RESEARCH LETTER

rptA, a novel gene from *Ensifer* (*Sinorhizobium*) *meliloti* involved in conjugal transfer

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Abstract

We approached the identification of *Ensifer (Sinorhizobium) meliloti* conjugal functions by random Tn*5*-B13 mutagenesis of the pSmeLPU88a plasmid of *E. meliloti* strain LPU88 and the subsequent selection of those mutants that had lost the ability to mobilize the small plasmid pSmeLPU88b. The Tn*5*-B13-insertion site of one of the mutants was cloned as an EcoRI-restricted DNA fragment that after subsequent isolation and sequencing demonstrated that a small open reading frame of 522 bp (designated *rptA*, for rhizobium plasmid transfer A) had been disrupted. The predicted gene product encoded by the *rptA* sequence shows a significant similarity to two hypothetical proteins of the plasmid pSmed03 of *Ensifer medicae* WSM419 and other rhizobia plasmids. No significant similarity was found to any protein sequence of known function registered in the databases. Although the *rptA* gene was required for pSmeLPU88b-plasmid mobilization in the strain 2011 background, it was not required in the original strain LPU88 background.

Introduction

Horizontal gene transfer is mediated by several mechanisms, with bacterial conjugation being the most widespread and the one that contributes the most to the horizontal gene pool within the prokaryotic world. Bacterial conjugation is a specialized process involving the unidirectional transfer of DNA from a donor to a recipient cell by a mechanism requiring a specific fruitful contact between the two bacteria (de la Cruz & Davies, 2000). The genetic information for conjugation is generally encoded in a plasmid, consisting of a circular double-stranded segment of naked DNA. Plasmids exist in the bacterial cell entirely separate from the bacterial chromosome. Conjugation systems are very efficient in mediating transfer over a wide range of bacterial genera; and, in some instances, conjugation goes beyond prokaryotes, e. g. plant cells (Zupan & Zambryski, 1995). The bacterial conjugation mechanism involves three steps. The first is the formation of a sex pilus to bring the participants into close contact. This hairlike bridge – a multiprotein complex, formed by about 10 different proteins – spans both the inner and outer membranes. The pilus components belong to a family of protein transporters known as type-IV secretion systems (T4SS; Alvarez-Martinez & Christie, 2009; Smillie *et al.*, 2010). Plasmid-encoded proteins then assemble at a unique locus, the origin of transfer, to form the relaxosome. Within this complex, the relaxase enzyme cleaves one of the DNA strands by a reversible transesterification (Parker *et al.*, 2005). In the third step, this severed DNA strand is

FEMS MICROBIOLOGY LETTERS

transferred from the donor to the recipient bacteria. The *coupling protein* brings the cleaved DNA and sex pilus together, thus moving into apposition both parts of the transfer machinery (Llosa & de la Cruz, 2005). The plasmids that possess complete sequence information for their own transfer are known as *self-transmissible*; whereas the so-called *mobilizable* plasmids, while possessing their own *oriT*, still need helper functions (e.g. the T4SS), with those usually being provided by a self-transmissible plasmid.

Bacteria belonging to the genera *Rhizobium*, *Ensifer (Sinorhizobium)*, *Azorhizobium* and *Mesorhizobium* can grow in the soil under free-living conditions or as nitrogen-fixing organisms in symbiotic association with the root of legumes. A general feature of the rhizobia is the presence of large amounts of plasmid DNA in their genomes (Torres Tejerizo *et al.*, 2011; López-Guerrero *et al.*, 2012). These associated plasmids vary in both number and size. In some rhizobia, most of the essential genes required for the symbiotic interaction are present in plasmids that are usually designated *symbiotic plasmids* or *pSyms*. In addition, rhizobia can bear plasmids whose role is not currently understood, referred to as *cryptic* or *nonsymbiotic plasmids*. Conjugal transfer of rhizobial plasmids has been well documented for decades (Hooykaas *et al.*, 1982, 1985; Kondorosi *et al.*, 1982; Truchet *et al.*, 1984) both for symbiotic and for cryptic plasmids, which in turn are either self-transmissible or mobilizable (Johnston *et al.*, 1978; Mercado-Blanco & Olivares, 1993; Rao *et al.*, 1994; Pérez-Mendoza *et al.*, 2004; Pistorio *et al.*, 2008; Torres Tejerizo *et al.*, 2010).

In recent years, considerable efforts have been made to characterize the conjugal functions in rhizobia at the molecular level, including both biochemical studies and sequencing approaches (Turner *et al.*, 2002; Tun-Garrido *et al.*, 2003; Pérez-Mendoza *et al.*, 2004, 2005, 2006; Stiens *et al.*, 2006, 2007; Giusti *et al.*, 2012). Ding & Hynes (2009) defined two major groups of conjugative plasmids of rhizobia based on the characteristics of the regulation of the expression of the *tra/trb* operons; Type I, which is regulated by conjugation quorum sensing (QS), and Type II plasmids, in which the conjugation is constantly suppressed by *rctA* gene. A third group (Type III) represented by some plasmids of *Rhizobium leguminosarum* bv *viciae* was proposed by Ding & Hynes (2009), based on the phylogenetic relationship between the relaxase sequences. Recently, another group (Type IV) was proposed, represented by plasmids of *E. meliloti* LPU88 and *R. leguminosarum* bv *viciae* strains (Giusti *et al.*, 2012; Ding *et al.*, 2013).

In a previous report we described the isolation and characterization of the conjugal properties of two cryptic plasmids from the *E. meliloti* strain LPU88 (Pistorio *et al.*, 2003). One of the plasmids, pSmeLPU88b (p88b, 42 kb), appeared to be mobilizable if helper functions were supplied by the accompanying plasmid pSmeLPU88a (p88a, 150 kb), i.e. in a binary conjugal system; this latter plasmid behaved as nontransmissible via conjugation. To further our knowledge of the helper functions encoded in p88a, we decided to perform a random Tn*5* mutagenesis so as to identify putative genes related to the conjugal–transfer process. We report here the identification of a novel genetic locus from *E. meliloti* that is involved in conjugal transfer, the rhizobium plasmid transfer A gene, *rptA*.

Materials and methods

Bacterial strains and plasmids

Table 1 lists the bacterial strains and plasmids used in this work. The *Escherichia coli* strains were grown at 37 °C on Luria–Bertani (LB) medium (Miller, 1971) and the *E. meliloti* and *Agrobacterium tumefaciens* at 28 °C on TY medium (Beringer, 1974). For the solid media, 15 g of agar was added per liter of medium. The final antibiotic concentrations per mL of medium were: 10 μg gentamicin (Gm), 10 μg tetracycline (Tc) and 200 μg ampicillin (Ap) for *E. coli*; 400 μg streptomycin (Sm), 50 μg gentamicin, 200 μg spectinomycin (Sp), 10 μg tetracycline, and 120 μg neomycin (Nm) for *E. meliloti*; and 200 μg rifampicin (Rif) and 100 μg spectinomycin (Sp) and 200 μg rifampicin (Rif) for *A. tumefaciens*.

Bacterial matings

The bacterial matings were performed as described by Simon *et al.* (1983). Stated in brief, 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. The donors and recipients were mixed in a microcentrifuge tube at a ratio of 1 : 1 (500 μL of each culture), the mating mixture concentrated by an 8-min centrifugation at 640× ***g***, and the pellet resuspended in 50 μL of the same medium for loading onto a Millipore filter (0.2 μm pore size). The filter-inoculated mating mixtures were placed on TY agar plates and incubated overnight at 28 °C.

Plasmid mutagenesis and forced mobilization through the use of transposon Tn*5*-B13

The T*n5*-B13 transposon (Simon *et al.*, 1989) from plasmid pSUP102::Tn*5*-B13 was first randomly introduced by conjugation into strain LPU88 with selection for streptomycin and tetracycline resistance. Tn*5*-B13 is a Tn*5* derivative carrying a Tc and mob cassette (Simon *et al.*, 1989). The Tn*5*-B13-containing rhizobia were then used *en masse* as donors in a triparental mating with *E. meliloti*

Table 1. Bacterial strains and plasmids used in this work

Strain and plasmids	Relevant properties	Reference
Agrobacterium tumefaciens		
UBAPF2	Plasmid-free strain, Rif^r	Hynes *et al.* (1985)
UBAPF2G	Plasmid-free strain, Rif^r, Gm^r	This work
GMI9023	Plasmid-free	Rosenberg & Huguet (1984)
GMI9023-Sp	Plasmid-free, Rif^r Sp^r	Althabegoiti, unpublished
Ensifer meliloti		
LPU88	Wild-type isolate from Argentina,	Pistorio *et al.*(2003)
LPU88b	LPU88 with a p88b::Tn*5*, Tra$^+$. Sm^r Nm^r.	Pistorio *et al.* (2003)
2011-Sp	Sp^r derivative of strain 2011, Sm^r	Pistorio *et al.* (2003)
GRM8	GR4 cured of plasmids pRmeGR4a and pRmeGR4b	Mercado-Blanco & Olivares (1993)
GRM8R	Rif^R derivative	This work
Escherichia coli		
DH5α	*recA*, *_lacU169*, F80d*lac* ZDM15	Invitrogen™
S17-1	*E. coli* 294 RP4-2-Tc::Mu-Km::Tn*7* integrated in the chromosome	Simon *et al.* (1983)
Plasmids		
pSUP102::B13	pSUP102 carrying a Tc-mob Tn*5* derivative, Tc^r.	Simon *et al.* (1989)
pK18mob	Cloning vector, Km^r	Schäfer *et al.*(1994)
pRK2013	RK2 conjugal functions, Km^r	Figurski & Helinski (1979)
pSUP102	Cloning vector, Tc^r	Simon *et al.* (1986)
pGEM-T easy	Cloning vector, Ap^r.	Promega
pCW504	Cloning vector, replicative in rhizobia Gm^r	Lenz *et al.* (2000)
pG18mob2	Cloning vector, Gm^r, suicide in rhizobia	Kirchner & Tauch (2003)
p88a::Tn*5*-B13-5	p88a with Tn*5*-B13 insertion	This work
p88a-*rptA*::pMP4	p88a with pMP4 inserted in *rptA* gene	This work
p88a-*rptA*::pMP7	p88a with pMP7 inserted in *rptA* gene	This work
pMP2	pK18mob with a EcoRI fragment containing Tn5-B13 from p88a::Tn*5*-B13-5, Km^r Tc^r	This work
pMP3	pCW504 with a complete *rptA* gene, Gm^r	This work
pMP4	pSUP102 with *rptA* gene internal fragment, Tc^r	This work
pMP7	pG18mob2 with *rptA* gene internal fragment, Gm^r	This work

Sm^r, Sp^r, Tc^r, Km^r, Ap^r, Rif^r, Nm^r and Gm^r = streptomycin, spectinomycin, tetracycline, kanamycin, ampicillin, rifampicin, neomycin and gentamicin resistance, respectively. Tra, properties of transfer pLPU88b::Tn*5*.

2011-Sp (recipient strain) and *E. coli* DH5α (pRK2013) (Figurski & Helinski, 1979), the latter providing the helper plasmid for conjugation. Transconjugants were first detected according to their expected tetracycline-, streptomycin-, and spectinomycin-resistant phenotypes, and the presence of mobilized plasmids in the transconjugants from the donor rhizobia was then evaluated by analysis in Eckhardt gels (Eckhardt, 1978).

Plasmid profiles and Eckhardt gels

From a culture grown to mid-log phase in TY medium, 100 μL of cells were collected in a microcentrifuge tube and mixed with 500 μL 0.3% (w/v) sarkosyl in TBE buffer (89 mM Tris, 2.5 mM EDTA, 89 mM boric acid). The suspension was centrifuged for 30 s at 14 000× ***g***, the supernatant discarded, and the cell pellet resuspended in 40 μL of loading buffer (10% w/v sucrose, 0.01 mg mL^{-1} ribonuclease A, and 1 mg mL^{-1} lysozyme) and applied to a 0.7% (w/v) agarose gel containing 1% w/v SDS in TBE buffer. Electrophoresis was run first at 30 V for 30 min and then at 110 V for 2 h. The plasmid bands were visualized under UV illumination after staining of the gel with 0.5–1.0 μg mL^{-1} ethidium bromide (Eckhardt, 1978).

DNA manipulation

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

(Sambrook *et al.*, 1989). Southern hybridization was carried out through the use of DNA probes labelled with digoxigenin. The probes were synthesized by PCR reactions with digoxigenin-dUTP (Boehringer Mannheim) and the appropriate primers to amplify the region of interest. For hybridization, DNA extracted from the bacteria was digested and transferred to nitrocellulose membranes (Hybond N, Amersham) as described by Chomczynski (1992). The digoxigenin-labelled DNA probes were then hybridized to the membranes overnight at 65 °C after blocking of nonspecific binding sites for 1 h at 68 °C by means of the solutions and experimental conditions specified by Boehringer Mannheim (Catalogue No. 1093 657). For the visualization of positive bands, the membranes were incubated with an antibody against the digoxigenin ligand and washed, and a final color reaction was initiated at alkaline pH by the addition of X-phosphate plus nitroblue tetrazolium chloride, as specified by the manufacturer.

DNA sequencing and sequence analysis

The EcoRI restriction fragment that contained the Tn*5*-B13 was cloned into pK18mob (Schäfer *et al.*, 1994) to generate pMP2. For sequencing, the DNA region bordering the transposon was subcloned into pUC19 (Yanisch-Perron *et al.*, 1985) to separate both IS*50* sequences of the Tn*5*-B13. The nucleotide sequence was obtained by means of a sequencing-walking strategy with specific deoxyoligonucleotides. The DNA was sequenced by Macrogen, Inc. (Korea) and the final sequence deposited in GenBank under the accession number JQ753316. Sequence comparisons and alignments within the NCBI database were performed through the use of the BLAST and ORF FINDER websites and default parameters.

DNA amplification

Deoxyoligonucleotide primers were synthesized by DNAgency (Malvern, PA). PCR amplifications were performed in 25 μL reactions containing 50 mM Tris, pH 8.3; 500 mg mL^{-1} bovine serum albumin; 3 mM $MgCl_2$; 200 μM dNTPs; 1 U Taq polymerase (Promega Corp.); 10 μM of each primer; and 10 μL of template DNA, previously obtained by heating a freshly isolated bacterial colony in 50 μL of distilled water at 100 °C for 15 min. The amplifications were carried out in capillary tubes in an Idaho 1605 Air Thermo Cycler (Idaho Technology). The cycling conditions were as follows: 94 °C for 30 s followed by 35 cycles at 94 °C for 10 s, at 53 °C for 10 s, and at 72 °C for 30 min. After the reaction, 10 μL of the PCR products was separated in 1% w/v agarose gels containing 0.5–1.0 μg mL^{-1} ethidium bromide and photographed with Polaroid 667 film.

Construction of the *E. meliloti* LPU88 *rptA* mutant

A 210-bp internal fragment of the *rptA* gene was amplified by PCR with the primers *rpt*-f (5′-TAG CTT CCG ACG GTA TTT CTC ATA-3′) and *rpt*-r (5′-AAA GTC GGT TCG CAT AGG TG-3′). The PCR fragment was then cloned into the pGEM®-T easy-shuttle vector (Ap^r, *lacZ*; Promega) and subsequently cloned into vector pSUP102 as an EcoRI fragment to generate pMP4. The resulting plasmid was transferred by conjugation to strain LPU88 to yield the p88a-*rptA*::pMP4 plasmid by site-specific insertional mutagenesis. The correct plasmid integration was confirmed by Southern blotting. Alternatively, the 210-bp internal fragment was cloned into the SmaI site of pG18mob2 plasmid to generate pMP7 and then transferred to strain LPU88 to yield p88a-*rptA*::pMP7.

Complementation of the *rptA* mutant

A 1.34-kbp DNA fragment containing the complete coding sequence and the intergenic region both upstream and downstream of *rptA* was amplified by PCR with the primers PTG-f (5′-ATC ATC GAC GGT AGC AGT CC-3′) and PTG-r (5′-ATA AGG TCG GAC ACT TCG C-3′). The resulting fragment was next cloned first in pGEM®-T Easy and then, as an EcoRI fragment, in the vector pCW504, previously digested with the same restriction enzyme. The resulting recombinant plasmid, pMP3, was finally transferred by mating from *E. coli* S17-1 to the *E. meliloti* 2011-Sp (p88a-*rptA*::pMP4, p88b::Tn*5*) and the presence of pMP3 in the recipient rhizobium was confirmed by Eckhardt gels.

Results

Isolation of a p88a plasmid lacking helper functions

As described previously (Pistorio *et al.*, 2003), plasmid p88a carries the helper functions necessary for the mobilization of p88b; however, p88a behaved as non-self-transmissible via conjugation. With the aim of investigating the conjugal helper functions encoded in p88a, strain LPU88 of *E. meliloti* was random-mutagenized with Tn*5*-B13 and used *en masse* as a potential donor of the Tn*5*-tetracycline resistance to the recipient strain *E. meliloti* 2011-Sp. Thus, via the triparental mating with the helper plasmid pRK2013, we were able to rescue p88a::Tn*5*-B13 mutant variants to be further

analyzed for the loss of the ability to mobilize p88b. Several tetracycline-resistant 2011-Sp transconjugants were obtained and analyzed for their plasmid content. In addition to the symbiotic megaplasmids present in strain 2011-Sp, transconjugants could potentially possess either p88a or p88b alone, or both plasmids together. Thus, clones harboring p88a, as determined by Eckhardt-type gel analysis, were selected. Upon construction of strain *E. meliloti* 2011-Sp (p88a::Tn*5*-B13, p88b::Tn*5*), these payload clones were tested for their efficiency in mobilizing p88b into *A. tumefaciens* UBAPF2. One strain of 2011-sp harboring p88a::Tn*5*-B13-5 was found that could not transfer p88b plasmid to UBAPF2 and this was selected for further characterization.

Characterization of the Tn*5* insertion on p88a::Tn*5*-B13-5

When strain 2011-sp (p88a::Tn*5*-B13-5) was examined by Southern-hybridization analysis with an IS*50* probe, a unique hybridizing EcoRI restriction fragment was found (not shown). To identify the interrupted gene in the p88a::Tn*5*-B13-5 plasmid, the EcoRI restriction fragment that contained the Tn*5*-B13 was cloned into pK18mob and the resultant plasmid pMP2 subcloned and sequenced beginning at each side of the insertion. The 6978-bp sequence obtained was analyzed to determine the type and number of open reading frames (ORFs), by ORF Finder (http://www.ncbi.nlm.nih.gov/projects/gorf/) and BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Figure 1 shows the deduced genetic structure.

The Tn*5* insertion had interrupted the coding sequence of a gene to be designated hereafter as *rptA* (rhizobium plasmid transfer A) that encodes a hypothetical protein of 173 amino acids lacking significant similarity to any protein sequence of known function described in the databases. However, this putative protein shows significant similarities to two hypothetical proteins coded by plasmid pSmed03 of *E. medicae* WSM419, Smed_6419 and Smed_6415, at respective sequence identities of 67% and 50%; two hypothetical proteins coded by pSM11c of *E. meliloti* SM11, SM11_pC0261 and SM11_pC0257, with 65% and 52% identity; two hypothetical proteins coded by chromosome 3 of *E. meliloti* AK83, Sinme_5726 and Sinme_5722, with respective identities of 66% and 52%; two hypothetical proteins coded by pRmeGR4a of *E. meliloti* GR4, C770_GR4pA112 and C770_GR4pA108, with respective identities of 64% and 54%; two hypothetical proteins coded by pSfHH103c of *Ensifer (Sinorhizobium) fredii* HH103, SFHH103_04101 and SFHH103_04097, with respective identities of 60% and 56%; and two hypothetical proteins coded by pAtS4a of *Agrobacterium vitis* S4, Avi_9832 and Avi_9884, with respective identities of 44% and 34%. Immediately upstream from the interrupted genes we could identify three ORFs (Fig. 1): ORF 1 encodes a protein with homology to the transposase ISRM17 (protein identity 100%), and the proteins of ORF 2 and ORF 3 have

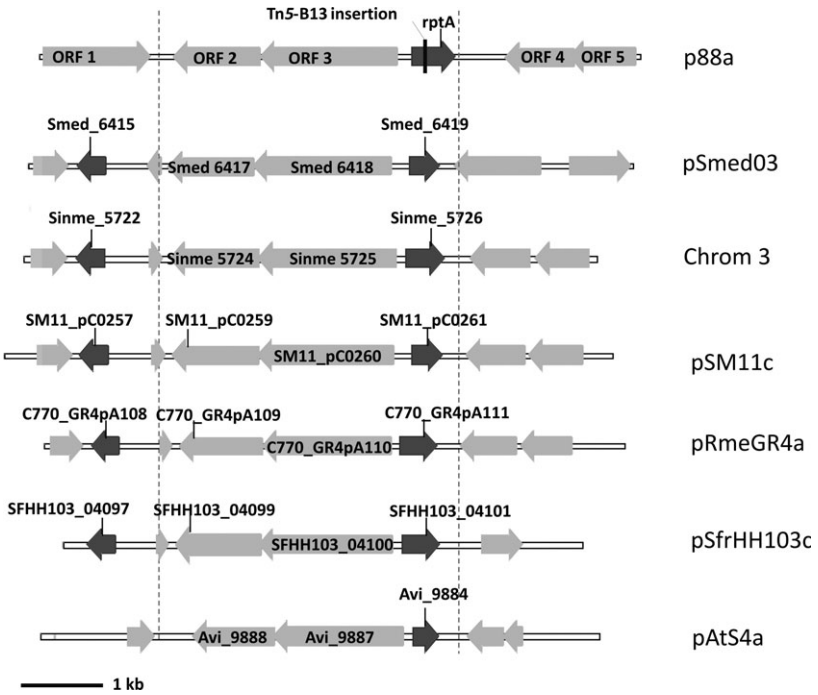

Fig. 1. Genetic organization of the 6.9-kb region containing the Tn*5*-B13 insertion present in p88a mutant compared to the same regions of other rhizobia. In dark gray are the *rptA* gene and homologous genes present in pSmed03 of *Ensifer medicae*, in chromosome 3 of *Ensifer meliloti* AK83, in pSM11c of *E. meliloti* SM11, in pRmeGR4a of *E. meliloti* GR4, in pSfrHH103c of *E. fredii* HH103 and in pAtS4 of *A. vitis*. The two vertical dotted lines indicate the synteny region.

respective homologies to Smed_6417 (protein identity 96%) and Smed_6418 (protein identity 87%). This region exhibited synteny with a homolog cluster located on plasmid pSMED03, pSM11c, chromosome 3 and pAtS4a interrupted by the gene for the transposase ISRM17 (Fig. 1). Downstream from *rptA* and transcribed in the opposite direction, two ORFs were identified (Fig. 1): ORF 4 (807 bp) encoding a protein with homology to a putative lipoprotein of *Anaeromyxobacter* sp. Fw109-5 (Anae109_2853, protein identity 32%) and – interrupted by the EcoRI restriction site – ORF 5, whose gene product is related to COG1858, the MauG–cytochrome-c peroxidase. No polar effects could be expected to cause the observed phenotype because the genes on either side of *rptA* are oriented in opposite directions.

Reverse genetics and complementation of the *rptA* gene

After the above genetic analyses, we performed reverse mutagenesis on strain LPU88b by homogenotization of the pMP2 plasmid. What was remarkable was that the mutation of the *rtpA* gene in strain LPU88 failed to lead to the loss of the helper function. This result raised the question of whether a cryptic mutation responsible for the loss of function and different from that generated by the transposon existed or whether we were in the presence of a mutation whose phenotype was dependent on the host strain, i.e. the mutation as expressed in the *E. meliloti* 2011-Sp background showed a transfer-minus phenotype but in the *E. meliloti* LPU88 background a transfer-plus phenotype. To answer this question we decided to (1) generate a new *rptA* mutant by integration into the p88a plasmid a suicide plasmid, pMP4 (pSUP102 carrying a 210-bp internal fragment of *rptA*); (2) transfer by triparental mating the resulting plasmid p88a-*rptA*::pMP4 from the LPU88 strain to the 2011-Sp strain; and (3) evaluate the helper phenotype after introducing the new p88b::Tn*5* construct from both genomic backgrounds. Before performing the conjugation assay, the correct integration of the plasmid was examined in both strains by Southern-hybridization analysis with a specific probe for the *rptA* gene. Figure 2 demonstrates that only one hybridization signal was present for the wild-type LPU88 strain, no signal for the 2011-Sp strain, and the same pattern of signals for the mutant plasmid in the two strains, thus confirming the absence of genetic rearrangements. Whereas the mobilization of the p88b::Tn*5* plasmid could be achieved from the LPU88 strain; consistent with the above observations, the transfer of this same plasmid from the *E. meliloti* strain 2011-Sp (p88a-*rptA*::pMP4) failed. These experiments were performed independently four times, and under no circumstances were transconjugants obtained. To determine whether the observed phenotypes were observed in other hosts, we performed the conjugation assays in other strains harboring the LPU88 plasmids. Upon construction of the strain *E. meliloti* GRM8 (p88a::Tn*5*-B13, p88b::Tn*5*) and *A. tumefaciens* UBAPF2 (p88a-*rptA*::pMP7, p88b::Tn*5*) the smaller plasmid p88b::Tn*5* could only be mobilized from strain GRM8.

Finally, to confirm the requirement of the *rptA* gene for the conjugal transfer of plasmid pLPU88b within an *E. meliloti* 2011-Sp background, a complementation assay was performed. A 1336-bp fragment including the coding sequence and the intergenic regions upstream and downstream from *rptA* was cloned in the replicative plasmid pCW504, with the resulting plasmid being designated pMP3. As expected, this pMP3 plasmid was able to complement *E. meliloti* 2011-Sp (p88a-*rptA*::pMP4), resulting in mobilization of p88b::Tn*5* (Fig. 3). The same strain carrying pCW504 without the insert was unable to do so. Furthermore, when *E. meliloti* 2011-Sp (p88a-*rptA*::pMP4, p88b::Tn*5*, pMP3) was cured of pMP3, the cured strain was rendered incapable of the transfer of p88b::Tn*5*.

Discussion

In this work we have addressed the molecular identification and characterization of a gene associated with the mobilization of plasmid p88b by the helper plasmid p88a. We first designed a strategy based on a random Tn*5*-B13 mutagenesis of LPU88 and a subsequent transfer of the tagged mutated plasmids to strain 2011-Sp, where the helper property of the transferred plasmid was assayed. With this strategy we were able to recover one clone possessing a Tn*5*-B13 that exhibited a transfer-minus phenotype: *E. meliloti* 2011 (p88a::Tn*5*-B13-5). A molecular analysis of the mutated region in the plasmid p88a::Tn*5*-B13-5 indicated that an open reading frame of 522 bp had been interrupted that was designated *rptA* (rhizobium plasmid transfer). The organization of neighboring genes revealed that the loss of conjugative function was not related to polar effects but to the *rptA* gene mutation itself. As mentioned above, the *rptA* gene product showed homology to hypothetical proteins of *E. medicae* WSM419, *E. meliloti* AK83, GR4 and SM11, *E. fredii* HH103 and *A. vitis* S4. In addition, analysis of the DNA sequence obtained results in 3200 bp that exhibited synteny with homolog regions in each of the previously mentioned strains. This synteny was interrupted upstream by the ISRm17 element (ORF 1) and downstream by the remnants of an IS66 element (upstream ORF 4) present in plasmid p88a. What was remarkable was that the LPU88 strain gave a single hybridization signal when an internal fragment of *rptA* was used as a probe, thus demonstrating that the *rptA* bearing the mutation was not

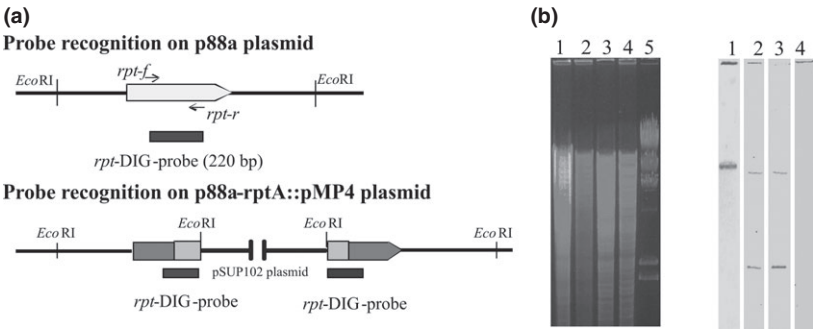

Fig. 2. Analysis of the new genomic structure in the *rptA* region of p88a plasmid generated by site-specific insertion of the pMP4 plasmid. (a) Recognition of the DIG-*rpt* probe in *Ensifer meliloti* strains LPU88 (p88a-*rptA*::pMP4) and 2011 (p88a-*rptA*::pMP4). (b) Southern-hybridization analysis by means of the DIG-*rpt* probe. The left panel shows the electrophoretic run of EcoRI-digested DNA in an 0.8% (w/v) agarose gel. The right side shows the membrane hybridized with the DIG-*rpt* probe. Lane 1, LPU88; lane 2, LPU88 (p88a-*rptA*::pMP4); lane 3, 2011 (p88a-rptA::pMP4); lane 4, 2011; lane 5, Lambda (HindIII).

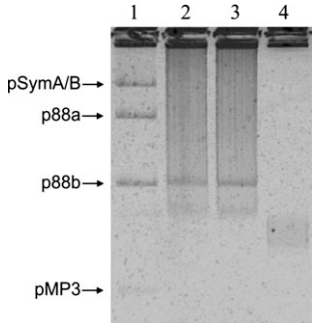

Fig. 3. Plasmid profiles of transconjugants obtained in mating *Ensifer meliloti* 2011 (p88a-rptA::pMP4, p88b::Tn*5*, pMP3) vs. *Agrobacterium tumefaciens UBAPF2*. Lanes 2 and 3 show the plasmid profile of *A. tumefaciens* UBAPF2 transconjugants (Rif^r Nm^r). Lanes 1 and 4 show the plasmid profile corresponding to the donor strain *E. meliloti* (p88a-*rptA*::pMP4, p88b::Tn*5*, pMP3) and the recipient strain *A. tumefaciens* UBAPF2, respectively. The arrow indicates the position of the plasmid pMP3, present as a faint band.

being functionally complemented by an extra copy of itself. If p88a also has two similar genes arranged near one another, such as occurs with *E. medicae* WSM419 or *E. meliloti* AK83, the putative second gene here must have evolved to perform a different function, as we observed no complementation of the mutation in the 2011-Sp strain.

In other conjugative systems genes have been described that are dispensable for conjugation within the same species but have been suggested possibly to be operative in interspecific conjugations (Lessl *et al.*, 1993; Miyazaki *et al.*, 2008). Using the RP4 plasmid, Lessl *et al.* (1993) showed that the genes *traA* to *traE*, as well as *traL* to *tra0*, are not absolutely required for transfer between *E. coli* cells but are necessary for efficient plasmid transfer to or from bacterial species different from *E. coli*. Recently, Miyazaki *et al.* (2008) demonstrated that a mutation in three genes of the *traD* operon of the self-transmissible plasmid NAH7 resulted in 10- to 10^5-fold decreases in the transfer frequencies of the plasmids from *Pseudomonas* to *Pseudomonas* or to *E. coli*, and from *E. coli* to *E. coli*. In contrast, the *traD* operon was essential for the transfer of NAH7 from *E. coli* to *Pseudomonas* strains, indicating that the *traD* operon was a host-range modifier in the conjugative transfer of NAH7.

The most interesting aspect of the *rptA* gene is that its requirement for mobilization by the p88b plasmid is dependent on the strain used as a host. The *rptA* locus exhibited a strict requirement in *E. meliloti* strain 2011 and *A. tumefaciens* UBAPF2 but not in *E. meliloti* strains LPU88 and GRM8. This observation suggests that the former strains lacks a structural and/or catalytic element that is either produced or activated by the presence of *rptA*. This raises questions as to how the functional requirement of the *rptA* product in LPU88 is replaced by another cell component, what the changes are that *rptA* generates in *E. meliloti* 2011-Sp to enable its conjugative capacity, and whether these changes are associated with T4SS or with the DNA-transfer and -replication systems. A more detailed elucidation of the translation product of the *rptA* locus as well as a characterization of the specific *E. meliloti*-associated factor(s) required for conjugal transfer should provide new insights into the determinants of host range in bacterial–plasmid conjugation systems.

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