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PCR Use of Highly Conserved DNA Regions for Identification of *Sinorhizobium meliloti*

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A PCR identification method in which four primers that recognize homologous conserved regions in the *Sinorhizobium meliloti* genome are used was developed and tested. The regions used for identification were the nodbox 4 locus, which is located in one of the symbiotic megaplasmids, and the *mucR* gene, which is located in the chromosome. The new method was used to establish a collection of *S. meliloti* strains from polluted soils.

Sinorhizobium meliloti forms a nitrogen-fixing symbiosis with plants of the genera *Medicago*, *Melilotus*, and *Trigonella*, including the crop alfalfa. During this symbiosis, the bacterium promotes the formation of a new plant organ, the root nodule, invades this organ, and differentiates into a nitrogen-fixing form, the bacteroid (15). Although isolation of *S. meliloti* from soil or nodule macerates is relatively easy, final identification of an isolate requires reisolation of the strain from nodules induced under axenic conditions, a time-consuming process which can take up to 3 weeks. Direct identification is hampered by the lack of a selective medium for this bacterium and by the presence of saprophytic bacteria in nodules, especially deteriorated nodules, which are typically found in plants collected in the field. An additional problem is the pleomorphism of the species; mucoid and dry strains are isolated frequently (12, 16).

PCR has proven to be an easy and reliable technique for identification of bacteria. However, in most studies of members of the *Rhizobiaceae* the workers have concentrated on identification of individual strains previously assigned to a species. In this type of work enterobacterial repetitive intergenic consensus (ERIC)-PCR and repetitive extragenic palindromic PCR have been used very successfully (8, 14). In a recent report Niemann et al. showed that the sequence of an ERIC amplicon is highly conserved in *S. meliloti* laboratory strains and proposed that this amplicon could be used for identification of the species by PCR (13). However, this amplicon encodes a 2-hydroxyacid dehydrogenase which is a widely distributed enzyme and has been tested with only a few laboratory strains and field isolates. Other primers used for identification of *S. meliloti* have targeted the *nodH* gene (4) and the insertion sequence element IS*Rm5* (5). In order to develop a method for rapid identification of *S. meliloti* field isolates, we investigated identification by PCR in which the following two regions of the *S. meliloti* genome were used: the nodbox 4 promoter (1) and the *mucR* gene (10). Both of these regions are specific for *S. meliloti* and are highly conserved and ubiquitous in the species (1, 12). Furthermore, they are located in different replicons; the nodbox 4 region is located in one of the symbiotic megaplasmids (1), and *mucR* is located in the chromosome (10). Simultaneous detection of both regions should increase the specificity of the method compared to methods based on use of a single primer pair.

The nodbox 4 region was amplified by using primers nodbox1 and nodbox2 (Table 1), which were derived from the previously published sequence of *S. meliloti* AK631 (1). PCR yielded a 138-bp band from the genomic DNA of three unrelated *S. meliloti* strains, strains AK631 (1), Rm2011 (6), and EFB1(11). The PCR products were cloned in the pCR2.1 vector and sequenced, and they exhibited 100% identity in the three strains. The sequence obtained differed at two places from the previously published sequence for the AK631 nodbox 4 region. Conservation of these mismatches in the three strains suggested that there was a sequencing mistake in the previously published AK631 sequence. A search performed with the BLAST program did not reveal homology with any other sequences in databases, indicating that the nodbox 4 region is specific for *S. meliloti* and not conserved in other rhizobia. The PCR product amplified with primers nodbox1 and nodbox2 might lead to confusing results due to its small size. For this reason a third primer, primer nodbox3 (Table 1), was designed for the adjacent *nodM* coding region. Amplification with nodbox1 and nodbox3 yielded a 646-bp fragment with the DNA of the three strains tested.

mucR codes for a regulatory zinc finger protein implicated in regulation of exopolysaccharides in *S. meliloti* (10). We have previously shown that the *mucR* gene is present in all strains irrespective of colony mucoidy (12). The *mucR* genes from Rm2011 and EFB1 have been sequenced and exhibit 96% identity (12). Homologues of *mucR* have been found in other members of the *Rhizobiaceae*, although with much lower levels of homology (2, 3, 7, 9). These characteristics make *mucR* a good candidate for PCR identification. When genomic DNA from laboratory *S. meliloti* strains were amplified by using primers mucRf and mucRr (Table 1), a 431-bp fragment was obtained.

Figure 1 shows the products amplified from genomic DNA of laboratory strains of *S. meliloti* and other related or soil bacteria with primers nodbox1, nodbox3, mucRf, and mucRr used simultaneously. PCR was performed with 1 U of Tth polymerase (Biotools, Madrid, Spain) in a 25-μl (final volume) reaction mixture. The conditions used were as follows: 95°C for 30 s, 53°C for 45 s, and 72°C for 30 s for 25 cycles, followed by a 7-min elongation step at 72°C. Each primer was added at a

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TABLE 1. Primers used for PCR amplification

Primer	Sequence (5′-3′)	Melting temp (°C)
nodbox1	TCTTTTCTTATCCATAGGGTGG	57.28
nodbox2	GAAATAATCTAGGCGCACGAGT	58.4
nodbox3	ACGGATCGTCCTCGAAG	57.10
mucRf	ATGACAGAGACTTCGCTCGGT	59.8
mucRr	TCACTTGCCGCGACGCTT	58.2

concentration of 0.4 μM. Two discrete bands at 646 and 431 bp were amplified from all of the *S. meliloti* strains but not from other bacteria, showing that amplification of *mucR* and nodbox 4 can be used for rapid identification of *S. meliloti*.

We used this approach to identify *S. meliloti* strains from nodules of *Medicago* sp. plants collected in fields around Madrid (Spain). Nodules were surface sterilized with 70% ethanol and crushed in a saline solution (0.9% NaCl), and dilutions were plated onto TY medium supplemented with 10 μg cycloheximide per ml to prevent fungal growth. A total of 240 colonies were isolated irrespective of colony morphology or pigmentation and were tested with the PCR. For each PCR a small amount of bacteria was collected with a toothpick from a fresh culture on a TY plate and resuspended in 6 μl of MilliQ water. The cells were broken by incubation at 94°C for 20 min, and a PCR was performed as described above. Fourteen colonies resulted in amplification of two bands with the appropriate sizes for nodbox 4 and *mucR* amplicons. None of the colonies produced a single band. ERIC-PCR experiments showed that the 14 positive isolates represented 13 independent strains (data not shown). Alfalfa nodulation assays were performed with the 13 strains by using Leonard jar systems containing perlite as the solid substrate and FP as the mineral medium (11). All of the PCR-positive strains induced root nodules within 3 weeks.

We also used this method to establish a collection of *S. meliloti* strains from hydrocarbon-polluted environments. Three sites with a history of contamination with polychlorinated biphenyls and/or polycyclic aromatic hydrocarbons in the Madrid region were sampled. The following strategies were used to isolate putative *S. meliloti* strains: (i) nodule macerates from *Medicago* sp. plants were directly plated onto TY medium, and colonies were isolated; (ii) nodule macerates were used to inoculate trap *Medicago sativa* plants in Leonard jars, and the nodules obtained were crushed and plated onto TY for colony isolation; and (iii) soil and soil infusions were inoculated onto trap *M. sativa* plants as described above. Sixty-eight colonies with morphology consistent with *S. meliloti* colony morphology were tested with PCR, and 46 positive strains were identified. Two isolates yielded a single band at ca. 700 bp that was considered nonspecific, and these isolates were considered negative. All of the isolates were inoculated onto *M. sativa* plants in Leonard jars, and only the PCR-positive strains induced nodule formation within 3 weeks. The relatively high number of negative isolates probably reflected the presence in the nodules of saprophytic nonrhizobial bacteria. It should be noted that in contrast to nodules induced with axenic cultures of *S. meliloti*, the nodules obtained often yielded more than one type of colony when they were crushed and plated onto TY medium.

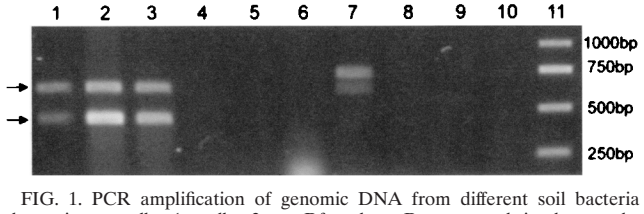

FIG. 1. PCR amplification of genomic DNA from different soil bacteria when primers nodbox1, nodbox3, mucRf, and mucRr were used simultaneously. The arrows indicate the positions of the 431-bp *mucR* amplicon and the 646-bp nodbox4 amplicon. Lane 1, *S. meliloti* EFB1; lane 2, *S. meliloti* 2011; lane 3, *S. meliloti* GR4; lane 4, *Rhizobium etli* CE3; lane 5, *Rhizobium leguminosarum* 3841; lane 6, *R. leguminosarum* STR6; lane 7, *Sinorhizobium fredii* HH103; lane 8, *Rhizobium* sp. strain NGR234; lane 9, *Pseudomonas fluorescens* F113; lane 10, negative control without DNA; lane 11, molecular weight markers.

The results presented here show that PCR amplification of the nodbox 4 and *mucR* loci is a reliable and rapid method for identification of *S. meliloti* strains. The method that we developed is useful for identification of *S. meliloti*, especially when high numbers of other bacteria are expected to be present in nodules. Therefore, this method can be used to generate a strain collection from field samples with reduced laboratory effort in a short period of time. Furthermore, the two amplicons used in this work add new probes to those already available for identification of *S. meliloti*. Optimization of this PCR procedure allowed simultaneous detection of the two loci with a single PCR. Redundant strains can eventually be detected by established ERIC or repetitive extragenic palindromic PCR methods.

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