**Rhizobium meliloti** lacking **mosA** synthesizes the rhizopine scyllo-inosamine in place of 3-O-methyl-scyllo-inosamine

J. Papa Rao, Wojciech Grzemski and Peter J. Murphy

The **Rhizobium meliloti** Rm220-3 **mos** locus producing the rhizopine scyllo-inosamine (**SI**) in nodules is shown to consist of three ORFs (ORF1, **mosB** and **mosC**) arranged in an operon structure. This differs from the **R. meliloti** L5-30 **mos** locus, which produces 3-O-methyl-scyllo-inosamine (**3-O-MSI**), by the complete absence of **mosA**. The deletion covers a region of 1235 nt and includes the entire **mosA** gene as well as the sequence both upstream and downstream. As a result, Rm220-3 **mos** ORF1 shares a 5' region common with L5-30 ORF1 but includes an additional 10 bp insertion and a section in the L5-30 **mosA** and **mosB** intercistronic region. Antibodies against L5-30 Mos proteins detected MosB and MosC proteins in Rm220-3-induced nodules but no translation product for either ORF1 or **mosA** was detected. A construct prepared from the L5-30 **mos** locus which has a truncated **mosA** gene produces **SI** rather than 3-O-MSI, confirming this gene is involved in a methylation step in the production of 3-O-MSI. Further, changes made to this ORF result in reduced levels of the rhizopine.

**Keywords**: rhizopine synthesis genes, *Rhizobium meliloti*, symbiosis

**INTRODUCTION**

Rhizopines are synthesized in bacteroids within nodules during the symbiotic interaction between legumes and certain rhizobia. To date two rhizopines have been found, 3-O-methyl-scyllo-inosamine (**3-O-MSI**) and scyllo-inosamine (**SI**) (Murphy et al., 1987, 1988, 1993, 1994; Murphy & Saint, 1992; Saint et al., 1993). In a recent survey about 10% of *Rhizobium meliloti* and *Rhizobium leguminosarum* bv. *viciae* but not *R. leguminosarum* bv. *trifolii* were found to produce one or the other of these rhizopines (Wexler et al., 1995). The genes for rhizopine biosynthesis (**mos** genes) have been investigated in two strains of *R. meliloti*, L5-30 and Rm220-3 which produce 3-O-MSI and SI, respectively. The **mos** genes from both of these strains are located on the *nod-nif* symbiotic plasmid and are regulated by NifA/NtrA type symbiotic promoters (Murphy et al., 1987; Murphy & Saint, 1992; Saint et al., 1993).

Where detailed studies have been conducted the rhizopine catabolism genes (**moc**) have been shown to be closely linked to the synthesis genes (Murphy et al., 1987; Murphy & Saint, 1992; Saint et al., 1993). Thus rhizopines, which are synthesized in nodules only by certain biovars, are catabolized by free-living bacteria and may result in a selective advantage for these bacteria. These strains have a competitive advantage in nodulation (Murphy & Saint, 1992; Murphy et al., 1995).

The L5-30 **mos** operon consists of four ORFs termed ORF1, **mosA**, **B** and **C**. Although ORF1 is transcribed as part of the **mos** operon no translation product can be found in nodules, nor is a functional protein from this ORF required for rhizopine biosynthesis (Murphy et al., 1993). The sequence of a 320 nucleotide region covering the promoter and part of ORF1 of the **SI**-producing strain Rm220-3 has been reported (Saint et al., 1993). Furthermore, regions equivalent to **mosB** and **C** but not **mosA** of L5-30 were detected by Southern hybridization in the Rm220-3 **mos** locus.

In this study, to provide further insight into the structure of **mos** genes, the complete sequence of the Rm220-3 **mos** locus is reported. The precise nature of the nucleotide sequence surrounding deletion breakpoints of a region equivalent to L5-30 **mosA** is defined in the Rm220-3 **mos** operon. Antibodies were also used to detect Mos proteins.
in nodules. We also provide direct evidence for the involvement of the L5-30 MosA protein in the conversion of SI to 3-O-MSI in symbiotic nodules.

**METHODS**

**Bacterial strains, plasmids and growth conditions.** *R. meliloti* and *Escherichia coli* strains, plasmids constructed and used in this study are listed in Table 1. *E. coli* was grown in Luria–Bertani (LB) medium (Miller, 1972) at 37 °C. Antibiotic concentrations used in the LB medium for *E. coli* cultures with plasmids were 100 μg ampicillin ml⁻¹, 12 μg tetracycline ml⁻¹ and 30 μg kanamycin ml⁻¹. *R. meliloti* Rm1021 was grown in TY complex medium (Beringer, 1974) at 28 °C. Bacterial triparental matings (Murphy et al., 1987) were performed at 28 °C on TY medium. *R. meliloti* strains containing plasmids with cloned L5-30 *mos* genes were selected and maintained on TY medium containing 100 μg rifampicin ml⁻¹ and 12 μg tetracycline ml⁻¹.

**Plasmid DNA extraction and manipulations.** Plasmid DNA extraction, manipulation and analysis was carried out by standard procedures (Sambrook et al., 1989). For generating nested deletions with exonuclease III (Henikoff, 1984), closed circular DNA was purified by acid phenol extraction as described (Zasloff et al., 1978). This procedure was also used to selectively remove traces of linear background DNA from plasmid DNA that was used for sequencing reactions. DNA ligation and restriction enzyme digestion of plasmid DNA were according to the manufacturer's instructions (Boehringer Mannheim). *E. coli* JM109 competent cells for plasmid DNA transformation were prepared as described (Alexander, 1987). DNA fragments from agarose gels were isolated and purified using the GenecleanII kit (Bio 101).

<table>
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<th>Table 1. Bacterial strains and plasmids</th>
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<td><strong>Strain or plasmid</strong></td>
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<td><strong>E. coli</strong></td>
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<td>JM109</td>
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<td>BL21(DE3)</td>
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<tr>
<td><strong>R. meliloti</strong></td>
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<td>Rm220-3</td>
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<td>Rm1021 Rif⁺</td>
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<td><strong>Plasmid</strong></td>
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<td>pPM1062</td>
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<td>pJPM5</td>
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<td>pJPM6</td>
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<td>pGEM-7Zf(+)</td>
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<td>pLAFR3</td>
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<td>pRK2013</td>
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<td>pVK102</td>
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<td>pGEMEX-1</td>
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Cloning and sequencing of DNA. A 3.65 kb KpnI–ApaI DNA fragment containing most of the Rm220-3 mos locus was isolated from pPM1169, a pVK102 based cosmid containing this locus. This fragment was cloned into the KpnI site of pGEM-7Zf(+) using a strategy (Fig. 1) designed to enable the nucleotide sequencing of both strands. The 3.65 kb KpnI–ApaI fragment was first ligated with KpnI-digested pGEM-7Zf(+) DNA, then the KpnI and ApaI cohesive ends were blunt-ended by the fill-in reaction using Klenow DNA polymerase (Promega), then ligated and the plasmid construct was introduced into E. coli JM109 by transformation. The resulting recombinant clones were found to have the DNA inserted in only one direction (pJPM1) as shown in Fig. 1. To provide a unique restriction site that protects DNA from exonuclease III activity, to facilitate the generation of unidirectional nested deletions for the second strand sequencing, the recombinant plasmid pJPM1 was cut with EcoRI and a 5.52 kb EcoRI–EcoRI fragment that contained a 2.52 kb mos EcoRI–ApaI region was isolated and re-ligated (pJPM2). The other 1.13 kb EcoRI–EcoRI fragment, containing a KpnI–EcoRI DNA segment of mos region, was cloned into the EcoRI site of pGEM-7Zf(+) and a clone having the insert in the required direction was selected (pJPM3).

Unidirectional deletions were generated in both orientations of the mos DNA by exonuclease III and S1 nuclease digestion (Henikoff, 1984), using the ‘Erase-a-Base’ kit (Promega). For pJPM1 and pJPM3 clones, the ApaI site was used to protect against exonuclease III activity and the Xbal site was used for unidirectional deletion. For the pJPM2 clone, the SacI site was used to block the exonuclease III activity and the Xbal site was used for exonuclease III activity. pJPM1 and pJPM3 clones and their nested deletions were sequenced using the M13 forward sequencing primer, and pJPM2 and its nested deletion clones were sequenced with the M13 reverse primer. Sequences were determined by the dideoxy method (Sanger et al., 1977), of double-stranded DNA templates using a Prism ready reaction dye primer cycle sequencing kit (Applied Biosystems) and were analysed with an Applied Biosystems DNA sequencer (model 373A).

**DNA sequence analysis.** Nucleotide sequences were examined and aligned using the SeqEd 1.0 computer program (Applied Biosystems). Further analysis was done by the University of Wisconsin GCG program version 7.0 (Devereux et al., 1984), and also by the DNA Strider PC program.

**Preparation of antibodies and Western blotting.** Antibodies were prepared from over-expressed fusion proteins of ORFl, MosA, -B and -C from R. meliloti L5-30 as described by Murphy et al. (1993). Rm220-3 nodule protein extracts were separated by SDS-PAGE and the gels were blotted onto nitrocellulose membranes and probed with antibodies prepared to L5-30 Mos proteins as described by Murphy et al. (1993)

**Construction of a L5-30 mosA deletion mutant.** An L5-30 mos 5.7 kb NdeI–ApaI DNA fragment obtained from pPM1062, a pLAFR1 clone containing this locus, was blunt-ended with T4 DNA polymerase (Boehringer Mannheim), then Xbal-liganded and cloned into the pGEM-7Zf(+) plasmid, termed pPM1242. An internal in-frame deletion of mosA was prepared utilizing two BamHI sites separated by 771 bp (Fig. 5). To avoid the BamHI site in the polycloning region of the pGEM-7Z(+) sequence, pPM1242 was cut with SacI, and the 3.25 kb SacI–Sal fragment containing the vector BamHI site was eliminated. The remaining 5.45 kb SacI–Sal fragment containing the vector and mosA sequence was ligated (creating pPM1242a), and the plasmid construct was introduced into E. coli JM109 by transformation.

The plasmid DNA from this clone was digested with BamHI, blunt-ended with 1 unit mung bean nuclease per µg DNA (New England Biolabs), and incubated at 30 °C for 30 min, then ligated and the plasmid construct was introduced into E. coli JM109 by transformation. To check that the remaining mosA region maintained the original reading frame, the region bordering the deletion was sequenced. For this purpose an ApaI–Sal fragment was removed from the BamHI–BamHI deletion clone (pPM1242b), blunt-ended with mung bean nuclease, ligated and sequenced using the forward sequencing primer as described above. To complete the construction of the mosA deletion mutant, a 1.6 kb ApaI–Sal fragment from pPM1242b was cloned into a 6.25 kb ApaI–Sal fragment (from pPM1242) that contained the pGEM-7Zf(+) sequence and a 3.2 kb fragment of the mos region. The resulting deletion construct of the mos operon, pJPM4, is a 4.85 kb Xbal–Xbal mos fragment cloned into the Xbal site of pGEM-7Zf(+).

The 4.85 kb Xbal–Xbal L5-30 mos fragment from pPM4 was further cloned into the Xbal site of pLAFR3, a broad host range vector to enable mobilization into R. meliloti, and this clone was termed pPM6. The 5.7 kb Xbal–Xbal L5-30 mos fragment from pPM1242d was cloned into the Xbal site of pLAFR3 vector, resulting in pJPM5, which served as the wild-type control clone for the deletion mutant of pPM6.

**Detection of rhizopines from nodules.** Plasmids pJPM5 and pPM6 were mobilized from E. coli to R. meliloti Rm1021 RifR by triparental mating (Murphy et al., 1987), with the helper plasmid pRK2013 on TY medium at 28 °C. Alfalfa plants, grown on agar medium (Kondorosi et al., 1977) in glass tubes, were inoculated separately with Rm1021 carrying either pJPM5 (a mosA control plasmid) or pPM6 (a mosA mutant construct) as described by Murphy et al. (1993).

**Preparation of antibodies and Western blotting.** Antibodies were prepared from over-expressed fusion proteins of ORFl, MosA, -B and -C from R. meliloti L5-30 as described by Murphy et al. (1993). Rm220-3 nodule protein extracts were separated by SDS-PAGE and the gels were blotted onto nitrocellulose membranes and probed with antibodies prepared to L5-30 Mos proteins as described by Murphy et al. (1993).
Fig. 2. Nucleotide and deduced amino acid sequences of Rm220-3 mos operon. The sequence is determined for both strands. The Hifa/Nitra-binding sites within the mos promoter are bold-faced. The amino acid sequences are shown for underneath that of Rm22O-3. The potential Shine-Dalgarno sequence for each gene is indicated by single underlining before the ATG start codon. The likely rho-independent terminator is indicated (> > > >). The sequence of the region

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**Note:** The text contains nucleotide and deduced amino acid sequences, along with various annotations and figures relevant to microbiology research.
incorporating part of the DNA sequence which is in the L5-30 region, Fig. 2), there are 12 single base changes and two regions common to L5-30 and Rm220-3 ORFl (227-11 kb). Consequently, the structure of Rm220-3 ORFl differs from that of L5-30. Both have very similar 5' regions but differ in the downstream (Figs 2, 3). The deleted region upstream of ORFl (including the stop codon) and 189 nt of the ORFl are boxed (2). The site of a novel 10 bp insertion in the Rm220-3 ORFl is shown. Structures of 3-O-MSI and SI are shown towards the right of L5-30 and Rm220-3 ORFs, respectively.

RESULTS AND DISCUSSION

Structure of the Rm220-3 mos locus

We have analysed the rhizopine SI synthesis genes (mos) from R. meliloti strain Rm220-3 and defined three ORFs that are organized in a 3-9 kb operon unit (Fig. 2). By analogy with the L5-30 mos locus which produces the rhizopine 3-O-MSI these ORFs have been termed ORFl (396 bp), mosB (1521 bp) and mosC (1221 bp). The Rm220-3 mos locus has a large deletion encompassing a region equivalent to the entire mosA gene of L5-30 with the remainder of the locus, including the 5' and 3' untranslated regions being similar to the L5-30 mos locus. Excluding the deleted region there is 98.26% sequence conservation between Rm220-3 and L5-30 mos operons. The 1235 bp deletion (1276-2510 nt, of the L5-30 mos sequence, GenBank accession number L17071) in the Rm220-3 mos locus spans a region equivalent to the entire L5-30 mosA gene as well as 203 nt upstream and 33 nt downstream (Figs 2, 3). The deleted region upstream of mosA consists of 14 nt of the 5' end of the L5-30 mos ORFl (including the stop codon) and 189 nt of the intercistronic region between ORFl and mosA. Consequently, the structure of Rm220-3 ORFl differs from that of L5-30. Both have very similar 5' regions but differ in their 3' region, with Rm220-3 ORFl being 96 nt longer, incorporating part of the DNA sequence which is in the L5-30 mosA and B intercistronic region. In the 286 bp region common to L5-30 and Rm220-3 ORFl (227-512 nt, Fig. 2), there are 12 single base changes and two deletions of 3 bases which are 14 bp apart. The majority of these changes occur just prior to the point where these sequences diverge. At the point of divergence in Rm220-3 (where the deletion starts) a 10 bp sequence (TATACGTCCA) not found in the L5-30 mosA region (Fig. 3) is present. Although this is suggestive of a footprint left by a departing transposon, the region in L5-30 corresponding to the deletion contains no repeated sequences characteristic of transposons (Döring & Starlinger, 1986).

Rm220-3 and L5-30 mosB and mosC are the same size and have extensive sequence identity. There are 18 single nucleotide changes in mosB and 17 single nt changes, one nt addition and one nt deletion in mosC of the Rm220-3 mos operon as compared with the corresponding regions in the L5-30 mos operon. There are no differences in potential Shine–Dalgarno sequences and initiation codons for ORFl, mosB and mosC in these operons. The sequence homology also extends to the untranslated 3' region with only 7 bp differences in a 357 bp region.

As with the L5-30 mos locus two in-frame translational initiation codons are present for mosC, ORFs C1 and C2. The molecular masses of polypeptides expected to be encoded by mos ORFs are 146 (ORFl), 56 (MosB), 43 (MosC1) and 40.9 kDa (MosC2), respectively. Putative Shine–Dalgarno sequences are present upstream of ORFl, mosB and mosC2 initiation codons. mosC1 translation may result from a read-through of ribosomes following on from the mosB termination codon.

A comparison of the amino acids coded by L5-30 and Rm220-3 mosB and mosC genes reveals that there are five amino acid changes for MosB proteins and 26 amino acid changes for MosC proteins (Fig. 2). Most of the changes in MosC are in the carboxy-terminal end of the protein and are the result of a frame-shift. In this region, the Rm220-3 Mos region reverts to the same frame as L5-30 MosC, 17 amino acids downstream of the point of divergence.

Does Rm220-3 mos ORFl produce a polypeptide in vivo?

Recently we reported that L5-30 MosA, B and C are translated in vivo, whereas the translation product of ORFl was not detected (Murphy et al., 1993). Rm220-3 ORFl has a similar 5' region (286 bp) to that of L5-30 but has an extended 3' region. Therefore, it was of interest to see if these changes resulted in a translation product being detectable in nodules. To detect the presence of Rm220-3 Mos polypeptides in nodules, nodule extracts were analysed by Western blotting using purified antibodies prepared against over-expressed L5-30 Mos fusion polypeptides. The results of Western blots of Rm220-3 nodule extracts (Fig. 4) reveal that 56 kDa MosB (lane 5),
43.1 kDa MosC1 (lanes 6 and 7) and 40.9 kDa MosC2 (lane 7) proteins are detected. As expected, due to the absence of mosA in the Rm220-3 mos locus the MosA antibody, which detects MosA in L5-30 (Murphy et al., 1993), did not detect a protein (Fig. 4; lane 4). This result also indicates that there is no other mosA gene elsewhere in the genome of Rm220-3. Similarly, with L5-30 no ORF1 protein (predicted to be 14.6 kDa) was detected in Rm220-3 (Fig. 4; lane 3). Since the antibody raised against ORF1 of L5-30 was prepared from a region common to L5-30 and Rm220-3, it would be expected to detect a protein if one were present. At present we cannot distinguish between the possibilities that this protein is absent because it is not synthesized or because it is rapidly degraded. Nevertheless, the conservation of the 5’-regions of ORF1 from Rm220-3 and L5-30, as well as with the nfeI locus (which is involved in nodulation competitiveness in R. meliloti GR4, Soto et al., 1993), suggests that this region has an important role; such as in mRNA stability.

Evidence for involvement of L5-30 MosA in the addition of a methyl group to the rhizopine 3-O-MSI

The rhizopines 3-O-MSI and SI produced by strains L5-30 and Rm220-3, respectively, only differ by the presence of a methyl group on the former compound (Fig. 3). At the genetic level, the Rm220-3 mos locus differs from L5-30 by having an extended ORFl and by the complete absence of mosA. Since an ORFl gene product is not required for rhizopine production in L5-30, it is most likely that MosA is a methylase involved in the conversion of SI to 3-O-MSI.

To test this we constructed an in-frame deletion of mosA from the L5-30 mos locus and investigated its effect on rhizopine production in nodules. This involved the removal of an internal 771 bp BamHI fragment from mosA. After blunt-ending and re-ligation of the construct, sequencing showed that an additional 84 nt were removed from around the BamHI site by this procedure. The deletion is from 1535–2389 nt of the L5-30 mos sequence and results in an extensively truncated in-frame mosA consisting of 144 bp (see Fig. 5). To investigate the effect of this mutation on the synthesis of the rhizopine, this R. meliloti L5-30 mos mutant and control strains producing 3-O-MSI and SI were separately inoculated onto alfalfa plants. Nodules were analysed for rhizopines. The results of HVPE analysis for rhizopine production in nodule extracts are shown in Fig. 6. The L5-30 mosA control construct pJPM5 (the starting clone for the mosA deletion) in R. meliloti Rm1021 produces 3-O-MSI (Fig. 6; lane 2) whereas the L5-30 mosA deletion construct pJPM6 in Rm1021 synthesizes SI (Fig. 6, lane 3) in nodules.

The MosA mutant strain produces SI rather than 3-O-MSI and, as mentioned above, there is no other copy of
mosA in the genome, therefore it is likely that mosA codes for a methylase involved in the addition of a methyl group to SI in the synthesis of 3-O-MSI in symbiotic nodules. This is despite the extensive amino acid similarity between MosA and DapA, a dihydrodipicolinate synthase, from E. coli (Richaud et al., 1986; Murphy et al., 1993). The possibility that MosA affects methylation indirectly rather than by acting as a methylase per se has not been eliminated.

Since no ORF1 translation product can be found, MosB and MosC must be involved in the production of SI. Sequence homology suggests MosB is a regulatory protein (Murphy et al., 1993), and the hydrophobicity profile, as well as immunogold studies (W. G. & P. J. M., unpublished results) implicate MosC as a transport protein in this process. Such a gene structure takes advantage of pre-existing pathways in bacteria to produce the rhizopine. A recent analysis of the L5-30 moc catabolism genes from L5-30 also indicates that this locus utilizes other pathways already present in bacteria (Rossbach et al., 1994).

**Level of rhizopine expression**

Strains containing the mosA deletion plasmid (pJPM6) did not produce as much rhizopine (SI) as did strains containing the parent plasmid (pJPM5, which produces 3-O-MSI), (Fig. 6, lanes 2 and 3). These plasmids differ only by the mosA gene being truncated to a small, non-functional 144 bp ORF.

As all rhizopine strains tested catabolized both SI and 3-O-MSI equally well (M. Wexler, personal communication) it is unlikely that the differing amounts of rhizopine is due to preferential catabolism of SI compared with 3-O-MSI. The differential expression of rhizopine may therefore be due to the reduction in translation of MosB and MosC as a result of the small non-functional upstream reading frame (present in pJPM6) as has been reported in polycistronic mRNA in eukaryotes (Futterer & Hohn, 1992). Alternatively, since SI is a likely precursor in the biosynthetic pathway of 3-O-MSI the absence of a functional mosA gene, and the resulting accumulation of SI, may result in end-product inhibition by SI.

**Evolution of rhizopine genes**

This study indicates that either the Rm220-3 mos locus has arisen as a result of mosA and the surrounding sequence being deleted from the L5-30 mos locus (or a closely related strain), or the L5-30 mos locus arose from an insertion within the Rm220-3 mos locus. As deletions in symbiotic plasmids are common (Romero et al., 1991), we consider it most likely that the Rm220-3 mos locus is the result of a deletion. Consistent with this suggestion a recent survey of the frequency of inositol rhizopines has shown that only one strain (Rm220-30) out of 22 rhizopine strains isolated produced SI (Wexler et al., 1995). Furthermore, Rm220-3 can catabolize 3-O-MSI as well as SI and restriction-fragment-length polymorphism of Rm220-3 and L5-30 catabolic genes shows them to be very similar (1.5% divergence, Saint et al., 1993). Together these data suggest Rm220-3 is derived from a deletion of a 3-O-MSI producing strain.

With the exception of the mosA deletion, the mos genes from these two rhizopine strains show a high degree of conservation. Similarly, restriction-fragment-length polymorphism indicates that other rhizopine mos genes are also similar (4-5% divergence in R. meliloti and 1-6% in R. leguminosarum bv. viciae, M. Wexler, personal communication). These rhizopine strains were isolated from geographically widespread regions. This may reflect the spread of rhizopine strains with the dissemination of cultivated pasture plants, followed by the rapid adaptation of the bacteria to the new environment.

Despite extensive DNA sequence conservation and geographical dispersal of rhizopine strains, only 10% of R. meliloti and R. leguminosarum bv. viciae strains are rhizopine strains. That the percentage is not higher might reflect a recent evolution and on-going dispersal of rhizopine genes in natural populations. The plasmid localization of rhizopine genes in all rhizopine strains studied to date (M. Wexler, personal communication) would aid in this...
process. Further explanations could be that rhizopine genes only function in strains with a particular chromosomal background, or the presence of different rhizopines in other strains. In this sense it should be noted that if all strains had the same rhizopine there would be no advantage for a particular strain in intra-species nodule competition.

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REFERENCES


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