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

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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 (*mos*) 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

Abbreviations: HVPE, high-voltage paper electrophoresis; 3-O-MSI, 3-O-methyl-*scyllo*-inosamine; *SI*, *scyllo*-inosamine.

The GenBank accession number for the *mos* sequence reported in this paper is U23753.

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

Strain or plasmid	Relevant characteristics	Reference/source
Strain		
<i>E. coli</i>		
JM109		Promega
BL21(DE3)		Novagene
<i>R. meliloti</i>		
Rm220-3	Mos ⁺ Moc ⁺ Str ^r , produces SI	Gift from A. Pühler, Saint <i>et al.</i> (1993)
L5-30	Mos ⁺ Moc ⁺ , produces 3-O-MSI	Dénarié, J. Kowalski (1967)
Rm1021	Mos ⁻ Moc ⁻	Meade <i>et al.</i> (1982)
Rm1021 Rif ^r	Mos ⁻ Moc ⁻ Rif ^r	This study
Plasmid		
pPM1169	Mos ⁺ Moc ⁻ , 3.5-, 1.0- and 7.3 kb <i>Hind</i> III Rm220-3 fragments of pPM1153 in pVK102, Km ^s , Tc ^r	Saint <i>et al.</i> (1993)
pPM1062	Mos ⁺ Moc ⁻ , 3.4- and 6.9 kb <i>Eco</i> RI fragments of L5-30 in pLAFR1, Tc ^r	Murphy <i>et al.</i> (1987)
pPM1242	5.7 kb <i>Nde</i> I- <i>Apa</i> I L5-30 <i>mos</i> fragment from pPM1062 cloned into pGEM-7Zf(+)	This study
pPM1242a	2.45 kb <i>Apa</i> I- <i>Sac</i> I L5-30 <i>mos</i> fragment of pPM1242 cloned into pGEM-7Zf(+)	This study
pPM1242b	Clone derived from pPM1242a after an in-frame deletion of L5-30 <i>mosA</i> ORF	This study
pJPM1	3.65 kb <i>Kpn</i> I- <i>Apa</i> I Rm220-3 <i>mos</i> fragment cloned into pGEM-7Zf(+)	This study
pJPM2	Obtained from pJPM1 with deletion of 1.13 kb <i>Eco</i> RI fragment	This study
pJPM3	1.13 kb <i>Eco</i> RI fragment of pJPM1 cloned into pGEM-7Zf(+)	This study
pJPM4	L5-30 <i>mosA</i> ORF in-frame deletion mutant derived from pPM1242	This study
pJPM5	5.7 kb L5-30 <i>mos</i> DNA from pPM1242 cloned into pLAFR3	This study
pJPM6	4.85 kb L5-30 <i>mos</i> DNA from pJPM4 cloned into pLAFR3	This study
pGEM-7Zf(+)	Cloning and sequencing vector	Promega
pLAFR3	pLAFRI, Tc ^r cosmid cloning vector, containing <i>Xba</i> I site	Friedman <i>et al.</i> (1982)
pRK2013	Km ^r helper plasmid	Figurski & Helinski (1979)
pVK102	Tc ^r Km ^r cosmid cloning vector	Knauf & Nester (1982)
pGEMEX-1	Expression cloning vector	Promega
pLysS		Novagene
pLysE		Novagene

Fig. 1. Partial restriction map of the *R. meliloti* Rm220-3 *mos* locus and cloning and sequencing strategy for both strands. ■, Previously sequenced 5' region; ▨, pGEM-7Zf(+) sequence; B, *ApaI*-*KpnI* blunt-end ligation site. The clones containing 3.65 kb *KpnI*-*ApaI*, 2.52 kb *EcoRI*-*ApaI* and 1.13 kb *KpnI*-*EcoRI* Rm220-3 *mos* fragments in pGEM-7Zf(+) are designated pJPM1, pJPM2 and pJPM3, respectively. Arrows show the direction of sequencing with nested deletions. Restriction sites are not drawn to scale on the vector sequence.

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 clone 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 *XbaI* site was used for unidirectional deletion. For the pJPM2 clone, the *SacI* site was used to block the exonuclease III activity and the *SmaI* 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 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 *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 *XbaI*-linked and cloned into the pGEM-7Zf(+); this plasmid was termed pPM1242. An internal in-frame deletion of *mosA* was prepared utilizing two *Bam*HI sites separated by 771 bp (Fig. 5). To avoid the *Bam*HI 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 *Bam*HI site was eliminated. The remaining 5.45 kb *SacI*-*SacI* fragment containing the vector and *mos* 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 *Bam*HI, 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*-*SmaI* fragment was removed from the *Bam*HI-*Bam*HI 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 *mos* 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 pJPM4 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^r 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-MST or SI, 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 [28.4 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 NifA/NtrA-binding sites within the *mos* promoter are bold-faced. The amino acid sequences are shown for *mosB* and *mosC*. Where the Rm220-3 amino acid sequence differs from that of L5-30 the L5-30 sequence is indicated underneath that of Rm220-3. 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. 3. Schematic representation of ORFs from *R. meliloti* L5-30 and Rm220-3 *mos* operons. The natural deletion of the L5-30 *mosA* region present in Rm220-3 is boxed with broken lines. The corresponding regions of the L5-30 *mos* operon which form the Rm220-3 *mos* ORF1 are boxed (▨). The site of a novel 10 bp insertion in the Rm220-3 ORF1 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 ORF1 (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* ORF1 (including the stop codon) and 189 nt of the intercistronic region between ORF1 and *mosA*. Consequently, the structure of Rm220-3 ORF1 differs from that of L5-30. Both have very similar 5' regions but differ in their 3' region, with Rm220-3 ORF1 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 ORF1 (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 ORF1, *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 (ORF1), 56 (MosB), 43.1 (MosC1) and 40.9 kDa (MosC2), respectively. Putative Shine–Dalgarno sequences are present upstream of ORF1, *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 MosC region reverts to the same frame as L5-30 MosC, 17 amino acids downstream of the point of divergence.

Does Rm220-3 *mos* ORF1 produce a polypeptide *in vivo*?

Recently we reported that L5-30 MosA, B and C are translated *in vivo*, whereas the translation product of ORF1 was not detected (Murphy *et al.*, 1993). Rm220-3 ORF1 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 *KpnI* site has been reported previously (Saint *et al.*, 1993). Stop codons of the ORFs are asterisked. The ATG for ORF1 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* ORF1 is indicated (△, △)

Fig. 4. Western blot of Rm220-3 *mos* polypeptides. Protein extracts from nodules induced by *R. meliloti* Rm220-3 were probed with antibodies raised to L5-30 *mos* proteins. Lane 1, marker proteins (Bio-Rad low range) stained with Coomassie blue; lanes 2–7, total protein, from *R. meliloti* Rm220-3 induced nodules, stained with Coomassie blue (lane 2) or blotted and probed with antibodies to: the C-terminal region of the deduced protein from L5-30 *mos* ORF1 (lane 3), *mosA* (lane 4), *mosB* (lane 5), the hydrophilic N-terminal region of *mosC* (lane 6) (detects MosC1), and the hydrophobic region of *mosC* (lane 7) (detects MosC1 and MosC2). For details of antibody preparation see Murphy *et al.* (1993).

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, as 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.

Fig. 5. Strategy for construction of the deletion within the *mosA* ORF of the L5-30 *mos* operon. pGEM-7Zf(+) DNA is indicated (■). An L5-30 5.7 kb *NdeI*–*Apal* *mos* DNA fragment was *XbaI*-linked and cloned into pGEM-7Zf(+), (pPM1242). A 2.45 kb *Apal*–*SacI* *mos* fragment in pGEM-7Zf(+) (pPM1242a) was used for preparing the *mosA* deletion within the *mosA* ORF which was then sequenced to verify the original reading frame maintained (see Methods). The 4.85 kb *XbaI*–*XbaI* *mosA* deletion construct in pGEM-7Zf(+) is pJPM4. The deleted sequence is represented (□) and the truncated *mosA* is shown (■). B indicates *Bam*HI site. Restriction sites are not drawn to scale on the vector sequence.

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 ORF1 and by the complete absence of *mosA*. Since an ORF1 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 *Bam*HI fragment from *mosA*. After blunt-ending and re-ligation of the construct, sequencing showed that an additional 84 nt were removed from around the *Bam*HI 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 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

Fig. 6. HVPE detection of rhizopines from nodule extracts. Lane 1, markers (A, 2-deoxy-adenosine; B, mannitol); lane 2, Rm1021(pJPM5) (L5-30 *mosA* control construct); lane 3, Rm1021(pJPM6) (L5-30 *mosA* deletion mutant); lane 4, Rm220-3 (SI control); lane 5, L5-30 (3-O-MSI control); and lane 6, Rm1021 (negative control). Each sample represents the rhizopine extract from 12.5 mg (wet wt) of nodules.

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 *mos* 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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