organization. Although pRetCFN42d had not previously been shown to be self-transmissible, mobilization of cosmids containing this *tra* region required the presence of a wild-type pRetCFN42d in the donor cell; the presence of multiple copies of this *mob* region in CFN42 also promoted conjugal transfer of the Sym plasmid pRetCFN42d. The overexpression of a small open reading frame, named *yp028*, located downstream of the putative relaxase gene *traA*, appeared to be responsible for promoting the conjugal transfer of the *R. etli* pSym under laboratory conditions. This *yp028*-dependent conjugal transfer required a wild-type pRetCFN42d *traA* gene. Our results suggest for the first time that the *R. etli* symbiotic plasmid is self-transmissible and that its transfer is subject to regulation. In wild-type CFN42, pRetCFN42d *tra* gene expression appears to be insufficient to promote plasmid transfer under standard laboratory conditions; gene *yp028* may play some role in the activation of conjugal transfer in response to as-yet-unknown environmental conditions.

Bacteria grouped within the *Rhizobiaceae*, *Phyllobacteriaceae*, and *Bradyrhizobiaceae* families, collectively known as rhizobia, are able to establish nitrogen-fixing symbiosis with leguminous plants. Many of these organisms contain complex genomes, with one chromosome and one or more large plasmids ranging in size from ca. 100 kb to >2 Mb. A common feature of the genomes of the rhizobia is that genes involved in the symbiotic process are located on independent replicons known as symbiotic plasmids (pSym) or in "symbiotic islands" within the chromosome. In addition to these symbiotic elements, rhizobia may carry additional plasmids, namely, nonsymbiotic or cryptic plasmids, that are not indispensable for symbiosis or simply with no specific function assigned (29, 38, 39, 45).

Rhizobia are difficult to isolate directly from the soil or rhizosphere; they are often isolated by virtue of their ability to nodulate specific legumes, although the presence of large numbers of nonsymbiotic rhizobia in soils is well recognized (32, 47, 52). Acquiring the ability to nodulate leguminous plants provides rhizobia with the capacity to exploit a very exclusive ecological niche and, therefore, some important 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.

There is abundant evidence for symbiotic gene transfer among different species or genera of rhizobia. Much of these data come from the analysis of soil populations and the finding that different bacterial species carry similar plasmids (10) or, vice versa, that different plasmids may associate with similar chromosomes (55). After introduction of inoculant strains in soils where no native symbiotic rhizobia are present, sometimes new rhizobial populations arise as a consequence of symbiotic gene transfer from the inoculant to nonsymbiotic rhizobia in the soil (51). However, direct experimental data are required to understand the dynamics of rhizobial DNA exchange. Furthermore, conjugal transfer of pSyms under laboratory conditions appears to occur at negligible frequencies or is undetectable, which raises questions about the actual capacity of these elements for lateral spread. Indeed, few rhizobial nodulation plasmids (pSyms) display consistent and high conjugation frequencies in normal laboratory media. One of these is pRL1JI from *Rhizobium leguminosarum*, a plasmid carrying genes for nodulation and nitrogen fixation on legumes such as pea, vetch, and lentil. pRL1JI conjugal transfer is regulated by quorum-sensing *N*-acyl-homoserine lactones (AHLs) through

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a novel regulatory relay (12). It seems likely that similar regulatory mechanisms operate in other rhizobial nonsymbiotic plasmids, such as pRme41a from *Sinorhizobium meliloti* (36) or pRetCFN42a from *R. etli* (53). In other cases, such as the Sym plasmid of *Rhizobium* sp. strain NGR234, *tra* gene expression may be regulated by AHLs, but conjugal transfer is negligible under laboratory conditions and not affected by quorum sensing AHLs, suggesting that additional upstream regulatory cascades control *tra* gene expression (21). Such a hypothetical cascade would perhaps be analogous to the opine response controlling conjugal transfer of *Agrobacterium tumefaciens* Ti plasmids (17, 26, 35).

In other cases, as for the *R. etli* symbiotic plasmid pRetCFN42d, the ability to cointegrate with a resident conjugative plasmid, pRetCFN42a, provides an alternative means for lateral spread (9, 53).

For most symbiotic plasmids or islands, it is clear that significant conjugal transfer does not occur under laboratory conditions; frequencies of transfer range from very low to undetectable (2, 21, 27, 41, 50), which suggests that either their transfer is precisely controlled or that these elements have lost the capacity for efficient lateral spread. However, genome sequencing is revealing that many of these elements do carry genes potentially involved in conjugal transfer (18, 19, 30, 31). Therefore, it seems necessary to investigate whether pSym plasmids that do not transfer at appreciable rates in the laboratory are actually proficient for self-transmissibility under natural conditions. For this purpose, approaches such as that reported by Turner et al. (54) do not provide an optimal solution, since the presence of *tra* genes in a particular replicon do not guarantee its conjugation proficiency, as indicated above. We have previously reported an approach for the identification of functional *mob* regions in *S. meliloti* (24). The use of merodiploid donor populations allowed the identification of clones carrying functional *oriT*s under the experimental conditions. This approach allowed us to characterize the *mob* region of plasmid pRmeGR4a, a cryptic, self-conjugative plasmid of *S. meliloti* GR4. Indeed, 10 other putative *oriTs* were identified. Here we report on the disadvantages of this original approach, where a *recA*-independent recombination event during the construction of merodiploid donor populations resulted in the downstream selection of false Mob^+ clones. The approach has been modified to prevent such disadvantages, and its efficacy was tested during the identification of *mob* regions in the *R. etli* CFN42 genome. This has allowed us to obtain the first evidence that the Sym plasmid of this strain may be self-transmissible and that its transfer is subject to regulation.

MATERIALS AND METHODS

Bacterial strains and plasmids. All bacteria and plasmids used in the present study are listed in Table 1. *R. etli* and *S. meliloti* strains were grown at 30°C on TY medium (tryptone-yeast extract-$CaCl_2$) (4). *Escherichia coli* and *A. tumefaciens* were grown on Luria-Bertani medium (44). When required, antibiotics were added at the following concentrations: nalidixic acid, 20 μg/ml; spectinomycin, 50 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 (Rif), 50 μg/ml; streptomycin (Sm), 100 μg/ml for *R. etli*, 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 of 0.2 and recipient *Rhizobium*, *Sinorhizobium*, or *E. coli* strains grown to late exponential phase were washed and mixed in a 1:1 donor/recipient ratio. Mating mixtures were resuspended in 50 μl of TY medium and loaded onto a sterile nitrocellulose filter of 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 spontaneous resistance to selective antibiotics was also determined.

Plasmid profiles. Plasmids profiles were visualized by the Eckhardt procedure (14) as modified by Hynes and McGregor (28).

DNA hybridizations. For DNA hybridization, total genomic DNAs of *S. meliloti* or *R. etli* strains were isolated by standard procedures (37) and digested with endonuclease EcoRI, electrophoresed on 0.8% agarose gels, and transferred to positively charged nylon membranes by the method of Southern (44). Blots of intact plasmid profiles electrophoresed in 0.8% agarose–1% sodium dodecyl sulfate–Eckhardt gels were transferred to positively charged nylon membranes as well (44). DNA hybridization probes were digoxigenin labeled according to manufacturer instructions (Roche, Barcelona, Spain). Hybridization and membrane washes were carried out under high-stringency conditions. Membranes were prepared for chemiluminiscent detection (Roche) and exposed to Kodak X-Omat film (Sigma).

PCR, cloning, and sequencing. Total DNA was prepared from mid-exponential-phase *R. etli* cells. PCR primers Yp028F and Yp028R were designed to match conserved regions of *yp028* gene of pRetCFN42d of *R. etli* (NC_004041). The primer sequences of Yp028F (GGATCCTCCATCACGTTGAGCAGC) and Yp028R (GGATCCGGCATCAACCTCTGAGAC) correspond to positions 140473 to 140490 and 141118 to 141135, respectively, of the *R. etli* pRetCFN42d replicon sequence (19), with BamHI restriction sites at the 5′ end of the primers (underlined) to facilitate subsequent clonings. These primers were used at a final concentration of 50 pmol in 50-μl amplification reactions containing 1× PCR buffer, 200 μM deoxynucleoside triphosphates, 1.5 mM $MgCl_2$, and 1 U of *Taq* polymerase (Sigma). The PCR profile used was as follows: an initial denaturation of 94°C for 10 min, followed by 30 cycles of 94°C for 60 s, 52°C for 60 s, and 72°C for 60 s, followed in turn by a final extension of 72°C for 10 min. The PCR product was checked and digested according to standard procedures (44). For all cloning procedures, standard DNA techniques were used as described previously by Sambrook et al. (44). Several EcoRI fragments from cosmid pRe182R1a were cloned into pBluescript (48). Sequencing was carried out with a Perkin-Elmer ABI Prism 373 automated sequencer. DNA sequence edition, translation, and analysis were performed by using the Vector NTI 5.0 software package and the program BLAST from the NCBI network service (1).

Construction of a *traA* mutant derivative of pRetCFN42d. Two primers, RetraAΔF (TCGTTGGTGTGGGCGAGCA) and RetraAΔR (GCGCAGCCG CCGATGCTCA), were used to amplify a 2,489-bp fragment from the *R. etli* CE3 pSym (from positions 143900 to 146388 of accession number NC_004041 [19]). The PCR product was cloned into pGEM-T Easy cloning vector; a 673-bp EcoRV fragment from *traA* was removed and replaced by a Gm resistance (Gm^r) gene cassette from SmaI-digested pMS255 (3). The construction was cloned as an EcoRI fragment into plasmid pK18*mobSacB* (46) and introduced by conjugation into strain CFNX195 of *R. etli*. Allele replacement was selected as described previously (46), and *traA* mutants were verified after hybridization with a labeled plasmid pK18Δ*traA*Gm BamHI digested as a probe.

Cell transformations. Bacterial transformation was carried out by electroporation by using an electro-cell manipulator apparatus (BTX 600; BTX, 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 and 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 field strength of 2.5 kV/cm, a 6.8-ms pulse length, and a 129-Ω set resistance was applied; cells were then immediately suspended in 1 ml of TY or Luria-Bertani medium and then incubated at 30°C for 15 h or 37°C for 1 h for *R. etli* or *E. coli*, respectively. Appropriate dilutions were plated on selective media.

RESULTS

Analysis of putative *mob* regions previously identified in *S. meliloti*. Herrera-Cervera et al. (24) reported an approach to

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant features	Reference or source
Strains		
S. meliloti		
GR4	Wild-type strain	38
GR4KLR	GR4 RecA$^-$ derivative with pRmeGR4a::Sm/Spc and pRmeGR4b::Km	23
GRM10KR	GR4KLR derivative cured of pRmeGR4a::Sm/Spc; Km^r	23
GRM8SR	GR4 derivative cured of pRmeGR4a and pRmeGR4b; Sm^r Rif^r	38
R. etli		
CE3	Sm^r derivative of wild-type strain CFN42	42
CFNX2001	CFN42 derivative (p42d$^-$/p42a$^-$)	34
CFNX667	CFN42 *recA* mutant derivative (p42a$^-$, p42d::Tn*5*mob)	J. Martinez (CIFN)
CFNX195	CE3 derivative (p42a$^-$, p42d::Tn*5*Mob)	9
CFNX182	CE3 derivative (p42a$^-$)	8
CFNX218Rif	CE3 derivative (p42a$^-$/p42b$^-$/p42c$^-$/p42d$^-$/p42eΔ/p42f$^-$); Rif^r	This work
CFNX195ΔTraAGm	CFNX195 derivative (Δ*tra*A::Gm^r)	This work
A. tumefaciens GMI9023	Plasmidless C58 derivative	43
E. coli		
DH5α	*supE44*, Δ*lacU169*, *ϕ80*, *lacZΔM 5hsdR171*, *recA1*, *endA1*, *gyrA96 thi-1 relA1*	20
HB101	*supE44*, *hsdS20*, *recA13*, *ara-14*, *proA2*, *lacY1*, *galK2*, *rpsL20*, *xyl-5mtl1*	6
S17-1	*thi*, *pro*, *recA*, *hsdR*, *hsdM*; RP4-2-Tc::Mu-Km::Tn*7*	49
Plasmids		
pJB3Tc19	IncP cloning vector; Tc^r Ap^r	5
pLAFR1	IncP cosmid vector; Tc^r	13
pTE3	IncP cloning vector carrying *Salmonella enterica* serovar Typhimurium *trp* promoter; Tc^r	15
pGEM-T Easy	PCR cloning vector; Ap^r	Promega
pK18*mobSacB*	Mobilizable Km^r suicide vector with a *sacB* gene of *Bacillus subtilis*	46
pBluescript II KS	2,961-bp phagemid cloning vector; Ap^r	Stratagene
pMS255	pUC derivative with the Gm^r cassette	3
pRK2013	RK2 derivative helper plasmid; Km^r	16
pRe182R1a	pLAFR1 derivative containing *mob* region from pRetCFN42d	This work
pRe182R1b-d	pReOR182R1a overlapping cosmids from the CFN42 library	This work
pRe182R1aΔ*Hin*dIII	pRe182R1a derivative with a 11-kb HindIII deletion	This work
pJBdp1	pJB3Tc19 carrying 7.7-kb HindIII fragment from pRe182R1a	This work
pJBdp2	BamHI deletion of pJBdp1	This work
pJBdp3	BglII deletion of pJBdp2	This work
pJBdp4	pJB3Tc19 carrying the 1.3-kb EcoRI fragment from pRe182R1a	This work
pTEYp028	pTE3 with *yp028* cloned in front of the *trp* promoter	This work
pTEYp028R	Same as pTEYp028 but with *yp028* in reverse orientation	This work

identify DNA regions with the ability to convert a nontransmissible vector into a mobilizable plasmid and thus to identify functional origins of conjugative transfer (*oriT* and *mob*). RecA$^-$ *S. meliloti* merodiploid populations were obtained by transferring an *S. meliloti* gene library from *E. coli* into *S. meliloti* by triparental matings using pRK2013 as a helper plasmid (16). The *S. meliloti* merodiploids were used as donors en masse in matings with *S. meliloti* recipient strains and transconjugants carrying vector-encoded antibiotic resistance selected. Eleven putative *mob* regions were identified, most of which originated from plasmid replicons. This approach allowed identification and further characterization of the *oriT* from the conjugative plasmid pRmeGR4a (24), as well as the *mob* region from pRmeGR4b cloned in cosmid pRmOR65, a nonsymbiotic plasmid that can be mobilized in *trans* by pRmeGR4a (22).

During the analysis of the remaining nine putative *mob* regions, we found that all of these nine cosmids showed unusually high transfer frequencies from either *S. meliloti* (24) or *E. coli* donors and therefore behaved as self-transmissible Mob$^+$ Tra$^+$ plasmids. In contrast, cosmids pRmOR69 (*oriT* from pRmeGR4a) and pRmOR65 (*oriT* from pRmeGR4b) displayed a Mob$^+$ Tra$^-$ phenotype. After removing all of the EcoRI insert from the nine Mob$^+$ cosmids to theoretically obtain the empty vector pLAFR1, we observed that the nine empty vectors (form here on named pLAFR1*) still maintained the Mob$^+$ Tra$^+$ character, in contrast to the Mob$^+$ Tra$^-$ properties of the original vector pLAFR1. This suggested that the Mob$^+$ Tra$^+$ capacity of the nine cosmids was due to a change in the cosmid vector pLAFR1. After digestion with Eco47III the restriction profiles of the pLAFR1* molecules were compared to those of vector pLAFR1 and plasmid pRK2013. Whereas the empty vectors derived from *mob* cosmids pRmOR69 and pRmOR65 had restriction profiles identical to the original pLAFR1 vector, the remaining nine pLAFR1* molecules with Mob$^+$ Tra$^+$ phenotype showed re-

TABLE 2. Isolation of Mob$^+$ cosmids from a *R. etli* cosmid library[a]

Merodiploid donor	Recipient	Frequency of transconjugants	No. of transconjugants analyzed	Cosmid types (no. of occurrences)	No. of nonoverlapping cosmids
CFNX182	*E. coli* HB101	1.6×10^{-4}	20	pC13a (18), pC13b (2)	1
	R. etli CFNX218Rif	3×10^{-5}	32	pC13a (11), pC13b (12), pC13c (4), pRe182R1b (2), pRe182R1c (1), pRe182R1d (2)	2
CE3	*E. coli* HB101	7.68×10^{-6}	32	pC13a (18), pC13b (5), pC13c (3), pRe182R1a (4), pRe182R1b (2)	2
	R. etli CFNX218Rif	1.17×10^{-4}	20	pC13a (18), pRe182R1a (1), pRe182R1d (1)	2

[a] An *R. etli* gene library was transferred by electroporation into CFNX182 or CE3 strains, and corresponding pools of merodiploids were used as donors in matings with HB101 and CFNX218Rif. Transconjugants were selected for Tc^r encoded by the vector supporting the gene library. Cosmids from transconjugants were isolated and identified by EcoRI restriction analysis.

striction patterns that appeared to be chimeras derived from both pLAFR1 and the mobilizing plasmid pRK2013 (data not shown). However, the pLAFR1* cosmids carried no resistance to Km, as does pRK2013. In conclusion, 9 of the 11 *mob* cosmids isolated by Herrera-Cervera et al. (24) did not contain any *S. meliloti oriT*. These cosmids probably were the result of recombination events between the pLAFR1 vector and the mobilizing plasmid pRK2013 during construction of the *S. meliloti* merodiploid populations. However, the recombination between the two plasmids must have been a *recA*-independent phenomenon, since all of the strains used by Herrera-Cervera et al. were RecA defective (24).

A modified approach to identify Mob$^+$ regions in rhizobial genomes. The above results indicated that, although the approach reported by Herrera-Cervera et al. might be useful for identifying rhizobial functional *oriT*s, a modification was needed in order to prevent or at least reduce the excessive selection of false Mob$^+$ clones. As a general rule, we recommend against using the suicide helper plasmid pRK2013 when the transfer of Mob$^+$ plasmids from *E. coli* to rhizobial strains is needed. Alternatively, this can be done by using mobilizing *E. coli* strains (i.e., S17-1 [49]) instead of mobilizing vectors or, when feasible, by direct introduction of cosmids or plasmids into the rhizobial strains by electroporation. We have tested both alternatives as a modification to the *oriT* cloning strategy of Herrera-Cervera et al. (24).

The cosmids from the *S. meliloti* GR4 gene library were isolated from pooled *E. coli* HB101 clones by standard methods and electroporated into the mobilizing strain S17-1. The resulting clones were pooled and used as donors en masse in matings with the *S. meliloti* strains GR4KLR and GRM10KR (24), yielding merodiploid populations derived from both rhizobial strains. From here, we repeated the experiments of Herrera-Cervera et al. (24). When we used the new GRM10KR merodiploid population as the donor in matings, no transconjugants were obtained, in contrast to the 10^{-5} transconjugants per recipient obtained by Herrera-Cervera et al. (24). However, when the GR4KLR merodiploid population was the donor, transconjugants arose at a frequency of 10^{-7}, a finding similar to that obtained by Herrera-Cervera et al. (24). The cosmids acquired by 24 of the transconjugants were studied by restriction analysis, and all of them were found to correspond to cosmid pRmOR69, carrying the *oriT* of plasmid pRmeGR4a (24). Thus, the modified approach resulted in the selection of the *mob* region of pRmeGR4a plasmid without the appearance of any false Mob$^+$ clones. We were unable to isolate additional putative *oriT*-containing regions from *S. meliloti* GR4. The fact that we could not isolate the *mob* region from plasmid pRmeGR4b in this experiment may be due to the fact that mobilization of this plasmid by pRmeGR4a is at least 10-fold less efficient than transfer of pRmeGR4a itself.

We also applied the modified approach to the isolation of *mob* regions from *R. etli* CE3. This strain carries six plasmids, ranging from 180 to 630 kb in size (9). To date, only the cryptic plasmid pRetCFN42a (abbreviated as p42a) has been shown to be self-transmissible (9, 53). Transfer of pRetCFN42d (the pSym; abbreviated as p42d) has also been detected (8), but this was shown to be fully dependent on the presence of p42a. The mechanism for pSym transfer appeared to require its cointegration with p42a.

Merodiploid populations of *R. etli* strains CE3 and CFNX182, a CE3 derivative cured of plasmid p42a (see Table 1), were obtained after introducing by electroporation cosmids from a *R. etli* CE3 gene library made in vector pLAFR1 (25) into each strain. Each merodiploid population was used as donor en masse in matings with *R. etli* CFNX218Rif or *E. coli* HB101 as recipients.

Using the CFNX182 merodiploids as donors, HB101 Tc^r transconjugants arose at frequency of 1.6×10^{-4} (Table 2). Cosmids from 20 of such transconjugants were isolated by standard procedures and subjected to restriction analysis with endonuclease EcoRI. All putative *mob* cosmids showed similar EcoRI patterns (data not shown), with at least five EcoRI fragments in common, indicating that all of them contained overlapping DNA inserts. Furthermore, one of these cosmids showed a restriction profile identical to cosmid pC-13, which was recently characterized by Tun-Garrido et al. (53), which contains the *oriT* and all transfer functions from plasmid p42a. To maintain the nomenclature used by Tun-Garrido et al. (53), we named the two different overlapping cosmids pC13a and pC13b, respectively. The selection of the p42a *mob* region demonstrated that our approach could also be applied to *R. etli*. When *R. etli* CFNX218Rif was used as a recipient in matings with the CFNX182 merodipoids, CFNX218Rif Tc^r transconjugants arose at frequency of 3×10^{-5} (Table 2). Cosmids from 32 such transconjugants were isolated and subjected to restriction analysis with EcoRI. Cosmids from 27 transconjugants showed similar EcoRI patterns to any one of members of the pC-13 cosmid family described above (with the appearance of a new member of this family, pC13c), whereas

the remaining five cosmids were of three overlapping types (which we designated pRe182R1b, pRe182R1c, and pRe182R1d), with restriction patterns completely different to the pC-13 family, suggesting that they could represent a new *mob* region.

Using the CE3-derived merodiploid population as donor, Tc^r transconjugants arose at frequencies of 7.68×10^{-6} and 1.17×10^{-4} when HB101 or *R. etli* CFNX218 Rif^r strains were used as recipients, respectively. Cosmids from the transconjugants obtained from each mating were analyzed and identified as members of either of the two cosmid families described above (Table 2). A new member of the pRe182R1 cosmid family, pRe182R1a, was identified.

In summary, our modified approach for identifying *oriT* regions appeared to work correctly in *R. etli*, since it had been able to select for the only previously known functional *mob* region in strain CE3, located in plasmid p42a. In addition, we were able to isolate a new functional *mob* region not reported previously.

Characterization of a *mob* region in the *R. etli* CFN42 symbiotic plasmid. The putative *mob* region present in the cosmid family pRe182R1 was found to belong to the symbiotic plasmid p42d after cosmid pRe182R1a was used as a probe in hybridizations either against blotted digested genomic DNAs from various *R. etli* strains or against blotted Eckhardt-type gels containing intact plasmids from these strains. Specific hybridization signals were absent only in strains cured of p42d (Fig. 1).

To confirm that the isolated cosmids of the pRe182R1 family could indeed be mobilized from *R. etli*, individual cosmids were introduced back into CFNX182, and the corresponding strains were separately used in matings with CFNX218Rif as recipient. All of the overlapping cosmids showed similar transfer frequencies of $\sim 10^{-4}$ transconjugants per recipient. To test the influence of p42d background on the mobilization of this cosmid family, pRe182R1a was introduced into strain CFNX2001 (cured of p42a and p42d), and the resulting strain was crossed with CFNX218Rif and HB101 as recipients. No transconjugants were obtained from these matings, indicating that mobilization of these cosmids required the presence of p42d in the donor cell. To further characterize this *oriT*, we cloned and end sequenced several EcoRI fragments from cosmid pRe182R1a. Comparison with the genome sequence of plasmid p42d (19) demonstrated that we had isolated a putative *mob* region containing two divergently transcribed *tra* operons and an *oriT* located in this symbiotic plasmid (Fig. 2A [19]). As described above, our data suggested that this cloned *mob* region was functional and that its mobilization required plasmid p42d, despite the fact that this plasmid has never been shown to be self-transmissible. This apparent paradox could be explained if both the intact plasmid and the cloned *mob* region (in multicopy) were necessary for the plasmid to show self-transfer in laboratory media. If so, then the cloned *mob* region would promote transfer of the entire pSym. Indeed, strain CFNX667 ($RecA^-$) carrying cosmid pRe182R1a was able to donate the pSym p42d to either *R. etli* CFNX218Rif (1.38×10^{-3} transconjugants per recipient) or *A. tumefaciens* GMI9023 (frequency of 2.56×10^{-5}) in media where the transfer of p42d is otherwise undetectable. This suggested that multiple copies of the p42d *mob* region allowed conjugation of the symbiotic plasmid to occur at detectable rates in standard laboratory conditions. Furthermore, a derivative of cosmid pRe182R1a carrying an 11-kb HindIII deletion that removed this *mob* region (plasmid pReOR182RaΔHindIII) also lost the ability to promote transfer of p42d.

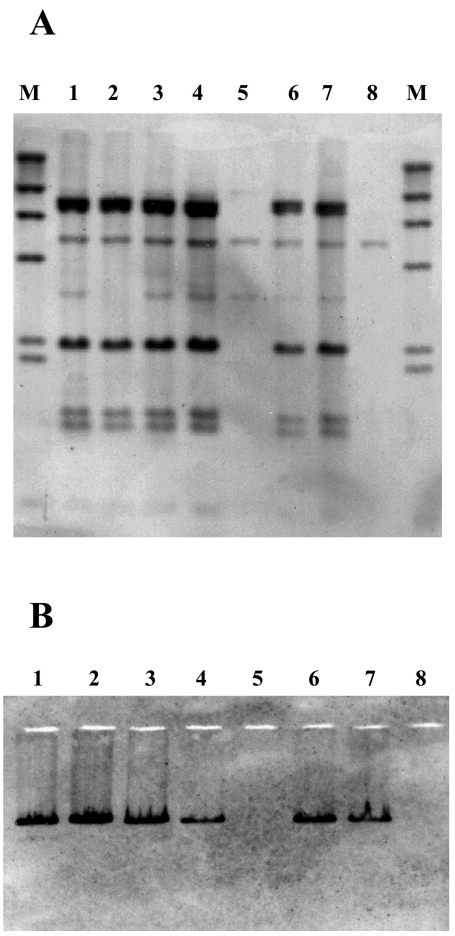

FIG. 1. Replicon localization of the Mob^+ DNA identified in cosmid pRe182R1a. Blots of EcoRI-digested genomic DNAs (A) and of intact plasmid profiles (Eckhardt gels) (B) hybridized against digoxigenin-labeled pRe182R1a as a probe are shown. Lanes: M, digoxigenin-labeled DNA molecular weight marker; 1, *R. etli* CE3 (wild type); 2, *R. etli* CFNX182(p42a$^-$); 3, *R. etli* CFNX183(p42b$^-$); 4, *R. etli* CFNX184(p42c$^-$); 5, *R. etli* CFNX89(p42d$^-$); 6 *R. etli* CFNX185(p42eΔ); 7, *R. etli* CFNX186(p42f$^-$); 8, *R. etli* CFNX218(p42a$^-$, p42b$^-$, p42c$^-$, p42d$^-$, p42eΔ, p42f$^-$).

Overexpression of the *yp028 gene* promotes *R. etli* pSym transfer in standard media. Since all overlapping cosmids pRe182R1a to pRe182R1d displayed the same capacity to promote p42d conjugation, it seemed obvious that the gene or genes responsible for this effect would be located in a DNA fragment common to all of them. This would be a DNA fragment flanked by *yp026* and *traD* (see Fig. 2A). Indeed, a pJB3 derivative including a 7.7-kb HindIII fragment carrying the *mob* region (pJBdp1) had the same effect as the entire cosmids in promoting p42d transfer (Fig. 2B and Table 3). Several fragments were subsequently subcloned into pJB3Tc19 and tested for promotion of p42d conjugal transfer. As outlined in Fig. 2B and Table 3, clone pJBdp2, containing an intact *yp028*

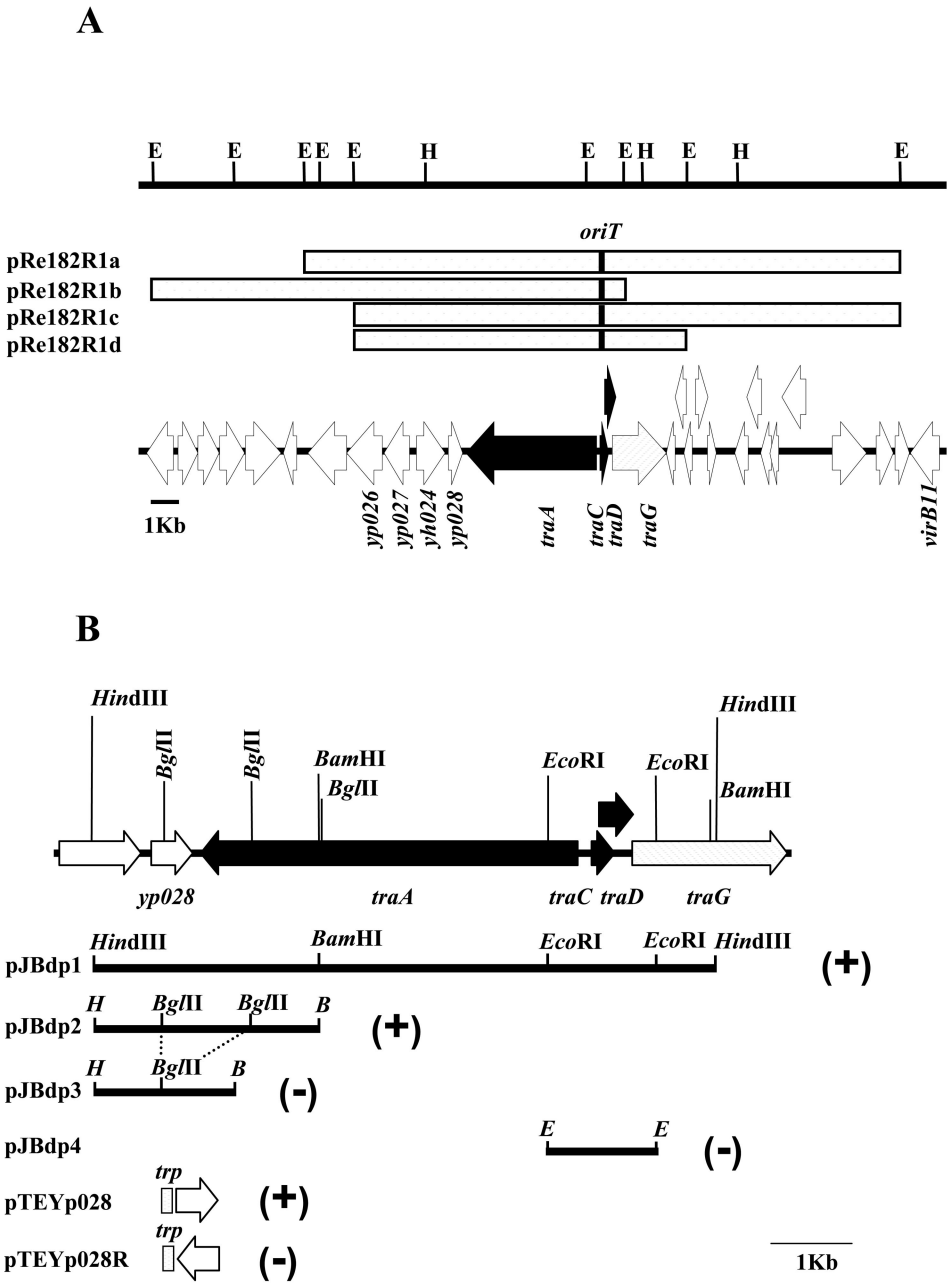

FIG. 2. (A) Physical map of the pRe182R1 cosmid family containing the *mob* region of the *R. etli* symbiotic plasmid p42d. Arrows indicate the transcription directions of the identified genes according to Gonzalez et al. (19). The Dtr (DNA transfer and replication) genes are shown as black arrows. The gene coding for the hypothetical coupling protein TraG gene is shown by a striped arrow. The DNA insert in each member of the cosmid family is indicated by dotted bars. *oriT*, origin of transfer; E, EcoRI; H, HindIII. (B) Identification of an ORF promoting conjugal transfer of the p42d symbiotic plasmid. Various constructs were derived from the 7.7-kb HindIII fragment cloned in pJBdp1 and tested for pSym transfer from strain CFNX667. "(+)" or "(−)" indicates promotion or no promotion, respectively, of p42d conjugal transfer. In pTEYp028 and pTEYp028R, the dotted box indicates the *trp* promoter, and the white arrow indicates the yp028 ORF. E, EcoRI; H, HindIII; B, BamHI.

open reading frame (ORF), maintained the ability to promote conjugation of p42d, but this property was lost when a BglII deletion removed the C-terminal half *yp028* (plasmid pJBdp3). The results indicated that it was the presence of this ORF *yp028* that was responsible for promoting conjugation of p42d. Since this effect was only observed when this gene was cloned in multicopy vectors (p42d contains an intact copy of *yp028*), we reasoned that a change in *yp028* expression resulted in the observed effects on p42d conjugal transfer. Indeed, when the *yp028* coding sequence was placed under the control of the *trp* promoter (plasmid pTEYp028), it had the effect of promoting conjugal transfer of p42d at high frequencies (Fig. 2B and Table 3). In contrast, plasmid pTEYp028R containing the same *yp028* cloned in the opposite orientation to the *trp* pro-

TABLE 3. Identification of an ORF promoting conjugal transfer of the *R. etli* symbiotic plasmid p42d

Donor	p42d transfer frequency[a]
CFNX667(pJBdp1)	2.3×10^{-5}
CFNX667(pJBdp2)	1.36×10^{-5}
CFNX667(pJBdp3)	ND
CFNX667(pJBdp4)	ND
CFNX667(pTE)	ND
CFNX667(pTEYp028)	2.63×10^{-5}
CFNX195(pTEYp028)	1.8×10^{-5}
CFNX667(pTEYp028R)	ND
CFNX195ΔtraAGm(pTEYp028)	ND

[a] The recipient was *A. tumefaciens* GMI9023. ND, no transconjugants detected.

moter (thus containing a promoterless *yp028*) was unable to promote conjugal transfer of the p42d Sym plasmid (Fig. 2B; Table 3). These results strongly suggested that both the presence of an intact *yp028* and expression of the ORF were necessary to promote conjugal transfer of the *R. etli* pSym. Transconjugants were verified to carry the p42d plasmid after the plasmid profiles were visualized (Fig. 3). We observed that most of the transconjugants had acquired only plasmid p42d from CFNX195(pTEyp028) (Fig. 3, lane 3), whereas in some cases both p42d and p42b were transferred (Fig. 3, lane 2). Cointegration of p42d and p42b has previously been observed (7). As a result, the transconjugants carrying these two plasmids may have been generated through the transfer of a p42b-p42d cointegrate and its subsequent resolution into the wild-type plasmids in the recipient.

yp028 is located downstream of *traA* and putatively encodes a protein of 171 amino acids that shows no sequence homology to any protein sequence of known function described in the databases. Yp028 displayed some sequence conservation (<30% sequence identity and 47% sequence similarity) with two small ORFs of unknown function, SMa0974 (1235562) from *S. meliloti* pSymA and Atu5116 (1136889), from the *A. tumefaciens* pAtC58 cryptic plasmid.

***yp028*-dependent transfer of p42d requires *traA*.** To determine whether the effect of overexpressing *yp028* on the promotion of conjugal transfer of p42d was indeed dependent on p42d conjugal transfer genes, we investigated the effect of a *traA* mutation on p42d conjugal transfer. A p42d-*traA* deletion mutant derived from CFNX195 was obtained as described in Materials and Methods. Plasmid pTEYp028 was introduced into the mutant strain by electroporation, and the transfer of p42d was determined in matings with *A. tumefaciens* GMI9023. No transconjugants were obtained (Table 3), demonstrating that the high-frequency conjugal transfer of the symbiotic plasmid p42d promoted by *yp028* required a wild-type *traA* gene, which likely encodes the relaxase of this pSym.

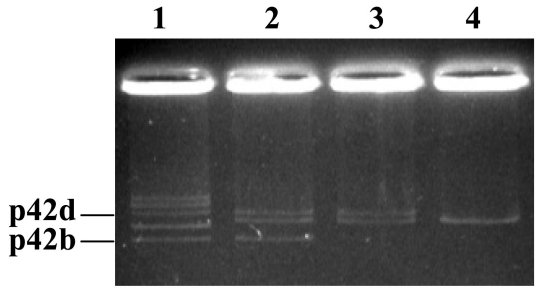

FIG. 3. Eckhardt-type gel electrophoresis showing plasmid profiles of transconjugants that had acquired the Sym plasmid p42d from CFNX195(pTEYp028) using CFNX218Rif(peΔ) as recipient. Lanes: 1, CFNX195(pTEYp028); 2, type I transconjugant; 3, type II transconjugant; 4, recipient strain CFNX218Rif. The bands corresponding to plasmids p42d and p42b are indicated.

DISCUSSION

The origin of transfer (*oriT* or *mob*) of conjugative or mobilizable elements is the only known *cis*-acting function required for DNA transfer. Its presence in a plasmid or a transposon is usually suggestive of the conjugative or mobilizable capacity of such genetic elements. Among the gram-negative bacteria known as rhizobia, genome sequencing is demonstrating that most, if not all plasmids and chromosomal islands in these bacteria contain an *oriT*, including the symbiotic plasmid and islands that carry genes important for the establishment of nitrogen-fixing symbiosis with legumes (18, 19, 30, 31, 40). In other cases, the search for *oriT*s by PCR amplification of putatively conserved *traC-traA* intergenic regions has provided similar results in *R. leguminosarum* (54). Neither approach, however, is able to determine whether the *mob* regions identified are actually functional in the genetic background where they are found. Indeed, relatively few rhizobial Sym plasmids and symbiotic islands have been found to conjugate at high frequencies under laboratory conditions (12, 21), despite the sequence data and other evidence indicating they may have self-transmissible (Mob^+ Tra^+) or mobilizable (Mob^+) capacities under natural conditions (51, 55). Therefore, reliable approaches are needed that allow the identification of Mob^+ capabilities in these bacteria, opening the possibility to study the putative regulation of their conjugal transfer. This was the aim of the approach described by Herrera-Cervera et al. (24), which allowed the identification of 11 putative *oriT*s in strain GR4 of *S. meliloti*. Among these were the *mob* region from the conjugative plasmid pRmeGR4a and that of the mobilizable plasmid pRmeGR4b. However, as shown in the present study, the remaining nine putative *oriT*-containing clones did not represent any functional *mob* region. These were actually false Mob^+ clones generated after a *recA*-independent recombination event between the cosmid vector pLAFR1 and the helper plasmid pRK2013 during the construction of merodiploid rhizobial populations used as donors of putative Mob^+ clones. Such recombination led to the generation of hybrid Mob^+ Tra^+ plasmids (resembling pRK2013) able to replicate in *S. meliloti* (as does pLAFR1). The subsequent experimental selection for Mob^+ clones converted a probably unusual event into a frequently selected one. We modified the procedure to obtain merodiploid populations, avoiding the use of helper plasmids during transfer of the rhizobial gene library from *E. coli* into rhizobial strains, and verified that for *S. meliloti* GR4 no false Mob^+ clones were ever obtained. Similarly, the procedure was applied to identify Mob^+ clones in a *R. etli* CFN42 cosmid library. In addition to showing that no artificial Mob^+ clones were isolated in this case, we demonstrated that there

are two functional *mob* regions in the genome of this bacterium that can be isolated by this procedure. One corresponded to the previously characterized transfer region of the cryptic, conjugative plasmid p42a (53), indicating the reliability of this approach and its application not only to *S. meliloti* but also to *R. etli*. The second was identified as a *mob* region in the symbiotic plasmid p42d of this strain. This was somewhat surprising since pSym p42d has never been shown to have conjugative capabilities (9, 19, 53). Although conjugal transfer of p42d has been detected previously under laboratory conditions, this transfer always relied on the cryptic plasmid p42a and required cointegration between p42d and p42a (9, 53). Furthermore, we observed that multiple copies of the *mob* region from p42d promoted conjugal transfer of this pSym in the absence of the cryptic plasmid p42a. Interestingly, we found that the presence of pSym was needed for mobilization of the cloned *mob* region. This apparent paradox was solved after identifying a small ORF, *yp028*, located adjacent to *traA*, which was responsible for this effect. Most likely, overexpression of *yp028* was leading to conjugal transfer of the Sym plasmid p42d, providing the first evidence that this plasmid may be self-transmissible. The second piece of evidence comes from the fact that conjugal transfer of the *R. etli* pSym promoted by *yp028* was dependent on the p42d *traA* gene. *traA* likely encodes the relaxase, which is essential for processing the *oriT* and for the initiation of DNA transfer. Thus, we have arrived at three conclusions: (i) the previously sequenced *mob* region of this pSym (19) is functional; (ii) conjugal transfer of this symbiotic plasmid under laboratory conditions can be promoted by overexpression of the *yp028* gene; and (iii) plasmid p42d probably contains all functions needed for conjugal transfer and therefore may be regarded as a Mob^+ Tra^+, self-transmissible plasmid. In addition to Dtr genes, p42d carries a complete set of *virB*-like genes (19), similar to those identified in *A. tumefaciens* and involved in DNA transfer to plants or in pAtC58 plasmid conjugation (11, 33). These results support the hypothesis that the presence of transfer-related genes and particularly *oriT*-processing genes in rhizobial plasmids probably indicates that these elements actually have conjugative capabilities. The fact that these elements often display negligible or undetectable conjugal transfer rates in standard laboratory media does not preclude their conjugative potential under natural conditions and probably reflects a rather strict regulation of transfer functions. Based on our results, we suggest that conjugal transfer functions of the *R. etli* pSym may be silent under laboratory conditions and that transfer can be activated upon an environmental signal with the participation of *yp028*. Since overexpression of *yp028* leads to pSym conjugal transfer, we speculate that activation of *yp028* gene expression is a critical point during activation of conjugal transfer. The pRetCFN42d genome sequence annotation (19) includes a computational prediction that the *yp028* promoter may be of the σ_{54} class, which would involve activation by a transcriptional regulator binding upstream of the promoter. Adequate experiments, however, are necessary to confirm this prediction.

At this point, we cannot speculate about the actual role of gene *yp028* or whether such regulation is exerted at transcriptional or posttranscriptional levels. However, our results open a new window for investigating the actual capabilities of rhizobial symbiotic plasmids for lateral spread.

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