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The Large Nonsymbiotic Plasmid pRmeGR4a of *Rhizobium meliloti* GR4 Encodes a Protein Involved in Replication That Has Homology with the RepC Protein of *Agrobacterium* Plasmids

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The large plasmid, pRmeGR4a, of *Rhizobium meliloti* GR4 is a nonsymbiotic, self-transmissible replicon that shows a high degree of stability. Plasmid replication and stabilization functions are located on a 4.8-kb *Pst*I fragment. Analysis of the nucleotide sequence of this DNA region revealed the presence of six open reading frames (ORFs) with coding capabilities; all are transcribed in the same direction. Only one of the ORFs (ORF3) seems to be essential for replication. Its predicted protein sequence shows homology with RepC, a protein that has been suggested to be involved in the replication of *Agrobacterium* Ri and Ti plasmids.

Bacteria of the genus *Rhizobium* usually harbour large plasmids with molecular weight larger than 100 MDa. In *R. meliloti* some of these plasmids reach a molecular weight of more than 1000 MDa (Rosenberg *et al.*, 1981) and carry essential symbiotic genes, hence they are also known as symbiotic plasmids or pSyms (Huguet *et al.*, 1983). Other plasmids present in *R. meliloti* strains, so-called pRmes, are dispensable and only a few phenotypic traits have been associated with their presence. Despite this fact, these plasmids show a high degree of stability, and may confer some environmental selective advantages to these bacterial strains. The replication and stabilization mechanisms of these plasmids are not known. So far, in rhizobia only the regions involved in the replication of plasmids from *R. leguminosarum* bv. trifolii G1027 (Neilan *et al.*, 1986), *Rhizobium* sp. (*Hedysarum*) UPM-Hc23 (Mozo *et al.*, 1990), and *R. meliloti* GR4 (Mercado-Blanco and Olivares, 1993) have been isolated.

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R. meliloti strain GR4 carries two highly stable pRme plasmids in addition to the pSyms. The larger one, pRmeGR4b (205 kb), contains the *mepA* gene responsible of the melanin synthesis (Mercado-Blanco *et al.*, 1993) and genes (*nfe*) involved in nodulation efficiency and competitiveness on alfalfa roots (Sanjuan and Olivares, 1989; Toro and Olivares, 1986). The smaller one, pRmeGR4a (175 kb), is a self-transmissible plasmid and is responsible for the cotransfer of pRmeGR4b (Mercado-Blanco and Olivares, 1993).

The minimal pRmeGR4a replicon is contained within a 4.8-kb *Pst*I fragment that was previously isolated and cloned in pBluescript in both orientations (plasmids pJMB40 and pJMB45, respectively) (Mercado-Blanco and Olivares, 1993). The genetic organization of this fragment is shown in Fig. 1A. Deletion derivative clones, obtained by the Erase-a-base system from Promega Biotech (Madison, WI), were used in stabilization assays. These experiments were carried out in the strain GRM8SR (a pRme cured GR4 derivative), and the stabilization data were calculated according to Durland and Helinski (Durland and Helinski, 1987). Results indicated that the minimal region involved in rep-

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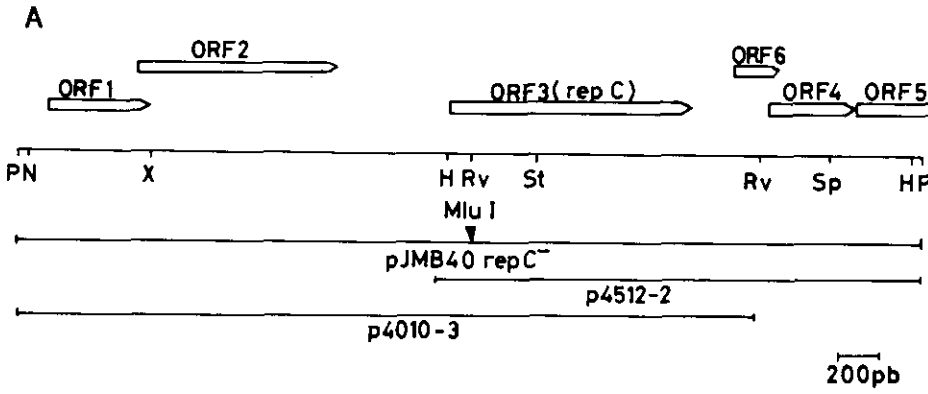

B

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CATAGCCCCCGCCGAGGCAGAACAGGAGCCTGAACGCGAGAGCATCGCCGAAGACGAGCAGTTCCCCCAAGCGGCTGAGTGAGTTCAGCCGCGGGCCGGT 1810
H  S  P  R  R  G  R  T  G  A  *  End ORF2

TACGTTCCTCCCGCGTGATCGGCCCTTCCCGATTGCACAACCGACGATGACCGTGGGCCAGGATCGACCTGGCCCACAATCATGTTGAGGGGAGGCCCGA 1910

TACCCCTCCCCTCAAACTCCCCATCCCACCCGCGTTTTCTTGCATTCAGGAATTGTAGTGCATCAAATTGCTTCAGTCTTGCGGCTCAAAGTCGGCGTTT 2010

CAAATCAGCATTAAAGCGGAAAGCCACTAAGAAATAAAGGCATTCTCGGCCTAACACACGCCCTACAGGCTTTTGCGTGTAGCACGTTGTTGCACCTTTT 2110

TGGTTTATCAGGTTAGTTTTCAAATGGCGGAATCACTCGTGATTCCGGCGCTCACGCATTCTTATCAAATTTCACGGAGATGACAGGCCAGACCAGAAAA 2210

GAAAAACCGCTCCCGAACAAGTCCGGAAGCGGGGGTCAAAGAGTAGATAAATAAGCCGAATCCTTTCTCGCAGACCCTCCCTTCCCTGTCAATTGCAAAA 2310

GCACCTGGTCGGTGAACGGCGAAGCTTTGCCTGGTCCCTAAATGGAGACGAGGAATGGGGAATACCCAAATTCATCAGCGGCCAATCGGCCGCAGGATAA 2410
                                                ORF3   M  G  N  T  Q  I  H  Q  R  P  I  G  R  R  I  T

CGCCGCAGCGTGCGCAATTCCGGCGTTTGGCAGAGTCGGCGGATATCGGAACCGTGACGCGTGCCCAATTGGCCGTGCTTGCGCAAAGCCTGACATGCAC 2510
  P  Q  R  A  Q  F  R  R  L  A  E  S  A  D  I  G  T  V  T  R  A  Q  L  A  V  L  A  Q  S  L  T  C  T

AGGGCTAATCAACGGGACCGAAAGCCACCTATTGGTAGCGCTGATCAATACGGTTCCCGCTGATTCGTTCGACAGGTCCGGCCGTCTGATCGTATTCAAG 2610
  G  L  I  N  G  T  E  S  H  L  L  V  A  L  I  N  T  V  P  A  D  S  F  D  R  S  G  R  L  I  V  F  K

TCGAACCAGCAACTGGCCTTCGAAATTGGCCGGTCGGTCGGTCGCGTCAGCCGCATGCTATCCAGCCTCTTCGACGCTGGCCTGATCACGATGCAGGACA 2710
S  N  Q  Q  L  A  F  E  I  G  R  S  V  G  R  V  S  R  M  L  S  S  L  F  D  A  G  L  I  T  M  Q  D  S

GCGGCAACTATAAGCGCTATCCCATCCGCAATGGCGACGGCGAGATCACGGACGGTTGCGGGATCGACCTTCGCATCTTGATCGTGCGATATGCCGAGCT 2810
  G  N  Y  K  R  Y  P  I  R  N  G  D  G  E  I  T  D  G  C  G  I  D  L  R  I  L  I  V  R  Y  A  E  L

TGACGAGCTCGTGAGACAGGCAAGGTCTCAGAAGAAAGCCCGCCGACGCGGCCTGCGGCGCTATCGTGGGGCGCTTCGCAATGCTCGCTATGCCCTCGCC 2910
  D  E  L  V  R  Q  A  R  S  Q  K  K  A  R  R  R  G  L  R  R  Y  R  G  A  L  R  N  A  R  Y  A  L  A

GCCGCCGCGCGTCTCTCGGAGGATTCTTTGAGCCGTCTCGGCGCCCGGATCGAGCGGGTGAGCAACATTGTCGGAATCGCCGGCAAGGCATCGAGTCGCG 3010
A  A  A  R  L  S  E  D  S  L  S  R  L  G  A  R  I  E  R  V  S  N  I  V  G  I  A  G  K  A  S  S  R  V

TGCTACGGCGGGCAACGGCTCTTCTGGAGTGGTTGATAGAGCGGATGATGCAGCTCCCGCGCCGATCGGCAGAAGCGCAGAAAACGCAAAATATGACATG 3110
  L  R  R  A  T  A  L  L  E  W  L  I  E  R  M  M  Q  L  P  R  R  S  A  E  A  Q  K  T  Q  N  M  T  C

CCCGTATGTCGAAAACGGCATGCACAAACAGATTACAACCCCTCATCCCTTTGACGATAGTAGTGACCAACGGCGTTCGGCTTACGCCGAACAACTCAGT 3210
  P  Y  V  E  N  G  M  H  K  Q  I  T  T  P  H  P  F  D  D  S  S  D  Q  R  R  S  A  Y  A  E  Q  L  S

TCGCCAAAGGCTGGCTCCGCCAGCAAATGGGCTTTTGAAGAAAGCCTGAGGCGGCGCCGGAGCGAAATCAACCAGCCACGGCGCTTGCGGCACGCAATGG 3310
S  P  K  A  G  S  A  S  K  W  A  F  E  E  S  L  R  R  R  R  S  E  I  N  Q  P  R  R  L  R  H  A  M  V

TGGCATTGCAAGACGTGCTTAGGGCGGTGCCAGCGTTGAAGACCTACGGGTTTGCCCCCACCACTTGGGCCGACCTGGCGCGGGCGGTGCCGCTGATGTG 3410
  A  L  Q  D  V  L  R  A  V  P  A  L  K  T  Y  G  F  A  P  T  T  W  A  D  L  A  R  A  V  P  L  M  C

CCGGTTTGCCGGGATCTCCGAGGATGCCCGCCGTCGCGCCGTGGAGCAGATGGGCGAGCAGCAAGCCGCCGTCGCCGTTGCCGTGACACTTGAGAAATAC 3510
R  F  A  G  I  S  E  D  A  R  R  R  A  V  E  Q  M  G  E  Q  Q  A  A  V  A  V  A  V  T  L  E  K  Y

GACCGGCAGGAAGTCAACTCGCCGGGAGGATATTTGCGGGCTATGACGGACCGCGCTAGACGGGGCGAATTGCATCTCGCCCGCTCTGTCTTCGGGCTCG 3610
D  R  Q  E  V  N  S  P  G  G  Y  L  R  A  M  T  D  R  A  R  R  G  E  L  H  L  A  R  S  V  F  G  L  A

CTGCCCGAAATTCCATGGAGATCAGACATTGAGACGTAACGCTTTGCTGGTGCTTTTCGCCGCCCTCGTCATTGTTCACG..                 3690
  A  R  N  S  M  E  I  R  H  *  End ORF3
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FIG. 1. (A) Physical map of the *ori* pRmeGR4a region. The six ORFs are indicated in the upper part by open arrows. Derivative clones p4512-2 and p4010-3 obtained by deletion construction are shown below the restriction map. pJMB40repC⁻, that carries a frameshift mutation introduced in the unique *Mlu*I restriction site present in the sequence (solid triangle) is also represented. (Abbreviations: P, *Pst*I; N, *Not*I; X, *Xho*I; H, *Hin*dIII; Rv, *Eco*RV; St, *Sst*I, Sp, *Ssp*I). (B) Nucleotide sequence of the ORF3 coding and upstream region. Below the DNA sequence and using one-letter symbols are represented the ORF2 C-terminal segment and ORF3 amino acid sequences are shown. A potential ribosome-binding site is underlined. Horizontal arrows indicate the three inverted repeats detected. Nucleotide positions 2097 to 2135 marked in bold type corresponds to an A + T-rich region (69.2%). The double-underlined segment is conserved in the upstream regions of *repC* genes found in Ri and Ti plasmids from *Agrobacterium* with a 60% identity.

lication is delimited by clones p4512-2 and p4010-3 Fig. 1A). Deletions carried out with plasmid p4010-3 did not result in a significant decrease of plasmid stability compared with plasmid pJMB40 (0.2% loss per generation). On the contrary, plasmid p4512-2 showed an instability of 1.7% loss per generation that it is considerably higher than that measured for plasmid pJMB45 (<0.2%). Further deletions resulted in the loss of the ability of the plasmid to replicate in GRM8SR.

The nucleotide sequence of the *Pst*I fragment (4844 bp) revealed, (using FASTA (Pearson and Lipman, 1988). PEPTIDE-STRUCTURE, PILEUP, and STEMLOOP programs), the presence of six open reading frames with high coding probability and rightward polarity (Fig. 1A). At 621 bp downstream of the stop codon of ORF2 is the starting ATG of ORF3 (Fig. 1B) which is 1274 bp long and potentially coding for a 425 amino acid residues protein (M_r = 47,594). This gene is essential for plasmid replication. The introduction of 4-bp (after Klenow treatment and further religation) in the unique *Mlu*I site (position 2467) located within the coding region of ORF3, abolished replication of a pJMB40 plasmid derivative in GRM8SR (Fig. 1A). The involvement, if any, of the ORFs other than ORF3 in pRmeGR4a stable maintenance is not clear and further studies are needed to elucidate their possible role.

The 621-bp intergenic region between ORF2 and ORF3 showed the presence of three contiguous inverted repeats between coordinate positions 1803 and 1925 (Fig. 1B). The inverted repeats are located in a G + C rich domain that may include a transcriptional terminator for ORF2 although typical polyU sequences are not present. A DNA segment with a high A + T content (69.2%) between nucleotide positions 2097 and 2135 was also found in the ORF2 and ORF3 intergenic region. A search for the presence of direct repeats or iterons (characteristics of some well studied replicons) within this DNA fragment and in the entire sequence was not successful. The possible role of the inverted repeats or the A + T-rich region in replication or stabilization is not yet known. The data available indicate that plasmids containing a deletion on this fragment show an instability level of about 2% loss per generation. However, effects on copy number cannot be ruled out and more studies are necessary to elucidate the importance of this region in stable plasmid maintenance. The functional origin of replication of plasmid pRmeGR4a may be located within the coding region of RepC, as it has been demonstrated for several other replicons.

Search in the Swissprot and PIR data bases for homologies to proteins encoded by the *ori* region of pRmeGR4a yielded relevant data only to the protein encoded by ORF3. This putative protein, which was identified using an *in vitro*-coupled transcription/translation system as a protein of a relative molecular weight of 46,000 Da (data not shown), showed homology (29.47% overall identity) with a possible replication protein (RepC) of plasmid pRiA4b of *Agrobacterium rhizogenes* (Nishigushi *et al.*, 1987). Furthermore, comparison with the RepC protein of *A. tumefaciens* pTiB6S3 plasmid (Tabata *et al.*, 1985) revealed an identity of 26.20%. The best alignment among the three sequences is shown in Fig. 2. Identities are located mainly in the N- and C-terminal domains of the proteins. The *Agrobacterium* RepC proteins as well as the GR4 RepC protein presented in this work are essential for replication of their respective plasmids, as revealed by deletions or frameshift mutations. However, their mode of action in replication is not known.

Sequence analysis of the GR4 RepC protein showed a putative helix–turn–helix (HTH)[2] motif according to Chou and Fasman (1978), between amino acid positions 83 to 105 that fit with the motifs found in certain prokaryotic regulatory proteins (Kondorosi *et al.*, 1991). On the other hand, examination of the α-helix domain present in the N-terminal portion of the protein indicated the existence of a putative leucine-zip-

[2] Abbreviations used: HTH, helix–turn–helix; L2, leucine zipper; ORF, open reading frame.

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Agrorhi  MMQTGSVTTP FGRRPMTLAL VRRQTALADI KQGKTADKWK VFRDASAAME
 Agrotu  .MQTHLSTTP FGRRPMTLGQ ISSQMSAKAV APDATANKWH VFQNIREARE
Oriorf3  MGNTQIHQRP IGRRITPQRA QFRRLAESAD IGTVTRAQLA VLAQSLTCTG

Agrorhi  LLGIQSNSLA VLDALLSFHP ETELRQEAQL IVFPSNAQLA LRAHGMAGAT
 Agrotu  LLGATDRSLA ILNALLTFHP ETTLTGDGEI IVWPSNEQLA ARANGMPATT
Oriorf3  LINGTESHL. .LVALINTVP ADSFDRSGRL IVFKSNQQLA F.EIGRSVGR

Agrorhi  LRRHIAMLVE SGLIVRKDSA NGKRYARKDG AGQIERAFGF DLSPLLARSE
 Agrotu  LRRHLAVLVE CGLVIRRDSP NGKRFARKGR GGEIEQAYGF DLSPIVARAK
Oriorf3  VSRMLSSLFD AGLITMQDSG NYKRYPIRNG DGEITDGCGI DLRILIVRYA

Agrorhi  ELAMMAQQVM ADRAAFRMAK ESLTICRRDV RKLITAA..M EEGAEGDWQA
 Agrotu  EFRDMAEAIQ AEKKAFRVAK ERLTLLRRDI VKLIDAG..I EEGVPGNWCG
Oriorf3  ELDELVRQAR SQKKARRRGL RRYRGALRNA RYALAAAARL SEDSLSRLGA

Agrorhi  VEEVYVELVG RIPRAPTL.. ....ADVESI LEEMWMLQEE IINRLEIRDN
 Agrotu  VQQVYQAIIG RLPRSAPR.. ....QLVEEI CIGLHALYIE IRDVLESFAK
Oriorf3  RIERVSNIVG IAGKASSRVL RRATALLEWL IERM....MQ LPRRSAEAQK

Agrorhi  SENNSTNAAQ SEQHIQNSKP ESV..NELEP R.......SE KEQGA.....
 Agrotu  TQIQDANESH FGRHIQNSKP DSIPESEYGF G.......NK PEAGG.....
Oriorf3  TQNMTCPYVE NGMHKQITTP HPFDDSSDQR RSAYAEQLSS PKAGSASKWA

Agrorhi  .......KPS EIDRARSEPI KAFPLGMILK ACPTIGNYGP SGAVASWRDL
 Agrotu  .......TVE EFDNVRSLPK RELPLGIVLN ACPSVLELAQ GGEIRHWRDF
Oriorf3  FEESLRRRRS EINQPRRLRH AMVALQDVLR AVPALKTYGF APTT..WADL

Agrorhi  MSAAVVVRSM LGVSPSAYQD ACEAMGPENA AAAMACILER AN..FINSPG
 Agrotu  LATVELARPM LGISQSAWRE ALDELGEQHA AITLAAIYQK AD..QIGSAG
Oriorf3  ARAVPLMCRF AGISEDARRR AVEQMGEQQA AVAVAVTLEK YDRQEVNSPG

Agrorhi  GYLRDLTRRS ELGKFSLGPM IMALLKA... ..SGQGTLRF G.........
 Agrotu  GYLRNLTDRA RDGKFSTWPM IMALLRAKLD AQKVKADDRL PALNETADNG
Oriorf3  GYLRAMTDRA RRGELHLARS VFGL...... AARNSMEIRH ..........

Agrorhi  .......... .........  405
 Agrotu  SGLRASDALL RTLGKSRPK  439
Oriorf3  .......... .........  425
```

FIG. 2. Protein sequence alignment of the RepC proteins. Identical residues are in boldface. Agrorhi, RepC protein of Ri plasmid of *A. rhizogenes;* Agrotu, RepC protein of Ti plasmid of *A. tumefaciens;* oriorf3, putative RepC protein of plasmid pRmeGR4a of *R. meliloti* GR4.

per (LZ) motif (aminoacid positions 25 to 46). These motifs have been also found in certain other proteins involved in plasmid replication (Giraldo *et al.*, 1989; Nieto *et al.*, 1992). Presence of a Gly residue in position 32 possibly does not destabilize the α-helix, as deduced from the Chou–Fasman (1978) prediction, because of the influence of the adjacent residues. Amino acid positions other than Leu in the LZ motif conserved the charge and chemical character of those reported for *Pseudomonas* plasmid pPS10 RepA protein (Nieto *et al.*, 1992). Nevertheless, site-directed mutations in this region will be needed to assess its function in plasmid replication.

Homology with the pRmeGR4a repC gene and adjacent regions has been detected in other plasmids of *R. meliloti* strains. Thus, hybridization experiments using an internal EcoRV restriction fragment that contains almost the entire sequence of *repC* as a probe showed homology with a high-molecular-weight *Eco*RI restriction fragment of plasmid pRmeGR4b (data not shown). Moreover, experiments using internal *repC* oligonucleotides and oligonucleotides flanking the ORF2 and *repC* intergenic region as primers in PCR experiments, produced DNA amplifications in GR4 derivatives harboring only plasmid pRmeGR4b and also in other *R. meliloti* strains field isolates (Toro and Villadas, unpublished data). These data, along with the observed homology with *Agrobacterium* RepC proteins, may indicate that some of the large plasmids in Rhizobiaceae have related replication proteins and adjacent regions, and that similar plasmid replication mechanisms may be functioning in these two species.

To our knowledge, this is the first replication region of a *Rhizobium* plasmid that has been characterized. We believe that this work could serve as a preliminary effort in the elucidation of replication and stabilization mechanisms in rhizobial plasmids, as well as to establish phylogenetic relationship among plasmids in Rhizobiaceae.

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