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Identification and Distribution of Plasmid-Type A Replicator Region in Rhizobia

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Sinorhizobium meliloti **strain GR4 harbors two cryptic plasmids, named pRmeGR4a and pRmeGR4b in addition to the symbiotic megaplasmids. The replicator region of plasmid pRmeGR4a has been recently cloned and sequenced. By DNA hybridization, homology to former replicator region was found on plasmid pRmeGR4b as well as on other plasmids harbored by *S . meliloti* and *S. fredii* strains. In these former bacteria, a PCR product of 362 bp was generated using pRmeGR4a-*repC* derived primers (C1 and C2). DNA sequence analysis showed that the amplified *repC* fragments were closely related being classified into two subgroups designated A(I) and A(II). Similarly, a PCR product of 482 bp was obtained when primers (C3 and C5) derived from pRmeGR4a *repC*-upstream noncoding DNA region (IR) were used. DNA sequence analysis of the corresponding amplified products showed that, as occurs, with *repC* the IRs were also conserved. In addition, we designed a set of primers (P2/P4), derived from the *S. meliloti* and *S. fredii* consensus sequence encompassing the IRs and *repC* loci, that are able to recognize homologous plasmid-type A replicator regions in *Rhizobium.* By using these primers, we determined that the former replicator region is widespread in *S. meliloti* indigenous populations and that its frequency within the infective isolates depends on the host plant. Furthermore, it is shown that the replicator region type A is linked to cryptic plasmids in *S. meliloti* and *S. fredii,* whereas it is located in the pSym of *R. tropici* strains.**

Additional keywords: symbiosis.

Sinorhizobium meliloti is a gram-negative soil bacteria able to elicit nitrogen-fixing root nodules on alfalfa (*Medicago sativa*). A general feature of *Rhizobium* species is the existence of a large amount of extrachromosomal DNA (for a review see Mercado-Blanco and Toro 1996). All *S. meliloti* strains studied so far harbor two large symbiotic megaplasmids designated pSym-a and pSym-b that contain most of the essential symbiotic genes (Banfalvi et al. 1985; Finan et al. 1986; Hynes et al. 1986). In addition, most of the strains harbor other large plasmids named cryptic or non-pSym plasmids. The pSym and cryptic plasmids are stably maintained through successive generations, which suggests the presence of an accurate mechanism(s) that ensures an equal partitioning among daughter cells. Whereas the *S. meliloti* symbiotic megaplasmids have not be cured, probably because they carry genes essential for free-living growth, it is possible to select derivative strains that have lost their cryptic plasmids. It is not known how these plasmids replicate or how they partition during the cell division. So far, only two *S. meliloti* regions involved in plasmid replication have been isolated: a 0.8-kb fragment of pSym-b that contains sequences sufficient for replication in *recA* derivative of *S. meliloti* strain 1021 (Margolin and Long 1993) and a 4.8-kb *Pst*I fragment responsible for the autonomous replication of *S. meliloti* strain GR4 cryptic plasmid pRmeGR4a (Mercado-Blanco and Olivares 1993). *S. meliloti* strain GR4 harbors two cryptic plasmids pRmeGR4a and pRmeGR4b of 175 and 205 kb, respectively (Toro and Olivares 1986). Plasmid pRmeGR4a is self-transmissible and responsible for the cotransfer of plasmid pRmeGR4b (Mercado-Blanco and Olivares 1993). Sequence analysis of the pRmeGR4a 4.8-kb *Pst*I fragment revealed the presence of an open reading frame (ORF) necessary for replication (Mercado-Blanco and Olivares 1994) which shows homology to RepC proteins coded by plasmid pRiA4b (29.47% identity) of *Agrobacterium rhizogenes* (Nishigushi et al. 1987) and plamid pTiB6S3 (26.20% identity) of *A. tumefaciens* (Tabata et al. 1985). From the evolutionary point of view these homologies indicate a certain degree of phylogenetic relationship. This sequence similarity could indicate a common ancestor of the corresponding plasmid replication mechanism as it was already pointed out for *Agrobacterium* plasmids (Otten et al. 1992), and can help us to understand mechanism that operate in plasmid evolution. Moreover, the RepC proteins, despite their sequence similarity, are highly specialized from the functional point of view and, in the case of the Rep proteins from *Agrobacterium* plasmids, it was not possible to achieve complementation (Tabata et al. 1985).

With the aim of a better understanding plasmid distribution and evolution in *Rhizobium*, using polymerase chain reaction (PCR)-based sequencing of *repC* and upstream DNA regions, we characterized a group of *Rhizobium* putative plasmid replicator regions which are closely related, named type A.

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The nucleotide sequences determined in this work are available on the EMBL GenBank Nucleotide Sequence Database under the accession number Y08790 to Y08803.

RESULTS AND DISCUSSION

Homology to the *S. meliloti* plasmid pRmeGR4a replicator region.

DNA hybridization experiments previously reported (Mercado-Blanco and Olivares 1994) suggested that the plasmid pRmeGR4a replicator region was highly homologous to the one located on plasmid pRmeGR4b, the other plasmid harbored by *S. meliloti* strain GR4. However, this *S. meliloti* plasmid-type replicator region is not present in all *S. meliloti* strains. As shown in Figure 1, neither L5-30 nor Rm 41 hybridized to the pRmeGR4a *repC*-containing DNA probe. Nevertheless, results from our laboratory using specific pRmeGR4a *repC* derived PCR primers (Villadas et al. 1995) showed that the former *repC* type is widespread in *S. meliloti* field populations. The hybridization of two PCR-selected (Villadas et al. 1995) *S. meliloti* field isolates (A13 and SA-Pae) from different locations in Spain with homology to the pRmeGR4a replicator region are also shown in Figure 1. We did not find homology to the former DNA region in other rhizobial population tested such as *R. leguminosarum* bvs. *viciae*, *phaseoli*, and *trifolii* isolated from the same field site as *S. meliloti* GR4 strain (data not shown).

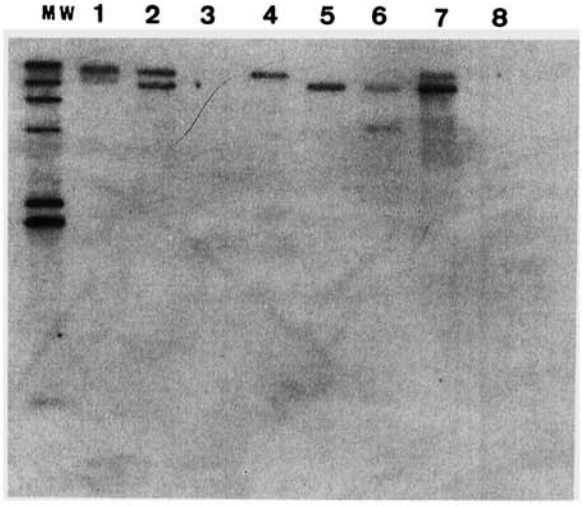

Fig. 1. Homology to *Sinorhizobium meliloti* pRmeGR4a replicator region. *S. meliloti* strains: A13 (lane 1), SAPae (lane 2), L5.30 (lane 3), GRM10 (lane4), GR4 (lane 7), and Rm41 (lane 8). *S. fredii* wild-type strains: HH003 (lane 5) and HH103 (lane 6). Total cellular DNA digested with *Eco*RI was electrophoretically separated, blotted and hybridized as described in Material and Methods. The *Eco*RV fragment (1505 bp) containing most of the pRmeGR4a *repC* loci (Fig. 3) was used as DNA probe.

It is well established that *S. meliloti* is closely related to *S. fredii*, a fast-growing bacteria able to nodulate soybean (Willems and Colling 1993; Laguerre et al. 1994). When we hybridized the pRmeGR4a *repC*-containing DNA probe against total restricted DNA from three different *S. fredii* strains (Table 1), two gave positive hybridization signals, strains HH103 and HH003 (Fig. 1). By Southern blot DNA hybridization of Eckhardt electrophoresis gels of the corresponding cell lysates, we found that the *S. meliloti* and *S. fredii* strains that hybridized to the *repC* probe (Fig. 1), the *repC*-homologous sequences were located on cryptic plasmids (examples are shown in Fig. 2).

Amplification and DNA sequence of the *S. meliloti* and *S. fredii* plasmid-type A *repC* loci.

PCR amplification using the pRmeGR4a *repC* derived primers C1 and C2 (Villadas et al. 1995) (Fig. 3) of cell lysate DNA from *S. meliloti* and *S. fredii* strains that hybridized to the pRmeGR4a *repC* probe (Fig. 1), resulted in a specific PCR product of similar size (362 bp) but with intensities different from that of *S. meliloti* strain GR4 (Fig. 4A). As it was reported (Villadas et al. 1995), C1 and C2 primers apparently are able to discriminate between pRmeGR4a and pRmeGR4b *repC* loci (Fig. 4A, lanes 1 and 3). In some amplification reactions with cell lysate DNA of GRM10 strain, a weak band of size similar to that of strain GR4 could be observed (data not shown). To analyze the homology among the different putative *repC* loci, the PCR products obtained after amplification (including GRM10) were cloned and sequenced. Alignment of the determined *repC* DNA sequences (322 bp, without primers) showed that they were extensively conserved with identities ranging from 83 to 100%. The evolutionary

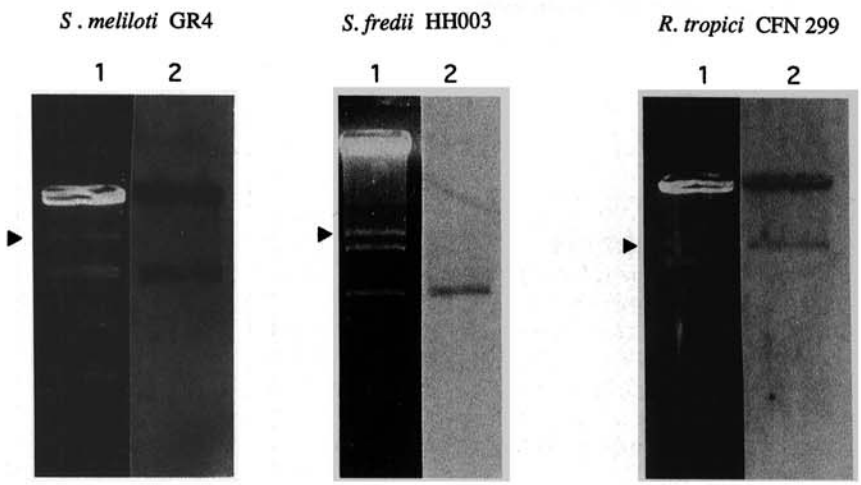

Fig. 2. Plasmid location of *repC* in *Sinorhizobium meliloti*, *S. fredii*, and *Rhizobium tropici* strains. Representative strains are shown. The Eckhardt-gel (lane 1) and the corresponding Southern blot hybridization (lane 2) are shown. Strain specific PCR-labeled probes were used for DNA hybridization. Similar hybridization was obtained using the *Eco*RV fragment (1,505 bp) as probe in the case of *S. meliloti* and *S. fredii* strains. The point arrows indicate the pSym plasmids.

tree constructed from such data analyzed by the unweight pair group method using arithmetic averages (UPGMA) (Nei 1975; Sneath and Sokal 1973) is shown in Figure 4. A similar tree was obtained by using the neighbor-joining method (NJ) (Saitou and Nei 1987) that does not require the assumption of a constant substitution rate (data not shown). Furthermore, trees constructed with the deduced *repC*-encoded amino acid sequences exhibited the same topology to that of the nucleotide sequences (data not shown). Thus, the data suggest that the *repC* loci analyzed evolved at a relatively constant rate. From the dendrogram showed in Figure 4A, it can be deduced the existence of two subgroups of closely related *repC* loci that may define two different, but very much related, plasmid replicator regions, one closer to pRmeGR4a, and the other to pRmeGR4b, named A(I) and A(II), respectively. The *S. fredii* plasmid *repC* homologs are classified within these two subgroups.

Amplification and DNA sequence of *S. meliloti* and *S. fredii* plasmid-type A *repC* upstream DNA region.

Upstream of the pRmeGR4a *repC* loci there is an intergenic region (IR) of 621 bp containing contiguous inverted repeats and a A+T-rich region of unknown function (Mercado-Blanco and Olivares 1994). To know whether in the *S. meliloti* and *S.*

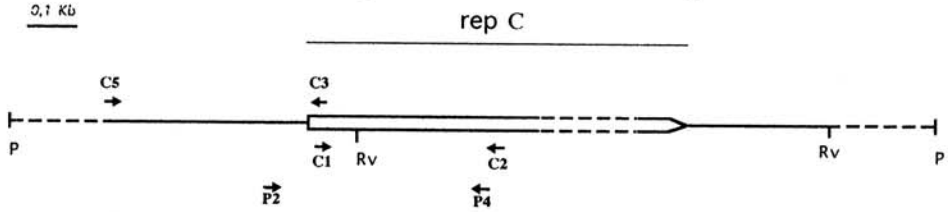

Fig. 3. Physical map of plasmid pRmeGR4a *repC* region. The location of the different primers used in this work are shown. The arrows indicate the amplification orientation. the restriction *Eco*RV(RV) and *Pst*I (P) sites are also shown.

Table 1. Bacterial strains used in this study

Species and strain	Isolation site and source	Source	Host plant	Characteristics
S. meliloti				
GR4	Granada	This lab.	*Medicago sativa*	Wild type
GRM10		J. Mercado and J. Olivares 1993	*Medicago sativa*	GR4 derivative strain cured of plasmid pRmeGR4a
L5.30		J. Dénairé	*Medicago sativa*	Wild type
Rm 41		A. Kondorosi	*Medicago sativa*	Wild type
A13		P. Villadas	*Medicago sativa*	Wild type
SAPae	Parada, Salamanca	P. Villadas et al. 1995	*Medicago sativa*	Wild type
SANab	Naharros, Salamanca	P. Villadas et al. 1995	*Medicago sativa*	Wild type
RMSA29	Leon	P. Villadas et al. 1995	*Medicago sativa*	Wild type
RMS11		P. Villadas et al. 1995	*Medicago sphaerocarpa*	Wild type
RMA24		P. Villadas et al. 1995	*Melilotus alba*	Wild type
LPU63	Argentina	A. Lagares	*Medicago sativa*	Wild type, acid tolerant
LPU66		A. Lagares	*Medicago sativa*	Wild type, acid tolerant
LPU119		A. Lagares	*Medicago sativa*	Wild type, acid tolerant
S. fredii				
HH003, 102, and 103	China	Dowdle and Bohlool 1985	*Glycine max*	Wild type
R. leguminosarum bv. *viciae*				
Field isolates	Granada, Spain	P. Villadas	*Vicia sativa*	Wild type
	Erlanger, Germany	W. Lotz	*Vicia sativa*	Wild type
	Dijon, France	N. Amarger	*Vicia sativa*	Wild type
	Rothamsted, England	P. Hirsch	*Vicia sativa*	Wild type
R. leguminosarum bv. *phaseoli*				
2616, 2630, 248, Sp18	Mexico	E. Martínez-Romero	*Phaseolus vulgaris*	Wild type
R. tropici				
Type A				
BR-835				
BR-828				
BR-833	Mexico	E. Martínez-Romero	*Phaseolus vulgaris*	Wild type
BR-836				
BR-10043				
CFN-299				
Type B				
BR-852				
BR-857	Mexico	E. Martínez-Romero	*Phaseolus vulgaris*	Wild type
BR-850				
BR-859				
CIAT899	Colombia	P. Graham	*Phaseolus vulgaris*	Wild type
R. etli				
F-16				
Viking 1				
F8	Mexico	E. Martínez-Romero	*Phaseolus vulgaris*	Wild type
CFN42				
Brasil 5				

fredii plasmid-types A(I) and A(II) replication regions, the *repC* loci and the upstream regions have evolved in a similar or different manner, we designed primers for the amplification of the corresponding DNA. To ensure that the amplified DNA was linked to the *repC* loci, one of the oligonucleotides, C3, was designed from the pRmeGR4a *repC* sequence, complementary to primer C1 (Fig. 3). PCR amplification of cell-lysate DNA from wild-type strain GR4 with the primers C5 and C3 (Fig. 3) resulted in a single PCR product of 482 bp (Fig. 4B). This DNA fragment was labeled with digoxigenin and used as a probe to test if similar sequences were located in the putative plasmid replicator regions of *S. meliloti* and *S. fredii* strains that showed homology at the level of *repC*. A similar pattern of DNA hybridization to that obtained with the pRmeGR4a *repC* probe (Fig. 1) was found, indicating that similar genetic organization are present in all these related plasmid putative replicator regions.

Amplifications with primers C5 and C3 resulted in a product similar to that of GR4 in *S. meliloti* as well as in the *S. fredii* strains tested (Fig. 4B). GRM10 strain gave a very weak amplification signal (Fig. 4A, lane 3). The amplified DNA fragments were cloned and sequenced. Alignment of the determined DNA sequences (442 bp, without primers) showed that they were extensively conserved like the *repC*, with identities ranging from 83 to 100%. The evolutionary trees constructed from the deduced sequences by the UPGMA (Fig. 4B) and NJ method showed also similar topology which again suggest that the IRs regions of these plasmids have evolved at a constant rate. The relationships of the *S. meliloti* strains plasmid replicator regions at the level of the *repC* upstream DNA are similar to those found for *repC*. However, major differences are observed in the case of *S. fredii* related plasmids. On one hand, whereas for the *repC* sequence (Fig. 4A), strain HH003 is within Group A(I), for the IR (Fig. 4B) is closer to strains belonging to Group A(II), GRM10 and A13, (Fig. 4A). On the other hand, strain HH103 seems to diverge more respect to the other *S. meliloti* plasmid replicator regions at the *repC* upstream DNA than for the *repC* sequence itself (Fig. 4A and B). Apparently in *S. fredii* plasmids-type A replicator regions analyzed the *repC* and IR sequences have evolved in a different manner.

Design of specific primers for the characterization of *Rhizobium* plasmids-type A replicator region.

As it was pointed out above, primers derived from the replicator region of plasmid pRmeGR4a (C1/C2 and C3/C5) gave poor amplification in some strains where DNA hybridization showed clear homology to pRmeGR4a *repC* loci. To avoid this problem and with the aim of performing a wider characterization of plasmid-type A homologs replicator regions in *Rhizobium*, we integrated the characterized *repC* and upstream sequences from *S. meliloti* and *S. fredii* strains, and designed primers encompassing the consensus sequence of both. The set of primers P2/P4 (Fig. 3) were tested in PCR reactions and compared their performance with those obtained with the set of primers C1/C2 and C3/C5 (Fig. 5). P2/P4 were able to amplify at similar level in *S. meliloti* and *S. fredii* strains, including GRM10, resulting a single-specific PCR product of

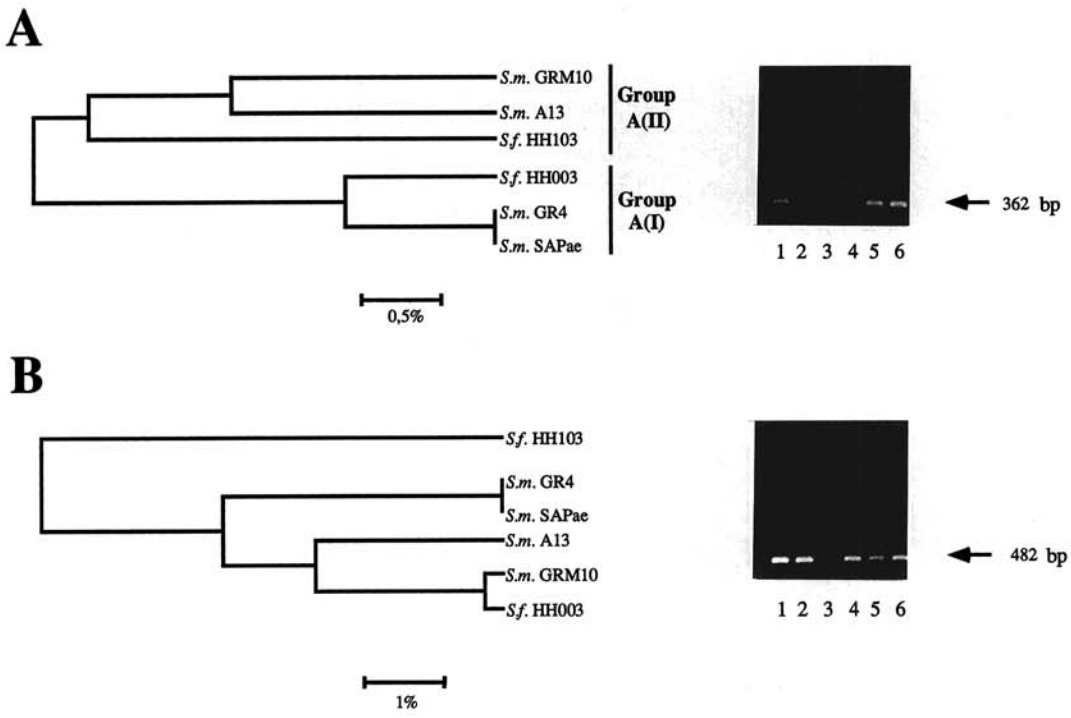

Fig. 4. PCR amplification and the deduced DNA evolutionary trees derived from *repC* **(A)** and upstream DNA regions **(B)** of *Sinorhizobium meliloti* (S.m.) and *S. fredii* (S.f.) strains. *S. meliloti* strains are: GR4 (lane 1), SAPae (lane 2), GRM10 (lane 3), A13 (lane 4). *S. fredii* strains are: HH003 (lane 5) and HH103 (lane 6). DNA sequences were aligned and analyzed by the unweight pair group method using arithmetics averages (UPGMA). the distance scale represents the percentage of divergence. Amplification and DNA sequence showed for strains GR4 and GRM10 correspond to that of plasmid pRmeGR4a and pRmeGR4b, respectively.

443 bp. In addition, we tested other *S. meliloti* field isolates from Granada (Spain) that were previously classified as containing pRmeGR4a, pRmeGR4b or both plasmid types (Villadas et al. 1995). Amplification of the corresponding cell lysate DNA with primers P2/P4 resulted in a PCR signal similar in size and intensity to that of GR4 in all the analyzed strains (data not shown). Therefore, we conclude that formers primers could be used for specific amplification of both plasmid-type A(I) and A(II) replicator regions.

Frequency of plasmid-type A replicator region in *S. meliloti* indigenous population and its distribution in *Rhizobium*.

The presence of plasmid-type A replicator region in five different *S. meliloti* soil populations in Spain was determined. PCR reactions were obtained using the set of primers P2/P4. The plasmid-type A replicator region was found in *M. sativa* root nodule isolates from the five field sites, at a rate ranging between 43% and 76% of the isolates (Table 2). However, root nodule isolates from *Melilotus alba* and *Medicago sphaerocarpa* harbor plasmid-type A replicator region at a significantly lower frequencies, 21% and 16% of the isolates, respectively (Table 2). This host plant effect was also observed for the presence of plasmid-type pRmeGR4b (Villadas et al. 1995). These results suggest a correlation between replicator region and plasmid-type with host dependent-infectiveness.

The presence of the plasmid-type A replicator region was also analyzed in *S. meliloti* strains isolated from acidic soils from Argentina. Four out of nine field isolates tested gave positive amplification with primers P2/P4. This result shows again that this replicator region is widely distributed within *S. meliloti* indigenous population. DNA hybridization showed that the strains from Argentina contain single copies of the *repC* type A located on cryptic plasmids (data not shown).

PCR amplification with primers P2/P4 could be achieved in other rhizobia species, as occurs with bean-nodulating strains, *R. tropici* type A and B (Table 1), including model strains CFN299 and CIAT899, respectively. The amplified fragments showed identical DNA sequences for all *R. tropici* strains analyzed. DNA hybridization with the amplified DNA as a probe, showed that *R. tropici* strains contain a single copy of the *repC* type A (data not shown), located in the Sym plasmid (Fig. 2). Interestingly, no amplification was obtained with primers P2/P4 with the *R. leguminosarum* bvs. *phaseoli, viciae* and *R. etli* strains analyzed (Table 1). These results may suggest some host restriction to plasmids containing replicator region-type A.

Table 2. Frequency of plasmid-type A replicator region in *Sinorhizobium meliloti* indigenous populations

Isolation site	Isolates analyzed (no.)	Host plant	Isolates harboring plasmid type A no.	%
Salamanca				
Naharros	37	*M. sativa*	26	70
Florida de Liébana	25	*M. sativa*	19	76
Parada	30	*M. sativa*	15	50
León	28	*M. sativa*	12	43
	19	*M. sphaerocarpa*	3	16
	33	*M. alba*	7	21

Genetic relationships between amplified *Rhizobium* plasmid-type A replicator region.

Figure 6 shows the phylogenetic tree derived by the UPGMA method from the partial sequence of PCR products obtained using primers P2/P4 with 11 *S. meliloti* and 2 *S. fredii* and *R. tropici* strains. A similar dendrogram was obtained when the neighbor-joining algorithms was used or when the deduced RepC amino acid sequences were analyzed (data not shown). At least three main groups could be delineated: se-

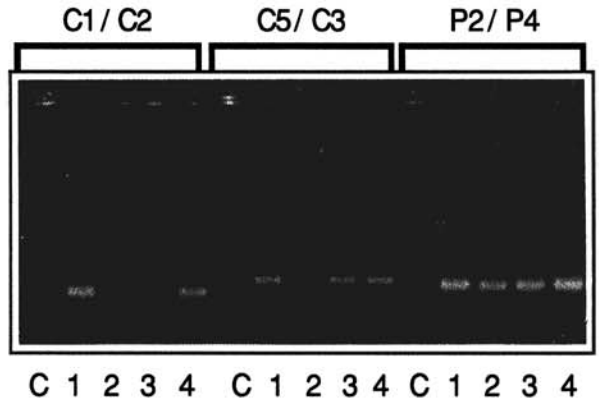

Fig. 5. PCR amplification of *Sinorhizobium meliloti* and *S. fredii* strains using the set of primers C1/C2, C3/C5 and P2/P4. *S. meliloti* strains are: GR4 (lane 1), GRM10 (lane 2), A13 (lane 3). The *S. fredii* strain tested was HH003 (lane 4). Strain which does not contain *repC* type A sequences by DNA hybridization was used as a negative control (lane C).

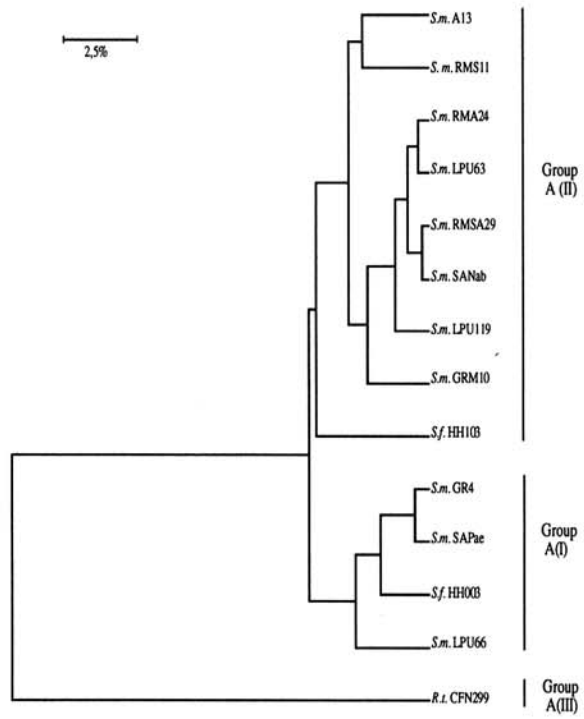

Fig. 6. DNA evolutionary tree derived from sequence analysis of P2/P4-PCR amplified fragments of *Sinorhizobium meliloti* (S.m.), *S. fredii* (S.f.) and *Rhizobium tropici* (R.t.) strains. The distance scale represents the percentage of divergence.

quences from *S. meliloti* and *S. fredii* strains comprising groups A (I) and A (II) and *R. tropici* forming a more divergent but linked group A(III). Comparison of the amino acid sequences from *Sinorhizobium* (consensus) and *R. tropici* RepC-type A with the published RepC from *R. leguminosarum* bv. *viciae* (Turner and Young 1995), *A. tumefaciens* (Tabata et al. 1985) and *A. rhizogenes* (Nishigushi et al. 1987) suggest that the RepC-type A belong to a different and distant RepC classes (Fig. 7). Recently, by using *repC*-derived conserved PCR primers, Turner and Young (1996) have characterized four different groups of plasmid replicator regions in *R. leguminosarum*. Future description of other rhizobia plasmid *repC*-types will lead to a better understanding of plasmid distribution and evolution in this bacterial specie.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

All bacterial strains used in this work are listed in Table 1. Strains were grown in TY medium at 28°C.

DNA preparation.

Total DNA was isolated according to Meade et al. (1982). For DNA hybridization total cellular DNA was digested with *Eco*RI and electrophoretically separated in a 0.8% agarose gel. DNAs were transferred from agarose gels to nylon filters according to the manufacturers' instructions using a VacuGene XL bloting apparatus (Pharmacia Biotech, Piscataway, NJ). As DNA probe we used a 1,505-bp *Eco*RV DNA fragment containing most of the pRmeGR4a *repC* loci

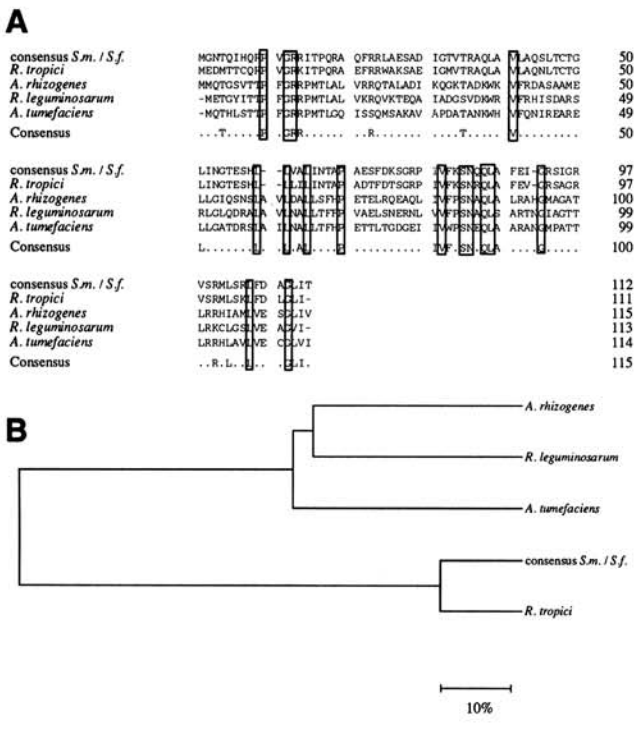

Fig. 7. Amino acid alignment **(A)** and deduced evolutionary tree **(B)** of the RepC N-terminal domain from *Agrobacterium tumefaciens*, *A. rhizogenes*, *R. leguminosarum* bv. *viciae*, *R. tropici* and the *S. meliloti* (S. m.)/ *S. fredii* (S.f.) consensus sequence. The distance scale represents the percentage of divergence.

(Mercado-Blanco and Olivares 1994). Labeling of the probe, hybridization and detection were carried out using a non-radioactive DNA labelling and detection Kit (Boehringer Mannheim) according to the manufacturers' instructions. Labeling during amplification was carried out by adding digoxigenin-11-dUTP to the reaction mixture (0.1 mM), using the dNTPs at a final concentration of 2 mM except the dTTP that was added at 1.9 mM. Eckhardt gel lysis electrophoresis (Eckhardt 1978) was carried out at 40 V for 30 min and then 120 V for 4 h.

PCR reactions.

The PCR reactions were carried out in 25-µl volume containing 4 µl of cell lysate obtained as described (Villadas et al. 1995), 50 pmol each of two opposing primers, 0.4 mM deoxinucleotide triphosphates, 2 U of *Taq* polymerase (Boehringer Mannheim) and 2.5 µl of 10× Taq polymerase buffer; overlaid with 20 µl of mineral oil. PCR reactions were performed in a termal cycler (Linus) as follow: an initial denaturation cycle (95°C, 6 min); 30 cycles of a denaturation step (94°C, 1 min), an annealing step (53°C for C1/C2, 55°C for P2/P4 and 56°C for C3/C5, 1 min) and an extension step (72°C, 1 min); and a final extension cycle (65°C, 15 min). Amplified DNA was examined by horizontal electrophoresis in 1% agarose with 7 µl of PCR products. The DNA sequence of the primers and the corresponding coordinate positions in pRmeGR4a plasmid origin of replication (Mercado-Blanco and Olivares 1994) are:

C1 (2371-2390 bp) 5′AATACCCAAATTCATCAGCG3′
C2 (2713-2732 bp) 5′GGATAGCGCTTATAGTTGCC3′
C3 (2371-2390 bp) 5′CGCTGATGAATTTGGGTATT3′
C5 (1911-1930 bp) 5′TACCCCTCCCCTCAAACTCC3′
P2 (2269-2288 bp) 5′AATCCTTTCTCGCAGACCCT 3′
P4 (2692-2711 bp) 5′CTGTCCTGCATCGTTGATCAG 3′.

DNA sequencing.

PCR products from *S. meliloti* and *S. fredii* (C1/C2, C5/C3) were cloned in pGEMT vector (Promega) and sequenced by the chain termination method (Sanger et al. 1977) using Sequenase T7-DNA polymerase, version 2.0 (United States Biochemical, Cleveland, OH).

PCR products of P2/P4 amplification were purified with the Magic PCR prep kit (Promega) and sequencing was performed in an Automatic Laser fluorescent DNA sequencer (Applied Biosystems, Foster City, CA) using primer P2.

DNA traslation and analysis were performed with the Gene Works sofware package (IntelliGenetics, Inc., Mountain View, CA)

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