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Sinorhizobium meliloti Plasmid pRm1132f Replicates by a Rolling-Circle Mechanism†

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pRm1132f isolated from *Sinorhizobium meliloti* is a group III rolling-circle-replicating (RCR) plasmid. At least seven of eight open reading frames in the nucleotide sequence represented coding regions. The minimal replicon contained a *rep* gene and single- and double-stranded origins of replication. Detection of single-stranded plasmid DNA confirmed that pRm1132f replicated via an RCR mechanism.

Sinorhizobium meliloti bacteria are gram-negative soil bacteria that fix nitrogen in symbiotic association with *Medicago* and *Melilotus* spp. In a previous study (14), an unusually small (7.2 kb) cryptic plasmid, pRm1132f, from *S. meliloti* strain 1132 was used to construct cloning vectors which were subsequently found to be destabilized upon cloning of a 12-kb DNA fragment. To ascertain the reason for this observed instability and to enhance our understanding of the replication, genomic organization, and function of *S. meliloti* cryptic plasmids, pRm1132f was characterized by nucleotide sequencing, cDNA hybridization, and deletion analysis.

Bacterial strains, growth conditions, plasmids, and recombinant DNA techniques. Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* bacteria were grown on Luria broth, and *S. meliloti* bacteria were grown on TY or TP medium (21). Plasmids resident in *E. coli* were introduced into *S. meliloti* by triparental mating (10). Standard recombinant DNA cloning techniques and Southern hybridization were carried out as described by Sambrook et al. (22).

DNA sequencing and analysis. Nested deletions were made in pNRRM1 and pNRRM2 (Table 1) using a Pharmacia kit. Nucleotide sequencing of both DNA strands was carried out by the University of Laval Sequencing Service, Quebec, Canada. DNA sequences were analyzed with the University of Wisconsin Genetics Computer software package (version 10) and DNAMAN (Lynon Biosoft Quebec). Database searches were carried out with GenBank (release 113), EMBL (release 59.0), and SWISSPROT.

The plasmid, pRm1132f, consisted of 7,212 bp with a GC content of 65%, and FRAME analysis (2) revealed the presence of eight open reading frames (ORFs) (Fig. 1). The predicted protein encoded by ORF2 (235 amino acids) shared 28.5% identity with the product of the homocitrate synthase gene from Frankia (EMBL accession no. P54610), and the ORF3 predicted protein (430 amino acids) shared 30 to 34% identity (42 to 46% similarity) with the initiator-replication (Rep) proteins of the group III (cluster III) rolling-circle-replicating (RCR) plasmids, as shown by comparison with an RCR plasmid replicon database (http://www.essex.ac.uk/bs/staff/osborn/DPR_home.htm). The putative protein encoded by ORF4 (335 amino acids) shared 24% identity (31% similarity) with the Mob protein of the group III (cluster I) RCR plasmid, pTA1040, from *Bacillus subtilis* in the RCR plasmid replicon database. No significant homology was found in the databases for the predicted proteins of the remaining ORFs.

ORF3 transcriptional start site. The transcriptional start site for the putative *rep* gene (ORF3) was determined by primer extension analysis (22) using the fluorescence (IRD800)-labeled primer X-100 (5′GTTTCGAGACGGTCA GATGGT-3′) from LI-COR (Lincoln, Nebr.). DNA sequencing was carried out according to the LI-COR protocol. To align the transcriptional start sites, the same primer was also used in conjunction with template DNA (pNRRM1) in a sequencing reaction. A single transcriptional start site with a GTG start codon was located at nucleotide 3241 of the plasmid sequence (Fig. 2).

***dso* and *sso*.** The double-stranded origins of replication (*dso*) of RCR plasmids are usually present either within or upstream of the *rep* gene in a region of strong secondary structure (8, 11, 18). Nucleotide sequences resembling the *dso* of a number of group III (cluster III) RCR plasmids in the RCR plasmid replicon database were present upstream of the putative *rep* gene of pRm1132f (Fig. 3a). The putative *dso* of pRm1132f contained imperfect repeat (IR) and direct repeat (iteron) nucleotide sequences (Fig. 3b) which have been implicated in the binding of the Rep protein prior to nicking of the duplex strand (8, 11, 18). Iterons at replication origins have also been implicated in copy number control of some plasmids (7). The single-stranded origins of replication (*sso*) of RCR plasmids exist in a region of strong secondary structure (8, 11, 17), as is the case for the putative *sso* of pRm1132f. A conserved six-nucleotide sequence, 5′-TAGCGt/a-3′, present in sso_A-type origins was shown to function as a transcriptional terminator for the synthesis of an RNA primer (19, 20). In pRm1132f, a TATCGT motif containing five of six nucleotides of the consensus sequence was located in the loop of a hairpin structure immediately upstream of the *dso* (Fig. 3c). The putative *sso* contained two imperfect repeat sequences (IR I and IR II) and a direct repeat sequence which may act as sites of attachment

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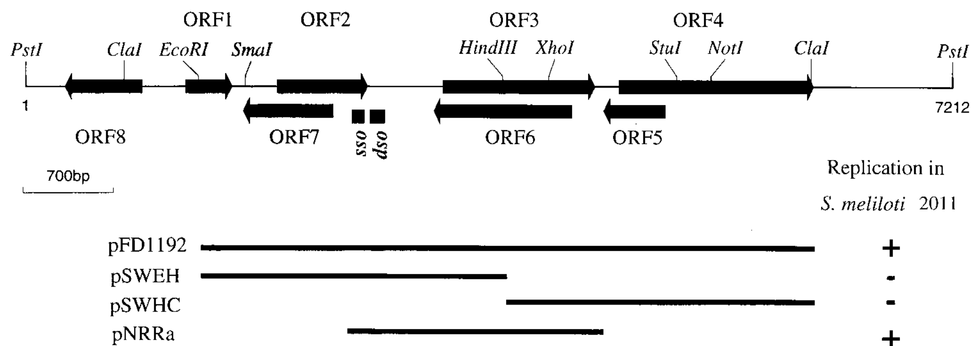

FIG. 1. Arrangement of ORFs in pRm1132f and the minimum replicon. For determination of the minimal replicon, the plasmid was subcloned into either pSUP301 or pBR322, and the composite plasmid was transferred in *S. meliloti* 2011 by mating in the presence of appropriate antibiotic selection pressure.

for host encoded factors during the conversion of single-stranded DNA (ssDNA) to double-stranded DNA (dsDNA).

cDNA detection. cDNA hybridization was used to determine whether the ORFs within the nucleotide sequence of pRm1132f represented coding regions. ORF-specific oligonucleotide primers (Table 2), total RNA from *S. meliloti* strain 1132, and reverse transcriptase were used to generate ^{32}P-labeled cDNA probes according to the procedure of Han et al. (15). ORF-specific DNA prepared by PCR using the above-described primers was subjected to agarose electrophoresis, blotted onto a nylon membrane, and hybridized to the labeled cDNA probes. Hybridization of cDNA was observed with all of the ORF-specific DNAs tested, indicating that the respective ORFs represented coding regions (Fig. 4); ORF6-specific DNA could not be tested, since it was completely overlapped by ORF3. To the best of our knowledge, substantial overlap of genes on opposing plasmid DNA strands has not been observed, and further experiments are required to confirm that ORF6 represents a coding region.

Minimal replicon. To determine the minimum plasmid replicon, DNA fragments of pRm1132f were ligated into either pBR322 or pSUP301 and tested for survival in *S. meliloti* 2011

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Reference or source
Strains		
E. coli		
DH5α	F$^-$ (φ80Δ*lacZ*ΔM15) *endA1 recA1 hsdR17 supE44 thi-1* λ$^-$ *gyrA96 relA1*Δ (*lacZYA-argF*)*U169*	26
HB101	F$^-$ *hsdS20* (r_B^- m_B^-) Δ(*gpt-proA*) *62 leu supE44* λ$^-$ *ara-14 galK2 lacY1* Δ (*merC-mrr*) *rpsL20* (Str^r) *xyl-5 mtl-1 recA13*	5
MT607	*pro-82 thi-1 hsdR17 supE44 rec A56*	13
S. meliloti		
1132	Wild-type *S. meliloti*	6
2011	*S. meliloti* SU47 (RCR2011)	Rothamsted. Experimental station
2011Rf-2	Rif mutant of 2011 nod$^+$ nif$^+$; Rif^r	This study
Plasmids		
pBSL15	3.8-kb plasmid: Ap^r Km^r	1
pFD1001	pRm1132f cloned into the *Pst*I site of pSUP301; Km^r	14
pFD1192	*Eco*RI/*Hin*dIII fragment of pRm1132f cloned into *Eco*RI/*Hin*dIII-digested pBR322 containing the *ori*T of pRK2; Tc^r	14
pNRRM01	pRm1132f cloned into the *Eco*RI site of pUC19, orientation +; Ap^r	This study
pNRRM2	pRm1132f cloned into the *Eco*RI site of pUC19, orientation −; Ap^r	This study
pNRRa	pRm1132f amplified fragment (nt 2408–4501) cloned into *Sma*I-digested pBSL15, excised with *Pst*I, and cloned into pSUP301; Km^r	This study
pNRSP1	pRm1132f linearized with *Eco*RI and cloned into pSPT18 transcription vector; Ap^r	This study
pRK600	pRK2013 *npt*::Tn9 Cm^r Nm-Km^s	13
pRm1132f	7.2-kb plasmid from *S. meliloti* 1132	6
pSPT18	Transcription vector; Ap^r	Roche
pSUP301	pACYC177 with *ori*T of RP4, ColE1 replicon; Ap^r Km^r	24
pSWEH	S1 nuclease-treated 2.4-kb *Eco*RI/*Hin*dIII fragment of pRm1132f and *Pst*I-linearized pSUP301 followed by blunt-end ligation; Km^r	This study
pSWHC	S1 nuclease-treated 2.4-kb *Hin*dIII/*Cla*I fragment of pRm1132f and linearized *Pst*I pSUP301 followed by blunt-end ligation; Km^r	This study

[a] nt, nucleotide.

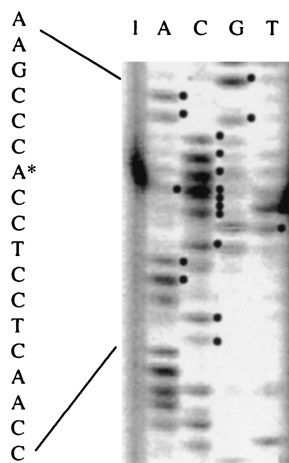

FIG. 2. Determination of the ORF3 transcriptional start site by the primer X-100 and using plasmid pNRRM1 as a template. The relevant sequence is shown on the left, with the asterisk indicating the position of the extension product.

in the presence of antibiotic selection (Fig. 1). The minimum replicon (pNRRa) consisted of the Rep protein, *dso*, and *sso*. Deletion of the *sso* of RCR plasmids results in plasmid instability (8), as was found to be the case for pRm1132f (data not shown).

Detection of *ss*DNA. To confirm that pRm1132f was an RCR plasmid, 10 μg of genomic DNA from mid-log-phase cells of *S. meliloti* 1132 grown on TY medium was treated without and with (100 U) S1 nuclease. The DNA was then subjected to agarose gel electrophoresis and transferred to a nylon membrane without prior denaturation. Hybridization with ^{32}P-labeled *Eco*RI-restricted pRm1132f DNA indicated that *S. meliloti* strain 1132 accumulated single-stranded plasmid DNA (Fig. 5). When rifampin and erythromycin (100 μg/ml of each) were added to the cells prior to harvesting, a lower-molecular-weight hybridizing band was evident, probably representing supercoiled ssDNA. Pretreatment of the DNA with S1 nuclease resulted in a loss of the hybridizing bands and confirmed that they consisted of ssDNA. The membrane-bound DNA was also hybridized to the ^{32}P-labeled strand-specific riboprobes

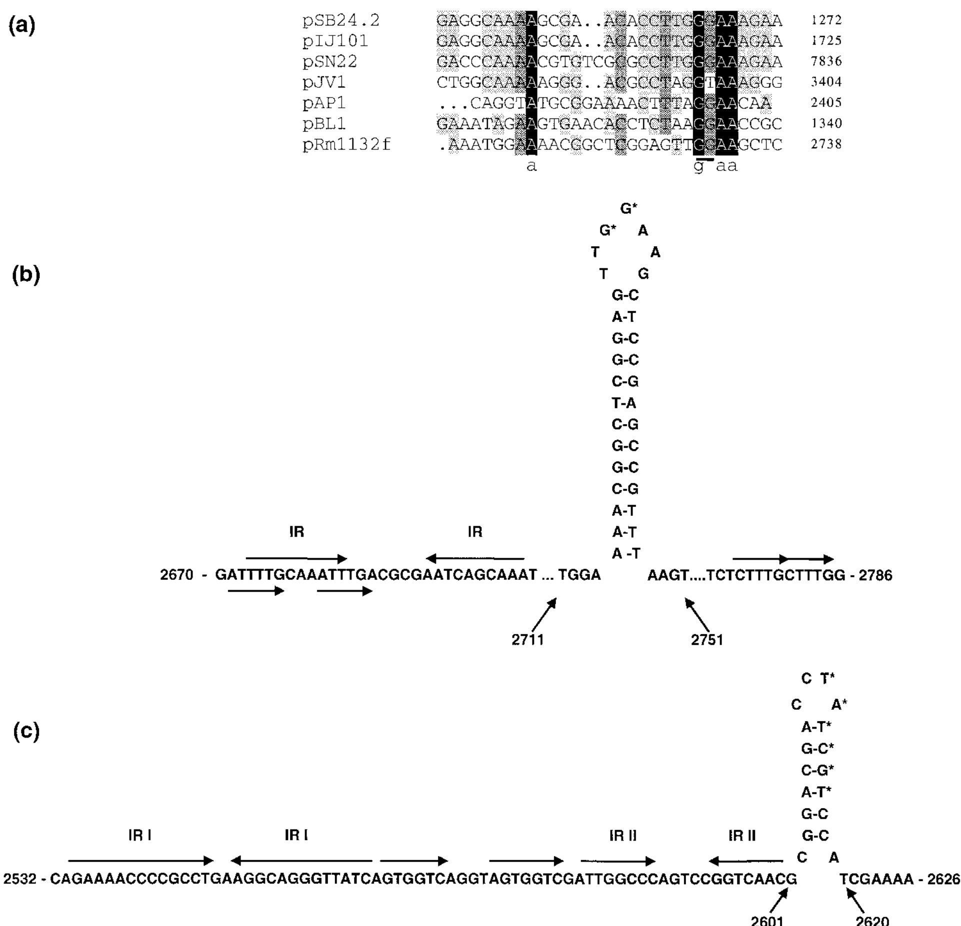

FIG. 3. *sso* and *dso* of pRm1132f. (a) Alignment of nucleotides in the *dso* of pRm1132f and group III RCR plasmids (from the RCR plasmid replicon database; see the text) pSB24.2 (4), pIJ101 (17), pSN22 (16), pJV1 (23), pAP1 (3), and pBL1 (12). The conserved GG dinucleotide (underlined) indicates the position of the nick site in pSN22, pJV1, and pIJ101 (23, 25). (b and c) Predicted secondary structures of the *dso* and *sso*, respectively; for the *dso*, a conserved GG dinucleotide in the loop of a hairpin structure is indicated by asterisks; for the *sso*, the nucleotide sequence TATCGT contains five of six of the nucleotides of the consensus sequence 5′-TAGCGt/a-3′ present in sso_A origins (19, 20). Direct repeats (unmarked arrows) and imperfect inverse repeats (IR) are indicated.

TABLE 2. Oligonucleotide primers for ORF-specific DNA

ORF[a]	Primer	Nucleotide no. in plasmid sequence
ORF1 (F)	5′-CCGAAAGCCTGACATATCG-3′	1242
ORF1 (R)	5′-GAGCGAACAGCCCTTTCTG-3′	1562
ORF2 (F)	5′-CATGCATGGGCGATCGACA-3′	2007
ORF2 (R)	5′-GCCAATCGACCACTACCTG-3′	2568
ORF3 (F)	5′-ATCGCGTGGGAAATGGCAGG-3′	3789
ORF3 (R)	5′-TTTCAGGCGGACCCTCTG-3′	4415
ORF4 (F)	5′-TATCGGGAGGCCATGAG-3′	5119
ORF4 (R)	5′-CTCGGGCACTGCGAATAT-3′	6059
ORF5 (F)	5′-CATCTCCAGAGCCAACGA-3′	4502
ORF5 (R)	5′-GTGCTGGTCCTTCCGCAC-3′	4783
ORF7 (F)	5′-ATTGACGGACCGAAGCGA-3′	1685
ORF7 (R)	5′-GGACGTCTGTCAGCGGAT-3′	2161
ORF8 (F)	5′-GACGAGCTCGGAACCGAA-3′	425
ORF8 (R)	5′-AACAGCTTGCAGCAGGCAC-3′	738

[a] F, forward; R, reverse.

complementary to each of the two DNA strands. Hybridization was observed only with the riboprobe complementary to the plus strand (data not shown), indicating that the ssDNA was generated from the plus strand. Under normal growth conditions, the accumulation of RCR plasmid ssDNA indicates that the plasmid *sso* is operating inefficiently in its bacterial host (11). Despite the accumulation of pRm1132f ssDNA observed in this study, the plasmid is stably maintained in the parental strain, *S. meliloti* 1132. This was also reported to be the case for the RCR plasmids, pRF22F and pC194, which were not segregationally unstable in *B. subtilis* despite the accumulation of ssDNA (9).

To date, the presence of RCR plasmids in many gram-positive and in some gram-negative bacteria has been documented in an RCR plasmid replicon database (see above). The present study indicating the presence of an RCR plasmid in a rhizobial species suggests that these plasmids are more widespread than originally thought, and this finding has implications with regard to the horizontal spread of RCR plasmids among soil bacteria.

Nucleotide sequence accession number. The nucleotide sequence of pRm1132f has been deposited in GenBank (accession no. AF327371).

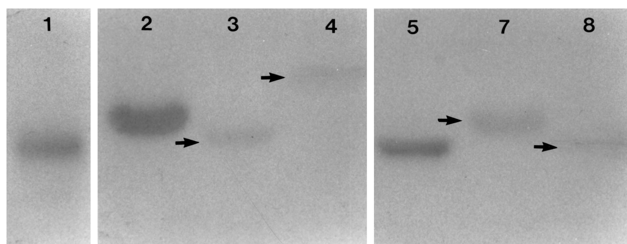

FIG. 4. Hybridization of ^{32}P-labeled cDNA with ORF-specific DNA of pRm1132f. cDNA probes prepared with ORF-specific oligonucleotide primers (see Table 2), total RNA from *S. meliloti* 1132, and reverse transcriptase were hybridized to ORF-specific DNA electrophoresed on agarose gels and blotted onto nylon filters. ORF-specific DNAs are numbered as indicated. Three separate hybridizations were carried out with cDNA probes derived from ORF1 (left panel), ORFs 2 to 4 (middle panel), and ORFs 5, 7, and 8 (right panel).

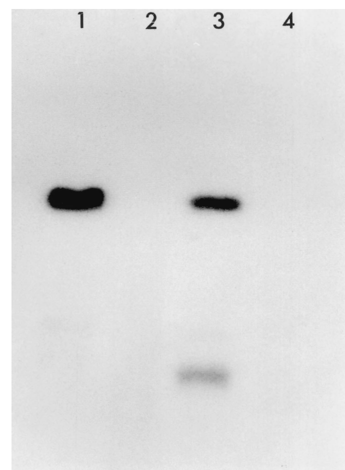

FIG. 5. Detection of ssDNA intermediates in the replication of pRm1132f. Genomic DNA from *S. meliloti* 1132 was blotted onto a nylon membrane without denaturation and hybridized to a ^{32}P-labeled pRm1132f DNA probe. DNA without (lane 1) and with (lane 2) S1 nuclease treatment prior to electrophoresis is shown. DNA from cells incubated with rifampin and erythromycin prior to harvesting, without (lane 3) and with (lane 4) S1 nuclease treatment, is shown.

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