Notas Científicas

Amplification of 16S rRNA gene sequences to differentiate two highly related bradyrhizobia species

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Abstract – A 16S rRNA gene PCR-based assay was developed aiming at a fast molecular diagnostic method to differentiate the two phylogenetically closely related species *Bradyrhizobium japonicum* and *B. elkanii*, isolated from soybean nodules, in order to identify those more competitive and comprising greater nitrogen fixation ability for use in the formulation of commercial inoculants. The assay used was able to discriminate ten reference strains belonging to these two *Bradyrhizobium* species, as well as to efficiently identify 37 strains isolated from fields cultivated with soybean.

Index terms: PCR, rhizobial diversity, commercial inoculants.

Amplificação de seqüências do gene RNAr 16S para diferenciar duas espécies de bradirrizóbios altamente relacionadas

Resumo – Foi avaliado um método baseado em PCR do gene do RNAr 16S, desenvolvido com a finalidade de um diagnóstico molecular rápido na identificação das espécies filogeneticamente relacionadas *Bradyrhizobium japonicum* e *B. elkanii*, isoladas de nódulos de soja, visando ao seu emprego na identificação de estirpes mais competitivas e com maior capacidade de fixação de nitrogênio para uso na formulação de inoculantes comerciais. O método usado foi capaz de discriminar dez estirpes-padrão pertencentes a essas duas espécies de *Bradyrhizobium*, assim como identificar eficientemente 37 estirpes isoladas de lavouras cultivadas com soja.

Termos para indexação: PCR, diversidade de rizóbios, inoculantes comerciais.

The massive inoculation of Brazilian soils comprising few bradyrhizobia strains belonging to *B. japonicum* and *B. elkanii* species has resulted in an established population in most soils cropped with this leguminous (Ferreira & Hungria, 2002). However, these populations may show morphological, biochemical, physiological, genetic and symbiotic variability related to adaptation processes and genetic transfer (Hungria & Vargas, 2000; Ferreira & Hungria, 2002). Literature data have shown that these new bacterial populations differed significantly from the original introduced strains (Ferreira & Hungria, 2002), including a high percentage of nodules occupied by strains with unknown serological reaction after some years of soybean cropping (Freire et al., 1983).

The fact that *B. japonicum* and *B. elkanii* species are largely used together in Brazilian soybean fields makes sometimes practically impossible to differentiate strains isolated from soybean nodules. This problem is particularly important when the objective of the screening is to identify strains more competitive and comprising greater nitrogen fixation ability to be used in the formulation of the commercial inoculants.

In this work, the development of a PCR-based assay targeting the 16S rRNA gene, which was able to discriminate the two related *B. japonicum* and *B. elkanii* species among 10 reference strains and to identify 37 strains isolated from fields cultivated with soybean is reported. Genomic DNA from these bacteria

was also used in RFLP analysis of 16S-23S rDNA ITS and *nifH* gene regions to confirm their taxonomic classification.

The bradyrhizobia reference strains used in this work were provided by Fepagro, Brazil: *B. elkanii* SEMIA 587, SEMIA 5019, SEMIA 5086 (USDA 31), SEMIA 5087 (USDA 76), and SEMIA 5088 (USDA 94); *B. japonicum*: SEMIA 586, SEMIA 5019, SEMIA 5080, SEMIA 5052 (USDA 6) and USDA 110. *B. betae* PL7HG1, *B. canariense* BTA-1 (provided by Universidad de Salamanca, Spain), *Rhizobium leguminosarum* bv. *trifolii*, as well as the strains isolated in this work and the reference strains, were cultivated in yeast extract-mannitol (YEM) broth (Somasegaran & Hoben, 1994) for seven days at 28ºC. *Agrobacterium tumefaciens* AGL1, *Escherichia coli* XL1-Blue, *Azospirillum brasilense* Sp7 ATCC 2914 and *Paenibacillus macerans* ATCC8244 were cultivated in LB medium (Sambrook & Russel, 2001) for 24 hours at 37ºC. Fifty microliters of the liquid cultures were heated in a boiling water bath. The supernatants obtained after a brief spin were used as a DNA template.

Partial nucleotide sequences of the 16S rRNA gene from 15 strains of *B. japonicum* (GenBank accession numbers: AB195991, AB195985, AB070570, AB070571, X66024, AF234888, DQ133343, AY996780, DQ133442, AB070569, AF236087, AF234884, AY904786, AY904732 and AY904774) and 11 strains from *B. elkanii* (GenBank accession numbers: AB195989, AY649438, AB195990, AY904780, AF417553, AY904789, AY904778, AY568513, AF234890, AF293380 and AF237422), which were isolated from diverse habitats all over the world were compared by ClustalX software v.1.81 (Thompson et al., 1997) to design three potentially specific primers. The forward primer sequence designed (Brady: 5'-AMTKCCTTTGAKWYTKAAGATCTTG-3') was the same for all DNAs analyzed. Reverse primers were specific for each *Bradyrhizobium* species [Bjap for *B. japonicum* (5'-GTCACATCTCTGCGACCGGTC-3') and Belk for *B. elkanii* (5'-AACTCCGTCTCTGGAGTCCGCGA-3')]. Amplification reactions (25 µL) contained 5 µL of the culture supernatant as the DNA template, 1 U Taq DNA polymerase (Invitrogen) with the correspondent 1 X Buffer containing 2.5 mM of $MgCl_2$, 5 mM of dNTP and 10 pmol of each primer (Brady combined with Belk or Bjap).

To determinate the most suitable annealing temperature, PCR reactions were performed using a temperature gradient varying from 45 to 64ºC, in which the temperature of 55ºC presented better amplification results. All final PCR amplifications were performed in a Thermo Hybaid thermal cycler using an initial denaturation step at 94ºC for 1 min, 30 cycles of 1 min of denaturation at 94ºC, 30 s of annealing at 55ºC, 1 min of extension at 72ºC, followed by a final extension for 5 min at 72ºC. The amplification products were electrophoresed in 1% agarose gel containing ethidium bromide in 1 X Tris-EDTA buffer (TBE) and the gels were photographed under UV light.

For the 16S-23S rDNA ITS spacer RFLP fingerprinting primers pHr (Massol-Deya et al., 1995) and p23S uni322anti (Honeycutt et al., 1995) were used. Fragments of *nifH* gene were amplified using the primers PolF and PolR defined by Poly et al. (2001) to amplify a 360 base pair *nifH* fragment from DNA isolated from a wide range of diazotrophic microorganisms. PCR were conducted as described above. Ten microliters of the purified amplification products were overnight digested using *Mbo*I and *Hae*III endonucleases (Promega Life Sciences) for ITS region and *Hae*III and *Sal*I endonucleases (Promega Life Sciences) for *nifH* gene fragment. The restriction fragments were separated on 8% acrylamide-bisacrylamide gel stained with silver nitrate (Sambrook & Russel, 2001).

The specificity of the PCR system developed in this work was confirmed by the application of the primer pairs (Brady + Belk and Brady + Bjap) to amplify DNA obtained from cultures of well-characterized *Bradyrhizobium* species, which are traditionally recommended as commercial inoculants in Brazilian fields (Figure 1 A). The reference strains were also subjected to the RFLP analysis using the molecular markers 16S-23S rDNA ITS spacer, as suggested by Willems et al. (2003), and the *nifH* gene, since its phylogeny has been reported to be largely consistent with the 16S rDNA tree (Zehr et al., 2003). The RLFP analysis of the *nifH* gene was able to discriminate strains belonging to *B. japonicum* or *B. elkanii* species, corroborating the results obtained with the *Bradyrhizobium*-specific PCR approach. However, the RFLP analysis of the 16S-23S rDNA ITS spacer presented variable patterns within the same specie, and could not discriminate these two bradyrhizobia species.

Group-specific primers developed in this work were also used with DNA obtained from other bacterial strains available in the laboratory: *Rhizobium leguminosarum* bv. *trifolii*, *Agrobacterium tumefaciens* AGL1, *Escherichia coli* XL1-Blue, *Azospirillum brasilense*

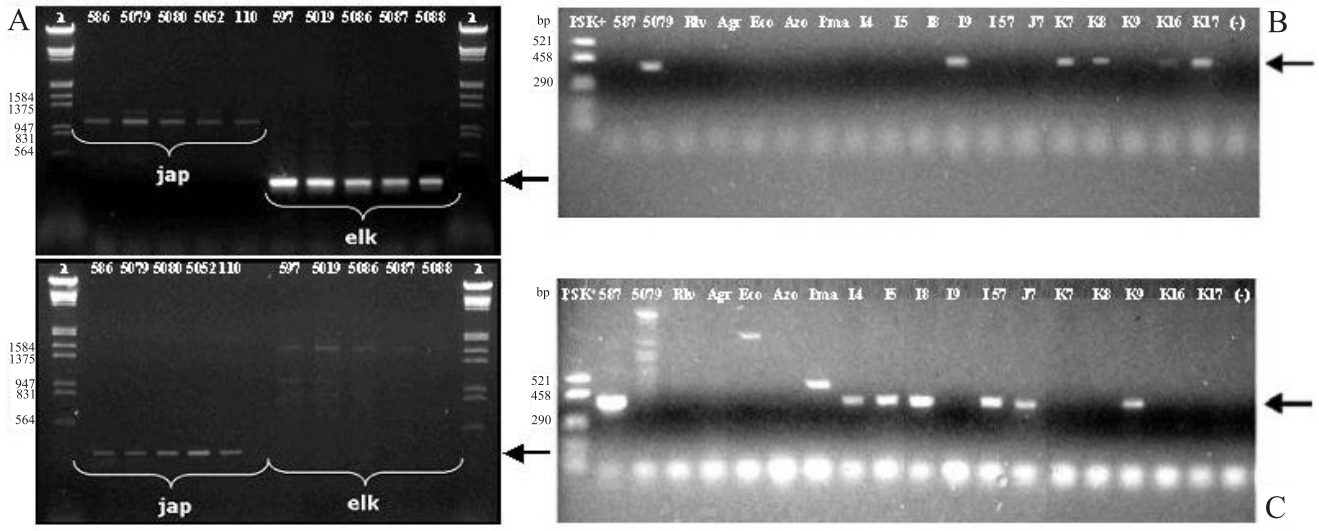

Figure 1. Amplification of the 16S rRNA gene region using the group-specific primers. (A) In the upper gel the primer combination was Brady + Bjap and in the lower gel was Brady + Belk. *B. japonicum* strains are: SEMIA 586 (586), SEMIA 5079 (5079), SEMIA 5080 (5080), SEMIA 5052 (5052), and USDA 110 (110). *B. elkanii* strains are: SEMIA 587 (587), SEMIA 5019 (5019), SEMIA 5086 (5086), SEMIA 5087 (5087), and SEMIA 5088 (5088). In (B) the primer combination was Brady + Bjap and in (C) was Brady + Belk. The bacterial strains are: *B. elkanii* SEMIA 587 (587); *B. japonicum* SEMIA 5079 (5019); *Rhizobium leguminosarum* bv. *trifolii* (Rlv); *Agrobacterium tumefaciens* AGL1 (Agr); *Escherichia coli* XL1-Blue (Eco); *Azospirillum brasilense* Sp7 ATCC 2914 (Azo); *Paenibacillus macerans* ATCC 8244 (Pma); I9, I57, K7, K8, K16 and K17 are strains identified as *B. japonicum*; I4, I5, I8, J7 and K9 are strains identified as *B. elkanii*. Molecular markers are λ DNA cleaved with *Eco*RI and *Hin*dIII in (A) and pBSSK$^+$ plasmid DNA cleaved with *Hae*III in (B) and (C). Negative control (-) was reaction without DNA. Arrows indicate the expected amplifications products comprising sizes of approximately 400 bp.

Sp7 ATCC 2914 and *Paenibacillus macerans* ATCC8244, as well as with DNA obtained from two other *Bradyrhizobium* species, *B. betae* PL7HG1 and *B. canariense* BTA-1. These bacteria were used as negative controls to check the specificity of the primer pairs developed. Either no PCR products were obtained or DNA fragments bigger than 400 bp were yielded (Figure 1 B and C).

After the confirmation of the PCR specificity with the reference strains, the method was used to identify isolates obtained from fields cultivated with soybean. From a total of 37 isolates, 24 were shown as belonging to the *B. japonicum* and 13 as belonging to the *B. elkanii* species (Figure 1 B and C). These 37 isolates were also subjected to the RFLP analysis using the molecular markers 16S-23S rDNA ITS spacer and the *nifH* gene. Exactly the same results to those obtained with the reference strains were achieved.

The assay was efficient in discriminating *Bradyrhizobium* field isolates, and proved to be of fast and relatively low cost. These features can be useful when the objective is to characterize genotipically bradyrhizobia species for agricultural practices.

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