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 Tn5-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 Tn5-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
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 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 conjugal plasmids of rhizobia based on the characteristics of the regulation of the expression of the tra/trb operons; Type I, which is regulated by conjuration 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 Tn5 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 Tn5-B13

The Tn5-B13 transposon (Simon et al., 1989) from plasmid pSUP102::Tn5-B13 was first randomly introduced by conjugation into strain LPU88 with selection for streptomycin and tetracycline resistance. Tn5-B13 is a Tn5 derivative carrying a Tc and mob cassette (Simon et al., 1989). The Tn5-B13-containing rhizobia were then used en masse as donors in a triparental mating with E. meliloti.
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.
DNA sequencing and sequence analysis

The EcoRI restriction fragment that contained the Tn5-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 IS50 sequences of the Tn5-B13. The nucleotide sequence was obtained by means of a sequencing-walking strategy with specific deoxyligonucleotides. 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 DNA-gency (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 MgCl₂; 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.

Complementation of the 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 (Ap⁵, 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.

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 Tn5-B13 and used en masse as a potential donor of the Tn5-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::Tn5-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::Tn5-B13, p88b::Tn5), these payload clones were tested for their efficiency in mobilizing p88b into *A. tumefaciens* UBAPF2. One strain of 2011-sp harboring p88a::Tn5-B13-5 was found that could not transfer p88b plasmid to UBAPF2 and this was selected for further characterization.

**Characterization of the Tn5 insertion on p88a::Tn5-B13-5**

When strain 2011-sp (p88a::Tn5-B13-5) was examined by Southern-hybridization analysis with an IS50 probe, a unique hybridizing EcoRI restriction fragment was found (not shown). To identify the interrupted gene in the p88a::Tn5-B13-5 plasmid, the EcoRI restriction fragment that contained the Tn5-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 Tn5 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 *Ensifer 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 pSHH103c of *Ensifer* (Sinorhizobium) *freddii* 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
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 pAt54a 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 (Anaee109_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 rpta 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::Tn5 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::Tn5 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::Tn5-B13, p88b::Tn5) and A. tumefaciens UBAFP2 (p88a-rpta::pMP7, p88b::Tn5) the smaller plasmid p88b::Tn5 could only be mobilized from strain GRM8.

Finally, to confirm the requirement of the rpta gene for the conjugal transfer of plasmid pLP88b 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::Tn5 (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::Tn5, pMP3) was cured of pMP3, the cured strain was rendered incapable of the transfer of p88b::Tn5.

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 Tn5-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 Tn5-B13 that exhibited a transfer-minus phenotype: E. meliloti 2011 (p88a::Tn5-B13-5). A molecular analysis of the mutated region in the plasmid p88a::Tn5-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
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 *traO*, 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 105-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-Sp 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 *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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