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Identification of a transmissible plasmid from an Argentine *Sinorhizobium meliloti* strain which can be mobilised by conjugative helper functions of the European strain *S. meliloti* GR4

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Abstract

We describe in this work the identification and the conjugal properties of two cryptic plasmids present in the strain *Sinorhizobium meliloti* LPU88 isolated from an Argentine soil. One of the plasmids, pSmeLPU88b (22 kb), could be mobilised from different *S. meliloti* strains to other bacteria by conjugation only if the other plasmid, pSmeLPU88a (139 kb), was present. This latter plasmid, however, could not be transferred via conjugation (frequency $<10^{-9}$ transconjugants per recipient) contrasting with the conjugal system from the previously described strain GR4, where one plasmid is mobilisable and a second one (helper) is self-transmissible. Despite the differences between the two systems, the conjugative helper functions present in the cryptic plasmids of strain GR4 were active in the mobilisation of plasmid pSmeLPU88b from strain LPU88. Contrasting with this, plasmid pSmeLPU88b was not mobilised by the helper functions of the broad-host-range plasmid RP4. Eckhardt gel analysis showed that none of the plasmids from strain GR4 were excluded in the presence of plasmid pSmeLPU88b suggesting that they all belong to different incompatibility groups for replication. The small plasmid from strain LPU88, pSmeLPU88b, was only able to replicate in members of the Rhizobiaceae family such as *Rhizobium leguminosarum*, *Rhizobium tropici* and *Agrobacterium tumefaciens*, but not in *Escherichia coli* or *Pseudomonas fluorescens*. The observation suggests that most likely plasmid pSmeLPU88b was not received from a phylogenetically distant bacterium.

Keywords: *Sinorhizobium meliloti*; Plasmid; Conjugation

1. Introduction

Bacteria belonging to the genera *Rhizobium*, *Sinorhizobium*, *Azorhizobium*, and *Bradyrhizobium* grow in the soil in free-living conditions, and in symbiosis associated with the root of legumes as nitrogen-fixing organisms. The nitrogen-fixing capacity of the rhizobium–legume symbioses enables their use for the introduction of nitrogen into agricultural soils avoiding the massive use of chemical fertilisers [1,2]. One aspect that should be considered in inoculation programmes is the potential risk of horizontal gene transfer from the introduced rhizobia to the native population of bacteria. Unfortunately, at present there are no molecular methods to detect conjugative plasmids and mobilisation systems in rhizobial strains. Even though gene transfer in soils may occur by natural transformation, transduction or conjugation [3–5], the last is considered the most efficient mechanism [6,7]. Most studies concerning conjugative gene transfer among rhizobia have focused on the qualitative and/or quantitative analysis of the genetic transfer in laboratory experiments, in the field, and also within root nodules [8–10]. However, there are few studies on the molecular characterisation of the rhizobial conjugal transfer systems. For this reason, little molecular information is currently available to approach the development of specific and reliable methods for the detection of transmissible plasmids in rhizobial strains of agricultural interest.

The types of self-transmissible replicons in rhizobia include a variety of cryptic plasmids (approx. 20–500 kb), and also symbiotic (mega)plasmids (approx. 400–1600 kb) [11–14]. In the case of *Sinorhizobium meliloti*, the natural

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symbiont of *Medicago* spp., neither of the symbiotic plasmids (pSym-a and pSym-b) could be spontaneously mobilised under laboratory conditions [10]. The introduction of mobilisation genes (*mob*) of plasmid RP4 into pSym-a, however, allowed its conjugative transfer to *Agrobacterium tumefaciens*. Contrasting with pSym-a and pSym-b, the occurrence of transmissible cryptic plasmids in *S. meliloti* is frequent. Unfortunately, so far only few functions have been associated with the presence of such cryptic plasmids, which have been little characterised [15].

The cryptic plasmids of *S. meliloti* GR4, pRmeGR4a and pRmeGR4b, are the best-characterised non-symbiotic plasmids in rhizobia [16–19]. It has been shown that plasmid pRmeGR4a is self-transmissible and able to mobilise the accompanying plasmid pRmeGR4b. This latter plasmid carries the nodule formation efficiency genes that are involved in the determination of the strain competitiveness for nodulation [20,21]. *S. meliloti* GR4 cosmid clones containing mobilisable DNA inserts from plasmids pRmeGR4a/b have already been identified [22], but their *oriT* sequences are not available yet. In addition, another nine DNA fragments with ability to behave as *oriT* regions were also recognised in strain *S. meliloti* GR4 [22].

The design of molecular tools for the detection of conjugal functions in rhizobial strains needs the isolation and characterisation of new plasmids that represent the diversity of the transfer systems towards the identification of common elements as detection targets. Despite the remarkable diversity of cryptic plasmids reported for *S. meliloti* [23], the plasmids of strain GR4 are the only ones analysed in their conjugal functions. In this paper, we present the isolation and functional characterisation of a binary conjugal system from *S. meliloti* where a small and narrow-host-range cryptic plasmid is mobilisable by a non-transmissible but bigger helper plasmid.

2. Materials and methods

2.1. Bacterial strains and plasmids

The strains and plasmid used in this work are listed in Table 1. *S. meliloti* isolates were obtained in the laboratory using soil samples collected at INTA Castelar (Buenos Aires, Argentina) and *Medicago sativa* cv. CUF101 as trapping plant. *Escherichia coli* strains were grown at 37°C on LB medium [24]. Rhizobia and *A. tumefaciens* were grown at 28°C on TY medium [25] or YEM [26] medium. For the solid media 15 g of agar per liter of medium was added. The final concentrations of antibiotics per milliliter medium were 10 μg gentamicin and 6 μg tetracycline (Tc) for *E. coli*; and 400 μg streptomycin (Sm), 50 μg gentamicin, and 6 μg tetracycline for *S. meliloti*.

2.2. Bacterial matings

Bacterial matings were performed as described by Simon et al. [28]. Briefly, liquid cultures were grown to early exponential phase for donor cells (optical density at 600 nm 0.1–0.2) and late exponential phase for recipient cells. Donor and recipient were mixed in a microcentrifuge tube in a ratio of 1:1. The mating mixture was concentrated by 8 min centrifugation at $640 \times g$. The pellet was finally suspended in 50 μl of the same medium and loaded onto a Millipore filter (0.2 mm pore size). Filter mating

Table 1
Bacterial strains and plasmids used in this work

Strain or plasmid	Description	Source
Bacterial strains		
E. coli S 17-1	*E. coli* 294 RP4-2-Tc::Mu-Km::Tn*7* integrated into the chromosome	[28]
S. meliloti 2011-Sp	Sp^r derivative of strain 2011 Sm^r Nod^+, Fix^+	This work
S. meliloti GR4	Wild-type strain	[41]
S. meliloti GRM6	GR4 cured of plasmid pRmeGR4b	[16]
S. meliloti LPU86	Wild-type isolate from Argentina	This work
S. meliloti LPU88	Wild-type isolate from Argentina	This work
S. meliloti 20MP6	Derivative of *S. meliloti* 2011, Sm^r, Tc^r, GFP	[38]
R. tropici CIAT 899	Wild-type, symbiont of *Phaseolus vulgaris*	P. van Berkum
R. etli CE3	Symbiont of *P. vulgaris*. Sm^r derivative of strain CFN42	E. Martínez
R. leguminosarum bv. *viciae* VF39	Wild-type, symbiont of *Pisum sativum*	U. Priefer
A. tumefaciens UBAPF2	Plasmid-free, Rif^r	[42]
P. fluorescens bv II LP19	Wild-type isolate	G. Favelukes
Plasmids		
pRK2013	RK2 conjugal functions, Km^r	[43]
pSUP1021	pSUP102 carrying a Tn*5* insertion	[31]
pSUP102::Tn*5*-B13	pSUP102 carrying a Tc-mob Tn*5* derivative	[32]
RP4	IncPα plasmid, Km^r Ap^r Tc^r	[44]
pSmeLPU88a	Cryptic plasmid from strain *S. meliloti* LPU88 (139 kb)	This work
pSmeLPU88b	Cryptic plasmid from strain *S. meliloti* LPU88 (22 kb)	This work
pSmeLPU88a::Tn*5*-B13	pSmeLPU88a carrying a Tn*5*-B13	This work
pSmeLPU88b::Tn*5*	pSmeLPU88b carrying a Tn*5*	This work

mixtures were placed on TY agar plates and incubated overnight at 28°C.

2.3. DNA manipulation

Plasmid DNA preparation, restriction enzyme analysis, cloning procedures, and *E. coli* transformation were performed according to previously established techniques [27].

2.4. Plasmid profiles – Eckhardt gels

Cells were grown in TY medium to mid-exponential phase. One hundred microliters of culture were collected in a microcentrifuge tube and mixed with 500 μl 0.3% Sarcosyl in TBE buffer. The cell suspension was centrifuged for 30 s at 14 000 × *g* and the supernatant discarded. The cell pellet was resuspended in 40 μl of loading buffer (10% sucrose, 0.01 mg ml^{-1} ribonuclease A, and 1 mg ml^{-1} lysozyme) and applied to a 0.7% agarose gel containing 1% sodium dodecyl sulfate in TBE buffer. Electrophoresis was run during 6 h at 80 V and 10°C. Plasmid bands were observed under UV illumination after staining of the gel with 0.5–1 μg ml^{-1} ethidium bromide [29,30].

2.5. Tn*5* plasmid tagging for conjugal transfer detection

First, Tn*5* from plasmid pSUP1021 [31] was randomly introduced by conjugation into strain LPU88 selecting for streptomycin and neomycin resistance. The transconjugants were then used en masse as donors in mating with strain 2011-Sp. Transfer of neomycin resistance to the recipient strain 2011-Sp was indicative of conjugal transfer.

2.6. Forced mobilisation of rhizobial plasmids using transposon Tn*5-B13, a* mob*-containing Tn5 derivative*

Rhizobia carrying the plasmid to be mobilised were mutagenised with the transposon Tn*5*-B13 (Tc-*mob*) [32]. The Tn*5*-B13-containing rhizobia were then used en masse as donors in a triparental mating with *S. meliloti* 2011-Sp (recipient strain) and *E. coli* DH5α (pRK2013) that provided the helper plasmid. Transconjugants were first detected according to their expected Tc^r Sm^r phenotype. The presence of mobilised plasmids in the transconjugants from the donor rhizobia was finally evaluated by analysis in Eckhardt gels.

2.7. Oligonucleotide primers and PCR conditions

2.7.1. DNA amplification fingerprints

Total DNA amplification fingerprints were performed using primer MBOREP1 as previously described [33,34]. The sequence of the primer is as follows: 3′-CCG CCG TTG CCG CCG TTG CCG CCG-5′. The deoxyoligonucleotide primers were synthesised by DNAgency (Malvern, PA, USA). Polymerase chain reactions (PCR) of 25 μl contained: 50 mM Tris, pH 8.3; 500 mg ml^{-1} bovine serum albumin (BSA); 3 mM $MgCl_2$; 200 μM dNTPs; 1 U Taq polymerase (Promega); 10 μM primer MBOR-EP1; and 10 μl of template DNA, previously obtained by heating a freshly isolated bacterial colony in 50 μl of distilled water to 100°C for 15 min. The amplifications were carried out in capillary tubes in an Idaho 1605 Air Thermo Cycler (ATC, Idaho Technology). The cycling conditions were as follows: 94°C for 7 min, 30 cycles of 94°C for 10 s, 52°C for 10 s, 72°C for 2 min, and a final extension at 72°C for 2 min. After the reaction, 10 μl of the PCR products were separated in 1% agarose gels containing 0.5–1 μg ml^{-1} ethidium bromide, and photographed using Polaroid 667 film.

2.7.2. Amplification of nptII sequences from Tn*5*

A 400-bp DNA fragment of the Tn*5* neomycin phosphotransferase (*nptII*) gene was PCR-amplified using the following primers: *nptII*-f, 5′-TGG GCA CAA CAG ACA ATC-3′; *nptII*-r, 5′-CCC CTG ATG CTC TTC GT-3′. PCR mixtures of 25 ml contained: 50 mM Tris, pH 8.3; 500 mg ml^{-1} BSA; 3 mM $MgCl_2$; 200 mM deoxynucleoside triphosphates; 1 U Taq polymerase (Promega); 0.5 μM concentration of each primer; and 10 ml of template DNA. Cycling conditions were as follows: 94°C for 15 s, followed by 35 cycles of 94°C for 10 s, 53°C for 10 s, 72°C for 20 s, and 72°C for 1 min. Amplification products were visualised in agar gels with ethidium bromide as described above.

2.8. Plant nodulation assays

M. sativa seeds (alfalfa, cv. CUF101, obtained from the Instituto Nacional de Tecnología Agropecuaria, La Pampa, Argentina) were surface-sterilised for 10 min with commercial bleach 20% v/v (NaClO concentration equivalent to 55 g active Cl_2 l^{-1}) followed by six washes with sterile distilled water. Surface-sterilised seeds were germinated on water-agar (1.5%, w/v). For competition studies 2-day-old seedlings were transferred to γ-irradiated sterilised plastic growth pouches (Mega Minneapolis International, Minneapolis, MN, USA) containing 10 ml of nitrogen-free Jensen mineral solution, pH 6.7 [35]. Three days later, roots were co-inoculated with a mixture of two microbial strains (10^6 cfu per 100 μl inoculant each) by dripping 100 μl of the bacterial suspension onto the root from the tip towards the base. The rhizobia were obtained from exponential-phase YEM cultures. The plants were cultured in a growth chamber at 22°C and a 16-h photoperiod. The CFU contained in the inocula were estimated by plate counts.

Since one of the inoculated rhizobia bears a chromosomal *gfp* (see Table 1), nodule occupancy was observed for 4 weeks by examination of plant roots under a Carl Zeiss-Jena fluorescent microscope either with visible light

or with UV with a filter set for fluorescence. In that case a 470–490-nm excitation filter and a 520–560-nm barrier filter were used. In addition nodules from the same experiments were removed from the roots, surface-sterilised with 30 vol. H_2O_2 for 10 min followed by washing with abundant sterile water, and crushed in 10 μl of sterile isotonic solution. Appropriate dilutions were plated on YEM Petri dishes with or without the appropriate antibiotic.

3. Results

3.1. Isolation of a transmissible cryptic plasmid from the strain S. meliloti LPU88

For the purpose of investigating the presence of transmissible plasmids in local *S. meliloti* strains we constructed a collection of 60 isolates recovered from neutral soils of the central area of Argentina. By analysis of plasmid profiles and MBOREP PCR fingerprints we detected that two isolates recovered from a same soil, LPU86 and LPU88, had indistinguishable DNA fingerprints but different plasmid contents. Isolate LPU88 had a small plasmid, designated pSmeLPU88b (22 kb), that was absent in isolate LPU86 (Fig. 1A). To assess if this plasmid was transmissible we used the strategy previously described by Mercado-Blanco and Olivares [16]. Strain LPU88 bearing the differential plasmid was mutagenised with Tn*5* and used en masse as putative donor of the Tn*5* neomycin resistance to the recipient strain *S. meliloti* 2011-Sp (see Section 2). Several neomycin-resistant 2011-Sp transconjugants were obtained and their plasmid content analysed as shown in the example in lane 1 of Fig. 1B. In addition to the symbiotic megaplasmids, transconjugants presented a small plasmid with a molecular mass of about 28 kb. According to the mobilisation strategy, this observation is compatible with the presence of a Tn*5* insertion in pSmeLPU88b (22 kb) and the subsequent mobilisation of the plasmid to strain *S. meliloti* 2011-Sp. In support of this, an internal sequence from the Tn*5* *nptII* gene could be amplified by PCR using specific primers and template DNA from the neomycin-resistant transconjugants (Fig. 2A). Positive amplification was also obtained when DNA from the 28-kb plasmid was extracted from the agarose gel and used as template for the same PCR reactions (Fig. 2B).

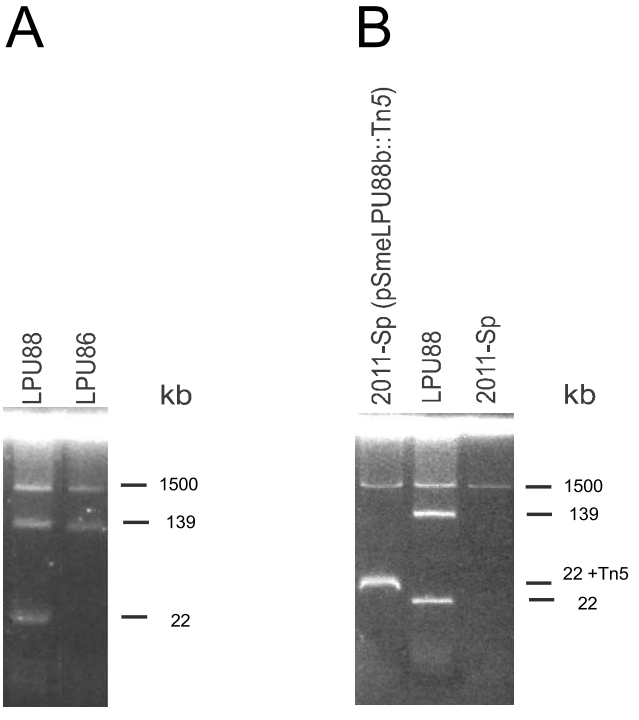

Fig. 1. Plasmid profiles of *S. meliloti* strains in Eckhardt-like gels. A: Agarose gel plasmid profiles of the wild-type strains *S. meliloti* LPU88 and *S. meliloti* LPU86. B: Plasmid profiles of transconjugants obtained in mating *S. meliloti* LPU88::Tn*5* (en masse)×*S. meliloti* 2011-Sp. The size of plasmids was estimated by comparison with previously reported plasmids from strain *S. meliloti* MVII-1 [45].

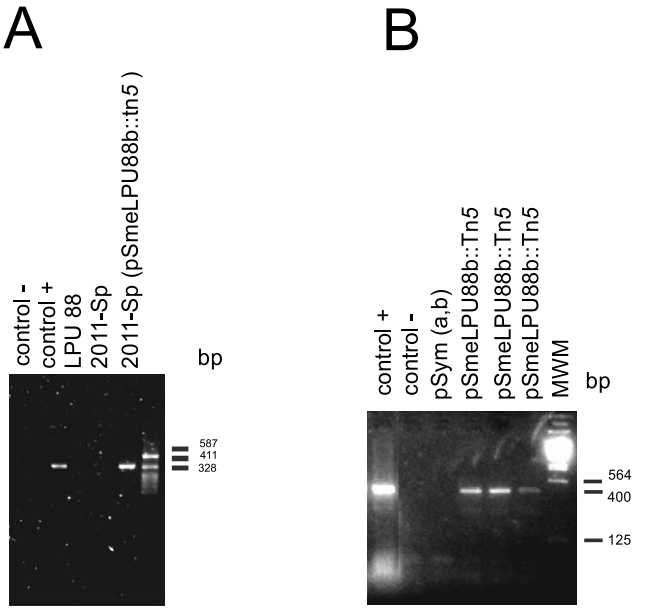

Fig. 2. PCR amplification of *nptII* sequences to assess the presence of Tn*5* in rhizobial strains and plasmid DNA samples. PCR amplifications were carried out using primers *ntpII*-f and *ntpII*-r as indicated in Section 2. A: Amplification using as template total DNA of the indicated strains. B: Amplification using as template plasmid DNA extracted from the region of agarose gels that corresponded to plasmids pSyma/b and the cryptic plasmid mobilised to strain 2011-Sp. Negative controls corresponded to PCR reactions of the material extracted from gel portions where no plasmid material was present.

We analysed over 30 neomycin-resistant transconjugants and no transfer of the 139-kb pSmeLPU88a was detected indicating that either the plasmid was non-transmissible or it was transmissible at very low frequency.

3.2. Mobilisation properties of plasmid pSmeLPU88b

In order to determine whether plasmid pSmeLPU88b was self-transmissible (conjugative) or needed helper func-

tions present *in trans* (mobilisable plasmid), conjugation tests were carried out using strain *S. meliloti* 2011-Sp (pSmeLPU88b::Tn*5*) as donor and *A. tumefaciens* UBAPF2 as recipient strain. Since no transconjugants were obtained (frequency $<10^{-9}$ transconjugants per recipient) we concluded that most likely plasmid pSmeLPU88b was not self-transmissible in the genomic background of strain *S. meliloti* 2011-Sp. As expected plasmid pSmeLPU88b::Tn*5* could be mobilised to different bacteria from the original strain LPU88 (frequency 6×10^{-3} transconjugants per recipient). These results suggest that strain LPU88 carries conjugative helper functions that are not present in strain 2011-Sp.

Since the presence of helper functions *in trans* had already been described for strain *S. meliloti* GR4 [19], we investigated if the conjugal functions required to mobilise plasmid pSmeLPU88b were present in the accompanying plasmid pSmeLPU88a. Upon construction of strain *S. meliloti* 2011-Sp (pSmeLPU88a::Tn*5*-B13, pSmeLPU88b::Tn*5*) the smaller plasmid could be efficiently transferred to *A. tumefaciens* UBAPF2. The collected evidence demonstrated that plasmid pSmeLPU88a was necessary to support the conjugal transfer of plasmid pSmeLPU88b.

3.3. *Functional complementation between the helper functions of* S. meliloti *plasmids from different geographic origins: plasmid pRmeGR4a from the European strain GR4 is able to mobilise plasmid pSmeLPU88b from the local strain LPU88*

During the construction of the genotypes presented above, we observed that the conjugal functions of the widely characterised plasmid RP4 (incPα group) did not promote the mobilisation of plasmid pSmeLPU88a/b, and vice versa. To get further insight into the functional relationship between the helper functions of different *S. meliloti* plasmids, we analysed the ability of plasmids from the European strain GR4 to promote the mobilisation of plasmid pSmeLPU88b. For this purpose we constructed strain *S. meliloti* GR4 (pSmeLPU88b::Tn*5*) which was assessed as donor of the received plasmid to *A. tumefaciens* UBAPF2. Eckhardt gel analysis showed that replication of plasmid pSmeLPU88b was compatible with the presence of plasmids pRmeGR4a and pRmeGR4b. Plasmid pSmeLPU88b was transferred from strain GR4 to strain UBAPF2 though with less efficiency than from strain LPU88. Interestingly, the use of strain GRM6 (a derivative cured of plasmid pRmeGR4b) instead of GR4 improved transfer efficiency of plasmid pSmeLPU88b::Tn*5* (Table 2).

Table 2
Mobilisation of plasmid pSmeLPU88b::Tn*5*

Donor strain	Transfer frequency[a]
LPU88 (pSmeLPU88b::Tn*5*)	6×10^{-3}
2011-Sp (pSmeLPU88a::Tn*5*-B13, pSmeLPU88b::Tn*5*)	5×10^{-4}
GR4 (pSmeLPU88b::Tn*5*)	3×10^{-7}
GRM6 (pSmeLPU88b::Tn*5*)	2×10^{-4}

[a]All conjugations were made using *A. tumefaciens* UBAPF2 as recipient strain. Results are given as the ratio of transconjugants to recipient.

3.4. *Replication host range of the 22-kb plasmid pSmeLPU88b*

Plasmid pSmeLPU88b::Tn*5* was transferred by conjugation from *S. meliloti* to different bacterial species and the neomycin-resistant transconjugants analysed in Eckhardt gels to confirm plasmid transfer (and to discard transposition). Plasmid replication was observed in *Rhizobium tropici*, *Rhizobium etli*, *Rhizobium leguminosarum*, and *A. tumefaciens*. The plasmid did not replicate either in *E. coli* or in *Pseudomonas fluorescens*. Results suggest that replication of plasmid pSmeLPU88b is possibly restricted to bacteria of the Rhizobiaceae family. Anyhow, it cannot be discarded that the Tn*5* insertion could affect the replication of the plasmid in certain bacterial species.

3.5. *Nodulation competitiveness of* S. meliloti *2011 carrying plasmid pSmeLPU88a or plasmid pSmeLPU88b*

It was previously shown that one of the cryptic plasmids present in strain GR4 modulates strain competitiveness for the nodulation of alfalfa [20,21,36]. Furthermore, previous evidence has shown that non-symbiotic cryptic plasmids may be involved in the biosynthesis of bacteriocins that can modulate competitiveness [11,37]. In order to assess whether the presence of any of the cryptic plasmids of strain LPU88 was associated with changes in nodulation competitiveness, strain *S. meliloti* 2011-Sp (pSmeLPU88a) and strain *S. meliloti* 2011-Sp (pSmeLPU88b) were separately co-inoculated with strain *S. meliloti* 20PM6 [38] on alfalfa roots. The nodule occupancy of each rhizobium was scored after a month as indicated in Section 2. No major differences between any of the bacterial genotypes were observed indicating that none of the plasmids significantly affected competitiveness.

4. Discussion

In this paper we describe the isolation and transmissibility properties of two cryptic plasmids from the strain *S. meliloti* LPU88. One of the plasmids, pSmeLPU88b (22 kb), appeared to be mobilisable if helper functions were supplied by the accompanying plasmid pSmeLPU88a (binary conjugal system). This latter plasmid behaved as non-transmissible via conjugation. It is unlikely that the non-transmissible phenotype of plasmid pSmLPU88a was due either to inappropriate quorum-sensing effects or to the lack of unknown host–plant factors: the plasmid Tra functions were functional in mobilising the accompanying

replicon pSmeLPU88b. According to these results plasmids pSmeLPU88a/b may have arisen from the resolution of an ancestral cointegrate with splitting of the *tra* and the *mob* regions into plasmid pSmeLPU88a and plasmid pSmeLPU88b, respectively. Alternatively, both plasmids may have separately entered strain LPU88 in independent conjugal events. If this is the case, the *oriT* from plasmid pSmeLPU88a must have been lost after the transfer.

The binary system pSmeLPU88a/b characterised in this work differs from the previous observation of strain *S. meliloti* GR4 [16,19]. While the helper plasmid pRmeGR4a is self-transmissible, the helper plasmid pSmeLPU88a from strain LPU88 is not. Another distinctive feature of the system characterised herein is the small size of plasmid pSmeLPU88b and its high mobilisation frequency (10^{-3} transconjugants per receptor) compared to the mobilisation frequency of plasmid pRmeGR4b from strain GR4 (10^{-5} transconjugants per receptor) [19]. Besides, we have not observed any effect of the cryptic plasmids from strain LPU88 on the nodulation competitiveness of *S. meliloti*.

Despite the differences between the two systems, we have observed that the conjugative functions of strain *S. meliloti* GR4 are active in the mobilisation of plasmid pSmeLPU88b of the local strain *S. meliloti* LPU88. Noteworthy, the GR4 derivative strain cured from plasmid pRmeGR4b mobilised plasmid pSmeLPU88b 1000 times more efficiently than the wild-type strain GR4. An explanation for this observation is through the possible existence of competition phenomena between the transfer origin (*oriT*) of plasmid pRmeGR4b and the *oriT* of plasmid pSmeLPU88b for the interaction with specific Tra-like proteins during conjugal events. Contrasting with this, no strong effects of the RP4-specific *oriT* on the mobilisation of plasmid pSmeLPU88b were observed. At the same time, conjugal functions of plasmid RP4 were unable to mobilise the *S. meliloti* plasmid pSmeLPU88b. Non-overlapped DNA regions bearing functional origins of transfer could be isolated from strain GR4 [22]. However, neither *oriT*s nor *tra*/*trb*-like sequences have been obtained yet in *S. meliloti* GR4 for their comparison to the conjugal genes from other related bacteria where several components of the DNA transfer and replication system and the mating pair formation complex have been identified [39]. Recently, putative *oriT* regions were identified within both megaplasmids of strain *S. meliloti* 1021 by sequence analysis [40]. The close functional characteristics between the cryptic plasmids present in the European strain GR4 and the local strain LPU88 make them a suitable target of analysis to get further insight into the genetic structure of conserved conjugal functions in *S. meliloti*. A better knowledge of the rhizobial conjugal systems should help to elucidate at the molecular level the evolutionary mechanisms through which very diverse chromosome genotypes acquired the traits that allow them to associate with legumes.

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