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

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

The Rhizobium meliloti Rm220-3 mos locus producing the rhizopine scylo-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-scylo-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

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**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-scylo-inosamine (3-O-MSI) and scylo-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 those 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 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Reference/source</th>
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<tr>
<td><strong>Strain</strong></td>
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<tr>
<td><em>E. coli</em></td>
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<td>JM109</td>
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<tr>
<td>BL21(DE3)</td>
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<td>Novagene</td>
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<td><strong>R. meliloti</strong></td>
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<tr>
<td>Rm220-3</td>
<td>Mos⁺ Moc⁺ Str⁺, produces SI</td>
<td>Gift from A. Pühler, Saint et al. (1993)</td>
</tr>
<tr>
<td>L5-30</td>
<td>Mos⁺ Moc⁺, produces 3-O-MSI</td>
<td>Dénarié, J. Kowalski (1967)</td>
</tr>
<tr>
<td>Rm1021</td>
<td>Mos⁺ Moc⁻</td>
<td>Meade et al. (1982)</td>
</tr>
<tr>
<td>Rm1021 Rif²</td>
<td>Mos⁺ Moc⁻ Rif²</td>
<td>This study</td>
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<td><strong>Plasmid</strong></td>
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<tr>
<td>pPM1169</td>
<td>Mos⁺ Moc⁺, 3-5-, 1-0- and 7-3 kb <em>HindIII</em> Rm220-3 fragments of pPM1153 in pVK102, Km⁺, Tc⁺</td>
<td>Saint et al. (1993)</td>
</tr>
<tr>
<td>pPM1062</td>
<td>Mos⁺ Moc⁺, 3-4- and 6-9 kb <em>EcoRI</em> fragments of L5-30 in pLAFR1, Tc⁺</td>
<td>Murphy et al. (1987)</td>
</tr>
<tr>
<td>pPM1242</td>
<td>5-7 kb <em>NdeI</em>- <em>ApaI</em> L5-30 mos fragment from pPM1062 cloned into pGEM-7Zf(+)</td>
<td>This study</td>
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<tr>
<td>pPM1242a</td>
<td>2-45 kb <em>ApaI</em>- <em>SacI</em> L5-30 mos fragment of pPM1242 cloned into pGEM-7Zf(+)</td>
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<tr>
<td>pPM1242b</td>
<td>Clone derived from pPM1242a after an in-frame deletion of L5-30 mosA ORF</td>
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<td>pJPM1</td>
<td>3-65 kb <em>KpnI</em>- <em>ApaI</em> Rm220-3 mos fragment cloned into pGEM-7Zf(+)</td>
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<td>pJPM2</td>
<td>Obtained from pJPM1 with deletion of 1-13 kb <em>EcoRI</em> fragment</td>
<td>This study</td>
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<td>1-13 kb <em>EcoRI</em> fragment of pJPM1 cloned into pGEM-7Zf(+)</td>
<td>This study</td>
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<tr>
<td>pJPM4</td>
<td>L5-30 mosA ORF in-frame deletion mutant derived from pPM1242</td>
<td>This study</td>
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<tr>
<td>pJPM5</td>
<td>5-7 kb L5-30 mos DNA from pPM1242 cloned into pLAFR3</td>
<td>This study</td>
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<td>pJPM6</td>
<td>4-85 kb L5-30 mos DNA from pJPM4 cloned into pLAFR3</td>
<td>This study</td>
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<tr>
<td>pGEM-7Zf(+)</td>
<td>Cloning and sequencing vector</td>
<td>Promega</td>
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<td>pLAFR1, Tc⁺ cosmid cloning vector, containing <em>XbaI</em> site</td>
<td>Friedman et al. (1982)</td>
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<td>pRK2013</td>
<td>Km⁺ helper plasmid</td>
<td>Figurski &amp; Helinski (1979)</td>
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<tr>
<td>pVK102</td>
<td>Tc⁺ Km⁺ cosmid cloning vector</td>
<td>Knauf &amp; Nester (1982)</td>
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<td>pGEMEX-1</td>
<td>Expression cloning vector</td>
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<td>pLysE</td>
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Rhizopine synthesis genes of *R. meliloti*

**Preparation of antibodies and Western blotting.** Antibodies were prepared from over-expressed fusion proteins of ORF1, 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 mosA 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 XbaI-linkered and cloned into the pGEM-7Zf(+) plasmid (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-7Zf(+) sequence, pPM1242 was cut with SacI, and the 3.25 kb SacI–SacI fragment containing the vector BamHI site was eliminated. The remaining 5.45 kb SacI–SacI 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–Stop* 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–SacI* fragment from pPM1242b was cloned into a 6.25 kb *ApaI–SacI* fragment (from pPM1242) that contained the pGEM-7Zf(+) sequence and a 3.2 kb fragment of the mosA region. The resulting deletion construct of the mos operon, pJPM4, is a 4.85 kb *XbaI–XbaI* mos fragment cloned into the *XbaI* site of pGEM-7Zf(+).

The 4.85 kb *XbaI–XbaI* L5-30 mos fragment from pPM4 was further cloned into the *XbaI* site of pLAFR3, a broad host range vector to enable mobilization into *R. meliloti*, and this clone was termed pJPM6. The 5.7 kb *XbaI–XbaI* L5-30 mos fragment from pPM1242 was cloned into the *XbaI* site of pLAFR3 vector, resulting in pJPM5, which served as the wild-type control clone for the deletion mutant of pJPM6.

**Detection of rhizopines from nodules.** Plasmids pJPM5 and pJPM6 were mobilized from *E. coli* to *R. meliloti* Rm1021 Rif 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 pJPM6 (a mosA mutant construct). Wild-type *R. meliloti* L5-30 and Rm220-3 strains producing 3-O-MJ-1 and 31, respectively, and strain Rm1021 (which does not produce any rhizopines), were also inoculated onto alfalfa plants to serve as controls. Nodules were harvested after 6 weeks. Nodule extracts were prepared in water and checked for rhizopine biosynthesis by high-voltage paper electrophoresis (HVPE) using formic acid/acetic acid buffer, pH 1.7 [284 ml 98% (v/v) formic acid and 59.2 ml glacial acetic acid per l], and staining with AgNO₃ as previously described (Murphy et al., 1987; Saint 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/NtrA-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

![Fig. 2](image-url)
incorporating part of the DNA sequence which is in the deletions of 3 bases which are 14 bp apart. The majority of L5-30 (512 nt, Fig. 2), there are 12 single base changes and two their 3' region, with Rm220-3 ORFl being 96 nt longer, the intercistronic region between ORFl and mosA downstream (Figs 2, 3). The deleted region upstream of mosA (including the stop codon) and 189 nt of the untranslated regions being similar to the L5-30 region, in Rm220-3 ORFl (227-10 bp insertion in the Rm220-3 ORFl is shown. Structures of 3-O-M5I 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-M5I 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 3' 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 14-6 (ORFl), 56 (MosB), 43-1 (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), upstream of the Koni site has been reported previously (Saint et al., 1993). Stop codons of the ORFs are asterisked. The ATG for ORFl is double underlined. The in-frame ATG start site for mosC2 initiation is bold-faced. The site of a 10 bp sequence insertion, that replaced the deletion of L5-30 mosA region, in Rm220-3 mos ORFl is indicated (∆. ∆).
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 mosA mutant and control strains producing 3-O-MSI and SI were separately inoculated onto alfalfa plants. Nodules were analysed for rhizopine production in 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 mosA, 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 catabolize 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 (Fütterer & 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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