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Structural and Functional Conservation of the Rhizopine Catabolism (*moc*) Locus Is Limited to Selected *Rhizobium meliloti* Strains and Unrelated to Their Geographical Origin

Silvia Rossbach,[1,2] Ghulam Rasul,[1] Maria Schneider,[1,2] Bertrand Eardly,[4] and Frans J. de Bruijn[1,2,3]

[1]NSF-Center for Microbial Ecology, [2]MSU-DOE Plant Research Laboratory, and [3]Department of Microbiology, Michigan State University, East Lansing 48824, U.S.A.; [4]Pennsylvania State University, Berks Campus, Reading, PA19610-6009, U.S.A.

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Rhizopine (L-3-*O*-methyl-*scyllo*-inosamine; 3-*O*-MSI) synthesis (*mos*) and catabolism (*moc*) genes were originally isolated from *Rhizobium meliloti* strain L5-30 (Murphy et al., Proc. Natl. Acad. Sci. U.S.A., 84:493, 1987). These genes have been postulated to give a competitive advantage to this strain in the rhizosphere, since the ability to utilize the unusual nutritional mediator rhizopine as nitrogen and carbon source appears to be correlated with the ability of Moc$^+$ bacteria to efficiently infect alfalfa plants. This study examines the distribution of rhizopine catabolism (*moc*) genes among different soil bacteria. By using oligonucleotide primers homologous to the *moc* genes and the polymerase chain reaction (PCR), *moc* genes were shown to be absent from a random collection of 100 different soil isolates. However, screening 50 different electrophoretic type strains of a worldwide *R. meliloti* collection (Eardly et al., Appl. Environ. Microbiol. 56:187, 1990) revealed the presence of *moc* genes in three additional strains, S33, 102F51, and 74B3. These three strains were found to be able to synthesize rhizopine in planta (Mos$^+$) and to catabolize it (Moc$^+$). To determine the relatedness of the Mos$^+$/Moc$^+$ strains to each other and to other *R. meliloti* strains, we used the rep-PCR method to generate genomic fingerprints, and to create a phylogenetic tree with the help of an optical imaging system and data analysis program (AMBIS). Because of the apparent infrequent occurrence of *moc* genes among soil bacteria, we suggest that the use of *moc* genes as a selectable marker trait for tracking genetically manipulated organisms is feasible.

Additional keywords: Medicago sativa, rep-PCR.

The potential of beneficial or genetically improved microorganisms for applications in agriculture or bioremediation has been generally recognized (see Tiedje et al. 1989). Examples of such organisms include plant growth-promoting bacteria (Davison 1988; Lambert and Joos 1989), biocontrol agents (see Cook 1993), nitrogen fixing bacteria (Bosworth et al. 1994), and microorganisms able to degrade environmental pollutants (see Chaudhry and Chapalamadugu 1991). However, the introduction of these microorganisms into the environment poses some major problems. Scientific knowledge is sparse concerning the short- and long-term effects of both native and genetically modified microorganisms that are introduced into the environment (see Kluepfel 1993). Little is known about the dissemination and the persistence of the introduced microorganisms, and even less about their interaction with already present bacterial communities.

One reason for the sparsity of studies is that only a few generally applicable marker traits are available for practical use in the environment. Antibiotic resistance genes, widely used in laboratories to mark bacteria, are not optimal for applications in nature, since the further spread of antibiotic resistance genes could endanger their use in human and animal therapy.

The *lacZ* gene, encoding β-galactosidase and originally isolated from *Escherichia coli*, was one of the first marker genes used to track bacterial strains of the genus *Pseudomonas* in the soil environment (Drahos et al. 1986). The gene product of this marker gene is easily recognizable via a colorimetric assay. Nevertheless, the use of this marker gene is restricted to soil environments which are free of organisms containing endogenous β-galactosidase activity. A similar limitation applies to the glucuronidase (*gus*) marker, which has been described as a useful system to monitor plant-associated bacteria (Wilson et al. 1994). Another chromogenic marker gene, encoding catechol 2,3 dioxygenase (*xylE*), has been proven to be useful to track bacteria in soil (see Pickup and Saunders 1990) and in the phyllosphere (Wilson and Lindow 1994). Also, the *lux* gene system, which was originally isolated from marine *Vibrio* species (see Meighen 1989; Prosser 1994), and the *luc* system from firefly species (Moeller et al. 1994; Cebolla et al. 1993) appear to be suitable for the use as marker genes in the soil environment. Provided with suitable substrates, the products of the *lux* and *luc* genes are responsible for bioluminescence, and the emit-

Corresponding author: Frans J. de Bruijn: E-mail: deBruijn@msu.edu

Present address of G. Rasul: National Institute for Biotechnology and Genetic Engineering, Jhang Road, P.O. Box 577, Faisalabad, Pakistan.

tance of light can be measured with any light-detecting device such as cameras, X-ray films, or fiber optics. However, the expression of the *lux* genes may be a energetic burden for the cell, and could result in a reduced growth rate. Thus, the data obtained may not reflect the actual behavior of the tagged organism in the environment.

Another problem related to microbial release into the environment is competition (see Dowling and Broughton 1986; Triplett 1990; Triplett and Sadowsky 1992). For example, highly efficient nitrogen-fixing strains of *Rhizobium* and *Bradyrhizobium* have been used to inoculate legume fields. However, these inoculation attempts often fail, because the introduced strains can not compete with the indigenous rhizobia which are well-adapted to their ecological niche (see Streeter 1994).

To address the complex questions of microbial competition in soil or in the rhizosphere of plants, we are developing a new genetic marker system for the use in the soil environment, which is based on the rhizopine catabolism (*moc*) genes. Rhizopine (L-3-*O*-methyl-*scyllo*-inosamine; 3-*O*-MSI) has been found to be present in nitrogen-fixing nodules, which are induced by *Rhizobium meliloti* strain L5-30 on its symbiotic host plant alfalfa (*Medicago sativa*; Tempe et al. 1982; Murphy et al. 1987). Rhizopine is only synthesized under nitrogen-fixing conditions in the nodule, but can be catabolized as a unique substrate by free-living L5-30 bacteria (Murphy et al. 1987). It has been hypothesized that the ability to convert plant-derived precursors into a specific nutrient source, which is only available for bacteria possessing the genes to catabolize it, may give them a competitive advantage (Murphy and Saints 1992). The genes for the synthesis (*mos*) as well as for the catabolism (*moc*) of rhizopine are both bacterial encoded and have been shown to be closely linked and located on the sym-plasmid of strain L5-30 (Murphy et al. 1987). The *mos* and *moc* genes have been cloned and their DNA sequence is known (Murphy et al. 1993; Rossbach et al. 1994). Four open reading frames have been shown to be essential for rhizopine degradation (*mocABR* and *mocC*, Rossbach et al. 1994). The gene product of *mocB* has been proposed to be involved in rhizopine uptake and transport, whereas MocR is likely to play a regulatory role. It has been suggested that *mocA* and *mocC* are structural genes encoding enzymes responsible for the degradation of 3-*O*-MSI (Rossbach et al. 1994).

Since the rhizopine catabolism genes confer upon their host the ability to utilize a rare substrate as sole nitrogen (N) and carbon (C) source, this trait should be easily recognizable by plating samples on minimal media containing rhizopine as sole N- and C-source. However, to construct a useful marker system, which is based on the catabolism of rhizopine, the prevalence of these genes in microorganisms present in the environment needed to be examined. In the initial screen for the presence of rhizopine in alfalfa nodules, 3-*O*-MSI was only found in nodules which were induced by one *R. meliloti* strain out of 20 strains tested, namely L5-30 (Tempe et al. 1982). Recently, in another study, a second *R. meliloti* strain has been discovered which is able to synthesize a related compound (*scyllo*-inosamine, SI) in alfalfa nodules (Rm 220-3, Saint et al. 1993). However, no other bacterial strain has been reported in the literature, which is able to synthesize and to catabolize rhizopine (3-*O*-MSI).

In this paper, we describe an extensive screen for the presence of rhizopine catabolism genes in common soil bacteria and more specifically in different *R. meliloti* strains. Employing PCR technology, we show that the presence of rhizopine catabolism genes is a relatively uncommon trait and limited to selected *R. meliloti* strains. To examine the relative phylogenetic relationship of these strains, a detailed analysis of the

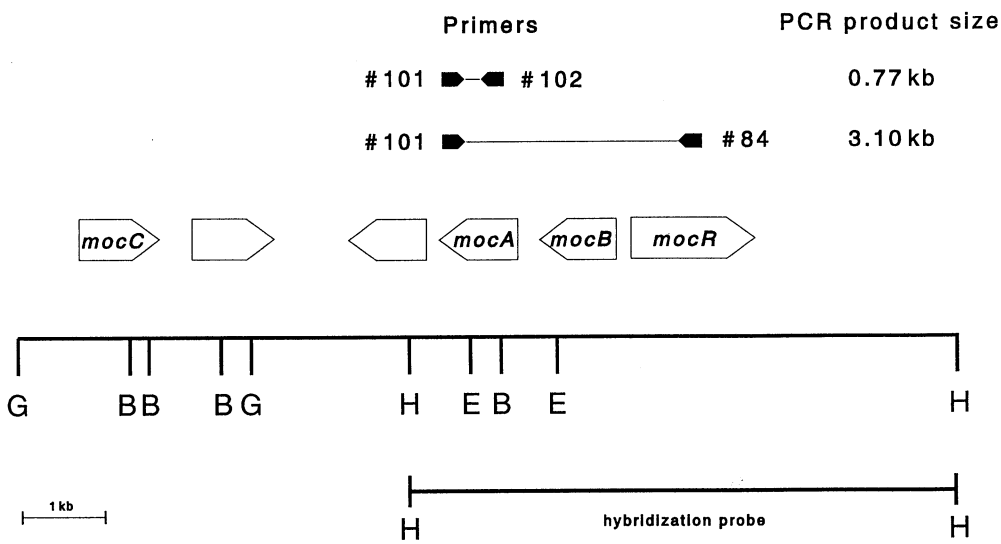

Fig. 1. Structure of the *Rhizobium* L5-30 *moc* locus and the location of oligonucleotide primers used for the polymerase chain reaction (PCR). In the middle, a physical map of the *moc* locus of *R. meliloti* strain L5-30 is represented by a horizontal line (flanked by "G" and "H"). The symbols used to indicate the following restriction enzyme cleavage sites are: B, *Bam*HI; E, *Eco*RI; G, *Bgl*II; H, *Hin*dIII. The potential open reading frames (ORF's), as deduced from the DNA sequence (Rossbach et al. 1994), and their orientations are indicated by open boxes with arrowheads. ORF's *mocABR* and *mocC* are essential for rhizopine catabolism (Rossbach et al. 1994). The location of the oligonucleotides corresponding to the *moc* DNA sequence and used as primers for PCR and the predicted sizes of the PCR products are indicated at the top of the figure. At the bottom, the map position of the 6.5-kb large *Hin*dIII fragment containing the *mocABR* genes used as a hybridization probe is indicated. A scale is provided in the bottom left hand corner in kilobases.

genome structure of 50 different *R. meliloti* strains was carried out, employing the recently described rep-PCR method (Versalovic et al. 1991; 1994; de Bruijn 1992). The phylogenetic tree based on rep-PCR genomic fingerprinting was found to show groupings of *R. meliloti* strains similar to those observed by Eardly et al. (1990), using multilocus enzyme electrophoresis (MLEE). However, the Moc$^+$ strains were not found to be clustered, nor could any relationship be established between their Moc phenotype and geographical origin.

RESULTS

Screening for *moc* genes by PCR.

The objective of this study was to find out how widespread rhizopine catabolism (*moc*) genes are in common soil bacteria. The Polymerase Chain Reaction (PCR) was used as a tool to screen large quantities of different bacterial strains for the presence of *moc* genes. Based on the known DNA sequence of the *moc* locus (Rossbach et al. 1994), oligonucleotides corresponding to specific DNA sequences of the *mocA* and *mocR* gene region were designed and synthesized (see Fig. 1). The two opposing oligonucleotides #101 and #102 correspond to the 5′- and 3′ ends of the open reading frame (ORF) corresponding to the *mocA* gene. Oligonucleotide #84 corresponds to the central region of the ORF corresponding to the *mocR* regulator gene and opposes primer #101 (see Fig. 1). When oligonucleotides #101 and #102 are used as PCR primers and incubated with the correct template DNA, a product of 770 bp is expected. Incubation with oligonucleotides #101 and #84 should result in a 3.1-kb PCR product.

As control reactions, total chromosomal DNA of *R. meliloti* strains L5-30 (Moc$^+$) and 1021 (Moc$^-$) were included in the PCR analysis. Incubation of primers #101 and #102 with DNA of strain L5-30 resulted in a single PCR product of 770 bp, whereas the use of primers #101 and #84 gave rise to a 3.1-kb DNA product, as expected. No PCR products were obtained when total chromosomal DNA of *R. meliloti* strain 1021 was used as template. In addition, it was tested whether cells taken from an agar plate and resuspended in PCR buffer could be used directly for PCR, without the need for DNA purification (Versalovic et al. 1994). Cells of strain L5-30, taken directly from a fresh agar plate, and incubated under the same conditions as purified DNA, displayed similar single PCR products of the expected sizes (770 bp and 3.1 kb, respectively; data not shown).

Screening the LTER100 collection for the presence of *moc* genes.

The LTER100 is a collection of 100 random bacterial strains, isolated from the Long Term Ecological Research (LTER) plot of the Kellog Biological Station (Hickory Corners, MI; see Table 1). The 100 strains of this collection have been extensively studied for their morphological and physiological traits, including fatty acid methyl ester profiles (FAME), utilization of 96 different carbon sources (BIOLOG), and colony morphology. It has been postulated that this collection contains a representative sample of bacterial strains commonly found in agricultural soils (M. Klug, personal communication).

Single colonies of the 100 LTER strains of the collection were taken directly from fresh solid medium and subjected to PCR. Oligonucleotides corresponding to *mocA* (#101 and #102) and *mocA-mocR* (#101 and #84) sequences were used as primers (see Fig. 1). The majority of the 100 strains did not display any distinctive PCR products using primers #101 and #102.

To verify that whole cell PCR was possible with the LTER isolates, we used rep-PCR (see below) to fingerprint the genome of these strains. Distinct rep-PCR fingerprinting patterns could be obtained with 73 out of 100 strains tested (data not shown), confirming the notion that other DNA sequences can indeed be amplified successfully from these strains. In addition, *mocA* PCR amplifications were carried out at lower annealing conditions (REP conditions; see Materials and Methods) and similar results as with the stringent *moc* primer conditions were obtained (data not shown).

With some of the strains (#4, 6, 35, 40, 50) minor PCR products ranging in the size from 700 to 800 bp were generated (data not shown). With other strains (#14, 25, 33, 37, 52, 59, 66, 79, 96) larger PCR products were obtained. No dis-

Table 1. Bacterial strains used in this study

Strains	Relevant genotype/phenotype	Source or reference
Rhizobium meliloti		
L5-30	Wild type, Sm^R, Mos$^+$, Moc$^+$	Kowalski 1970
1021	Wild type, Sm^R, Mos$^-$, Moc$^-$	Meade et al. 1982
M275, CC2003, CC2013, 15A6, 74B12, 102F51, ATCC9930, M56, M95, M98, M119, M248, M257, M270, M286, M289, M294, A145, N6B1, N6B4, N6B5, N6B9, N6B11, S33, 15A5, 15B4, 17B6, 56A14, 74B3, 74B4, 74B12, 128A7, 102F85, 128A10, 1322	Different electrophoretic type strains—grouped into Division A	Eardly et al. 1990
M1, M3, M102, M161, M205, M7, M16, M58, M75, M158, M214, M254, M278, M280, CC169	Different electrophoretic type strains—grouped into Division B	Eardly et al. 1990
Other species		
LTER1, 2, 3, ... 99, 100	100 different soil isolates, collected at the Long Term Ecological Research Plot of the Kellog Biological Station, Hickory Corners, Michigan	M. Klug, Michigan State University
Rhizobium sp. IRBG233	Internal reference strain for REP-PCR	J. K. Ladha, International Rice Research Institut, The Philippines

tinct PCR products were observed for any of the LTER isolates when primers #101 and #84, covering the *mocA-mocR* region were used (data not shown).

Representatives of strains that resulted in the generation of PCR products in the expected size range with primers #101 and #102, were tested for their ability to catabolize rhizopine. Strains 4, 6, 35, and 40 were subjected to the rhizopine catabolism assay (see Materials and Methods). None of these strains were found to be able to catabolize rhizopine (Moc$^-$; data not shown).

Screening 50 different *R. meliloti* strains for the presence of *moc* genes.

Based on the results obtained with the LTER100 collection, we assumed that the occurrence of the *moc* genes is rather infrequent in soil bacteria. Therefore, the frequency of *moc* genes in other *R. meliloti* strains was examined. Eardly et al. (1990) have described a collection of 232 different *R. meliloti* strains, which have been isolated from nodules of different *Medicago* species growing in a variety of countries located on different continents. The 232 *R. meliloti* strains have been grouped into 50 distinct electrophoretic types (ET's), according to their distinctive multilocus enzyme electrophoresis profiles. The 50 different *R. meliloti* ET's fall into two primary phylogenetic divisions, A and B (Eardly et al. 1990; see Table 1). We used these 50 electrophoretic type strains for PCR analysis with *moc* primers. Cells from single colonies were taken directly from fresh TY plates and employed for PCR with *moc* primer combinations #101 and #102, as well as #101 and #84.

When *moc* primers #101 and #102 were used, no distinct PCR product was generated with 22 of these *R. meliloti* strains. With 24 strains, weakly amplified DNA products of various sizes were observed. With four strains, one major PCR product was generated. Out of these four strains, three (S33, 74B3, and 102F51) displayed a single, 770-bp PCR product. Strain 74B4 showed a slightly larger PCR product of 900 bp and a minor product of 850 bp. Some representative examples of the PCR amplification products are shown in Figure 2. The 770-bp, large DNA products observed in strains S33, 74B3, 102F51 and L5-30 can be seen in the middle of Figure 2, as well as the slightly larger product of strain 74B4.

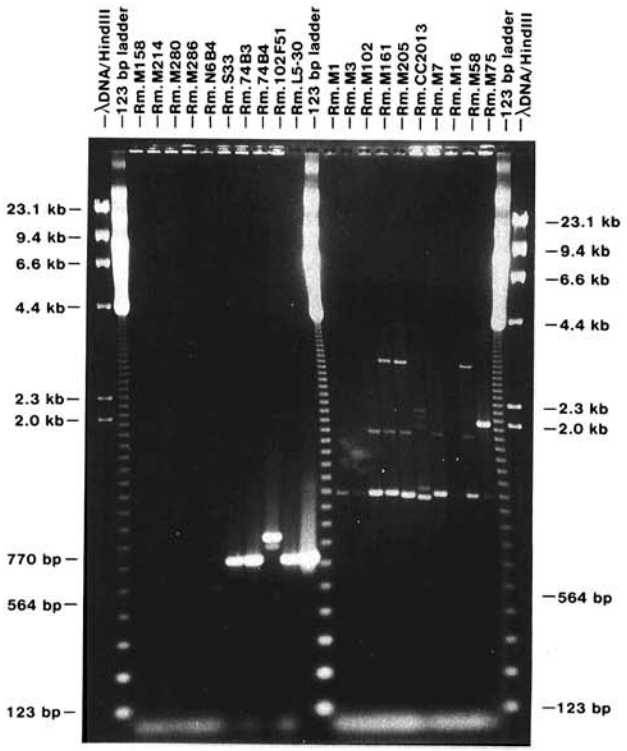

Fig. 2. Polymerase chain reaction (PCR) products generated with oligonucleotide primers corresponding to the rhizopine catabolism (*moc*) genes and whole cells of different *Rhizobium meliloti* strains. A photograph of an ethidium bromide stained 1.5% agarose gel is shown. At the top, the names of the *R. meliloti* strains used as templates are indicated. As size markers λ DNA restricted with *Hin*dIII and the 123-bp ladder were used (outermost lanes). The sizes of relevant marker fragments are indicated to the right and to the left of the panel. In the lanes where PCR products of whole cells of strains S33, 74B3, 74B4, 102F51, and L5-30 were loaded, strongly amplified PCR products are present that resulted from incubations with primers #101 and #102 (see Fig. 1). In the lanes where PCR products of whole cells of strains M1, M3, M102, M161, M205, CC2013, M7, M16, M58, and M75 were loaded, PCR products of one to three different size classes are present.

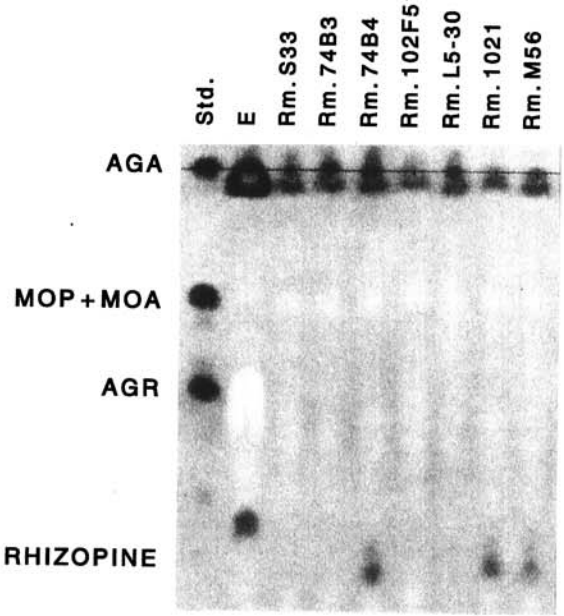

Fig. 3. High-voltage paper electrophoregram (HVPE) of a rhizopine catabolism assay after alkaline silver staining. Half-strength minimal medium containing rhizopine was inoculated with different strains and incubated for 3 days at 28°C. Fifteen microliters of the supernatant of each culture was loaded on the origin of the paper and electrophoresed. The origin is located at the top of the figure and the cathode is positioned at the bottom of the figure. The reference substances in the left lane (Std.) are, in order of their mobility, agropinic acid (AGA), mannopine and mannopinic acid (MOP + MOA), and agropine (AGR). In the second lane on the left (E), the supernatant of the uninoculated control was loaded. The other lanes show the supernatants of the cultures inoculated with the *Rhizbium meliloti* strains S33 (Moc$^+$), 74B3 (Moc$^+$), 74B4 (Moc$^-$), 102F51 (Moc$^+$), L5-30 (Moc$^+$), 1021 (Moc$^-$), and M56 (Moc$^-$). A dark-stained spot representing the rhizopine can be seen at the bottom of the figure in those lanes where supernatants of cultures inoculated with Moc$^-$ strains were loaded (strains 74B4, 1021, and M56). The minor mobility shift of rhizopine after incubation with Moc$^-$ strains is generally observed after incubation with rhizobial strains.

The right half of Figure 2 shows some strains with PCR products falling into one to three distinct size classes, but all larger than the 770 bp product observed with strain L5-30. All 50 *R. meliloti* strains were also examined using primers #101 and #84. No specific *moc* PCR product could be observed in any of the strains, with the exception of strain 74B3, which displayed a single 3.1-kb PCR product (data not shown) corresponding in length to the size of the *mocA-mocR* PCR product of strain L5-30 (see Fig. 1).

To show that the 770-bp PCR product indeed carries *mocA* sequences, we repeated the PCR with the strains shown in Figure 2, blotted a gel containing the 770 bp and other PCR products, and hybridized the blot with a *mocA* probe. Only the 770 bp products of the strains S33, 74B3, 102F51, and L5-30 gave a positive hybridization signal when probed with *mocA*. Neither the 850/900-bp products of strain 74B4, nor any of the PCR products of the different size classes from the other strains were found to hybridize with the *mocA* probe (data not shown). Thus, the observed 770-bp PCR products indeed carry *mocA* gene-specific DNA sequences.

Rhizopine catabolism studies.

The correlation between the appearance of a PCR product of the predicted size of the *mocA* gene (770 bp) with the ability to catabolize rhizopine was subsequently examined. Nodule extract containing 3-*O*-MSI was provided as sole nitrogen and carbon source to the strains to be tested. Strains M1, M3, M102, M161, M205, CC2013, and M75, representing strains which displayed PCR products of various sizes with primers #101 and #102, were not able to catabolize rhizopine (Moc$^-$; data not shown). In contrast, strains S33, 74B3, and 102F51 (Fig. 3), which displayed the 770-bp large PCR products hybridizing with *mocA* DNA sequences, were found to be able to catabolize rhizopine (Moc$^+$). Strain 74B4, which was observed to generate a major PCR product of around 900 bp, was not able to catabolize rhizopine (Moc$^-$; Fig. 3). Thus, the appearance of a 770-bp large PCR product when using primers corresponding to the *mocA* gene was found to be directly correlated with the phenotypic ability of these strains to catabolize rhizopine.

Rhizopine synthesis studies.

In *R. meliloti* strain L5-30 the ability to catabolize rhizopine is correlated with the ability to synthesize it in planta (Moc$^+$, Mos$^+$ phenotype). In addition, *mos* and *moc* genes are located 5-kb apart from each other (Murphy et al. 1987). This prompted us to examine if the newly discovered Moc$^+$ strains were able to synthesize rhizopine in alfalfa nodules (Mos$^+$ phenotype). Therefore, we inoculated *Medicago sativa* plants with cultures of strains S33, 74B3, 102F51, 74B4, and control strains L5-30 (Mos$^+$) and 1021 (Mos$^-$). The nodules were harvested after 8 weeks and extracts were subjected to high voltage paper electrophoresis (see Materials and Methods). In nodules induced by the Mos$^+$ control strain L5-30 or strains S33, 74B3, and 102F51, rhizopine could be easily detected. No rhizopine could be detected in nodules induced by the Mos$^-$ control strain 1021 or strain 74B4 (data not shown). Thus, the ability to catabolize rhizopine by the newly discovered Moc$^+$ strains S33, 74B3, and 102F51 is directly correlated with their ability to synthesize rhizopine in alfalfa nodules.

Conservation of the *moc* locus in the Moc$^+$ *R. meliloti* strains.

To examine how conserved the *moc* locus was in the newly discovered Moc$^+$ strains, we isolated total chromosomal DNA of the strains L5-30, 102F51, 74B3, and S33, and of the Moc$^-$ strains 74B4 and 1021. The DNA was digested with the restriction endonucleases *Eco*RI, *Bam*HI, and *Hin*dIII, separated by agarose gel electrophoresis and Southern blotted. The filter was hybridized with the 6.5-kb *Hin*dIII fragment carrying the *mocABR* genes as a probe (see Fig. 1). Figure 4 shows that the Moc$^+$ strains 74B3, S33, and 102F51 displayed the same *Eco*RI and *Hin*dIII restriction pattern as strain L5-30. In the case of the *Bam*HI-digestion, the DNA of strain L5-30 displayed a slightly different hybridization pattern than the DNA of the other Moc$^+$ strains (L5-30: 8-kb *Bam*HI fragment; others: 9.4-kb *Bam*HI fragment). Thus, a single restriction fragment length polymorphism (RFLP) could be detected in the *moc* locus. The Moc$^-$ strains 74B4 and 1021 showed only weakly hybridizing fragments. Therefore, the results from the hybridization with the *moc* genes as probe confirmed the data obtained by PCR with *moc*-specific primers and the catabolism assay. The strains that displayed a PCR product of 770 bp and were Moc$^+$ gave a positive hybridization signal in the Southern blot. Moreover, only limited RFLP's were detectable in the Moc$^+$ strains, suggesting a high degree of relatedness between the rhizopine catabolism loci in the different Moc$^+$ *R. meliloti* strains.

Relatedness of the Moc$^+$ *R. meliloti* strains.

To determine how closely related the newly discovered Moc$^+$ strains were, and to deduce their phylogenetic relation-

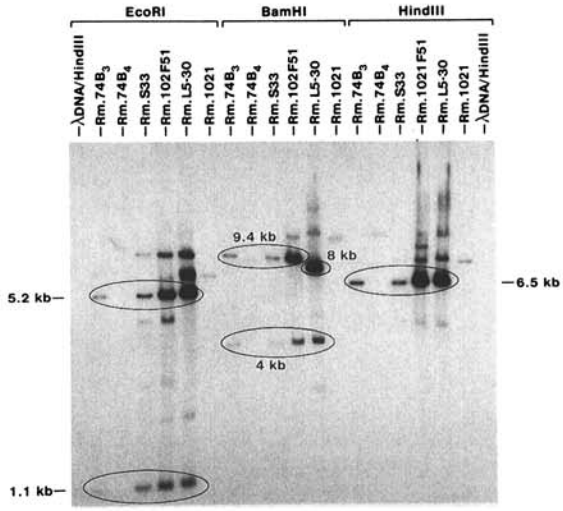

Fig. 4. Southern blot of the total chromosomal DNA from different *Rhizobium meliloti* strains hybridized with the rhizopine catabolism (*moc*) genes. The 6.5-kb *Hin*dII fragment (see Fig. 1), isolated from strain L5-30, was used as a hybridization probe. The names of the *R. meliloti* strains, from which the total chromosomal DNA had been isolated, are indicated on the top. The DNA was restricted with three different restriction enzymes (*Eco*RI, *Bam*HI, or *Hin*dIII). The strong hybridization of the L5-30 and 102F51 DNA with the probe correlates with the higher amount of DNA loaded in these lanes. The λ/*Hin*dIII size marker, visible on the original agarose gel, was used to determine the sizes of the hybridizing restriction fragments. The circled fragments denote the major fragments hybridizing with the *moc* gene probe.

ship to the other *R. meliloti* isolates, the recently described rep-PCR fingerprinting method was used (Versalovic et al. 1991, 1994; de Bruijn 1992). The rep-PCR genomic fingerprinting method is based on the use of both degenerate (REP) and nondegenerate (ERIC; BOX) primers corresponding to the inverted repeats of naturally occurring repetitive sequences in bacteria (see Versalovic et al. 1994). By amplifying specific DNA sequences located inbetween the REP-, ERIC-, and BOX-like elements, unique DNA fingerprint patterns can be generated for each strain, and their patterns can be used for phylogenetic analyses (see Versalovic et al. 1994). This method has been successfully employed to identify and classify different soil bacteria, including rhizobia (de Bruijn 1992; Judd et al. 1993; Leung et al. 1994; Louws et al. 1994; Nick and Lindstrom 1994).

We used REP- and ERIC-PCR primers (see Materials and Methods) to perform rep-PCR with cells from single colonies from those *R. meliloti* strains which showed distinct PCR products with the *moc* primers (see Fig. 2). In Figure 5A, the results of the REP-PCR, and in Figure 5B the results of the ERIC-PCR are presented. The strains shown are arranged in the same order as in Figure 2. The Moc$^+$ strains L5-30, 102F51, 74B4 and S33 displayed related, but different REP- (Fig. 5A) and ERIC-patterns (Fig. 5B), suggesting that all four Moc$^+$ isolates analyzed represent distinct strains of *R. meliloti*. To further examine the phylogenetic relationship of the four Moc$^+$ strains to each other and to the worldwide collection of *R. meliloti* strains classified by Eardly et al. (1990), we carried out a rep-PCR analysis of all representatives of the 50 different electrophoretic type strains (ET's). The resulting gels were scanned and a phylogenetic tree was generated using the AMBIS system, as described by Versalovic et al. (1994). The data of the experiment using REP primers are shown in Figure 6. The REP-PCR fingerprints of the 50 ET's, including the newly identified Moc$^+$ strains S33 (#11), 102F51 (#38), and 74B3 (#44) are shown in random order in the top panels of Figure 6. The dendrogram generated from these two gels by the AMBIS system is shown below the top panels. Based on the relative order derived from this dendrogram, aliquots of the same REP-PCR products shown in the top panel were reloaded on a gel to generate the bottom two panels. Visual inspection of the bottom two panels in Fig. 6 revealed clear grouping of the strains into two major divisions. These two divisions correspond almost exactly to the divisions A and B identified by Eardly et al. (1990). The only exception was strain M245 (#24), which is placed into division B by MLEE (Eardly et al. 1990), but into division A by REP-PCR. Another difference observed pertained to strain #8, which still had been positioned in division A—but as an outlier—by MLEE analysis (Eardly et al. 1990). However, it was found to be clearly integrated in division A by the REP-PCR analysis.

Both MLEE and REP-PCR derived dendrograms clearly showed that the Mos$^+$/Moc$^+$ strains were present only in division A. Two strains, S33 (#11) and 102F51 (#38) were nearest neighbors in both dendrograms, suggesting that these two strains are very closely related. Strain 74B3 (#44) was found to be in a different part of the A division. It is interesting to note that strain M56 (#15), which is the nearest neighbor of the Moc$^+$ strain 74B3 (#44) in the REP-PCR generated dendrogram, was not able to catabolize rhizopine in the catabolism assay (see Fig. 3). The first Mos$^+$/Moc$^+$ strain isolated

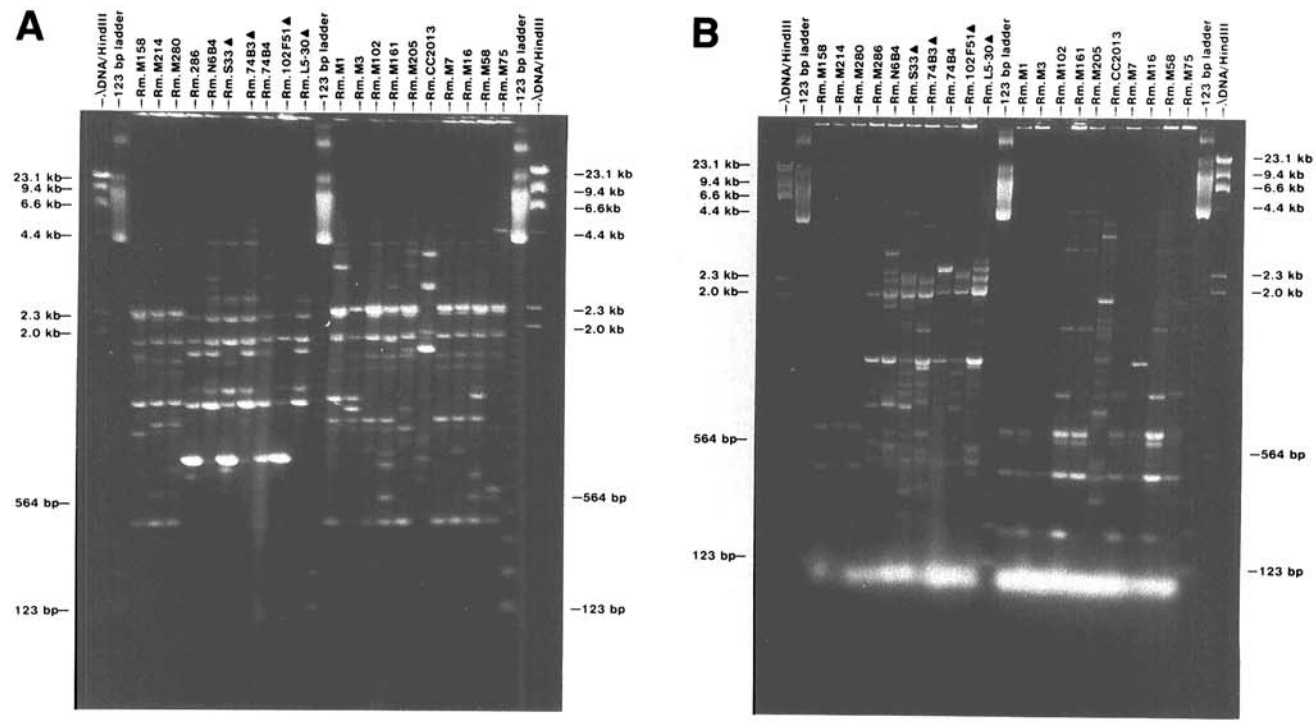

Fig. 5. REP-PCR **(A)** and ERIC-PCR **(B)** generated genomic fingerprints of different *Rhizobium meliloti* strains. Photographs of ethidium bromide stained 1.5% agarose gels are shown, on which the REP-PCR (A) and ERIC-PCR (B) products of 20 different *R. meliloti* strains were separated. The names of the strains are shown at the top and they are arranged in the same order as in Figure 2. Size markers were λ DNA/*Hin*dIII and the 123 bp ladder. The sizes of the most relevant marker fragments are indicated to the left and to the right side of the panels. The triangles indicate the Moc$^+$ strains.

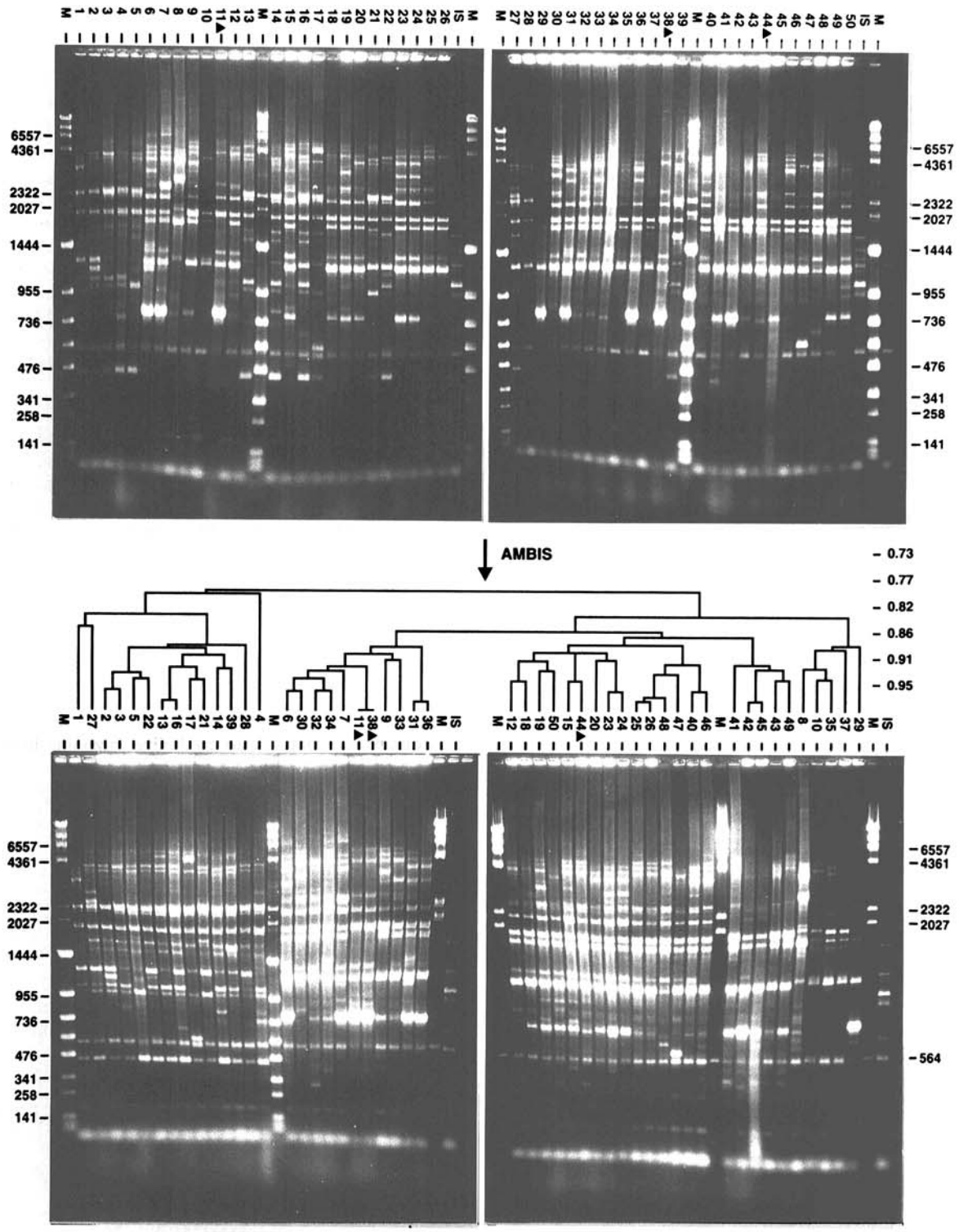

Fig. 6. REP-PCR generated genomic fingerprints of *Rhizobium meliloti* strains and the corresponding dendrogram. The top panel shows the photographs of two ethidium bromide stained 1.5% agarose gels, on which the REP-PCR products of 50 different type strains of *R. meliloti* were separated, loaded in random order. The dendrogram derived from these two gels by using the AMBIS system with the "simple" algorithm (Versalovic et al. 1994) is shown in the middle section. The bottom panel shows two 1.5% agarose gels with aliquots of the same REP-PCR products as in the top panel, reloaded according to the order predicted by the dendrogram. "M" indicates the size marker lanes (λ*Hin*dIII or λ*Hin*dIII+ pUC19/*Taq*I+pUC19/*Sau*IIIA) and the sizes of the most relevant marker fragments are indicated at the side of the panels (in bp). "IS" indicates the internal standard reaction lane, which consists of REP-PCR products derived from chromosomal DNA of *Rhizobium* strain IRBG233 (Table 1) generated with the REP primer. The relative similarity coefficients of the genetic distances between the different strains are indicated numerically on the right side of the dendrogram (0.73–0.95). The numbers above the lanes correspond to the following strain designations: 1, M1; 2, M3; 3, M102; 4, M161, 5, M205; 6, M275; 7, CC2003; 8, CC2013; 9, 15A6; 10, 74B12; 11, 102F51; 12, ATCC9930; 13, M7; 14, M16; 15, M56; 16, M58; 17, M75, 18, M95; 19, M98; 20, M119; 21, M158; 22, M214; 23, M248; 24, M254; 25, M257; 26, M270; 27, M278; 28, M280; 29, M286; 30, M289; 31, M294; 32, A145; 33, N6B1; 34, N6B4; 35, N6B9; 36, N6B11; 38, S33; 39, CC169; 40, 15A5; 41, 15B4; 42, 17B6; 43, 56A14; 44, 74B3; 45, 74B4; 46, 74B12; 47, 128A7; 48, 102F85; 49, 128A10; 50, 1322 (see also Table 1). The triangles denote the strains able to synthesize and catabolize rhizopine (Mos$^+$/Moc$^+$).

(L5-30) was not included in this dendrogram, but has been shown to be of the same electrophoretic type as strain ATCC9930. This strain is also a member of division A, confirming that Mos$^+$/Moc$^+$ strains are limited to this division.

The question whether the different Mos$^+$/Moc$^+$ strains possess a common geographical origin was also examined. Strain L5-30 was first described in Poland (Kowalski 1970). Strains 102F51 and S33 were isolated in the United States, whereas 74B3 was isolated in Pakistan (Eardly et al. 1990). Thus, the Mos$^+$/Moc$^+$ strains were found on three different continents. We also evaluated the effects of the host plants, from which the Mos$^+$/Moc$^+$ strains had been isolated. Interestingly, all Mos$^+$/Moc$^+$ strains were isolated from nodules induced on a single species, namely *Medicago sativa*, while the 50 different electrophoretic type strains used in this study were isolated from 11 different *Medicago* species (Eardly et al. 1990). Thus, there appears to be a lack of correlation of the occurrence of *moc* genes and geographic origin, but we can conclude that the nodules of *M. sativa* seem to be a favorable environment for the production of rhizopine.

DISCUSSION

In this study, we have shown that the occurrence of *moc* genes is infrequent among soil bacteria. We screened 100 different, random isolates from an agricultural field site (LTER100) for the presence of *moc* genes, but we did not find *moc* gene containing strains. Also, selected representatives of the LTER collection were tested for their ability to catabolize rhizopine, but none of them were found to be able to degrade this nutritional mediator. We also screened arbitrary laboratory collections of soil microbes via Southern blotting with the *moc* genes as probes, but did not find DNA sequences sharing homology with the *moc* genes in strains of the genera *Agrobacterium*, *Bradyrhizobium*, *Azorhizobium*, *Azospirillum*, *Escherichia*, and *Pseudomonas* (S. Rossbach, G. Rasul, and F. J. de Bruijn, unpublished data). Lastly, we screened for the presence of *moc* genes in a worldwide collection of 50 different electrophoretic type strains of *R. meliloti* (Eardly et al. 1990), using PCR and *moc*-specific primers. The *mocA* gene of strain L5-30 encodes a presumptive dehydrogenase involved in catabolism of 3-*O*-MSI (Rossbach et al. 1994). The primers homologous to the L5-30 *mocA* gene DNA sequence (primers #101 and #102; Fig. 1), were successfully used to identify three additional strains (102F51, S33, and 74B3) carrying *moc*-gene sequences. The employment of the primers #101 and #84 (which are homologous to DNA sequences in *mocA* and *mocR*, respectively), resulted in the positive identification of only one new strain (74B3). The PCR product obtained by the use of this primer combination comprises, in addition to *mocA*, a gene encoding a putative transport protein (*mocB*) and the putative regulator gene (*mocR*; Rossbach et al. 1994). We postulate that in the other Moc$^+$ strains, the regulatory gene *mocR* might have a slightly diverged DNA sequence, thus inhibiting the correct annealing of primer #84. We do not think that *mocR* is completely absent or has another location, because our hybridization data speak against a major rearrangement of the rhizopine catabolism genes in the other Moc$^+$ strains (see Fig. 4).

The presence of the 770-bp PCR product, which hybridizes with *mocA* DNA, was correlated with the ability of the corresponding strains to catabolize rhizopine (Moc$^+$, see Fig. 3) and to synthesize rhizopine in planta (Mos$^+$). It is interesting to note, that strain 74B4, which displayed a strongly amplified PCR product of a slightly different than the expected size with the #101 and #102 primers, did not reveal any *moc* homologous sequences, when probed with the *moc* genes. Also, this strain displayed a Mos$^-$/Moc$^-$ phenotype.

An independent study (P. J. Murphy, personal communication), has confirmed that the Moc$^+$/Mos$^+$ *R. meliloti* strains identified in this study (102F51, S33, and 74B3) are able to synthesize and catabolize rhizopine. In addition, seven additional Mos$^+$/Moc$^+$ *R. meliloti* strains (out of 117) have been identified by Murphy. These strains have also been classified as members of division A, according to the grouping of Eardly et al. (1990). Moreover, 10 (out of 73) *R. leguminosarum* bv. *viciae* strains and 2 (out of 9) *Rhizobium* sp. (*Phaseolus*) strains were found to be able to catabolize rhizopine. However, neither strains of the related species *R. l.* bv. *trifolii* or *phaseoli*, nor strains of *R. etli* or *R. tropicii* were shown to be able to catabolize rhizopine (P. J. Murphy, personal communication). Thus, although the ability to catabolize rhizopines can be observed in species other than *R. meliloti*, it still nevertheless appears to be an infrequent trait, even among rhizobia.

The REP and ERIC-PCR genomic fingerprinting analysis showed clearly that the Mos$^+$/Moc$^+$ strains we identified are different strains. For an extended analysis of their relatedness, we used the rep-PCR method and the AMBIS system to generate a phylogenetic tree. All Mos$^+$/Moc$^+$ strains were located relatively closely together in one of the two major divisions (division A), into which the *R. meliloti* strains had previously been classified by multilocus enzyme electrophoresis (Eardly et al. 1990). In general, the resulting dendrograms obtained with MLEE and REP-PCR are remarkably similar. Both methods show a major partitioning of all *R. meliloti* strains tested into one of the two phylogenetic divisions, A or B. One exception is #24, which was grouped into division B by MLEE analysis, but into division A with the REP-PCR method. We do not know the reason for this difference, but in the case of #24, the PCR experiments carried out with other primers corresponding to repetitive sequences (ERIC, BOX) resulted in identical grouping for #24 (our unpublished results). Another exception is #8, which fell a little bit apart from division A in the MLEE analysis, but is clearly integrated into division A based on the REP-PCR analysis. However, in other dendrograms, generated by PCR analysis carried out with BOX and ERIC primers, #8 also tends to be an outlier, and does not show a consistent grouping with either division A or B.

The Mos$^+$/Moc$^+$ strains therefore appear to be phylogenetically related, but only two of them are nearest neighbors in this phylogenetic tree (102F51, S33). The nearest neighbor of Mos$^+$/Moc$^+$ strain 74B3 (strain M56), was shown to be unable to catabolize rhizopine and did not show any *moc*-specific DNA amplification product after being subjected to PCR. An interesting question arises regarding the nature of the mechanism that directed the acquisition of the *mos/moc* genes by the different *R. meliloti* strains. Our analysis does not allow us to distinguish whether they were horizontally transferred or arose from a common ancestor. It should be considered, however, that the *mos* and *moc* genes of L5-30 are located on

the sym plasmid. Sym-plasmid transfer between closely related rhizobia might be quite common in nature (Young and Wexler 1988), and this could have facilitated a concurrent transfer of *mos/moc* genes together with the nitrogen-fixation genes.

Our analysis did not reveal a common geographic origin for the Mos^+/Moc^+ *R. meliloti* strains. Indeed, they have been isolated from three different continents (Asia, Europe, and North America; see Eardly et al. 1990). However, one common feature of the Mos^+/Moc^+ strains identified in this study is the host plant. They all have been isolated from *M. sativa* nodules. In contrast, the 50 electrophoretic type strains examined originate from 11 different *Medicago* species (Eardly et al. 1990). The question arises, whether rhizopine production relies on specific precursors, which can be exclusively provided in *M. sativa* nodules. The only observation speaking against this argument is that we could detect rhizopine in nodules of *Lotus corniculatus*, which had been infected with *Rhizobium loti* harboring the rhizopine synthesis genes (B. McSpadden, S. Rossbach, and F. J. de Bruijn; unpublished observation).

It has been hypothesized that the *mos/moc* system confers a competitive advantage upon the bacterial strains harboring it (Murphy and Saints 1992). In this respect it is interesting to note that strain 102F51 is used as part of a commercial inoculant for legume inoculation (see Eardly et al. 1990). Bacterial inoculants introduced into the field face a strong competition problem (Triplett 1990; Triplett and Sadowsky 1992). It is possible that strain 102F51 was initially chosen for use in commercial inoculants, because of its higher competitive ability. This, in turn, could be related to the presence of the *moc/mos* system in this strain.

Since the rhizopine catabolism (*moc*) genes confer the ability to utilize rhizopine as sole nitrogen (N) and carbon (C) source upon its host, we have previously proposed to use these genes as a selectable marker trait (Rossbach et al. 1994; 1995). The *moc* genes could be useful for tracing genetically modified microorganisms in the soil environment, since a simple selective plating of soil samples on minimal medium containing rhizopine as sole N and C source could serve as detection method. A similar use of the agrobacterial mannopine and agropine catabolism genes has recently been described (Hwang and Farrand 1994). Although various soil bacteria have been found to be able to degrade mannopine (Nautiyal et al. 1991; Beauchamp et al. 1990), the ability to catabolize agropine seems to be restricted to certain agrobacterial strains (Dessaux et al. 1986). The scarcity of the rhizopine compound, which is isolated from alfalfa nodules, has precluded larger scale screening studies of this type. Nevertheless, experiments are in progress to chemically synthesize 3-*O*-MSI (in collaboration with R. Hollingsworth, Department of Biochemistry, MSU), To obtain large quantities of 3-*O*-MSI, which are suitable for the use as sole N- and C-source in minimal medium.

The most important precondition for the use of the rhizopine catabolism (*moc*) genes as a selectable marker trait, namely the infrequent distribution of *moc* genes among soil bacteria, has been addressed in this study. Our conclusion is that the *moc* genes do not appear to be widely distributed in soil bacteria and therefore should be quite useful as selectable marker to track genetically modified microbes. In addition, during the course of this work, a rapid PCR-based method to identify *moc*-gene sequences in microorganisms has been developed. This should allow us to design tracking studies in which we will be able to compare selective plating and PCR-based detection methods directly.

MATERIALS AND METHODS

Bacterial strains.

The bacterial strains used in this study are shown in Table 1.

Media and growth conditions.

R. meliloti strains were grown on TY medium (Beringer 1974) and the medium used for the LTER100 collection was R2A agar from Difco (Detroit, MI). All strains were incubated at 28°C.

DNA manipulations.

DNA isolations and manipulations (Southern blotting, hybridizations) were carried out using standard protocols (Sambrook et al. 1989).

Oligonucleotide primers and polymerase chain reaction (PCR).

The following oligonucleotides were synthesized by the Macromolecular Facility at Michigan State University: #84, 5′TGCCTCTCTCCAATCTG3′; #101, 5′GTTACCTTCCGCCTTATTTA3′, and #102, 5′TGATAGAAGCAGGCGAAGTT3′. Their sequences are based on the published DNA sequence of the *moc* gene cluster (Rossbach et al. 1994; GenBank accession no. X78503). The oligonucleotides used as primers in rep-PCR were: 5′-IIIICGICGICATCIGGC-3′ (REP1R-I), 5′-ICGICTTATCIGGCCTAC-3′ (REP2-I), 5′-ATGTAAGCTCCTGGGGATTCAC-3′ (ERIC1R), and 5′-AAGTAAGTGACTGGGGTGAGCG-3′ (ERIC2; see Versalovic et al. 1994). The reaction volume for PCR was 25 µl containing 50 pmol each of two opposing primers, 1.25 mM of each of four dNTP's (dATP, dCTP, dGTP, and dTTP), 2 units of AmpliTaq DNA polymerase (Perkin Elmer) in a reaction buffer with 10% DMSO. The 5× reaction buffer stock solution contained 83 mM ammonium acetate, 335 mM Tris-HCl, 33.5 mM magnesium chloride, 33.5 µM EDTA, 150 mM β-mercaptoethanol, 850 µg/ml bovine serum albumin, pH 8.8 (see Versalovic et al. 1994). Either 50 ng of total chromosomal DNA served as template, or a resuspension of bacteria from a single colony directly taken from a freshly incubated agar plate with an inoculation loop (whole cell rep-PCR; Versalovic et al. 1994). The amplifications were performed in a DNA thermal cycler (Perkin Elmer Cetus). REP (ERIC) conditions were: 1 cycle at 95°C for 6(7) min, then a three-step program with 35 cycles at 94°C for 1 min, at 40°(52°)C for 1 min, and at 65°C for 8 min; then 1 final cycle at 65°C for 16 min. The screening for the presence of *moc* homologous sequences was carried out under ERIC conditions. A negative control consisting of all ingredients, but without template DNA, was routinely run with each PCR amplification.

Analysis of PCR products.

One fifth to one half of the 25-µl PCR mix was loaded on a 1.5% agarose gel and electrophoresed at 5 V/cm in 1/2

strength TAE buffer. Size markers used were λ/*Hin*dIII (Boehringer Mannheim), the 123-bp ladder (Boehringer Mannheim) and the mixture λ/*Hin*dIII;pUC19/*Sau*IIIA; pUC19/*Taq*I (Stratagene, La Jolla, CA). For the analysis of the REP-PCR generated fingerprints, a photograph of the ethidium bromide stained gel was taken and scanned into the AMBIS system (San Diego, CA), using the AMBIS optical imaging system and the AMBIS MicroPM software as described by Versalovic et al. (1994). The banding pattern of each lane was converted into bar code patterns. A similarity matrix was constructed from the data using the "simple" coefficient. Similarity groups were derived using the Unweighted Pair Group Mathematical Averaging method (UPGMA) and presented as a dendrogram of relatedness (Versalovic et al. 1994).

Rhizopine catabolism studies and high-voltage paper electrophoresis.

Rhizopine catabolism studies and high voltage paper electrophoresis were carried out as described by Rossbach et al. (1994).

Rhizopine synthesis studies.

Surface-sterilized seeds of *Medicago sativa* cv. Cardinal were germinated in test tubes (200 × 25 mm) on Whatman 3 MM paper wetted with 15 ml of B&D nutrient solution (Broughton and Dilworth 1971). After 1 week, the germinated seedlings were inoculated with 1 ml of washed cells of late logarithmic cultures of the different *R. meliloti* strains to be tested. Nodules were harvested after 8 weeks and rhizopine synthesis was assayed by high-voltage paper electrophoresis as described by Murphy et al. (1987).

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