JOURNAL OF BACTERIOLOGY, Sept. 1998, p. 4583–4590 Vol. 180, No. 17
0021-9193/98/$04.00+0

Cloning and Identification of Conjugative Transfer Origins in the *Rhizobium meliloti* Genome

JOSE A. HERRERA-CERVERA, JULIO M. SANJUAN-PINILLA, JOSE OLIVARES, AND JUAN SANJUAN*

Departamento de Microbiología del Suelo y Sistemas Simbióticos, Estación Experimental del Zaidín, CSIC, E-18008 Granada, Spain

Received 6 February 1998/Accepted 12 June 1998

A simple approach was used to identify *Rhizobium meliloti* DNA regions with the ability to convert a nontransmissible vector into a mobilizable plasmid, i.e., to contain origins of conjugative transfer (*oriT*, *mob*). RecA-defective *R. meliloti* merodiploid populations, where each individual contained a hybrid cosmid from an *R. meliloti* GR4 gene library, were used as donors en masse in conjugation with another *R. meliloti* recipient strain, selecting transconjugants for vector-encoded antibiotic resistance. Restriction analysis of cosmids isolated from individual transconjugants resulted in the identification of 11 nonoverlapping DNA regions containing potential *oriT*s. Individual hybrid cosmids were confirmed to be mobilized from the original *recA* donors at frequencies ranging from 10^{-2} to 10^{-5} per recipient cell. DNA hybridization experiments showed that seven *mob* DNA regions correspond to plasmid replicons: four on symbiotic megaplasmid 1 (pSym1), one on pSym2, and another two on each of the two cryptic plasmids harbored by *R. meliloti* GR4. Another three *mob* clones could not be located to any plasmid and were therefore preliminarily assigned to the chromosome. With this strategy, we were able to characterize the *oriT* of the conjugative plasmid pRmeGR4a, which confirmed the reliability of the approach to select for *oriT*s. Moreover, transfer of the 11 *mob* cosmids from *R. meliloti* into *Escherichia coli* occurred at frequencies as high as 10^{-1}, demonstrating the *R. meliloti* gene transfer capacity is not limited to the family *Rhizobiaceae*. Our results show that the *R. meliloti* genome contains multiple *oriT*s that allow efficient DNA mobilization to rhizobia as well as to phylogenetically distant gram-negative bacteria.

Rhizobium species are gram-negative soil bacteria able to establish nitrogen-fixing symbiotic associations with legume hosts. Many genes required for symbiosis are located in one or more large plasmids, so-called symbiotic plasmids (pSyms). In several *Rhizobium* species, pSyms have been shown to be self-transmissible at variable frequencies under laboratory conditions, but only in few cases have they been demonstrated to transfer to native bacteria in soil microcosms (17, 21, 24). Attempts to quantify plasmid transfer under field conditions have usually given negative results (15, 21), even though there is substantial evidence for plasmid transfer between rhizobia in soil. For instance, among native field populations, the same symbiotic plasmid can be found in otherwise unrelated strains; vice versa, chromosomally related strains may harbor different symbiotic plasmids (reviewed in reference 37). After introduction of inoculant *Rhizobium* strains in soils where no native rhizobia are present, sometimes a rhizobial population that is different from the original inoculant arises. Sullivan et al. (33) have recently demonstrated that in a field of *Lotus corniculatus* where 7 years earlier a defined *Rhizobium loti* strain was introduced, genetically different strains which contained a large chromosomal symbiotic DNA region identical to that of the introduced strain could be isolated. There is now evidence indicating that transfer of this chromosomal DNA may involve a conjugative mechanism (32). This report further strengthens the idea that genetic exchange between rhizobia may be relatively abundant under natural conditions and not limited to plasmid DNA.

Rhizobia are difficult to isolate directly from the soil or rhizosphere; they are often isolated by virtue of their ability to nodulate specific legumes, although the presence of large numbers of nonsymbiotic rhizobia in soils is well recognized (18, 28, 35).

Studies on gene transfer in rhizobia often deal with large plasmids, usually pSyms, and no environmental conditions, other than the presence of a specific host plant, indicative that the acquisition of a symbiotic plasmid represents an adaptive advantage leading to the rapid development of a putative transconjugant population are known to exist in the soil. Thus, plasmid recipients are often searched for as nodule-forming bacteria on a particular host plant. Such transconjugant bacteria may be difficult to detect, as they must outcompete the donor population for nodule occupancy, and this requires not only the optimal expression of symbiotic genes but probably also that the transconjugant populations reach a critical density. Therefore, potential transfer of a DNA not directly involved in symbiosis can go undetected or simply not be investigated.

An additional question relates to the forces driving gene transfer. For instance, although Sullivan and coworkers (33) isolated symbiotic transconjugants by their ability to nodulate a particular host plant, these new symbiotic bacteria had apparently also gained prototrophy for certain vitamins (34). Thus, although transconjugants were isolated by their ability to nodulate *Lotus* plants, growth of the transconjugant population could have been favored by an improved saprophytic competence.

In summary, although there is abundant evidence for extensive gene exchange among rhizobia, direct experimental data are required to understand the dynamics of rhizobial DNA exchange. Recently, the complete sequence of the 536-kb sym-

* Corresponding author. Mailing address: Departamento de Microbiología, Estación Experimental del Zaidín-CSIC, Prof. Albareda 1, E-18008 Granada, Spain. Phone: 34-958-121011. Fax: 34-958-129600. E-mail: Juan.Sanjuan@eez.csic.es.

biotic plasmid from *Rhizobium* sp. strain NGR234 has been reported (10); sequence analysis showed the presence of a cluster of genes homologous to the conjugal transfer genes of *Agrobacterium* Ti plasmids. Surprisingly, no one had previously noticed that this plasmid could be self-transmissible, and as Downie pointed out (8), sequencing of such a large plasmid seems a rather complicated way to find this out. Thus, feasible approaches are needed to identify what replicons are transferable by conjugation so that information about their transfer dynamics can be obtained quickly. In this work, we have developed an approach to identify replicons or parts of them that are susceptible of conjugative transfer, regardless of their stability or expression within the recipient cells. For this purpose, we have cloned DNA regions containing an origin of conjugative transfer (*oriT* or *mob*), the only known *cis*-acting function required for conjugative DNA transfer, defined by its ability to convert a nontransmissible vector into a mobilizable plasmid (19). We have used *Rhizobium meliloti* as an experimental model. All strains belonging to this species harbor two symbiotic megaplasmids; pSym1 (1,400 kb) carries nodulation and nitrogen fixation genes, whereas pSym2 (1,700 kb) carries genes required for exopolysaccharide production and dicarboxylate transport, also essential for symbiotic establishment (4, 5, 9, 21, 29). Given the sizes of these plasmids, symbiosis-related genes represent only a small portion of their genomes. Besides encoding essential symbiotic functions, pSyms carry nonsymbiotic genes, often involved in the metabolism of specific nutritional compounds (5). The fact that no *R. meliloti* strains cured of their pSyms have been obtained suggests that these plasmids also encode functions important for viability. Self-transfer of *R. meliloti* symbiotic megaplasmids in laboratory matings is barely detectable, although mobilization by IncP1-type plasmids has been shown, provided that a specific IncP1 mobilization (*mob* or *oriT*) region is previously incorporated in *cis* (9, 23). Thus, there is a general belief that conjugal transfer of *R. meliloti* pSyms occurs at very low frequencies in nature, probably because of their very large sizes. In addition to the pSyms, some strains of *R. meliloti* carry other plasmids, namely, nonsymbiotic or cryptic plasmids, which are not essential for symbiosis and in many cases have no specific function assigned. Cryptic plasmids may be self-transmissible at relatively high frequencies, and in some cases they have been shown to promote cotransfer of other accompanying plasmids (13, 21). *R. meliloti* GR4 carries, in addition to the two symbiotic megaplasmids, two nonsymbiotic large plasmids, pRmeGR4a and pRmeGR4b, of 110 and 140 MDa, respectively. pRmeGR4a has been shown to be self-transmissible to other *Rhizobium* and *Agrobacterium* species, and it can also support mobilization of plasmid pRmeGR4b (13). We have taken advantage of a strain GR4 gene library to identify potential DNA regions carrying *oriT*s. Our results show that despite their large sizes, *R. meliloti* replicons may be much more promiscuous than previously believed, and they provide new tools for studying and understanding the dynamics of rhizobial DNA exchange. (Part of this work was presented at the 16th North American Conference on Symbiotic Nitrogen Fixation, held in Cancun, Mexico, 1 to 6 February 1998.)

MATERIALS AND METHODS

Bacterial strains. *R. meliloti* GR4 and 2011 are wild-type strains. Strain 2011 carries three replicons, the chromosome and the two symbiotic megaplasmids, whereas strain GR4 carries two additional nonsymbiotic plasmids, pRmeGR4a and pRmeGR4b. GRM8SR is a Sm^r Rif^r GR4 derivative cured of cryptic plasmids pRmeGR4a and pRmeGR4b (13). Strain GR4KLR carries a β-glucuronidase cassette inserted within the *recA* gene. GR4KLR is therefore a $RecA^-$ derivative of strain GR4, which also carries a *lucOR*-Sm/Sp mini-Tn*5* insertion on plasmid pRmeGR4a and a kanamycin resistance cassette on plasmid pRmeGR4b (13, 14). Derivatives of GR4KLR are strains GRM6LR (cured of pRmeGR4b), GRM10KR (cured of pRmeGR4a), and GRM8R (cured of both plasmids pRmeGR4a and pRmeGR4b), which were obtained by heat treatment at 37°C (14). Strain GRT3 is a GR4 derivative carrying a deletion of pSym1 spanning the *nod-nif-fix* gene cluster (36). *Agrobacterium tumefaciens* C58 and C58(pRme2011a), which carries the *nod-nif* megaplasmid from *R. meliloti* 2011 (16), were provided by A. Pühler (University of Bielefeld, Bielefeld, Germany). *A. tumefaciens* 104 and 117 are also C58 derivatives carrying symbiotic megaplasmid pRmeSU47b (also known as pRme2011b), the pSym2 of *R. meliloti* SU47, or pRmeSU47a, respectively (9), and were provided by T. M. Finan (McMaster University, Hamilton, Ontario, Canada). The *R. meliloti* cosmid library used is constructed on cosmid vector pLAFR1 (Tc^r, RK2 Tra^- Mob^+ [7, 30]) and maintained in *Escherichia coli* HB101 (3). Cloning vector pJB3Tc19 encodes resistance to tetracycline and ampicillin (1) and is also a Tra^- Mob^+ RK2 derivative.

Bacterial matings. *R. meliloti* donor strains grown to an approximate optical density at 600 nm of 0.2, and recipient *R. meliloti* GRM8SR or *E. coli* HB101 strains grown to late exponential phase, were washed and mixed in a donor/recipient ratio of 1:1. Mating mixtures were resuspended in 50 μl of TY (tryptone-yeast extract-$CaCl_2$ [13, 14]) medium and loaded onto a sterile 0.45-μm-pore-size nitrocellulose filter. Filter mating mixtures were deposited on TY agar plates and incubated overnight at 30°C. Cells were resuspended by vigorous vortexing and diluted on liquid medium. *R. meliloti* GRM8SR transconjugants were selected on TY plates supplemented with appropriate antibiotics: streptomycin (200 mg/liter), rifampin (20 mg/liter), and tetracycline (10 mg/liter). *E. coli* HB101 transconjugants were selected on Endo agar (Difco) plates with antibiotics (tetracycline [10 mg/liter] and streptomycin [50 mg/liter]). To calculate transfer frequencies, donor, recipient and transconjugant CFU were counted after mating disruption and plating of appropriate dilutions. Donor and recipient spontaneous resistances to selective antibiotics were also determined. *R. meliloti* Rif^r strains arose at frequencies of 10^{-7} or lower, whereas resistance to tetracycline alone or to two antibiotics was undetectable ($<10^{-9}$).

DNA hybridizations and sequencing. Total genomic DNAs of *R. meliloti* or *A. tumefaciens* strains were isolated and digested with endonuclease *Eco*RI, electrophoresed on 0.7% agarose gels, and transferred to positively charged nylon membranes by the method of Southern (31). DNA hybridization probes were digoxigenin-labeled according to standard protocols (Boehringer, Mannheim, Germany). Hybridizations and membrane washes were carried out under high-stringency conditions. Membranes were prepared for chemoluminiscence detection (Boehringer) and exposed to Kodak X-Omat film. A 2,459-bp fragment from plasmid pRmOR69 was cloned into pUC18 (40) and sequenced with a Perkin-Elmer ABI Prism 373 automated sequencer. Samples were prepared for cycle sequencing according to the manufacturer's instructions. Sequencing was initiated with universal and reverse primers and continued with sequence-specific primers. Sequence was obtained for both strands. The Genetics Computer Group (University of Wisconsin) package was used in sequence analysis.

Nucleotide sequence accession number. The nucleotide sequence of the 2,459-bp *Bam*HI-*Eco*RI fragment has been deposited at the EMBL nucleotide sequence database under accession no. AJ223303.

RESULTS

Selection and identification of *mob* cosmids from an *R. meliloti* GR4 gene library. To identify *R. meliloti* GR4 DNA regions able to convert a nontransmissible vector into a mobilizable plasmid, we took advantage of a strain GR4 gene library constructed on pLAFR1. This cosmid vector is a RK2 derivative encoding tetracycline resistance and contains plasmid RK2 *oriT* (*mob*) but lacks all other *tra* functions (7). pLAFR1 can be mobilized in *trans* by plasmids carrying RK2-specific *tra* functions but cannot be mobilized from an *R. meliloti* genetic background. Similarly, we observed that randomly chosen cosmid clones could not be mobilized from this bacterium in the absence of a RK2-like (Tra^+) helper plasmid. The GR4 cosmid library was transferred from *E. coli* HB101 into each of the *recA* strains GR4KLR, GRM6LR, and GRM10KR by triparental matings, using pRK2013 as the helper plasmid (7), obtaining corresponding merodiploid populations where each individual carries a hybrid cosmid. The rationale of the approach consisted in using each merodiploid population as donors en masse in conjugation with the recipient strain *R. meliloti* GRM8SR. Transfer of pLAFR1-encoded tetracycline resistance into GRM8SR should occur only in the case of cosmids containing a *R. meliloti* DNA sequence serving as a functional transfer origin that can be recognized by *R. meliloti* Tra functions provided in *trans*. The use of *recA* donor strains was

TABLE 1. Identification of cosmids in GRM8SR transconjugants arising from matings using merodiploid donors[a]

Merodiploid donor	Frequency of transconjugants	No. of transconjugants analyzed	Cosmid type (occurrence)	No. of nonoverlapping cosmids
GR4KLR	6.0×10^{-7}	24	pRmOR69 (24)	1
GRM6LR	8.2×10^{-7}	24	pRmOR69 (10)	2
			pRmOR610 (4)	
			pRmOR612 (8)	
			pRmOR65 (2)	
GRM10KR	1×10^{-5}	44[b]	pRmOR106 (1)	9
			pRmOR1012 (1)	
			pRmOR1026 (1)	
			pRmOR1030 (1)	
			pRmOR1033 (1)	
			pRmOR1034 (3)	
			pRmOR1035 (1)	
			pRmOR1041 (1)	
			pRmOR1042 (4)	

[a] An *R. meliloti* GR4 gene library was transferred into each of the *recA* strains, and corresponding pools of merodiploids were used as donors in matings with strain GRM8SR. Transconjugants were selected for tetracycline resistance, encoded by the vector supporting the gene library. Cosmids from transconjugants were isolated and identified by *Eco*RI restriction analysis.

[b] Only a limited number of transconjugant cosmids could be analyzed by digestion with restriction endonucleases (see text for details).

TABLE 2. Frequencies of transfer of individual *mob* cosmids from different *R. meliloti recA* donor strains into *R. meliloti* GRM8SR as recipient

Cosmid	No. of transconjugants/recipient cell[a]		
	GR4KLR	GRM6LR	GRM10KR
pRmOR69	1.4×10^{-4}	8.3×10^{-5}	$<10^{-8}$
pRmOR65	7.5×10^{-5}	6.2×10^{-6}	$<10^{-8}$
pRmOR106	1.7×10^{-4}	1.3×10^{-4}	1.2×10^{-2}
pRmOR1012	1.8×10^{-3}	4.0×10^{-3}	3.7×10^{-2}
pRmOR1026	2.7×10^{-3}	1.8×10^{-3}	3.0×10^{-2}
pRmOR1030	3.0×10^{-3}	8.5×10^{-4}	6.0×10^{-2}
pRmOR1033	2.4×10^{-4}	1.5×10^{-4}	1.3×10^{-2}
pRmOR1034	9.3×10^{-4}	1.7×10^{-4}	4.0×10^{-2}
pRmOR1035	3.2×10^{-4}	4.0×10^{-4}	4.0×10^{-3}
pRmOR1041	5.4×10^{-4}	5.0×10^{-4}	9.0×10^{-3}
pRmOR1042	2.0×10^{-4}	3.4×10^{-4}	5.5×10^{-3}

[a] Average of at least two independent matings.

implemented to limit false mobilization due to cointegration, via homologous recombination, of hybrid cosmids with any of the self-transmissible or mobilizable replicons of *R. meliloti* GR4.

Using the GR4KLR merodiploids as donors, GRM8SR tetracycline-resistant transconjugants arose at a frequency of 6×10^{-7}. Cosmids from 24 such transconjugants were isolated by standard procedures and subjected to restriction analysis with endonuclease *Eco*RI (Table 1). All cosmids presented the same *Eco*RI pattern, suggesting that the hybrid cosmid pRmOR69 had been mobilized at high frequency. With merodiploid GRM6LR cells used as donors, we identified four different cosmids among GRM8SR transconjugants. One of them was identical to the previously isolated cosmid pRmOR69 and shared two *Eco*RI fragments with two other cosmids, pRmOR610 and pRmOR612, indicating that these three plasmids contained overlapping DNA inserts. The fourth cosmid had a completely different restriction pattern and was present in only 2 of 24 transconjugants analyzed (Table 1).

To verify that the isolated cosmids could indeed be mobilized from *R. meliloti*, individual plasmids were introduced back into each of the *R. meliloti recA* donors, and the corresponding strains were separately used in matings with the recipient GRM8SR. Cosmids pRmeOR69 and pRmOR65 could be mobilized only from strains carrying plasmid pRmeGR4a (Table 2, donors GR4KLR and GRM6LR), indicating that Tra functions required for their mobilization were provided by the conjugative plasmid pRmeGR4a. This finding suggested that these two cosmids contained the *oriT*s of plasmids pRmeGR4a and/or pRmeGR4b.

To determine the genomic locations of the cloned *mob* DNAs, each cosmid was used as a hybridization probe against genomic DNAs from several *R. meliloti* strains with different plasmid contents, as well as DNAs from *A. tumefaciens* strains carrying pSym1 or pSym2 of *R. meliloti*. We used genomic DNAs from these strains (i) to ascertain the locations of the different cosmid insert DNAs among the various replicons of *R. meliloti*, as a strain lacking a particular plasmid replicon should not give specific hybridization signals, thus enabling us to discern possible cross-hybridization with other replicons; and (ii) to verify that the cloned DNAs had not undergone any rearrangements due to the various manipulations to which they had been subjected (e.g., genomic DNA of the wild-type strain GR4 and cosmid DNAs should give identical hybridization profiles). As shown in Fig. 1, plasmid pRmOR69 contains DNA from the cryptic plasmid pRmeGR4a, as hybridizing bands corresponding to the cosmid probe (lane 9) were present only in strains carrying plasmid pRmeGR4a (lanes 4, 5, and 8), although the cloned DNA cross-hybridized with plasmid pRmeGR4b (lanes 4, 6, and 8). The insert DNA in pRmOR65 belongs to the nonsymbiotic plasmid pRmeGR4b (lanes 4, 6, and 8) and also cross-hybridizes with plasmid pRmeGR4a (Fig. 1, noncosmid bands in lanes 4, 5, and 8). Although pRmeGR4b is not a self-transmissible plasmid, we have previously shown that it can be mobilized by pRmeGR4a in *trans* (13), which implies that pRmeGR4b must contain at least an origin of transfer that is recognized by pRmeGR4a-specific Tra system. Thus, the replicon localization of *mob* cosmids pRmOR69 and pRmOR65 explains why they cannot be mobilized from strains lacking pRmeGR4a (donor GRM10KR [Table 2]). Furthermore, transfer frequencies of *mob* cosmids pRmOR69 and

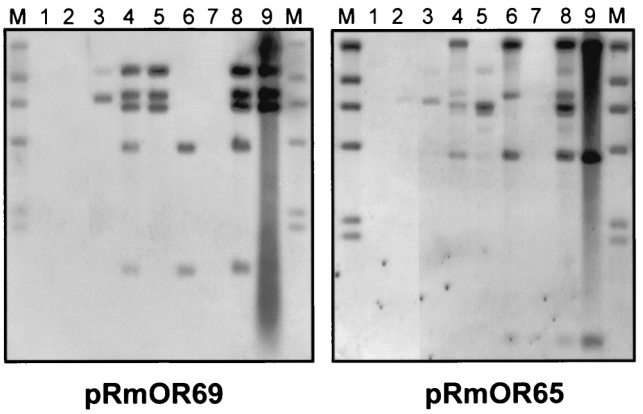

FIG. 1. Replicon localization of *mob* DNAs in cosmids pRmOR69 and pRmOR65. Blots of *Eco*RI-digested genomic DNAs were hybridized against digoxigenin-labeled cosmid probes. Lanes: M, digoxigenin-labeled molecular weight marker; 1, *R. meliloti* 2011 (wild-type); 2, *A. tumefaciens* C58; 3, *A. tumefaciens* C58(pRme2011a); 4, *R. meliloti* GR4 (wild type); 5, *R. meliloti* GRM6 (cured of plasmid pRmeGR4b); 6, *R. meliloti* GRM10 (cured of plasmid pRmeGR4a); 7, *R. meliloti* GRM8 (cured of pRmeGR4a and pRmeGR4b); 8, *R. meliloti* GRT3 (carrying pSym1 deletion); 9, *Eco*RI-digested cosmid DNA.

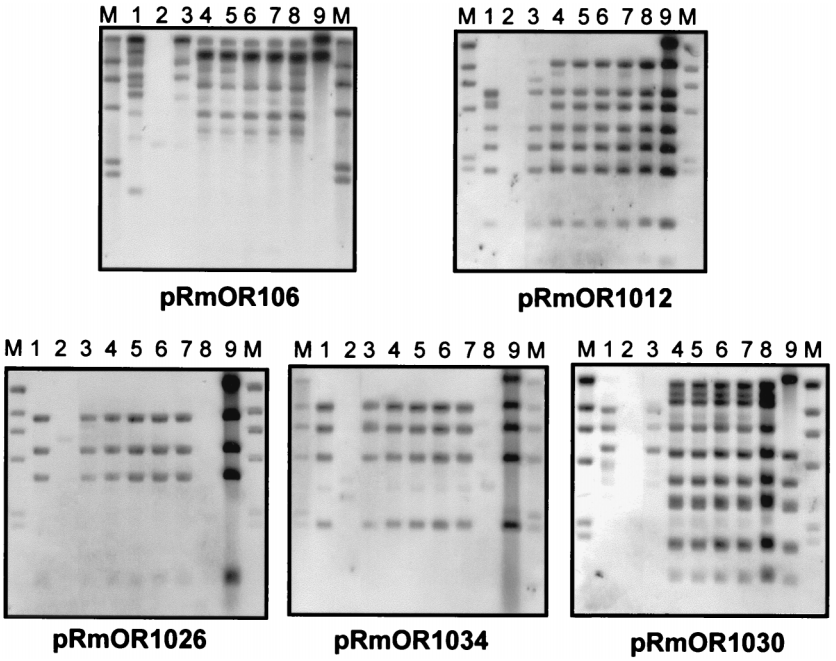

FIG. 2. Replicon localization of *mob* DNAs in cosmids pRmOR106, -1012, -1026, -1034, and -1030. Blots of *Eco*RI-digested genomic DNAs were hybridized against digoxigenin-labeled cosmid probes. Lanes: M, digoxigenin-labeled molecular weight marker; 1, *R. meliloti* 2011 (wild type); 2, *A. tumefaciens* C58; 3, *A. tumefaciens* C58(pRme2011a); 4, *R. meliloti* GR4 (wild type); 5, *R. meliloti* GRM6 (cured of plasmid pRmeGR4b); 6, *R. meliloti* GRM10 (cured of plasmid pRmeGR4a); 7, *R. meliloti* GRM8 (cured of pRmeGR4a and pRmeGR4b); 8, *R. meliloti* GRT3 (carrying pSym1 deletion); 9, *Eco*RI-digested cosmid DNA.

pRmOR65 were similar to those of plasmids pRmeGR4a and pRmeGR4b, respectively (data not shown and reference 13).

When the donor was the GRM10KR (which lacks the conjugative cryptic plasmid pRmeGR4a) merodiploid population, GRM8SR transconjugants appeared at a frequency of 10^{-5} (Table 1). In this case, most of the isolated transconjugant cosmids could not be completely digested with *Eco*RI or several other restriction enzymes. We have no explanation for this failure in digesting cosmid DNAs, but it may be related to a particular protection of rhizobial insert DNAs, since pLAFR1-specific restriction fragments could be detected. We then decided to use these DNAs to transform *E. coli* HB101 competent cells. Successful restriction analysis of 14 transforming cosmids identified nine nonoverlapping *Eco*RI restriction patterns (Table 1, cosmids pRmOR106 to pRmOR1042), all different from the previously identified *mob* cosmids pRmeOR69 and pRmeOR65.

All of these nine nonoverlapping hybrid cosmids were confirmed to be mobilized at high frequency from all *recA* donors (Table 2). The transfer efficiency, however, varied according to the genetic background of the donor, and they were more efficiently mobilized from GRM10KR than from GR4KLR or GRM6LR (Table 2), which explains their preferential occurrence among transconjugants derived from the mating using GRM10K merodiploids as donors (Table 1).

Genomic localization demonstrated that four *mob* plasmids, pRmOR106, -1012, -1026, and -1034, contain DNA from pSym1 (Fig. 2). Similar or identical hybridization patterns were found in *R. meliloti* GR4, *R. meliloti* 2011, and *A. tumefaciens* C58 (pRme2011a) (lanes 1, 3, and 4 of all blots in Fig. 2). Moreover, strain GRT3, a GR4 derivative carrying a pSym1 deletion spanning a few hundred kilobases around the *nod-nif* cluster (36), did not hybridize with cosmids pRmOR1026 and pRmOR1034 (lanes 8 of corresponding blots in Fig. 2), which further confirms that the cloned DNAs in these two cosmids belong to pSym1. The insert DNA in cosmid pRmOR106 contains two *Eco*RI fragments of approximately 13 kb, both present in strain GR4 and derivatives, whereas only one strongly hybridizing fragment of about 25 kb was found in strains 2011 and C58(pRme2011a) (lanes 1 and 3). This difference may be due to variations in restriction sites between the two wild-type strains 2011 and GR4. Another cosmid, pRmOR1030, appears to contain DNA that might be repeated within the genome of strain GR4, as multiple cross-hybridization bands were visible (Fig. 2, blot pRmOR1030, lanes 4 to 8). At least some of the nonspecific bands may be due to pSym1 DNA, since several hybridizing bands were detected in strains 2011 and C58(pRme2011a) (lanes 1 and 3). However, the DNA cloned in pRmOR1030 may be unique to strain GR4, as cosmid-specific hybridization fragments were detected in strain GR4 and its derivatives (lanes 4 to 9) but not in the wild-type strain 2011 (lane 1). Therefore, it is not possible to assign a specific genome location to the insert DNA in cosmid pRmOR1030.

As shown in Fig. 3, cosmid pRmOR1035 clearly carries DNA from pSym2, as identical hybridization profiles were found in *R. meliloti* 2011 and *A. tumefaciens* 104 (lanes 1 and 3), although this profile coincided only partially with strain GR4 and the cosmid probe (lanes 5 and 6). Finally, cosmids pRmOR1033, pRmOR1041, and pRmOR1042 clearly contain DNA present in both of the *R. meliloti* wild-type strains 2011 and GR4 with almost identical restriction profiles (lanes 1, 5, and 6 of the corresponding blots in Fig. 3), but these DNAs do not correspond to any of the plasmid replicons harbored by these strains (no signals in lanes 3 and 4). Since no additional plasmid replicons are known to exist in *R. meliloti*, they are preliminarily considered to be chromosomal *mob* DNA regions, although direct demonstration of their genomic locations will be required to confirm this point.

The results indicated that on the one hand, our approach was effectively selecting for mobilizable hybrid cosmids present

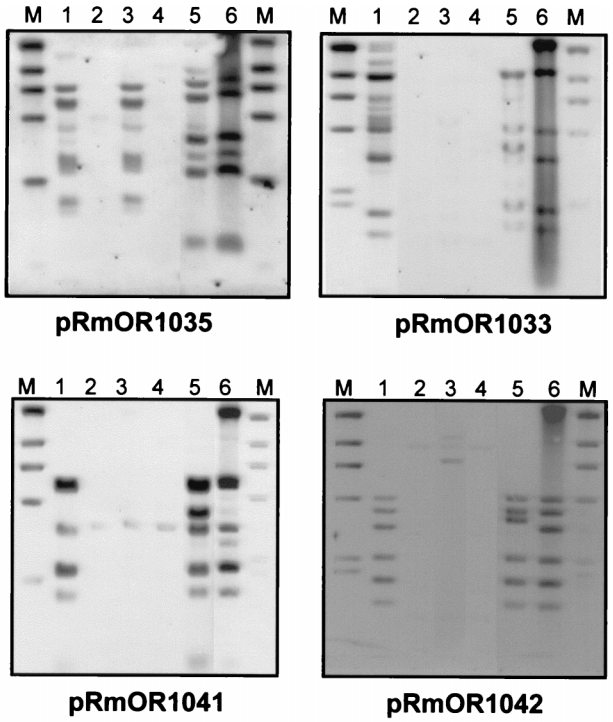

FIG. 3. Genome localization of *mob* DNAs in cosmids pRmOR1035, -1033, -1041, and -1042. Blots of *Eco*RI-digested genomic DNAs were hybridized against digoxigenin-labeled cosmid probes. Lanes: M, digoxigenin-labeled molecular weight marker; 1, *R. meliloti* 2011 (wild type); 2, *A. tumefaciens* C58; 3, *A. tumefaciens* 104 (C58 carrying pRmeSU47b); 4, *A. tumefaciens* 117 (C58 carrying pRmeSU47a); 5, *R. meliloti* GR4 (wild type); 6, *Eco*RI-digested cosmid DNA.

in the strain GR4 gene library, and on the other hand, the transfer efficiency of these cloned DNAs depended on the plasmid content of the donor strain. It is noteworthy that most (7 of 11) of the selected *mob* cosmids carried DNA from plasmid replicons. However, given its rationale, our strategy had also been expected to select for any existing chromosomal DNAs able to serve as conjugal transfer origins.

Characterization of the *oriT* of plasmid pRmeGR4a. In strain GR4, the only known important conjugative replicon is the self-conjugative cryptic plasmid pRmeGR4a, which can conjugate into rhizobia as well as into agrobacterial strains at relatively high frequencies (13). Thus, to further confirm that our approach selects for *oriT*-containing cosmids from the strain GR4 gene library, we characterized the *oriT* of plasmid pRmeGR4a. As shown above (Fig. 1), cosmid pRmOR69 was identified as putatively containing the *oriT* of this plasmid. As indicated in Table 1, the *mob* cosmids pRmOR69, pRmOR610, and pRm612 shared two *Eco*RI fragments of 7 and 6 kb, respectively. Thus, the *mob* function present in these three cosmids should be localized in one of these two fragments. To verify which fragment contains the *mob* function, each *Eco*RI fragment was subcloned into vector pJB3Tc19 (1). Like pLAFR1, pJB3Tc19 is an RK2 derivative which carries a *mob* site but cannot be mobilized from *R. meliloti* unless RK2-specific *tra* functions are provided in *trans*. By functional subcloning, we found that only the 6-kb *Eco*RI fragment conserved the ability to convert vector pJB3Tc19 into a mobilizable plasmid from *R. meliloti* GRM6LR (Fig. 4A). Further subcloning demonstrated that this *mob* function resides within a 2.5-kb *Bam*HI-*Eco*RI fragment. Sequence analysis showed that it is 2,459 bp long and has a genetic organization strongly resembling that of plasmid *oriT*s. Two divergently transcribed open reading frames (ORFs) separated by an A+T-rich sequence stretch were identified (Fig. 4B). At positions 900 to 907 we identified the sequence 5′-TATCCTGC-3′, which matches the consensus found in the nick region, the recognition site for relaxase of plasmid *oriT*s of the P group, which includes plasmids RP4/RK2 and R751 as well as plasmid pTF-FC2 of *Thiobacillus ferrooxidans* and the T-DNA borders of the Ti plasmid of *A. tumefaciens* (reviewed in reference 19). The putative *nic* site within this sequence would be located between nucleotides (nt) G-906 and C-907. Immediately upstream of this 8-nt sequence

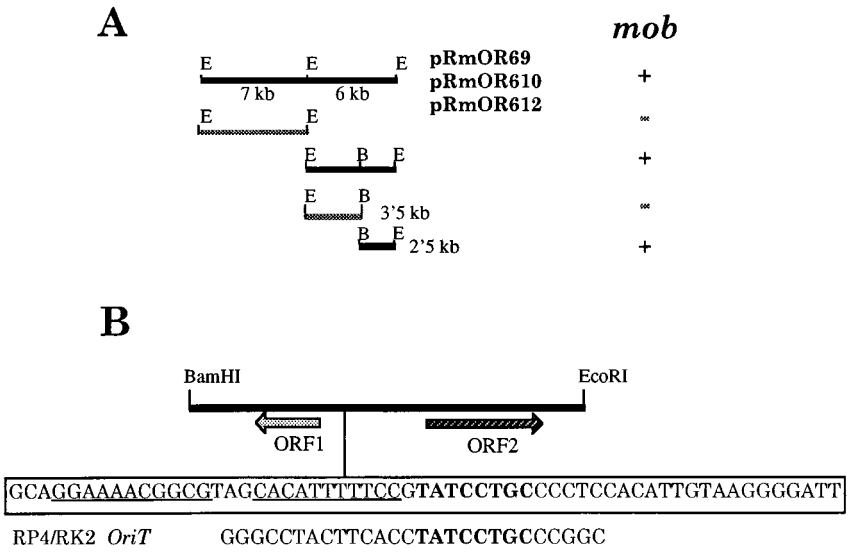

FIG. 4. Characterization of the *mob* site (*oriT*) of plasmid pRmeGR4a. (A) Identification of a 2.5-kb *Bam*HI-*Eco*RI fragment from cosmids pRmOR69, -610, and -612 that contains the *mob* function. Functional subcloning identified the smaller fragment with the ability to convert vector pJB3Tc19 into a mobilizable plasmid from *R. meliloti* GRM6LR (fragments marked mob^+). (B) Genetic organization of the *Bam*HI-*Eco*RI fragment containing the *mob* site of pRmeGR4a. The directions of transcription of ORF1 and ORF2 are indicated. The 8-nt sequence matching the nick region within P-type plasmid *oriT*s is shown in boldface, as is the corresponding sequence in plasmid RP4/RK2 (19). An upstream imperfect inverted repeat is underlined.

there is a imperfect inverted repeat (Fig. 4B), a situation also found in all nick regions of known conjugative or mobilizable plasmids. These structural elements are abundant within *oriT*s and are believed to function as recognition sites for specific DNA-binding proteins (19). Whereas the A+T content of the entire 2,459-bp fragment is 41%, it is greater in sequence near the putative nick region (50% A+T between nt 850 to 950).

ORF1 (Fig. 4B) encodes a protein of 133 amino acids (aa) with an estimated M_r of 14,632. Orf1 showed significant homology with MbeC from plasmid ColE1 (2), a protein involved in conjugal transfer of plasmid ColE1 that is part of the relaxosome complex required for transfer initiation at *oriT*. Over a length of 74 aa (corresponding to approximately 70% of the length of MbeC), Orf1 and MbeC are 40% identical. The Orf1 protein is also 36% identical to *T. ferrooxidans* plasmid pTF4.1 Orf4 (GenBank accession no. X96982), which is located adjacent to the nick region within the *oriT* of pTF4.1. Likewise, Orf1 shows significant homology with MobC from plasmid pEC3 of *Erwinia carotovora* (22), which is also located within the *mob* region of this plasmid.

ORF2 is transcribed divergently from ORF1 (Fig. 4) and putatively encodes a protein of 240 aa. Orf2 has in its N-terminal region the sequence GKGGAGKT, which corresponds to the P-loop consensus present in many ATP/GTP-binding proteins (27, 38). Orf2 has homology with ParA of plasmid pTAR (11) and VirC1 of plasmids pTiA6NC (41) and pTiC58 (6) of *A. tumefaciens*. These homologies were more significant within the N-terminal halves of the proteins. Over a 120-aa range, the Orf1 sequence is 27% identical to ParA and 34% identical to VirC1 from pTiA6NC. ParA is involved in the efficient distribution of the plasmid molecules to the daughter cells, thus ensuring plasmid stability (11), whereas VirC1 is required for efficient processing of Ti DNA by VirD proteins, probably by binding to overdrive sequences located to the right of the T_R and T_L borders of Ti DNA, thus contributing to the formation of the T strand that is transferred to the plant cell (39, 41). Both ParA and VirC1 appear to be DNA-binding proteins, although their mechanisms of action are not completely clear. Orf1 also has discrete homology with a large number of bacterial proteins that bind ATP or GTP. These homologies were restricted around the ATP/GTP-binding sequence found in Orf2.

Thus, this 2,459-bp fragment is capable of converting vectors pLAFR1 and pJB3Tc19 into mobilizable plasmids from *R. meliloti*, and it also has a genetic structure typical of conjugative plasmid *oriT*s. These findings strongly suggest that it contains the *oriT* of the conjugative plasmid pRmeGR4a. However, more detailed genetic analysis is required to verify the specific roles of the sequences and genes identified in transfer of pRmeGR4a.

Conjugal transfer of *R. meliloti mob* plasmids into *E. coli*. It is known that rhizobial plasmids are capable of conjugal transfer to species within the family *Rhizobiaceae*, where they can stably replicate (21). However, it is not known whether rhizobial plasmids can transfer into bacterial hosts unable to support their replication. Thus, the cloning of *R. meliloti* mobilizable DNA regions into a broad-host-range cloning vector such as pLAFR1 provided a unique opportunity to verify if this species can transfer its plasmid DNA to bacteria where they cannot replicate. As an extreme example, we chose *E. coli*, a gram-negative bacteria unrelated to the *Rhizobiaceae* that cannot support stable replication of rhizobial plasmids.

We set up matings between *R. meliloti recA* donors carrying individual *mob* cosmids and *E. coli* HB101 as the recipient. The pLAFR1 vector can replicate in both *Rhizobium* spp. and *E. coli*. Transconjugants were selected for the acquisition of the pLAFR1-encoded tetracycline resistance on Endo agar, a medium specific for the coliforms where *R. meliloti* is unable to grow. All plasmids were found to be transferable from *R. meliloti* to *E. coli* HB101. As with *R. meliloti* recipients, the efficiency of transfer to *E. coli* was greatly dependent on the donor plasmid content. Efficiencies of transfer from strains GR4KLR and GRM10KR (both carry the nonsymbiotic plasmid pRmeGR4b) were several orders of magnitude lower than from strains GRM6LR (which lacks pRmeGR4b) and GRM8R (which lacks both pRmeGR4a and pRmeGR4b), indicating that the presence of plasmid pRmeGR4b in the donor strain was responsible for a significant reduction of transfer efficiency. On the other hand, it was surprising that in most cases, transfer from strain GRM6LR into *E. coli* was more efficient than transfer into *R. meliloti* GRM8SR. Except for plasmid pRmeGR4a, the *recA* mutation, and the different antibiotic resistances, strains GRM6LR and GRM8SR are genetically identical (both carry the two pSyms and the chromosome). Therefore, it is possible that in the recipient strain GRM8SR there are some surface exclusion mechanisms which could be responsible for a reduction in transfer efficiency. As shown in Table 3, chromosomal *mob* cosmids were transferred to *E. coli* as efficiently as those carrying *mob* sites of plasmid localization.

TABLE 3. Frequencies of transfer of individual *mob* cosmids from different *R. meliloti recA* donors into *E. coli* HB101[a] as recipient

Cosmid	No. of transconjugants/recipient cell[b]			
	GR4KLR	GRM6LR	GRM10KR	GRM8R
pRmOR69	4.1×10^{-6}	3.3×10^{-4}	NT	NT
pRmOR65	4.1×10^{-8}	1.0×10^{-3}	NT	NT
pRmOR106	3.1×10^{-7}	5.8×10^{-1}	2.1×10^{-7}	2.3×10^{-1}
pRmOR1012	1.0×10^{-7}	3.9×10^{-1}	5.6×10^{-4}	1.6×10^{-1}
pRmOR1026	8.3×10^{-8}	1.5×10^{-1}	2.1×10^{-4}	8.3×10^{-2}
pRmOR1030	3.3×10^{-6}	2.1×10^{-1}	8.9×10^{-5}	9.1×10^{-5}
pRmOR1033	8.3×10^{-8}	5.6×10^{-2}	$<10^{-8}$	1.4×10^{-1}
pRmOR1034	5.4×10^{-7}	1.8×10^{-1}	1.0×10^{-6}	1.1×10^{-1}
pRmOR1035	3.3×10^{-7}	2.1×10^{-1}	2.3×10^{-6}	3.7×10^{-2}
pRmOR1041	$<10^{-8}$	3.5×10^{-1}	3.3×10^{-5}	3.3×10^{-1}
pRmOR1042	1.9×10^{-7}	8.3×10^{-1}	4.5×10^{-4}	2.6×10^{-1}

[a] HB101 transconjugants were selected on Endo agar plates supplemented with tetracycline.
[b] Average of two independent matings; NT, not tested.

The fact that *mob* plasmids can be mobilized into *E. coli*, a phylogenetically distant gram-negative bacterium, shows that *R. meliloti* can transfer DNA into bacterial species outside the family *Rhizobiaceae* and indicates that transfer promiscuity of rhizobial replicons may be much broader than their known replication host range.

DISCUSSION

The initiation complex for conjugative transfer of transmissible plasmids is called relaxosome, a specific DNA-protein structure that includes the relaxase, a protein that catalyzes the specific cleavage of a phosphodiester bond at the *nic* site within the origin of transfer, as well as accessory DNA-binding proteins (19). The transfer origin (*oriT* or *mob*) from conjugative or mobilizable plasmids is the only known *cis*-acting function required for DNA transfer. We have devised a simple approach to identify *R. meliloti* DNA regions that comply with the definition of *oriT*, i.e., have the capacity to convert a nontransmissible vector into a mobilizable plasmid (19). According to this definition, potential sequences able to serve as *oriT*s must be present in a genomic library from the desired organism, and therefore can be selected by taking advantage of their intrinsic

properties, provided that the cloning vector supporting the gene library cannot be mobilized from a given genetic background. This is the case for cosmid pLAFR1, which cannot be mobilized from an *R. meliloti* background in the absence of RK2-specific Tra functions. Moreover, the use of a gene library with a large insert average allows cloning of not only these *oriT*s but also genes related to transfer that are likely to be located next to the nick region (19). Using this strategy, we have been able to identify 11 DNA regions from *R. meliloti* GR4 that are able to convert pLAFR1 into a mobilizable plasmid and therefore must contain origins of conjugative transfer or *mob* sites. The use of *recA* strains has undoubtedly facilitated this selection, by limiting the possibility of homologous recombination between hybrid cosmids and conjugative resident replicons, which could have led to replicon cointegration, giving rise to false transconjugants containing no mobilizable plasmids. Interestingly, seven of the cloned regions clearly correspond to plasmid replicons harbored by *R. meliloti* GR4. This finding further strengthens the reliability of our approach, as it was devised to select for plasmid *oriT*s. Moreover, from one of the *mob* cosmids we were able to identify and characterize a 2,459-bp fragment containing the *oriT* of the conjugative plasmid pRmeGR4a, thus confirming that this approach selects for *mob* sites. At least three of the cloned *mob* regions could not be assigned to any plasmid replicon and therefore appear to correspond to the chromosome. No specific location within the strain GR4 genome could be assigned to the DNA cloned in cosmid pRmOR1030, which seemed to contain DNA that is present in *R. meliloti* GR4 but not in *R. meliloti* 2011. To our knowledge, natural occurrence of *oriT*s in the bacterial chromosome has not been reported. Nevertheless, if chromosomal *oriT*s were to occur, a strategy like the one used here should have been able to select for them. It is well known that stable integration of a conjugative plasmid into the bacterial chromosome gives rise to high-frequency-of-recombination (Hfr) donor strains (25). It is possible that *R. meliloti* strains used in this work are naturally occurring Hfr strains, containing one or more episomes. Alternatively, the cloned *mob* regions may correspond to conjugative transposons that could be present in these strains, as has been shown for other gram-negative bacteria (26). It is possible that conjugative elements are not rare in rhizobial chromosomes. It seems that conjugal transfer may be operating in the case reported by Sullivan and colleagues (32–34), identifying lateral transfer of a chromosomally located symbiotic region from *R. loti* to bacteria resident in the soil.

It is not known if all *R. meliloti* GR4 *mob* regions have been identified. Since we have analyzed limited numbers of transconjugants, and given the unexpected problems found with the restriction analysis of certain cosmids, it is possible that there are additional *mob* regions in the *R. meliloti* genome. On the other hand, given the size of the selected hybrid cosmids (average insert size of 25 kb), it is likely that other genes related to the transfer process are cloned along with their *oriT*s, as in many other systems where *oriT*s are usually located within the transfer gene complex (19). This should facilitate further characterization of the various conjugative systems.

A single *mob* region was identified for each of the *R. meliloti* GR4-resident cryptic plasmids, pRmeGR4a and pRmeGR4b, cloned in pRmOR69 and pRmOR65, respectively. pRmeGR4a is known to be self-transmissible and able to mobilize pRmeGR4b in *trans* (13). Efficient transfer of both *mob* cosmids to *R. meliloti* recipients required the presence of plasmid pRmeGR4a in the donor strain. All of these results show that cosmids pRmOR69 and pRmOR65 contain the *oriT*s of plasmids pRmeGR4a and pRmeGR4b, respectively. In fact, we were able to delimit the *mob* function of cosmid pRmOR69 to a 2,459-bp *Bam*HI-*Eco*RI fragment, which has a genetic organization typical of plasmid *oriT*s (19). Two divergently transcribed ORFs were identified, one encoding a putative protein with homology to proteins involved in *oriT* DNA processing during conjugation (Orf1) and another encoding a protein homologus to plasmid processing and stabilization proteins (Orf2); the two ORFs are separated by an A+T-rich stretch that includes an 8-nt sequence identical to the consensus of nick regions of a family of conjugative plasmids (Fig. 4). If the putative *nic* site identified within this fragment is indeed functional, ORF2 would be the first gene to enter the recipient cell whereas ORF1 would enter the recipient last. These data strongly support the conclusion that this fragment contains the *oriT* of plasmid pRmeGR4a and provide further evidence for the efficacy of our approach in selecting for *R. meliloti oriT*s.

Several *mob* cosmids were found to contain DNA from symbiotic pSym1, but only one (pRmOR1035) corresponded to pSym2. By analogy to pSym1, it is possible that pSym2 also carries more than one *oriT* which could not be identified in this work, as discussed above. The existence of multiple *oriT*s in pSyms may relate to the large size of these molecules, in line with the experimental evidence indicating that *R. meliloti* megaplasmids contain more than one origin of replication (20). Thus, *R. meliloti* pSyms may represent the evolutionary cointegration of several plasmids that have maintained some or all their *rep* and *tra* functions. Compared to previous reports investigating self-transfer of *R. meliloti* megaplasmids, we found that transfer of individual *mob* cosmids is up to 10^6-fold more efficient than transfer of the entire plasmids (9, 23). Although the *mob* cosmids in this work are about 30 to 40 times smaller than pSyms, this could only partially explain the difference in transfer efficiency. It seems then that transfer of the complete plasmid is inefficient compared to the rates of transfer initiation. The presence of several *oriT*s may represent a drawback for transfer of the entire plasmid, as DNA mobilization could simultaneously initiate (and terminate) at different sites. This possibility suggests that transfer of the plasmid can take place as separate DNA fragments (albeit of several hundreds kilobases each) and that reconstitution of the entire plasmid after transfer to the recipient cell would be required. This possibility is not without precedents, as several catabolic and R plasmids have been shown to form cointegrates that dissociate or reassociate after transfer (12). On the other hand, the existence of multiple functional *oriT*s in the same plasmid has been shown to produce preferential transfer of deletant plasmid versions which contain hybrid *oriT*s and lack the spacer regions located between *oriT*s (reference 19 and references therein). Since termination of transfer at *oriT* is not 100% effective, full-length plasmid copies are eventually transferred (19). The occurrence of multiple *oriT*s in *R. meliloti* pSyms could thus explain the great difference in transfer efficiency of the entire replicon and that of the *mob* regions identified in this work, as the existence of multiple *oriT*s would lead to preferential transfer of deletant plasmid versions, which will not replicate in the recipient unless an origin of replication and stability functions are transferred as well. On the other hand, the existence of multiple *oriT*s may be related to the plasmid promiscuity, allowing transfer to a more diverse set of recipient species. For instance, mobilization of the entire plasmid could preferentially initiate at a particular *oriT* depending on the environmental conditions and/or the recipient available, thus increasing opportunities for the plasmid to spread.

All *mob* plasmids identified in this work were efficiently mobilized from *R. meliloti* into *E. coli*. To our knowledge, this is the first report demonstrating physical transfer of resident

DNA from *R. meliloti* into *E. coli* without the assistance of nonrhizobial plasmid-encoded Tra functions. However, efficiency of transfer into *E. coli* greatly depended on the plasmid content of the donor strain, which could reflect the complex interactions between different replicons, carrying mostly DNA of unknown function, residing in the same bacterium. In terms of horizontal gene transfer, our results show that *R. meliloti* plasmids, and likely the chromosome, are capable of promoting efficient DNA transfer to species where replicons cannot be stably maintained and therefore can be the subject of "suicide" conjugal transfer. The fate of the DNA transferred into such genetic backgrounds would be either its loss or its incorporation into the recipient genome by homologous recombination or by other means such as those involving insertion elements, which are known to be abundant in *R. meliloti* (21). The result, nevertheless, is a situation where opportunities for DNA exchange are much higher than previously observed.

The approach used here has demonstrated its reliability in selecting mobilizable DNA from different replicons and should be easily applicable to other bacteria. Future characterization of the *mob* regions identified in this work will provide a better understanding of conjugal gene transfer in rhizobia.

ACKNOWLEDGMENTS

This work was supported in part by grant BIO2-CT93-0053 (IMPACT project) of the EU Biotechnology Action Programme and the Plan Andaluz de Investigación of the Junta de Andalucia (Spain).

We are grateful to A. Pühler and T. M. Finan for providing agrobacterial strains and to J. A. Acevedo and S. Muñoz for excellent assistance during experimental setup.

REFERENCES

1. **Blatny, J. M., T. Brautaset, H. C. Winther-Larsen, K. Haugan, and S. Valla.** 1997. Construction and use of a versatile set of broad-host-range cloning and expresion vectors based on the RK2 replicon. Appl. Environ. Microbiol. **63:** 370–379.
2. **Boyd, A. C., J. A. K. Archer, and D. J. Sherratt.** 1989. Characterization of the ColE1 mobilization region and its proteins products. Mol. Gen. Genet. **217:** 488–498.
3. **Boyer, H. W., and D. Roulland-Dussoix.** 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. **41:**459–465.
4. **Burkhardt, B., D. Schillik, and A. Pühler.** 1987. Physical characterization of *Rhizobium meliloti* megaplasmids. Plasmid **17:**13–25.
5. **Charles, T. C., and T. M. Finan.** 1991. Analysis of a 1600-kilobase *Rhizobium meliloti* megaplasmid using defined deletions generated *in vivo*. Genetics **127:** 5–20.
6. **Close, T. J., R. C. Tait, H. C. Rempel, T. Hirooka, L. Kim, and C. I. Kado.** 1987. Molecular characterization of the *virC* genes of the Ti plasmid. J. Bacteriol. **169:**2336–2344.
7. **Ditta, G., S. Stanfield, D. Corbin, and D. R. Helinski.** 1980. Broad host range DNA cloning system for Gram-negative bacteria. Construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. USA **77:**7347–7351.
8. **Downie, A.** 1997. Fixing a symbiotic circle. Nature **387:**352–354.
9. **Finan, T. M., B. Kunkel, G. F. de Vos, and E. R. Signer.** 1986. Second symbiotic megaplasmid in *Rhizobium meliloti* carrying exopolysaccharide and thiamine synthesis genes. J. Bacteriol. **167:**66–72.
10. **Freiberg, C., R. Fellay, A. Bairoch, W. J. Broughton, A. Rosenthal, and X. Perret.** 1997. Molecular basis of symbiosis between *Rhizobium* and legumes. Nature **387:**394–401.
11. **Gallie, D. R., and C. I. Kado.** 1987. *Agrobacterium tumefaciens* pTAR *parA* promoter region involved in autorregulation, incompatibility and plasmid partitioning. J. Mol. Biol. **193:**465–478.
12. **Hardman, D. J., and P. C. Gowland.** 1985. Large plasmids in bacteria. Part 2. Genetics and evolution. Microbiol. Sci. **2:**184–190.
13. **Herrera-Cervera, J. A., J. Olivares, and J. Sanjuan.** 1996. Ammonia inhibition of plasmid pRmeGR4a conjugal transfer between *Rhizobium meliloti* strains. Appl. Environ. Microbiol. **62:**1145–1150.
14. **Herrera-Cervera, J. A., F. I. Rodriguez-Alonso, J. Olivares, and J. Sanjuan.** 1997. Evaluation of the *rec*A-based containment system in *Rhizobium meliloti* GR4. FEMS Microbiol. Ecol. **22:**49–56.
15. **Hirsch, P. R., and J. D. Spokes.** 1994. Survival and dispersion of genetically modified rhizobia in the field and genetic interactions with native strains. FEMS Microbiol. Ecol. **15:**147–160.
16. **Hynes, M. F., R. Simon, and A. Pühler.** 1985. The development of plasmid-free strains of *Agrobacterium tumefaciens* by using incompatibility with *Rhizobium meliloti* plasmid to eliminate pAtC58. Plasmid **13:**99–105.
17. **Kinkle, B. K., and E. L. Schmidt.** 1991. Transfer of the pea symbiotic plasmid pJB5JI in nonsterile soil. Appl. Environ. Microbiol. **57:**3264–3269.
18. **Laguerre, G., E. Geniaux, S. I. Mazurier, R. R. Casartelli, and N. Amarger.** 1993. Conformity and diversity among field isolates of *Rhizobium leguminosarum* bv. *viciae*, bv. *trifolii* and bv. *phaseoli* revealed by DNA hybridization using chromosome and plasmid probes. Can. J. Microbiol. **39:**412–419.
19. **Lanka, E., and B. M. Wilkins.** 1995. DNA processing reactions in bacterial conjugation. Annu. Rev. Biochem. **64:**141–169.
20. **Margolin, W., and S. R. Long.** 1993. Isolation and characterization of a DNA replication origin from the 1,700-kilobase-pair symbiotic megaplasmid pSym-b of *Rhizobium meliloti*. J. Bacteriol. **175:**6553–6561.
21. **Mercado-Blanco, J., and N. Toro.** 1996. Plasmids in rhizobia: the role of nonsymbiotic plasmids. Mol. Plant-Microbe Interact. **9:**535–545.
22. **Nomura, N., M. Yamashita, and Y. Murooka.** 1996. Genetic organization of a DNA-processing region required for mobilization of a non-self-transmissible plasmid, pEC3, isolated from *Erwinia carotovora* subsp. *carotovora*. Gene **170:**57–62.
23. **Pretorius-Güth, I.-M., A. Pühler, and R. Simon.** 1990. Conjugal transfer of megaplasmid 2 between *Rhizobium meliloti* strains in alfalfa nodules. Appl. Environ. Microbiol. **56:**2354–2359.
24. **Rao, J. R., M. Fenton, and B. D. W. Jarvis.** 1994. Symbiotic plasmid transfer in *Rhizobium leguminosarum* biovar *trifolii* and competition between the inoculant strain ICMP2163 and transconjugants soil bacteria. Soil Biol. Biochem. **26:**339–351.
25. **Reimmann, C., and D. Haas.** 1993. Mobilization of chromosomes and nonconjugative plasmids by cointegrative mechanisms, p. 137–188. *In* D. B. Clewell (ed.), Bacterial conjugation. Plenum Press, New York, N.Y.
26. **Salyers, A. A., N. B. Shoemaker, A. M. Stevens, and L.-Y. Li.** 1995. Conjugative transposons: an unusual and diverse set of integrated gene transfer elements. Microbiol. Rev. **59:**579–590.
27. **Saraste, M., P. R. Sibbald, and A. Wittinghofer.** 1990. The P-loop—a common motif in ATP- and GTP-binding proteins. Trends Biochem. Sci. **15:**430–434.
28. **Segovia, L., D. Piñero, R. Palacios, and E. Martinez-Romero.** 1991. Genetic structure of a soil population of nonsymbiotic *Rhizobium leguminosarum*. Appl. Environ. Microbiol. **57:**426–433.
29. **Sobral, B. W. S., R. J. Honeycutt, A. G. Atherly, and M. McClelland.** 1991. Electrophoretic separation of the three *Rhizobium meliloti* replicons. J. Bacteriol. **173:**5173–5180.
30. **Soto, M. J., V. Lepek, I. M. Lopez-Lara, J. Olivares, and N. Toro.** 1992. Characterization of a *Rhizobium meliloti ndvB* mutant and a symbiotic revertant that regains wild-type properties. Mol. Plant-Microbe Interact. **5:** 288–293.
31. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98:**503–517.
32. **Sullivan, J. T., and C. W. Ronson.** 1997. Towards characterization of the transferable chromosomal symbiotic element of a *Lotus Rhizobium* strain, abstr. 07.02, p. 89. *In* Abstracts of the 11th International Congress on Nitrogen Fixation 1997. Institute Pasteur, Paris, France.
33. **Sullivan, J. T., H. N. Patrick, W. L. Lowther, D. B. Scott, and C. W. Ronson.** 1995. Nodulating strains of *Rhizobium loti* arise through chromosomal symbiotic gene transfer in the environment. Proc. Natl. Acad. Sci. USA **92:**8985–8989.
34. **Sullivan, J. T., B. D. Eardly, P. van Berkun, and C. W. Ronson.** 1996. Four unnamed species of nonsymbiotic rhizobia isolated from rhizosphere of *Lotus corniculatus*. Appl. Environ. Microbiol. **62:**2818–2825.
35. **Tong, Z., and M. J. Sadowsky.** 1994. A selective medium for the isolation and quantification of *Bradyrhizobium japonicum* and *Bradyrhizobium elkanii* strains from soils and inoculants. Appl. Environ. Microbiol. **60:**581–586.
36. **Toro, N., and J. Olivares.** 1986. Analysis of *Rhizobium meliloti* Sym mutants obtained by heat treatment. Appl. Environ. Microbiol. **51:**1148–1150.
37. **Vlassak, K. M., and J. Vanderleyden.** 1997. Factors influencing nodule occupancy by inoculant *Rhizobia*. Crit. Rev. Plant Sci. **16:**163–229.
38. **Walker, J. E., M. Saraste, M. J. Runswick, and N. J. Gay.** 1982. Distantly related sequences in the alpha- and beta-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. EMBO J. **1:**945–951.
39. **Winans, S. C.** 1992. Two-way chemical signaling in *Agrobacterium*-plant interactions. Microbiol. Rev. **56:**12–31.
40. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.
41. **Yanofsky, M., and E. W. Nester.** 1986. Molecular characterization of a host-range-determining locus from *Agrobacterium tumefaciens*. J. Bacteriol. **168:**244–250.