Identification of Casuarina-Frankia strains by use of polymerase chain reaction (PCR) with arbitrary primers

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1. SUMMARY

Free-living N₂-fixing Frankia strains isolated from Casuarina sp. were investigated for genomic polymorphism. We used six 10-mer oligonucleotides as single arbitrary primers (AP) for the polymerase chain reaction (PCR) in order to amplify random DNA fragments in the genome of free-living Frankia strains. Agarose-gels of the amplified genomic DNA revealed that two of the six arbitrary primers showed polymorphism in the eight different Frankia genomes. Analysis of the AP-PCR products showed 9 polymorphic bands ranging from 4.1–0.60 kb. We conclude that single arbitrary primers can be used to amplify genomic DNA, and that polymorphism can be detected between the amplification products of the different Frankia genomes.

2. INTRODUCTION

The symbioses between plants and microorganisms, e.g., Casuarina and the actinomycete Frankia, are unique systems that can be used in production of fuelwood, to prevent soil erosion and to stabilize sand dunes [1]. Also, these symbioses can be used to increase nitrogen in soil [2], due to the decay of symbiotic plant residues. Considerable differences in nitrogen-fixation rates have been recorded in Casuarina symbioses [3,4]. Optimizing the Casuarina-Frankia symbioses implies increasing nitrogen fixation and thereby also nitrogen content in soil. In order to optimize the symbiosis it is necessary to combine the right Frankia with the right Casuarina. This would require methods to identify different Frankia genotypes.

The genus Frankia has been subjected to taxonomical identification by use of different techniques. Attempts to identify Frankia strains have been based on e.g. host specificity, morphology,
biochemistry [5] and polypeptide patterns of soluble proteins [6]. The knowledge of *Frankia* genomes is still incomplete and therefore techniques based on genomic DNA are relatively recent. Examples are the use of amplified 16S rDNA sequences [7] and hybridization of PCR products with strain-specific probes [8]. Polymerase chain reaction (PCR) has successfully been used to amplify DNA from some microorganisms [9,10], but most often it is used to amplify the target DNA in vitro before detection by hybridization with a suitable DNA probe [8]. However, detection of restriction-fragment-length polymorphism (RFLP) by Southern-blot hybridization is very laborious and conventional PCR requires information about the target DNA sequence. Very recently, arbitrarily primed (AP) PCR have been used in order to detect polymorphisms in strains of *Staphylococcus* sp. and *Streptococcus* sp. [11,12]. The advantage of using AP-PCR is that it does not require information about the genome of the organisms studied.

This is, to our knowledge, the first report on the use of arbitrary primers in the polymerase chain reaction to detect polymorphism in genomic DNA from *Frankia*.

3. MATERIALS AND METHODS

3.1. Strains and media

*Frankia* strains that were isolated from *C. equisetifolia*, *C. equisetifolia* ssp. *incana*, *C. cunninghamiana* and *C. glauca* are listed in Table 1. For DNA extraction, strains were grown for 14 days at 28°C in a nitrogen-containing medium [13], and for 5 days in the above mentioned medium without nitrogen.

3.2. DNA extraction

*Frankia* strains were harvested by centrifugation (15,340 × g, 10 min), washed once in 1 × TE buffer (10 mM Tris (2-amino-2-hydroxymethyl)-1,3-propanediol), pH 8.0, with 1.0 mM EDTA. Cells were digested by lysozyme (3 mg/ml; Sigma) and achromopeptidase (0.5 mg/ml Sigma). EDTA was added to a final concentration of 50 mM and the cells were kept at 37°C for 90 min. Lysis was completed by the addition of sodium dodecyl sulphate (SDS; Sigma electrophoresis purity gradient) at a final concentration of 0.5%, Protease K (final concentration 1 mg/ml) and NaCl (final concentration 125 mM), and incubated at 65°C for 30 min. DNA extractions were made once with phenol-chloroform (1/1, v/v) and once with plain chloroform (1/1, v/v). The aqueous phase was extracted with 99.5% ethanol (20°C), and centrifuged (13,000 × g, 30 min). After addition of sodium acetate to a final concentration of 0.3 M, the aqueous phase was precipitated with ice-cold 95% ethanol, stored in −20°C for 30 min or overnight and centrifuged (14,300 × g, 30 min). The pellet was washed in 70% ethanol, dissolved, dried and resuspended in 1 × TE buffer pH 8.0. After addition of RNAseA to a final concentration of 40 μg/ml, the micro-tube was incubated for 1 h at 37°C followed by 30 min in 65°C. Re-extraction and precipitation was done as described above and the pellet was resuspended in 1 × TE buffer.
Table 2
Sequences of 10-mer oligonucleotides used as arbitrary primers in the PCR

<table>
<thead>
<tr>
<th>Code</th>
<th>5' to 3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-01</td>
<td>CAGGCGTTTC</td>
</tr>
<tr>
<td>A-02</td>
<td>TGCCGAGCTG</td>
</tr>
<tr>
<td>A-03</td>
<td>AGTCAGCCAC</td>
</tr>
<tr>
<td>A-04</td>
<td>AATCGGGCTG</td>
</tr>
<tr>
<td>A-05</td>
<td>AGGGGTCTTG</td>
</tr>
<tr>
<td>A-06</td>
<td>GGTCCCCTGAC</td>
</tr>
</tbody>
</table>

3.3. PCR-amplification

Part of the *Frankia* genome was randomly amplified using a modification of the polymerase chain reaction as described earlier [14]. The primers used were six 10-mer oligonucleotides (Table 2) and were obtained from Operon (Operon Technologies, Alameda, CA). Two concentrations of *Frankia* DNA, 10 and 100 ng, were amplified in 10 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂ and 0.001% (w/v) gelatine, dNTP-mixture (Pharmacia) containing 125 μM of each dATP, dTTP, dCTP and dGTP, 0.2 μM of each primer (Table 2), template DNA (1 μg ml⁻¹), and finally 25 U ml⁻¹ amplitaq ™ DNA polymerase (Perkin Elmer) and sterile distilled H₂O. The PCR reaction was performed in a total volume of 100 μl in 0.5 ml-Eppendorf tubes under a layer of mineral oil (Sigma M-5904). The tubes were transformed automatically through heat blocks for 50 cycles in a Perkin Elmer Cetus DNA Thermal Cycler; denaturation temperature was 92°C for 1 min, primer annealing at 35°C for 1 min, and extension from the primers at 72°C for 2 min. Thirty μl of the PCR-product was analyzed by electrophoresis on a 1.2% (w/v) agarose (IBI electrophoresis grade) gel.

4. RESULTS AND DISCUSSION

4.1. DNA preparation

Two concentrations of target DNA were tried, 10 and 100 ng. The efficiency of amplification was

![Fig. 1. Amplification of *Frankia* genomic DNA by PCR with arbitrary primers A-01 (A) and A-05 (B) at a concentration of 100 ng DNA. The PCR products were resolved by electrophoresis on a 1.2% agarose gel, which was stained with ethidium bromide, visualised in UV-light and photographed. Lanes 1–8 refer to *Frankia* strains 1–8 as described in Table 1. M refers to kb ladders (Pharmacia), and fragment sizes are indicated in the figure. Amplification was repeated twice with DNA extracted at different occasions, and the same result was obtained. PCR performed at a concentration of 10 ng of target DNA showed the same polymorphic pattern, but was not as easily visualised in UV-light.](image-url)
slightly greater in the higher concentration of target DNA (see text to Fig. 1). This result indicated that the DNA preparation was pure and did not contain phenolic compounds, as have been found in Frankia nodules [8]. The result also showed that the same polymorphic markers were obtained irrespective of the DNA concentration.

4.2. Polymerase chain reaction

PCR amplification of genomic Frankia DNA using arbitrary primers was carried out on eight free-living Frankia strains isolated from C. equisetifolia, C. equisetifolia ssp. incana, C. cunninghamiana and C. glauca. Primers were chosen in the absence of any nucleotide sequence information about the Frankia strains tested. The six primers used were all 10-mer oligonucleotides (Table 2). In this PCR we have used low temperature for the initial two cycles, so that the primers would anneal to as many sequences as possible in the Frankia genome. Also, Welsh and McClelland [11,12] used a low temperature (40°C) for low-stringency annealing of primers.

No amplification products were seen for any primers but A-01 and A-05 (Fig. 1A and B). It was shown that primers A-01 and A-05 annealed to the Frankia genome in a way that polymorphism was obtained (Fig. 1A and B). Detection of polymorphism was also found by Welsh and McClelland [11], who were able to generate discrete fingerprints from the genomes of viruses, humans and plants.

We compared the pattern of AP-PCR products from eight Frankia strains (Fig. 1A and B). The eight different strains showed different band pattern both with primer A-01 (Fig. 1A) and with primer A-05 (Fig. 1B). The differences in PCR products were more obvious though when using primer A-05. Analysis of AP-PCR products achieved using primer A-05 is shown in Table 3. Nine bands were used for analysis of polymorphic pattern. Bands designated 1–6 were all observed in Frankia strains 1, 2 and 3, but three polymorphisms were detected when comparing the AP-PCR products. The greatest difference was seen in AP-PCR products between strains 1 and 8. Thus, polymorphism was detected between the PCR-products of the different Frankia strains. The result probably reflects sequence differences between the genomes of the Frankia strains. The experiment was repeated twice and the same results were obtained both times.

PCR is based on the enzymatic amplification of a DNA fragment that is flanked by oligonucleotide primers [17]. Consequently, PCR is capable of increasing the amount of a target DNA, but the use of conventional primers for PCR requires sequence information for the relevant genes. The actinomycete Frankia is a microorganism that was isolated as late as 1978 [19], and knowledge about the Frankia genome is there-

<table>
<thead>
<tr>
<th>Bands</th>
<th>Frankia strains</th>
<th>M</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+ + + - - + - +</td>
<td>4.1</td>
</tr>
<tr>
<td>2</td>
<td>+ + + - - + + -</td>
<td>3.9</td>
</tr>
<tr>
<td>3</td>
<td>+ + + - - + - +</td>
<td>3.4</td>
</tr>
<tr>
<td>4</td>
<td>+ + + - - + - +</td>
<td>3.1</td>
</tr>
<tr>
<td>5</td>
<td>+ + + - - + + +</td>
<td>2.2</td>
</tr>
<tr>
<td>6</td>
<td>+ + + - - + - +</td>
<td>2.1</td>
</tr>
<tr>
<td>7</td>
<td>- - - - - - - -</td>
<td>1.8</td>
</tr>
<tr>
<td>8</td>
<td>+ - + + + + + +</td>
<td>0.95</td>
</tr>
<tr>
<td>9</td>
<td>+ - - - - - - -</td>
<td>0.60</td>
</tr>
</tbody>
</table>

See Table 2, Fig. 1B. Lanes 1–8 designate Frankia strains listed in Table 1. M refers to kb ladders (Pharmacia).
fore incomplete. The PCR method involving arbitrary primers does not require a special set of primers with known sequences [11,18]. This method can accordingly be performed with incomplete or even without knowledge of the genome of the organisms. The use of arbitrary primers is therefore of great use and of advantage over the use of conventional primers when new microorganisms are to be differentiated genetically from each other. The primers A-01 and A-05 obviously bind in regions of the *Frankia* DNA which is not conserved.

We conclude that arbitrary primers can be used to generate polymorphic genomic fingerprints by PCR, as was also shown earlier in other organisms [11,12,18].

**ACKNOWLEDGEMENTS**

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**REFERENCES**


