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## NUCLEIC ACIDS, RECOVERY, AND DETERMINATION

## A Simple Method for Obtaining DNA Suitable for RAPD Analysis From *Azospirillum*

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### 1. Introduction

The random amplified polymorphic DNA (RAPD) technique has increasingly been used in the last decade as a simple, low-cost, and time-effective technique for the analysis of genomic polymorphism among related organisms. This method proved to be particularly useful in genomic fingerprinting and phylogenetic studies of microorganisms that present taxonomic problems or a large diversity within the taxon, such as clinical and soil microorganisms (1–5). However, this technique presents persistent problems of reproducibility that have been attributed to a high sensitivity to subtle procedure changes and to the quality of the DNA used as template (6–8). For this reason, other techniques that are more time and budget demanding, such as restriction fragment-length polymorphism (RFLP) and amplified fragment-length polymorphism (AFLP) have been used as valid and more reliable alternatives to assess genomic diversity (9,10). Although DNA fingerprints obtained by RFLP and AFLP yield excellent results, often cost considerations make RAPD the technique of choice. Additionally, because the RAPD method is much faster and simpler than RFLP and AFLP, it is also well-suited when large numbers of specimens, or microbial isolates that require previous laborious manipulations, have to be analyzed. Thus, any procedural simplification of tedious and time-consuming steps is welcome, provided that the information quality does not decrease due to technical artifacts or loss of reproducibility; the latter is precisely the most sensitive issue of the RAPD technique, attributed mainly to DNA quality. Hence, obtaining DNA of such a quality that it can be directly used in RAPD

experiments is the most critical and time-consuming step required in this technique, which has to be carefully adjusted and optimized for every case under study.

The genus *Azospirillum* belongs to a group of rhizosphere bacteria often referred to as plant-growth-promoting rhizobacteria (**11**). They are free-living nitrogen-fixing bacteria closely associated with grasses (**12**). Owing to the increasing interest in making world agriculture sustainable, they are used as inocula for enhancing crop productivity and to replace chemical nitrogen fertilizers (**13**).

With the aim of assessing azospirilla diversity in soils, a short protocol based on direct cell disruption (**14**) was tried in our laboratory; although it proved to work quite well with specific gene primers (15- and 20-mer oligonucleotides), the method failed to give reliable profiles when using the random decanucleotide primers utilized in the RAPD technique. In this chapter, we present a modification of the aforementioned protocol (**14**). The method involves DNA extraction based on cell lysis by thermal disruption of bacterial suspensions, which does not require any extra purification step and yields a DNA suitable for RAPD experiments. This technique has been successfully used in our laboratory to assess the diversity of *Azospirillum* sp. and other soil microorganisms present in the area and associated with sugarcane plantations.

## 2. Materials

1. Strain: *Azospirillum brasilense* Sp7 (ATCC 29145) (EMBRAPA-CNPq, Seropédica Km 47, R.J., Brazil).
2. LB medium (**15**): 10.0 g tryptone, 5.0 g yeast extract, 3.0 g NaCl, and distilled water to 1000 mL, pH 7.0, adjusted with NaOH. For solid medium, add 15.0 g agar.
3. N-free malate medium (NFb) (**16**): 5.0 g malic acid, 0.5 g K<sub>2</sub>HPO<sub>4</sub>, 0.2 g MgSO<sub>4</sub>•7H<sub>2</sub>O, 0.1 g NaCl, 0.02 g CaCl<sub>2</sub>•2H<sub>2</sub>O, 2.0 mL micronutrients solution, 2.0 mL bromothymol blue (0.5% w/v in 0.2 N KOH), 4.0 mL FeEDTA (1.64% w/v, aqueous), 1.0 mL vitamins solution, 4.5 g KOH, and distilled water to 1000 mL, pH 6.5–6.8, adjusted with NaOH. For semisolid NFb medium add 1.75 g agar.
4. Micronutrients solution (**16**): 0.04 g CuSO<sub>4</sub>•5H<sub>2</sub>O, 1.20 g ZnSO<sub>4</sub>•7H<sub>2</sub>O, 1.40 g H<sub>3</sub>BO<sub>3</sub>, 1.00 g NaMoO<sub>4</sub>•2H<sub>2</sub>O, 1.175 g MnSO<sub>4</sub>•H<sub>2</sub>O, and distilled water to 1000 mL.
5. Vitamins solution (**16**): 10 mg biotin, 20 mg piridoxal-HCl, and distilled water to 100 mL. Store at 4°C.
6. Chelex 100 resin (Bio-Rad).
7. Wizard® Genomic DNA Purification Kit (Promega).
8. Spectrophotometer.

9. RAPD mixture: MgCl<sub>2</sub>, 0.1 mM of each dNTP (deoxyribonucleotides), 0.2 μM of random-sequence 10-mer primers (OPJ series from Operon Technologies), 0.75 U *Taq* polymerase (Promega), and 2 μL *Taq* buffer (10X).
10. Thermocycler.
11. 0.5X Tris-borate-EDTA buffer (TBE).
12. Agarose and electrophoresis equipment.
13. UV transilluminator analyzer.

### 3. Methods

The methods described below outline (1) *Azospirillum* DNA extraction by cellular thermal disruption based on the direct lysis of bacterial suspensions, (2) other DNA extraction techniques to compare the quality and reproducibility of the DNA obtained by the thermal cellular technique, and (3) the RAPD method used in this assay.

#### 3.1. Cell Culture of *Azospirillum* and DNA Extraction by Cellular Thermal Disruption

1. The starting material is an overnight culture of *A. brasilense* Sp7 in NFB liquid medium (DO<sub>600</sub> = 1) (see **Note 1**).
2. Inoculate 10 μL of the starting culture into 6 mL of LB broth.
3. Inoculate 10 μL of the starting culture into 6 mL NFB liquid medium supplemented with 1 g/L NH<sub>4</sub>Cl and into 5 mL NFB semisolid medium.
4. Likewise, plate a loopful of the inoculum on LB solid medium.
5. Incubate liquid cultures overnight at 30°C in water-bath shaker at 200 rpm.
6. Incubate solid and semisolid cultures at 37°C for 48 h in oven.
7. Pick up with a sterile toothpick one or two colonies from solid culture and suspend it thoroughly in 30 μL of double-distilled sterile water contained in a 1.5-mL microtube.
8. Put 6 μL of cell suspensions coming from liquid or semisolid media in 30 μL of double-distilled sterile water in a 1.5-mL microtube.
9. Place the microtubes in upright position into a container of tap water and boil at 95°C for 10 min (see **Note 2**).
10. After thermal treatment, remove the microtubes from the water and cool down at room temperature (25°C). DNA template samples obtained by this method can be stored at -20°C for months to be used in further experiments.

#### 3.2. Other DNA Extraction Techniques to Compare the Quality and Reproducibility of the DNA Obtained by Thermal Cellular Method

In order to test the influence of the DNA quality on the reproducibility of RAPD profiles, experiments and controls were carried out with DNA coming from the same cultures of *A. brasilense* Sp7 previously described, but extracted and purified according to the following protocols: Dellaporta et al. (17); Walsh

et al. (18), which includes the use of Chelex 100 resin (BioRad); and the Wizard™ Genomic DNA Purification Kit (Promega). The DNA was quantified with a Beckman 7000 spectrophotometer.

### 3.3. RAPD Assay

#### 3.3.1. PCR Reaction

Reaction mix contains: 0.5 mM MgCl<sub>2</sub>, 0.1 mM of each dNTP (deoxyribonucleotides), 0.2 μM of random-sequence 10-mer primers (see Note 3), 0.75 U of *Taq* polymerase (Promega), 2 μL *Taq* buffer (10X), 1 μL of the suspension obtained by thermal cell disruption or 20 ng DNA obtained with other methods (see Note 4). Final volume: 20 μL.

#### 3.3.2. Amplification Program

Denaturation, 30 s at 92°C; annealing, 1 min at 35°C; extension, 2 min at 72°C; number of cycles: 45. The program started with a thermal treatment of 3 min at 94°C and finished with an extension of 5 min at 72°C. The reaction was carried out in a MJ Research thermocycler.

#### 3.3.3. Electrophoresis

Amplification products were separated by electrophoresis at 4 V/cm in a 1.5% agarose gel in 0.5X TBE running buffer. Gels were stained with 0.5 μg/mL ethidium bromide for 30 min, washed three times with distilled water, and photographed with a Polaroid camera (Polaroid film 667). RAPD experiments were repeated three times to avoid false results and to assure reproducibility. Negative controls, without DNA, were included in each experiment. In Fig. 1 we show RAPD profiles of the strain Sp7 of *Azospirillum brasilense* when using the decanucleotide OPJ20 (Operon) as random primer and with DNA templates obtained according to the methods mentioned above. As seen in Fig. 1, there is no visible difference between the size and number of bands obtained using DNA prepared with different degrees of purification, and the profile obtained using DNA from thermal cell disruption (see Note 5). Although we show here a single example, we have tested this procedure many times using different primers and azospirilla isolates.

### 3.4. Results

The modification introduced in the original protocol (14) allowed us to evaluate the diversity of *Azospirilla* genus in soils of the sugarcane crop area of the region by using a simple and fast procedure. RAPD analysis and genome fingerprinting with this procedure showed no significant differences with con-

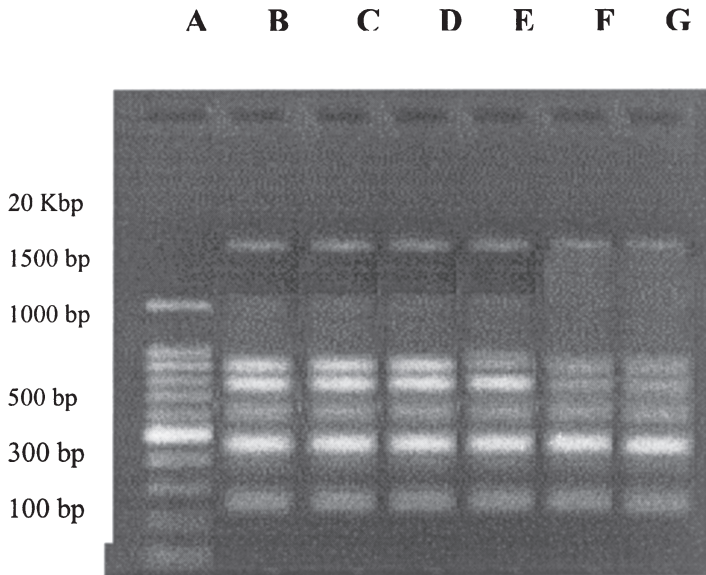


Fig. 1. RAPD profiles of the strain Sp7 of *A. brasilense* (ATCC 29145) when using DNA obtained with different methods. (A) Molecular weight marker (Ladder 100 bp, Promega); (B) DNA extracted and purified according to Dellaporta et al. (17); (C) DNA obtained according to Walsh et al. (18), with Chelex 100 resin (Bio-Rad); (D) DNA obtained by using the Wizzard™ Genomic DNA Purification kit from Promega; (E) DNA obtained by thermal cell disruption from solid media; (F) DNA obtained by thermal cell disruption from semisolid media; (G) DNA obtained by thermal cell disruption from liquid media.

controls carried out with DNA obtained and purified with standard methods (see Fig. 1).

#### 4. Notes

1. To obtain reliable results with the cellular thermal disruption method described here, it is important to control the purity of all cultures coming from liquid or semisolid media before extracting the DNA; owing to the conspicuous shape and motility features of these bacteria, the latter can be done microscopically.
2. Instead of the boiling water procedure, cell disruption can be carried out in a thermocycler using a one-cycle program of 95°C for 10 min.
3. The success of the amplification and reproducibility by RAPD, relies on many factors: quality and amount of DNA template, mix composition (i.e., Mg<sup>2+</sup> concentration), primers used, and adjustment of the amplification program. Therefore, these factors need to be optimized before starting the RAPD analysis. Particular attention must be paid to the selection of the RAPD primers, as not all of them would yield a similar number and size of polymorphic bands. There are

primers that would not amplify any band at all. An ideal primer should yield highly reproducible DNA profiles, containing monomorphic and polymorphic bands, with a sufficient number to permit discrimination of samples.

4. As mentioned in **Note 3**, another important aspect to consider in RAPD analysis is the amount of DNA used in the amplification. An excess of DNA will produce a high-molecular-weight band (approx 20 Kbp) regardless the primer used (**Fig. 1**). The latter can be eliminated from the gel by reducing the concentration of DNA in the amplification reaction.
5. The procedure described here to obtain DNA in quality and quantity suitable for RAPD analysis has several advantages over previously mentioned techniques:
  - a. It avoids the cumbersome procedure of DNA extraction and purification included in other protocols.
  - b. The quality of DNA renders identical results to those from DNA obtained with more sophisticated and time-consuming procedures.
  - c. The procedure allows analysis of DNA from different sources and culture media without any apparent loss of reproducibility of the fingerprint profile.
  - d. The thermal cell disruption followed by PCR thermal cycling seems to eliminate contamination and other technical artifacts that usually appear as serious nuisances in standard RAPD procedures.
  - e. This protocol is rather insensitive to changes in DNA load, in contrast to regular protocols that usually require thorough optimization to avoid negative effects on fingerprint reproducibility.
  - f. This procedure eliminates the use of chemicals used in standard DNA extraction and purification protocols, therefore reducing the chance of DNA or any other unknown contaminants that often annoy RAPD users.
  - g. This protocol also proved to be useful for ARDRA analysis (**19**), which requires DNA digestion with specific endonucleases after PCR amplification. The latter confirms that after the thermal treatment the DNA quality is still good enough to be used as a substrate for further DNA manipulations.

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