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RESEARCH ARTICLE

Rhizobial plasmid pLPU83a is able to switch between different transfer machineries depending on its genomic background

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Abstract

Plasmids have played a major role in bacterial evolution, mainly by their capacity to perform horizontal gene transfer (HGT). Their conjugative transfer (CT) properties are usually described in terms of the plasmid itself. In this work, we analyzed structural and functional aspects of the CT of pLPU83a, an accessory replicon from *Rhizobium* sp. LPU83, able to transfer from its parental strain, from *Ensifer meliloti*, or from *Rhizobium etli*. pLPU83a contains a complete set of transfer genes, featuring a particular organization, shared with only two other rhizobial plasmids. These plasmids contain a TraR quorum-sensing (QS) transcriptional regulator, but lack an acyl-homoserine lactone (AHL) synthase gene. We also determined that the ability of pLPU83a to transfer from *R. etli* CFN42 genomic background was mainly achieved through mobilization, employing the machinery of the endogenous plasmid pRetCFN42a, falling under control of the QS regulators from pRetCFN42a. In contrast, from its native or from the *E. meliloti* background, pLPU83a utilized its own machinery for conjugation, requiring the plasmid-encoded *traR*. Activation of TraR seemed to be AHL independent. The results obtained indicate that the CT phenotype of a plasmid is dictated not only by the genes it carries, but by their interaction with its genomic context.

Introduction

Biologic evolution began about 3.5 billion years ago. In addition to the classical Darwinian mechanism of mutant selection, HGT has also been implicated in microbial evolution (Davison, 1999). HGT between bacteria is mainly accomplished by three mechanisms: transformation, transduction, and conjugation (Hall, 1984; Slater, 1984). Conjugative transfer (CT) is considered to be one of the most efficient means of DNA exchange among prokaryotes; thus, special attention has been paid to the transfer of genetic material via plasmid conjugation. Plasmids are found in almost all the bacterial communities studied including water and soils (Sorensen *et al.*, 2005). The molecular mechanism for conjugation has been widely studied in proteobacteria. In general, CT requires the participation of proteins involved in Dtr (DNA transfer replication) and Mpf (Mating pair formation) as well as an *oriT* (origin of transfer) site (de la Cruz *et al.*, 2010). Plasmids are classified either as conjugative (self-transmissible), when they harbor all the elements needed to perform CT, or as mobilizable, when they need proteins provided *in trans* by another conjugative plasmid (Ding & Hynes, 2009).

Rhizobia are common inhabitants of the soil, able to form symbiotic associations with the roots of legumes, as nitrogen-fixing organisms. In their genomes, they harbor a variable number of plasmids. Genes involved in the symbiotic process are usually present on symbiotic plasmids (pSym). In addition, some accessory plasmids have been described in rhizobia. Some of these plasmids are able to move by conjugation, either as self-transmissible

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or as mobilizable plasmids, in the last case exploiting helper functions provided *in trans* by other replicons (Johnston *et al.*, 1978; Brewin *et al.*, 1980; Hooykaas *et al.*, 1981; Brom *et al.*, 1992, 2000; Mercado-Blanco & Olivares, 1993; Pistorio *et al.*, 2003). Nevertheless, some plasmids have features that do not allow them to be clearly defined as conjugative or mobilizable (Torres Tejerizo *et al.*, 2010; Cervantes *et al.*, 2011).

The regulation of plasmid transfer in *Rhizobiaceae* has recently been reviewed by Ding & Hynes (2009). Two regulatory mechanisms have been described, one by quorum-sensing (QS) (Piper *et al.*, 1993; Zhang *et al.*, 1993; Tun-Garrido *et al.*, 2003) and another one by *rctA*/*rctB* genes (Pérez-Mendoza *et al.*, 2005; Sepúlveda *et al.*, 2008). Briefly, the regulation by QS is performed by the TraI enzyme [acyl-homoserine lactone (AHL) synthase], which synthesizes AHL molecules at high population densities. AHLs bind to the TraR regulator and activate transcription of genes involved in CT (McAnulla *et al.*, 2007). In the group of plasmids regulated by QS, two subtypes have been described. The first one comprises plasmids originating from *Agrobacterium tumefaciens*, where plant-secreted opines are necessary to activate the transcription of *traR*. The TraM anti-activator antagonizes the activity of TraR in *A. tumefaciens* (Ellis *et al.*, 1982; Fuqua *et al.*, 1995). In the other group of plasmids, different AHL regulation circuits have been described, which involve other, as yet unidentified, molecules (Danino *et al.*, 2003; He *et al.*, 2003; Tun-Garrido *et al.*, 2003).

Rhizobium sp. LPU83 strain is acid tolerant and has an extended nodulation host range (Del Papa *et al.*, 1999). The genetic structure of this strain is unique in that it has a chromosome related to the bean-, pea-, and clover-nodulating rhizobia, while the pSym genes participating in nodulation and nitrogen fixation are more similar to those present in *Medicago* symbionts (Laguerre *et al.*, 2001; Del Papa *et al.*, 2007; Torres Tejerizo *et al.*, 2011a). Previously, we described the conjugative properties of plasmids from *Rhizobium* sp. LPU83: the symbiotic plasmid (pLPU83b) was mobilizable at a very low frequency, only in the presence of pLPU83a. Plasmid pLPU83a was able to perform CT from genomic backgrounds such as *Rhizobium* sp. LPU83 or *E. meliloti*, but it was unable to transfer from plasmid-free *A. tumefaciens* UBAPF2 (Torres Tejerizo *et al.*, 2010). These results have hindered a clear classification of pLPU83a, either as a self-transmissible or as mobilizable plasmid. In this work, we aim to elucidate this issue, through the analysis of the mechanisms involved in the regulation of CT of plasmid pLPU83a from its original as well as from other host genomic backgrounds.

Materials and methods

Bacterial strains and plasmids

The strains and plasmids used in this work are listed in Table 1. *Escherichia coli* was grown on Luria–Bertani [LB (Miller, 1972)] medium at 37 °C. Rhizobial and *A. tumefaciens* strains were grown on PY [peptone of casein – yeast extract (Noel *et al.*, 1984)] at 30 °C. For solid media, 15 g of agar L^{-1} of medium was added. The final concentration of antibiotics used for *E. coli* was as follows (in μg mL^{-1}): gentamicin (Gm) 10, kanamycin (Km) 25, and tetracycline (Tc) 10 and for *Rhizobia* and *A. tumefaciens*: streptomycin (Sm) 400, nalidixic acid (Nal) 20, neomycin (Nm) 60, rifampicin (Rif) 100, spectinomycin (Sp) 100, Gm 30, and Tc 5.

Bacterial matings

Conjugation experiments were performed as described by Cervantes *et al.* (2011). Briefly, overnight cultures were grown to stationary phase. Donor and recipient strains were mixed in a 1 : 2 ratio and incubated overnight on PY plates at 30 °C. The mixtures were resuspended in 1 mL of 10 mM $MgSO_4$-0.01% Tween 40 (v/v). For triparental matings, pRK2013 was used as helper plasmid. Serial dilutions were plated on selective PY medium supplemented with the corresponding antibiotics to quantify the number of donor, recipient, and transconjugant cells. The conjugation frequencies are expressed as the number of transconjugants per donor cell. The visualization of plasmids in the transconjugants (plasmid profiles) was evaluated on Eckhardt gels (Eckhardt, 1978) as modified by Hynes and McGregor (Hynes & McGregor, 1990).

DNA manipulation and genetic procedures

Procedures to obtain total DNA, plasmid purification, restriction enzyme analysis, cloning, and *E. coli* transformation were performed according to previously established techniques (Sambrook *et al.*, 1989).

Primers were purchased from Operon or from Unidad de Síntesis Química IBT-UNAM. PCR amplification was carried out with recombinant *Taq* DNA polymerase or *Pfu* DNA polymerase as specified by the manufacturers in a Mastercycler 5330 (Eppendorf) or in an iCycler (Bio-Rad) thermocycler.

Construction of a pLPU83a-*traR* mutant

To construct an insertional mutation in *traR* of pLPU83a, an internal fragment of 275 bp of the *traR* gene was amplified with primers traR-Fw (5′– ATCGGATC

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Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant properties	Reference
Agrobacterium tumefaciens		
UBAPF2	Plasmid free, Rif^r	Hynes *et al.* (1985)
GMI9320	C58 cured of its native plasmids. Rif^r	Rosenberg & Huguet (1984)
UBAPF2G	Tn*5*-M8S labeled derivative of UBAPF2 (Gm^r)	Torres Tejerizo *et al.* (2010)
UIA143	*recA*, pTi-cured derivative of C58, Erythromycin resistant	Farrand *et al.* (1989)
NTL4 (pZLR4)	NT1 derivative carrying a plasmid that harbors *traR* and a *traG*::*lacZ* reporter fusion from pTiC58, independently cloned into the broad-host-range vector pBBR1MCS5	Cha *et al.* (1998)
Rhizobium sp.		
LPU83	Sm^r	Del Papa *et al.* (1999)
LPU83-13	LPU83 derivative with Tn5-B10 inserted in pLPU83a	Torres Tejerizo *et al.* (2010)
LPU83-*traR*	Derivative of LPU83 *traR*::pK18mob	This work
Rhizobium etli		
CFNX89	CFN42 derivative, cured of pRetCFN42d	Brom *et al.* (1992)
CFNX89R	CFNX89 derivative, Rif^r	This work
CFN2001	CFN42 derivative, cured of pRetCFN42a and pRetCFN42d, Rif^r	Leemans *et al.* (1983)
CFNX107	CFN2001 derivative, *recA*	Martínez-Salazar *et al.* (1991)
CFNX661	CFN42 *intA*::pJQ200mp18, Gm^r	Brom *et al.* (2004)
CFNX669	CFN42 derivative, *traI*::pSUPΩSp	Tun-Garrido *et al.* (2003)
CFNX670	CFN42 derivative, *traR*::*loxP*Sp	Tun-Garrido *et al.* (2003)
CFNX671	CFN42 derivative, *cinR*::*loxP*Sp	Tun-Garrido *et al.* (2003)
CFN42-Sp	CFN42 derivative with pRetCFN42a tagged with an mCherry-ΩSp cassette	Torres Tejerizo, in preparation
CFN42-*traA*	CFN42 derivative, *traAa*::pG18mob2	This work
CFN2001-G1	CFN2001 derivative, harboring plasmids pLPU83a-13 and pRetCFN42a::TnGm	This work
CFN2001-G2	CFN2001 derivative, harboring plasmids pLPU83a-13 and pRetCFN42a::*intA*::pJQ200mp18	This work
CFN107-G1	CFN107 derivative, *recA*, harboring plasmids pLPU83a-13 and pRetCFN42a::TnGm	This work
CFN107-G2	CFN107 derivative, *recA*, harboring plasmids pLPU83a-13 and pRetCFN42a::*intA*::pJQ200mp18	This work
Ensifer meliloti		
20MP6	Derivative of *E. mel.* 2011, Sm^r, Tc^r, GFP	Pistorio *et al.* (2002)
Rm11512	Derivative of *E. mel.* 1021 *sinR*::Gm	Marketon *et al.* (2002)
Escherichia coli		
E. coli DH5α	*recA*, Δ*lacU169*, F80d*lac* ZDM15	Bethesda Res. Lab.
E. coli S17–1	*E. coli* 294 RP4-2-Tc::Mu-Km::Tn7 integrated into the chromosome	Simon *et al.* (1983)
Plasmids		
pLPU83a-13	Tn*5*-B10 inserted in pLPU83a	Torres Tejerizo *et al.* (2010)
pK18mob	Km^r, high copy number cloning vector.	Schäfer *et al.* (1994)
pG18mob2	Gm^r, high copy number cloning vector.	Kirchner & Tauch (2003)
pBBR1MCS5	Gm^r, broad-host-range cloning vector	Kovach *et al.* (1995)
TOPO 2.1	Amp^r, high copy number cloning vector	Invitrogen
pLPU83a-13	Tn*5*-B10 inserted in pLPU83a	Torres Tejerizo *et al.* (2010)
pRK2013	Km^r; helper plasmid in triparental matings	Figurski & Helinski (1979)
pK-traR	Derivative of pK18mob with an internal fragment of *traR* of pLPU83a	This work
pG-traA	Derivative of pG18mob2 with an internal fragment of *traA* of pRetCFN42a	This work
pBBR1MCS5::*traR*	Derivative of pBBR1MCS5 with a full copy of *traR* of pLPU83a under control of the promoter Lac	This work
pBBR1MCS5::ori83a	Derivative of pBBR1MCS5 with the intergenic region of *traA-traC* of pLPU83a	This work

GATTTCGTCGT –3′) and traR-Rv (5′– AGACGAAAGTT GGACCTGGA –3′), with *Pfu* polymerase. This fragment was cloned in the SmaI site of pK18mob (a suicide vector in rhizobia) and sequenced to check its fidelity. The resulting plasmid, pK-traR, was introduced into LPU83 by conjugation from *E. coli* S17-1. pK-traR is unable to replicate in the rhizobial background; thus, the selected transconjugants are those where the plasmid containing the fragment of *traR* recombines with the host *traR* gene, generating two incomplete copies of the gene, interrupted

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by the vector. The insertion was checked by PCR with primers traR-Fw-out (5′– GGGACGGGAGGAGCAG –3′) and M13-Fw (5′– GTTTTCCCAGTCACGAC –3′).

Construction of pBBR1MCS5::*traR*

The complete wild-type *traR* gene of pLPU83a was obtained using primers traR-Fw-out (5′– GGGACGG-GAGGAGCAG –3′) and traR-Rv-out (5′– CCGGTGACC-GAGACGA –3′) amplifying an 876-bp fragment with *Pfu*. This fragment was cloned in the SmaI site of pBBR1MCS5 (this vector is able to replicate in rhizobia). The accuracy of the sequence of the resulting plasmid, pBBR1MCS5::*traR*, was checked. Plasmid pBBR1MCS5::*traR* was introduced into the LPU83 *traR* mutant by conjugation, using *E. coli* S17-1 as donor, to determine whether it was able to complement the mutant phenotype. Expression of *traR* was under the *lac* promoter of the cloning vector.

Construction of a pRetCFN42a-*traA* mutant

A mutant in *traA* of pRetCFN42a was generated by insertion of a suicide vector, using the same strategy employed to construct the LPU83 *traR* mutant. 278 bp were amplified with primers p42a-traA-Fw (5′– CACTGCGCCA AGATGGA –3′) and p42a-traA-Rv (5′– ACGAAATCCC GCACCAG –3′) to generate an internal fragment of the *traA* gene, using *Pfu* polymerase. This fragment was cloned into the SmaI site of pG18mob2 – a suicide vector in rhizobia – and sequenced to check the accuracy of the cloning procedure. The resulting plasmid, pG-traA, was introduced into CFN42 by conjugation, generating two incomplete copies of the gene. The insertion was checked by PCR with primers p42a-traAout-Fw (5′– GACGCC CTGCTTTTTGG –3′) and M13-Fw. The mutant was evaluated for CT. In four independent experiments, no transconjugants were detected (conjugation frequency was lower than 10^{-9} transconjugants per donor cell).

Cloning of the *traA-traC* intergenic region of pLPU83a

In QS-regulated rhizobial plasmids, the *oriT* is usually present in the intergenic region between *traA* and *traC*. To clone the putative *oriT* site of pLPU83a, we amplified this region with oligonucleotides 83a-traA-Fw (5′– AAAA TCTAGATGAGCGTTGCTTCCTTGTT –3′) and 83a-traA-Rv (5′– AAAGAATTCCGCAGTGCCGGTAGG –3′), to obtain a 359-bp PCR product using Taq polymerase. This fragment was cloned in TOPO 2.1. The resulting plasmid was restricted with EcoRI, and a 362-bp fragment was obtained and cloned in the EcoRI site of pBBR1MCS5. This construction was called pBBR1MCS5::ori83a.

N-Acyl-HSL detection

Autoinducers were detected through thin-layer chromatography (TLC) analysis with the reporter plasmid pZLR4 as described previously (Shaw *et al.*, 1997; Cha *et al.*, 1998). This plasmid contains the *traR* gene and a *traG::lacZ* reporter fusion from *A. tumefaciens* pTiC58, independently cloned into the broad-host-range vector pBBR1MCS5. Extracts were prepared from 5-mL cultures grown in PY medium. The presence of autoinducer molecules in the tested extracts is detected because they are able to form a complex with TraR and induce expression of the *traG::lacZ* reporter.

Identification of pLPU83a transfer genes

The draft genome sequence of *Rhizobium* sp. LPU83 has been established (Torres Tejerizo *et al.*, 2011b). Subsequently, this draft genome sequence has been improved by performing a paired-end sequencing run on the genome sequencer FLX platform using titanium chemistry. This approach led to the complete Dtr and Mpf gene region of pLPU83a. Annotation was performed by applying the annotation system GenDB (Meyer *et al.*, 2003). The finished sequence of pLPU83a was deposited in the GenBank database under the accession number KF647254.

Sequences used and phylogenetic analyses

For the construction of the relaxase (TraA) and the transcriptional regulator (TraR) phylogenetic trees, the proteins were aligned with the module of CLUSTAL implemented in MEGA5 (Tamura *et al.*, 2011). The models of protein evolution for our sequences were selected with ProtTest 2.4 (Abascal *et al.*, 2005). In both cases, the best model was JTT + I + G + F. Maximum likelihood (ML) trees were inferred under the selected model using PHYML v3.1 (Guindon & Gascuel, 2003). The robustness of the ML topologies was evaluated by bootstrap analysis implemented in PHYML v3.1 (100 replicates). We employed the best of NNIs and SPRs algorithms to search the tree topology and 100 random trees as initial trees.

Many draft sequences are available nowadays; however, some of them are not useful for the analysis of gene clusters; thus, for the data set of the phylogenetic approaches, we included only the homologous proteins of completely sequenced plasmids. The only exception was the TraR of pRi10.

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The accession numbers for the proteins selected for the TraA phylogeny were YP_771015.1 (*R. leguminosarum* bv. *viciae* 3841 pRL8), YP_001965642.1 (*Sinorhizobium meliloti* SM11 pSmeSM11b), YP_002540050.1 (*A. vitis* S4 pTiS4), YP_002551269.1 (*A. radiobacter* K84 pAtK84b), YP_0025 39500.1 (*A. vitis* S4 pAtS4e), YP_770819.1 (*R. leguminosarum* bv. *viciae* 3841 pRL7), NP_396650.2 (*A. fabrum* str. C58 pTiC58), YP_002542670.1 (*A. vitis* S4 pAtS4c), NP_443828.1 (*S. fredii* NGR234 pNGR234a), YP_001314094.1 (*S. medicae* WSM419 pSMED02), YP_004716816 (*S. fredii* GR64 pSfr64a), YP_471748.1 (*R. etli* CFN42 pRetCFN42a), NP_06 6693.1 (*A. rhizogenes* pRi1724), YP_001961052.1 (*A. rhizogenes* pRi2659), YP_002978881.1 (*R. leguminosarum* bv. *trifolii* WSM1325 pR132503), and YP_001312323.1 (*S. medicae* WSM419 pSMED01). For the TraR phylogeny, we used YP_771022.1 (*R. leguminosarum* bv. *viciae* 3841 pRL8), YP_001965652.1 (*S. meliloti* SM11 pSmeSM11b), YP_00254 0056.1 (*A. vitis* S4 pTiS4), YP_002551281.1 (*A. radiobacter* K84 pAtK84b), NP_396657.1 (*A. fabrum* str. C58 pTiC58), YP_002542675.1 (*A. vitis* S4 pAtS4c), NP_443817.1 (*S. fredii* NGR234 pNGR234a), YP_001314089.1 (*S. medicae* WSM 419 pSMED02), YP_004716824.1 (*S. fredii* GR64 pSfr64a), YP_471756.1 (*R. etli* CFN42 pRetCFN42a), NP_066700.1 (*A. rhizogenes* pRi1724), YP_001961058.1 (*A. rhizogenes* pRi2659), YP_002978861 (*R. leguminosarum* bv. *trifolii* WSM1325 pR132503), and AAA64793.1 (*A. tumefaciens* R10 pTiR10).

The accession numbers of the sequences used in the synteny analysis were as follows: AM236082.1 (pRL8), EF066650.1 (pSmeSM11b), NC_007762.1 (pRetCFN42a), (pNGR234a), NC_003065.3 (pTiC58), and NC_011982.1 (pTiS4).

Results

Phylogenetic analyses of the CT region of pLPU83a

Ding and Hynes classified the rhizobial plasmids into three different classes (I, II, and III) according to a phylogenetic analysis of the *traA*-encoded conjugative relaxases (Ding & Hynes, 2009). This classification correlates with the CT regulation of the plasmids – that is, group I contains plasmids regulated by QS; group II plasmids are regulated by *rctA* repression; and group III is formed by plasmids that have a Dtr system but no Mpf. These plasmids are mobilizable, but it is still unclear whether this mobilization is due to *in trans* effectors or to cointegration with a conjugative plasmid. A fourth group was recently described, where two different genes were identified as CT regulators: *rptA* in *Ensifer (Sinorhizobium) meliloti* LPU88 and *trbR* in *R. leguminosarum* bv. *viciae* VF39SM (Giusti *et al.*, 2012; Ding *et al.*, 2013; Pistorio *et al.*, 2013). In order to elucidate to which group pLPU83a belongs, we performed phylogenetic analyses of its CT relaxase TraA, and of the CT regulator TraR.

The draft genome sequence of *Rhizobium* sp. LPU83 (Torres Tejerizo *et al.*, 2011b) was used to identify the relaxase as well as the rest of Dtr and Mpf genes. Its analysis revealed a region involved in CT, located on plasmid pLPU83a. It is worth mentioning that the nomenclature of rhizobial plasmids is based on the genus, species, strain name, and size; the plasmids are labeled in alphabetical order, from smallest to biggest (Casse *et al.*, 1979). Thirty-two open-reading frames were predicted in a contig of *c.* 34 kbp assigned to pLPU83a. These ORFs showed a high degree of similarity to known Dtr and Mpf genes of other rhizobial strains. The highest degree of similarity was found to the genes from pRL8 of *R. leguminosarum* bv. *viciae* (Young *et al.*, 2006) (more than 90% of identity for each protein) and pSmeSM11b of *E. meliloti* SM11 (Stiens *et al.*, 2007).

Phylogenetic analyses were conducted by the ML method. The results showed that the relaxase of pLPU83a is closely related to those of group I plasmids (not shown). Moreover, the topology of group I seemed to include different subgroups. To get a sharper definition, another phylogenetic analysis was performed, using only the relaxases of plasmids that belong to group I. With this analysis (Fig. 1a), we could define three subgroups, which we designated as I-A, I-B, and I-C. Additionally, we performed a phylogenetic analysis of the global regulator of CT, TraR, to determine whether the phylogenies for TraR and the relaxase are congruent. Orthologs of TraR from other plasmids were chosen, and a ML phylogeny was calculated (Fig. 1b). The results clearly showed three clusters of regulators, similar to those observed with the relaxase phylogeny, except for the TraR from pR132503. Subgroup I-A includes TraR from *Agrobacterium* plasmids, whose CT regulation operates at two hierarchical levels. In the second clade (I-B), the TraR proteins of pRetCFN42a, pNGR234a and pSfr64a group together; the CT of these plasmids depends on the *traI* gene localized next to the *trb* genes (Mpf). In the third group (I-C), pLPU83a clusters with TraR from plasmids pRL8 and pSmeSM11b. Interestingly, these plasmids lack a *traI* gene (Fig. 2a), and their conjugative properties have not been clearly defined. pRL8 has been transferred at low frequency from the wild-type strain (Johnston *et al.*, 1982), but it is not clear whether it is able to transfer from another host genomic background. Transfer of pSmeSM11b has not been achieved under laboratory conditions (discussed below) (Stiens *et al.*, 2007). These results suggest that group I-C plasmids could feature novel regulatory properties. Hence, we employed

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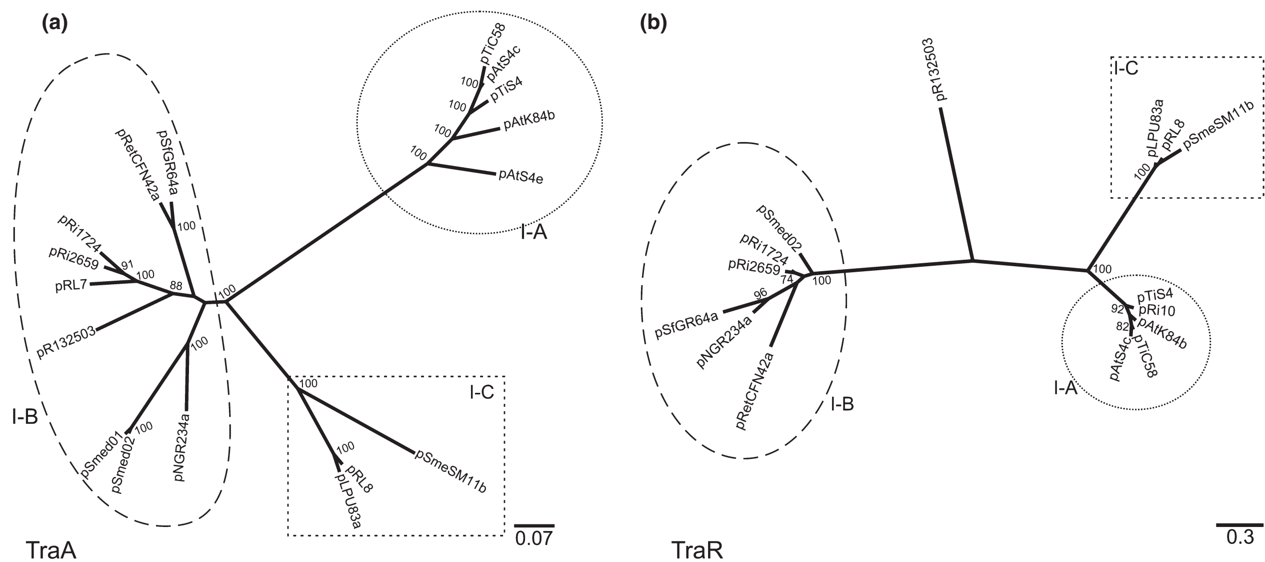

Fig. 1. Phylogenetic analysis of rhizobial group I. Phylogenetic trees based on (a) TraA, and (b) TraR proteins encoded in plasmids that belong to rhizobial group I according to Ding & Hynes (2009). Analyses were conducted by means of the ML method. The accession numbers are mentioned in 'Materials and methods'. Bootstrap values higher that 70 are shown at the nodes. Bar indicates substitution/site.

pLPU83a as a model to determine how different genomic backgrounds may modulate CT.

Structural organization of the CT region of pLPU83a

A comparative analysis of the Dtr and Mpf regions of pLPU83a and representative clusters of plasmids belonging to the same subgroup as defined above was performed. Figure 2a shows a remarkable synteny of the Dtr and Mpf regions of pLPU83a with the transfer regions from the other two related plasmids (pRL8 and pSmeSM11b) and also with the QS-regulated plasmids from group I-B. The most relevant differences were the lack of a *traI* gene and an inversion of the *traR* and *traM* genes. Plasmids pTiC58 and pTiS4 of group I-A also show a high degree of synteny; however, the localization of *traR and traM* differs in that they are closer to the Dtr region, at a distance of *c.* 100–150 kbp from the Mpf genes, while the distance between Dtr and Mpf genes in the other plasmids is between 7 and 1.5 kbp. Orthologs of *intA*, previously described as necessary for recombination and CT of the symbiotic plasmid of *R. etli* CFN42 (Brom *et al.*, 2004), are also present in pLPU83a, pRL8, and pSmeSM11b.

TraR-mediated induction of CT has been associated with the presence of *tra* boxes, which are usually located upstream of *traA*, *repABC,* and *traI* (Li & Farrand, 2000). In pLPU83a, we found *tra* boxes upstream of *trbB* and in the *traA*-*traC* intergenic region. An alignment performed with other boxes (Fig. 2b) showed that they were strongly conserved between plasmids from groups I-A and I-C, and not very similar to those from group I-B plasmids. This relationship is consistent with the phylogenetic distances of corresponding TraR regulators. In group I-B, the *tra* boxes located adjacent to *traI* were conserved, but to detect a *tra* box in the *traA*-*traC* intergenic region, we had to allow mismatches, because it is much less conserved and presents no palindrome (Fig. 2c).

TraR is required for CT of pLPU83a from its native host

Despite the absence of a *traI* gene in pLPU83a, the plasmid is able to transfer from its native host *Rhizobium* sp. LPU83 and from *E. meliloti* 20MP6 (Torres Tejerizo *et al.*, 2010). Although a *traR* gene is present, the absence of *traI* raises the question whether this *traR* is functional or not. To test this, we constructed a derivative mutated in *traR*, as described in 'Materials and methods'. The *traR* mutant lost its ability to transfer. Complementation of the mutant with a functional copy of the *traR* gene restored its CT. These results confirm that the *traR* gene encoded on pLPU83a is functional. However, identification of the inducer-activating TraR is an unresolved issue (see below).

Previously, it has been shown that *traR* gene products involved in CT of rhizobial plasmids regulate the activation of *traI* and, thus, induce the production of different AHLs. Mutations in the *traR* genes of those plasmids that have a corresponding *traI* gene abolish the production of some AHLs (Wilkinson *et al.*, 2002; Tun-Garrido *et al.*,

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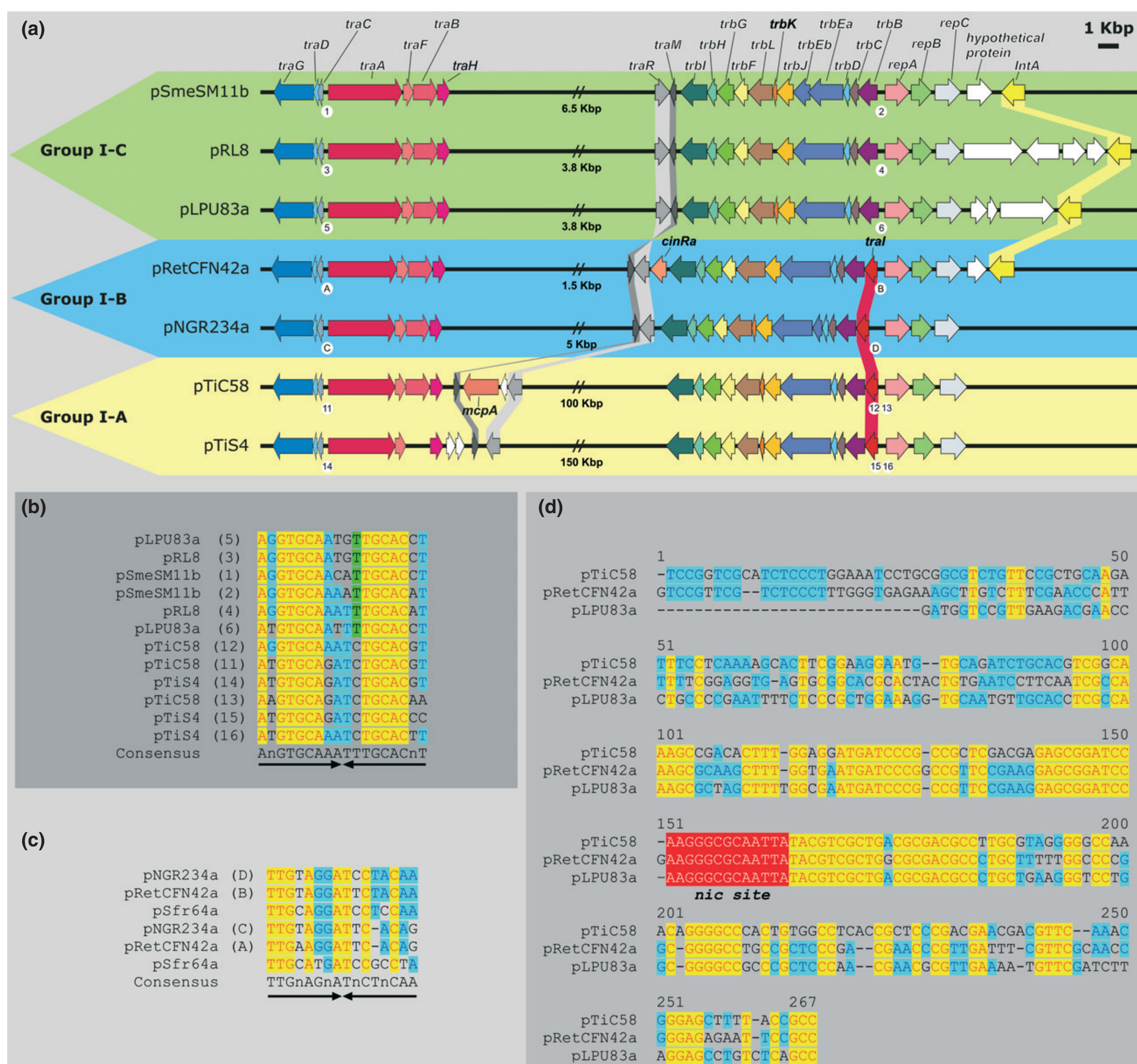

Fig. 2. Structural organization of rhizobial group I transfer regions. (a) Genetic organization of the Dtr and Mpf genes presents in pLPU83a, compared with clusters present in other rhizobial group I plasmids. Orthologs are marked with the same color. Important traits are highlighted between plasmids (*traR, traM, traI,* and *intA*). The black numbers inside white circles indicate a *tra* box. (b) Sequence alignment of *tra* boxes of groups I-C and I-A, the numbers between parentheses correspond to the number of the *tra* box, as labeled in (a). (c) Alignment of *tra* boxes of group I-B. The arrows in (b) and (c) show the palindromes in the consensus sequence. (d) Alignment of the intergenic region between *traA* and *traC* of one representative plasmid of each group. The predicted *oriT*-nick site is indicated. Color key in (b, c, and d): red or yellow, invariant nucleotides; blue on light blue, strongly conserved, black on green, block of similar nucleotides.

2003). To assess whether we could find such a phenotype in LPU83, we prepared extracts from the wild-type strain and the *traR* mutant and examined their AHL patterns. We did not observe any changes in the patterns of AHLs nor in the intensity of the spots in an *A. tumefaciens* NTL4 overlay detection assay (not shown).

CT of pLPU83a from an *E. meliloti* donor also depends on the presence of the pLPU83a encoded TraR

Plasmid pSymA of *E. meliloti* 1021 possesses all the elements needed for CT (*traA1*, *traCDG* and *virB* operon) (Blanca-Ordóñez *et al.*, 2010). It belongs to group II

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plasmids, according to the classification proposed by Ding & Hynes (Ding & Hynes, 2009), and its CT is repressed by RctA (Pérez-Mendoza *et al.*, 2005). Strain SmA818 [a pSymA-cured derivative of *E. meliloti* (Oresnik *et al.*, 2000)] lacks all these elements; it only has an *oriT* and a *traA2* gene, localized on pSymB. Previously, we showed that pLPU83a is able to perform CT from *E. meliloti* SmA818 (Torres Tejerizo *et al.*, 2010), suggesting that the genes allowing CT are those present in pLPU83a.

To determine which elements are involved in the transfer of pLPU83a from *E. meliloti*, we introduced the pLPU83a derivative with the mutation in *traR* (see 'Materials and methods') into *E. meliloti* and tested it for CT. The results showed that the *traR* mutant was unable to perform CT (Table 2). The inability to get transconjugants from *E. meliloti* clearly demonstrates that *traR* is involved in the activation of the CT machinery. In *E. meliloti*, the only AHL-synthase gene (*sinI*) forms part of the *sinR-sinI* operon (Marketon *et al.*, 2002; Gao *et al.*, 2005). To elucidate whether the AHLs synthesized by SinI are responsible for TraR activation, we introduced pLPU83a into the *E. meliloti sinR* mutant Rm11512 (which is unable to produce AHL). Unexpectedly, CT frequency was similar to that obtained from the wild-type donor (Table 2). This result indicates that *E. meliloti* AHLs are not involved in the activation of the TraR from pLPU83a and suggests that it may be activated in an AHLs-independent manner.

CT of pLPU83a from *R. etli* CFN42 depends on the transfer machinery encoded in pRetCFN42a

As mentioned in the Introduction, the chromosome of *Rhizobium* sp. LPU83 is more closely related to *R. etli* than to *E. meliloti* (Eardly *et al.*, 1992; Del Papa *et al.*, 1999; Torres Tejerizo *et al.*, 2011b). Plasmids pRetCFN42a and pRetCFN42d (pSym) from *R. etli* CFN42 harbor gene clusters involved in CT. The CT regulation of these plasmids has been described previously (Tun-Garrido *et al.*, 2003; Brom *et al.*, 2004; Pérez-Mendoza *et al.*, 2005; Sepúlveda *et al.*, 2008). Due to the relationship among the chromosomal sequences of *Rhizobium* sp. LPU83 and *R. etli* CFN42, we evaluated the CT of pLPU83a from *R. etli* CFN42 and its plasmid-cured derivatives. Plasmid pLPU83a was able to transfer from wild-type *R. etli* CFN42, at a frequency *c.* 100-fold higher than from its own genomic background (Table 2). A pRetCFN42d-cured derivative of CFN42 (CFNX89)-harboring pLPU83a as donor strain showed a CT frequency similar to that from the wild-type *R. etli* strain; however, when a derivative lacking both pRetCFN42a and pRetCFN42d (CFN2001) was used as donor for pLPU83a, no transconjugants were obtained (Table 2). These results indicate that pRetCFN42a is required for CT of pLPU83a from the *R. etli* genomic background. We propose three possible explanations for this behavior: (A) pLPU83a and pRetCFN42a form a cointegrate which allows CT of both plasmids, (B) regulators encoded on pRetCFN42a are able to activate the CT machinery of pLPU83a, or (C) the CT machinery of pRetCFN42a recognizes the *oriT* from pLPU83a and initiates transfer of the plasmid. Accordingly, pLPU83 would be mobilized by pRetCFN42a.

(A) Transfer of pLPU83a does not depend on cointegration with pRetCFN42a

In *R. etli* CFN42, pRetCFN42a and pRetCFN42d are able to cointegrate, either through RecA-dependent homologous recombination or through IntA-mediated site-specific recombination between attachment sites *attA* and *attD* (Brom *et al.*, 2004). In pLPU83a, we found an attachment site similar to *attA* from pRetCFN42a. To test whether

Table 2. Conjugation frequencies of pLPU83a from different genomic backgrounds*

Donor strains	Relevant phenotype: Background	Relevant phenotype: Plasmid	Transfer frequency[†]
1. *Rhizobium* sp. LPU83 (pLPU83a-13)	wt	wt	$2.77 \pm 5.41 \times 10^{-06}$
2. *E. mel.* 20MP6 (pLPU83a-13)	wt	wt	$4.85 \pm 3.90 \times 10^{-07}$
3. *E. mel.* Rm11512 (pLPU83a-13)	*sinR* (lacks AHLs)	wt	$6.61 \pm 5.69 \times 10^{-07}$
4. *E. mel.* 20MP6 (pLPU83a-*traR*)	wt	*traR* mutant	n.d.[‡]
5. *R. etli* CFN42-Sp (pLPU83a-13)	wt	wt	$0.91 \pm 0.77 \times 10^{-03}$
6. *R. etli* CFNX89R (pLPU83a-13)	pRetCFN42d^{-}	wt	$7.61 \pm 6.08 \times 10^{-03}$
7. *R. etli* CFN2001 (pLPU83a-13)	pRetCFN42a^{-}, pRetCFN42d^{-}	wt	n.d.[‡]
8. *A.t.* GMI9023 (pLPU83a-13)	Plasmid free	wt	n.d.[‡]

*All crosses were repeated at least three times. *Agrobacterium tumefaciens* strain UIA143 was used as recipient in crosses 1, 6 and 7; *A. tumefaciens* UBAPF2G was used as recipient in crosses 2, 4, 5 and 8; and *A. tumefaciens* GMI9320 was used in cross 3.

[†]Conjugation frequencies are expressed as transconjugants per donor cells.

[‡]n.d., not detectable (below 1×10^{-9}).

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cointegration events are required for CT of pLPU83a from the *R. etli* background, we introduced pLPU83a into a *recA* and/or *intA* mutant derivative of CFN42 and tested the CT of pLPU83a and the cotransfer of pRetCFN42a (Table 3). The results showed that the mutations in *recA* and/or *intA* did not significantly alter the CT frequency of pLPU83a, suggesting that cointegration does not contribute to pLPU83a transfer from the *R. etli* genomic background. Supporting this is the fact that we never detected cointegration of the plasmids when visualized on Eckhardt-type gels (not shown). Cotransfer of pRetCFN42a was always higher than 45%.

(B) Regulatory signals of *R. etli* activate the CT machinery of pLPU83a at low levels

To determine whether the regulatory system of pRetCFN42a participates in activation of pLPU83a CT, we used *traR*, *traI,* and *cinR* mutants, which are unable to activate their own Dtr and, thus, transfer of pRetCFN42a (Tun-Garrido *et al.*, 2003). We introduced pLPU83a into *R. etli* harboring these mutant pRetCFN42a derivatives and tested them for CT of pLPU83a. We were unable to detect CT of pLPU83a (Table 4: lines 2–4). This shows that the QS regulators of pRetCFN42a are needed for pLPU83a CT. To elucidate whether the Dtr and Mpf genes of pLPU83a are directly activated by the *R. etli* QS regulators, we employed a *traA* mutant of pRetCFN42a. This mutant can activate the transcription of the Dtr and Mpf genes, through TraI, TraR, and CinR, but is unable to process the DNA for CT (pRetCFN42a-*traA* shows no CT, see 'Materials and methods'). Plasmid pLPU83a was still able to transfer from this background, although at reduced levels compared with the wild-type. These results indicate that the signals of pRetCFN42a are able to activate the pLPU83a machinery, albeit at a low level. Also, we tested whether the pLPU83a encoded *traR* participated in promoting CT of the plasmid in the *R. etli* background. No significant difference in transfer frequency was observed between the wild-type and the *traR* mutant, either in the wild-type (Table 4: lines 1 and 5) or in the *traA* mutant (Table 4: lines 6 and 7), indicating that the *traR* of pLPU83a has a minor role in this background. The highest CT frequency was obtained with the wild-type *R. etli* donor for pLPU83a, suggesting that the machinery of pRetCFN42a also participates directly in processing the *oriT* of pLPU83a.

(C) CT of pLPU83a from a *R. etli* donor is mainly achieved through mobilization

To analyze whether pRetCFN42a could promote mobilization of pLPU83a, we compared the intergenic sequences between *traA* and *traC*, where the *oriT* is usually localized (Tun-Garrido *et al.*, 2003). We detected 63.3% identity between pRetCFN42a and pLPU83a. The previously proposed *nic*-site is highly conserved and present in all the sequences (Fig. 2d). To determine whether the *oriT* of pLPU83a could be processed by the pRetCFN42a machinery, we cloned a 359-bp sequence containing the pLPU83a *oriT* into the pBBR1MCS5 vector, which is able to replicate in rhizobia, to obtain pBBR1MCS5::ori83a. We introduced pBBR1MCS5::ori83a into LPU83 and CFN42. As a control, we used plasmid pCT11, which contains the *oriT* of pRetCFN42a (Tun-Garrido *et al.*, 2003). Conjugations from LPU83 were performed, and transconjugants were detected at a frequency of $1.16 \pm 1.11 \times 10^{-8}$ for pBBR1MCS5::ori83a and $3.05 \pm 4.58 \times 10^{-8}$ for pCT11, demonstrating that the cloned fragment harbors the *oriT* of pLPU83a and that the *oriT* of pRetCFN42a can be processed by the relaxase encoded in pLPU83a. We detected a much higher CT frequency ($7.05 \pm 5.47 \times 10^{-3}$) for pBBR1MCS5::ori83a from CFN42, confirming that mobilization is the main pathway contributing to CT of pLPU83a from the genomic background of CFN42.

Discussion

The presence of plasmids in microorganisms from different environments and their role in bacterial evolution have been exhibited in several works (reviewed by Sorensen *et al.*, 2005; Heuer & Smalla, 2012). Numerous plasmids are able to transfer by conjugation, and they may

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Table 3. Conjugation frequencies of pLPU83a from strains with mutations in *R. etli* CFN42 recombination genes*

Donor strains	Relevant phenotype	Transfer frequency[†]	Cotransfer[‡] %
R. etli CFN2001-G1	*recA*⁺, pLPU83a-13; pRetCFN42a::TnGm	$2.79 \pm 2.43 \times 10^{-03}$	44.9 ± 9.5
R. etli CFN2001-G2	*recA*⁺, pLPU83a-13; pRetCFN42a::*intA*::pJQ200mp18	$1.38 \pm 0.18 \times 10^{-04}$	54.7 ± 16.0
R. etli CFN107-G1	*recA*, pLPU83a-13; pRetCFN42a::TnGm	$2.89 \pm 0.93 \times 10^{-03}$	74.4 ± 5.9
R. etli CFN107-G2	*recA*, pLPU83a-13; pRetCFN42a::*intA*::pJQ200mp18	$1.91 \pm 2.84 \times 10^{-03}$	63.2 ± 7.7

*All crosses were repeated at least three times. *Agrobacterium tumefaciens* strain UIA143 was used as recipient.
[†]Conjugation frequencies of pLPU83a are expressed as transconjugants per donor cells.
[‡]Cotransfer of pRetCFN42a.

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Table 4. CT of wild-type and the *traR* mutant of pLPU83a from pRetCFN42a derivatives with mutations in QS regulators*

Donor strains	Relevant phenotype		Transfer frequency†
	Background	Plasmid	
1. *R. etli* CFN42-Sp (pLPU83a-13)	wt	wt	$0.91 \pm 0.77 \times 10^{-03}$
2. *R. etli* CFN669 (pLPU83a-13)	pRetCFN42a-*traI*::ΩSp	wt	n.d.‡
3. *R. etli* CFN670 (pLPU83a-13)	pRetCFN42a-*traR*::*loxP*Sp	wt	n.d.‡
4. *R. etli* CFN671 (pLPU83a-13)	pRetCFN42a-*cinR*::*loxP*Sp	wt	n.d.‡
5. *R. etli* CFN42-Sp (pLPU83a-*traR*)	wt	*traR*::pK18mob	$1.18 \pm 1.03 \times 10^{-03}$
6. *R. etli* CFN42-*traA* (pLPU83a-13)	pRetCFN42a-*traA*::pG18mob2	wt	$7.88 \pm 2.97 \times 10^{-05}$
7. *R. etli* CFN42-*traA* (pLPU83a-*traR*)	pRetCFN42a-*traA*::pG18mob2	*traR*::pK18mob	$1.64 \pm 0.31 \times 10^{-06}$

*All crosses were repeated at least three times. *Agrobacterium tumefaciens* strain UBAPF2G was used as recipient in crosses 1–5. *Agrobacterium tumefaciens* GMI9320 was used in crosses 6 and 7.
†Conjugation frequency are expressed as transconjugants per receptor cells.
‡n.d., not detectable (below 1×10^{-9}).

perform this process by themselves (conjugative or self-transmissible) or by the CT machinery of another plasmid (mobilizable). Usually, these features have been employed to allocate plasmids into different groups. In this work, we described a plasmid that is able to switch among these groups. Previously, we analyzed the conjugation properties of pLPU83a (Torres Tejerizo *et al.*, 2010). Here, we extended the characterization of the genes involved in CT, to better understand the mechanisms that regulate CT from the different genomic backgrounds. With this purpose, we studied pLPU83a as a model of a new group of rhizobial plasmids, by means of phylogenetic and phenomenological experiments.

In the genome sequence of *Rhizobium* sp. LPU83, a complete Dtr/Mpf cluster was found in pLPU83a, similar to that of pRL8, pSmeSM11b, and other rhizobial plasmids. Nevertheless, some striking differences were observed, such as the lack of a *traI* gene, and a change in the relative position of *traR* and *traM* regulatory genes. According to the phylogenetic groups proposed by Ding & Hynes (2009) and Ding *et al.* (2013), pLPU83a, pRL8, and pSmeSM11b should be included in group I of rhizobial plasmids. However, the differences observed between the phylogenetic and synteny analysis of pLPU83a, pRL8, and pSmeSM11b in comparison with pTiC58, pTiS4, pNGR234a, and pRetCFN42a allowed us to define three subgroups of type I plasmids: group I-A, formed by plasmids from *Agrobacterium* species, where *traR* and *traM* are near to the Dtr locus; group I-B which includes plasmids from *S. fredii* and *R. etli,* that have a *traI* gene within the Mpf cluster and *traR* is next to *trbB*; and group I-C, with plasmids that lack a *traI* gene, and *traM* is localized next to *trbI* which is the case of pLPU83a. Furthermore, these groups are supported by the phylogenetic analyses of TraR and TraA.

In plasmids where CT is regulated by QS, TraR and TraI are the LuxR-LuxI-type regulators that activate the expression of the target genes (i.e. genes involved in CT). We evaluated whether the *traR* gene localized on pLPU83a is involved in CT of the plasmid. The results showed that the pLPU83a-encoded TraR is necessary for CT of the plasmid from LPU83 or *E. meliloti* donors, indicating that in these genomic backgrounds, the conjugative machinery of pLPU83a is functional. Previously, Marketon and González (2002) described two QS systems in *E. meliloti* 1021, one encoded by the *sinR-sinI* locus (long-chain AHLs) and another by the '*mel system*' (short-chain AHLs). However, later on, Gao *et al.* (2005) demonstrated that only long-chain AHLs are found in *E. meliloti* 1021 encoded by *sinI* and that the '*mel system*' does not lead to production of AHLs, but to unidentified molecular signals that can be detected by the reporter strain. A mutant in *sinR* does not produce any AHLs; nevertheless, pLPU83a was able to transfer from this genomic background. Recently, there have been reports of LuxR orthologs that do not have a cognate LuxI protein. These 'solos' or orphan *luxR* genes are widespread in *Proteobacteria* (Patankar & Gonzalez, 2009; Subramoni & Venturi, 2009). The activation of some of these LuxR genes has been shown to be independent of AHLs (Fernández-Pinar *et al.*, 2011; Charoenpanich *et al.*, 2013). For example, *Pseudomonas fluorescens* PsoR (LuxR-like regulator) responds to some plant compounds (Subramoni *et al.*, 2011); α-pyrones act as signaling molecules in *Photorhabdus luminescens* by means of an orphan LuxR gene (Brachmann *et al.*, 2013). Furthermore, addition of AHL-containing ethyl acetate extracts from *E. meliloti* or LPU83 cultures to *A. tumefaciens* (pLPU83a) did not lead to CT of pLPU83a (not shown). Correlation of the results obtained in this work with the participation of non-AHL molecules as QS signaling molecules evokes the possibility that the TraR encoded in pLPU83a responds to new signals, different from AHLs. The results suggest that these signals are absent in *A. tumefaciens*, but present in LPU83 and *E. meliloti,* and therefore responsible for CT of pLPU83a from these hosts. Previously, it was

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shown that an *A. tumefaciens* donor containing pLPU83a was unable to transfer pLPU83a to an *E. meliloti* recipient strain (Torres Tejerizo *et al.*, 2010); however, transfer is accomplished from an *E. meliloti* donor, suggesting that the signal allowing transfer is not diffussible. Future work will focus on elucidating the chemical nature of those signals. The panorama is different in the *R. etli* genomic background, where the pLPU83a-encoded TraR is not necessary for its CT; instead, pLPU83a CT from CFN42 depended on the machinery encoded in pRetCFN42a, as pRetCFN42a mutant derivatives unable to activate their Mpf/Dtr genes (*traR*, *cinR,* and *traI* mutants) were unable to transfer pLPU83a. We rejected the hypothesis that promotion of transfer was achieved through cointegration among the plasmids. The combination of a pRetCFN42a with a mutation in *traA*, with the pLPU83a containing a mutation in *traR*, indicated that the CT machinery of pLPU83a may be turned on by the pLPU83a *traR*, but the CT frequency is much lower than from the wild-type *R. etli* donor. Finally, we evaluated the possibility of CT induction through mobilization. Mobilizable plasmids only need an *oriT in cis* and an active conjugative machinery for the transfer process *in trans*. Our results confirmed that the relaxase of pRetCFN42a is able to process the pLPU83a *oriT*, allowing CT of the plasmid at frequencies comparable to those of the endogenous pRetCFN42a, establishing mobilization as the main mechanism for CT induction of pLPU83a in the *R. etli* background.

In the new group of rhizobial plasmids proposed in this work, pLPU83a and pRL8 are able to perform CT from their own genomic backgrounds, but attempts to transfer pSmeSM11b have not been successful (Stiens *et al.*, 2007). This may be due to the experimental conditions used, or to inactivation of *trbE*, whose sequence seems to be interrupted (Fig. 2). Another possible explanation is that plasmid pSmeSM11b was acquired from another strain to *E. meliloti* SM11 and that in this new genomic environment, it is unable to perform CT.

Our results indicate that the CT phenotype (conjugative or mobilizable) could depend on the interaction among elements from different replicons, and not only on the plasmid itself; therefore, the wide-spread assumption that a plasmid has a defined CT behavior should be carefully evaluated from several related hosts. A plasmid may perform CT using regulatory elements carried on the plasmid, interacting with elements from other replicons; alternatively, in another genomic background, the same plasmid may achieve CT relying exclusively on the intrinsic machinery. Elucidation of the mechanisms that differentially regulate plasmid CT in different genomic backgrounds will be helpful to understand the boundaries of plasmid exchange in bacteria.

From an ecological point of view, our data suggest that the population of a niche serves not only as recipient for plasmid-encoded genetic information, but may have an active role in the determination of the future distribution of this information. One interesting issue is that in broad-host-range IncP-1 plasmids, replication, partitioning, and transfer are coordinately regulated in a negative manner. These plasmids are considered the most promiscuous (Heuer & Smalla, 2012). Nevertheless, it cannot be discarded that some, not yet described, genomic backgrounds may also control Inc-P1-type plasmid transfer. On the other hand, transfer of most rhizobial plasmids seems to be positively regulated. It will be interesting to elucidate whether the regulatory pattern is related to the host's influence on plasmid dissemination.

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