Gene probe designing for evaluation of the diversity of *Bradyrhizobium japonicum* isolates

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Abstract
Many researchers consider the use of different probes for hybridization assays as suitable for studying the genetic diversity of nitrogen fixing bacteria. In this study for asessing genetic diversity among *Bradyrhizobium japonicum* isolates, two different probes (*sucA* and *topA*) chosen from the chromosomal genome of *Bradyrhizobium* strain USDA 110 were designed, evaluated by DNAMAN software and implemented in the DNA/DNA hybridization of eighteen isolates. Hybridization patterns of the *sucA* and *topA* probes showed that all *B. japonicum* isolates were clustered into 4 and 5 groups, respectively. It was also found that the sequences of these genes among the isolates were different, thus making them suitable for studying the genetic diversity of rhizobial bacteria. The sequence diversity of *topA* gene was more variable than that of the *sucA* gene.
Keywords: Probe designing; DNA hybridization; *Bradyrhizobium japonicum*; Diversity.

INTRODUCTION

Among living organisms, there are certain genera of prokaryotes which are able to biologically fix nitrogen. The genus *Bradyrhizobium* is capable of nitrogen fixation and nodule formation in leguminous plants (Jordan, 1982). Most of these isolated strains are associated with nodules on *Glycine* spp. (Giongo *et al*., 2008; Sameshima *et al*., 2003; Zakhia and Lajudie 2001).

Nitrogen fixing bacteria, especially *Bradyrhizobium* and *Rhizobium* are of great importance in sustainable agriculture. Nowadays many different techniques are used for studying bacterial genetic diversity, especially that of rhizobial bacteria. Evaluation of bacterial plasmid profiles (number and size of plasmids) is an example of such techniques. However, Plasmid profiling is a time consuming procedure, the results of which are usually considered as morphological characteristics. Therefore, researchers do not use it as a routine method for genetic diversity studies (Shishido and Pepper 1990; Pepper *et al*., 1989). Multi locus enzyme electrophoresis (MLEE) is another technique for evaluating the genetic diversity of bacteria. This technique is based on the relative movement of intercellular enzymes. By using this method, different protein profiles can be produced which can reveal genetic relationships among different genera of bacteria (Wise *et al*., 1996 and 1995).

The amplification of 16S rRNA gene by using polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) of the PCR products is also routinely used by many scientists for studying bacterial genetic diversity and their genetic relationships. A wide range of genes in accordance with the aim of research are used in the PCR/RFLP technique (Giongo *et al*., 2008). In the case of bacteria belonging to different genera with less genetic relatedness, amplification of only small parts of the 16S rRNA gene is performed (Herrera-Cervera *et al*., 1997). Intergenic spacer regions of the 16S-23S rRNA gene are generally used in the PCR/RFLP procedure for studying genetic diversity within a genus of bacteria (Willems *et al*., 2001). In recent years, this technique has extensively been used because of its good discriminative characteristics.

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Another method is RFLP of different genes such as *nif* and *nod*, which are involved in nitrogen fixation and process of nodulation by *Rhizobium* and *Bradyrhizobium* bacteria. In this technique, the bacterial genome is hybridized by different probes and the resulting patterns are studied for the presence of genetic relationships among bacteria. The aim of this investigation was to design, use and evaluate *sucA* (alpha-ketoglutarate dehydrogenase gene consists of 2955 bp) and *topA* (topoisomerase gene consists of 2484 bp) probes for the purpose of revealing genetic relationships among a certain number of *B. japonicum isolates*.

MATERIALS AND METHODS

Bacterial isolates and DNA extraction: Eighteen isolates of *Bradyrhizobium japonicum* were obtained from Prof. Broomfield's collection, Agriculture andAgri-Food (AAFC), Quebec, Canada. Small-scale preparations of total bacterial DNA were obtained by growing each isolate in 5 ml of tryptone yeast (TY) broth medium at 28ºC for 7 days.

Bacterial cell pellets of each individual isolate were obtained by spinning 2 ml of bacterial culture in eppendorf tubes at 4ºC, 9,750 ×*g* for 5 min. The pellets were then resuspended in 250 µl of resuspension buffer (50 mM Tris-Cl, pH 8; 10 mM EDTA). Resuspended cells were treated with 250 µl of lysis buffer (200 mM NaOH; 1% (w/v) SDS) and then mixed by inverting tubes until the solution became viscous and slightly clear. The resulting solution was then treated with 350 µl of neutralization buffer (3 M potassium acetate, pH 5.5) and centrifuged at 9,750 ×*g* for 10 min. The supernatant from each isolate was collected in a fresh tube and the extracted DNA was precipitated by adding isopropanol, followed by washing with 70% (v/v) ethanol. The DNA of each individual isolate was dried under vacuum and re-dissolved in 80 µl of TE buffer (pH 8) (Cullen and Hirsch, 1992).

PCR procedure: PCR reaction was performed as described by Williams *et al*. (1990), but with some modifications. The DNA amplification reaction wascarried out in a 50 µl volume containing: 5 µl of 10X PCR buffer, 1 µl of each dNTP (10 mM), 1 µl of each of the forward and reverse primers (5 µM) (*sucA* 5′gagtggcaggagttcttca3′ and 5′gttcagcacccactccagata3′, *topA* 5′ttcaacgccatcaccaagc3′ and 5′ggcataggtcttctcgtgctt3′), 41 µl of deionized water, 0.5 units of *Taq* DNA polymerase per reaction and 0.5 µl of the DNA sample. The thermal program for the PCR reactions was carried out by denaturation at 95ºC for 3 min followed by 35 cycles of denaturation at 95ºC for 1 min, annealing at 54ºC for 1 min, extension 72ºC for 2 min and a final cycle of extension at 72ºC for 3 minutes. The PCR products were analyzed on a 1.4% (w/v) agarose gel and visualized under ultraviolet light.

Digestion conditions: PCR products (10 µl) were digested in a reaction mixture consisting of 1.5 µl of specific buffer as recommended for each of the *Sac*I and *Xho*I endonuclease enzymes, and 3.5 µl of deionized water and incubated at 37ºC for 3h. Digested PCR products were separated on a 2% (w/v) agarose gel running at 70 V, for 6 h.

Southern blotting: Bacterial genomic DNA was digested with *Sac*I and separated by gel electrophoresis. The denatured DNA (by using 0.5 N NaOH and 1.5 M NaCl) was transferred from the electrophoresis gel to a nitrocellulose membrane. The DNA was then permanently attached to the nitrocellulose membrane by exposing to UV for 60 sec.

Hybridization: The Nitrocellulose membrane was incubated with the labeled probes (*sucA* and *topA*). Hybridization patterns were studied only after the removal of excess probe and poorly bound probe molecules, by washing briefly in 3×SSC/0.5% SDS.

RESULTS

PCR amplification of *suc*A and *top*A genes showed that the PCR reactions were carried out successfully. The PCR generated fragments were of the appropriate sizes and the intensity and purity of bands were acceptable (Fig. 1). The amplified fragment size of the *suc*A and *top*A genes were 2712 and 1869 bp, respectively.

The digestion of the PCR products of the *sucA* and *topA* genes, using the *Sac*I enzyme, produced three (1267, 1104 and 341 bp, Lane 3) and five (212, 636, 266, 654 and 124 bp, Lane 4) fragments, respectively (Fig. 1). However, digestion of the same products with *Xho*I produced six (765, 691, 486, 338, 261 and 171 bp Lane 5) and four (918, 636, 213 and 104 bp Lane 6) fragments, respectively (Fig. 1).

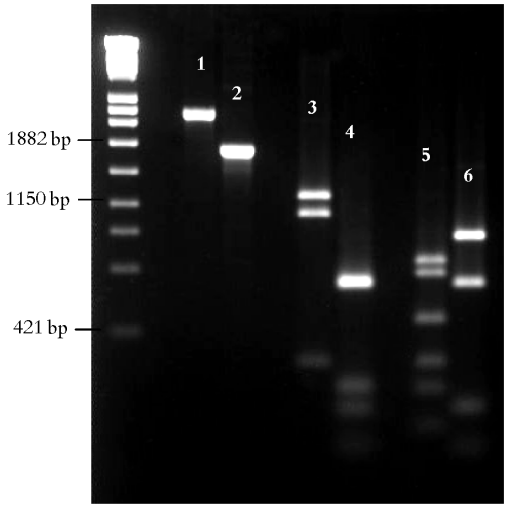

Figure 1. Amplification of *sucA* and *topA* genes using the PCR technique [Lanes 1 and 2, respectively]. *sucA* and *topA* genes digested by *Sac*I [Lanes 3 and 4 respectively], *Xho*I [Lanes 5 and 6 respectively]. Lambda-PUC mix was used as molecular weight size marker.

A total of 18 *Sac*I digested genomic DNA samples, belonging to the *B. japonicum* isolates are shown in Figure 2. The digested genomic DNA of the USDA110 strain as shown in lane 4 was selected as the reference strain, and the two *sucA* and *topA* probes were designed according to its DNA sequence . The results showed that the concentrations of genomic DNAs present in all lanes were similar, therefore making them suitable for the southern blot assay.

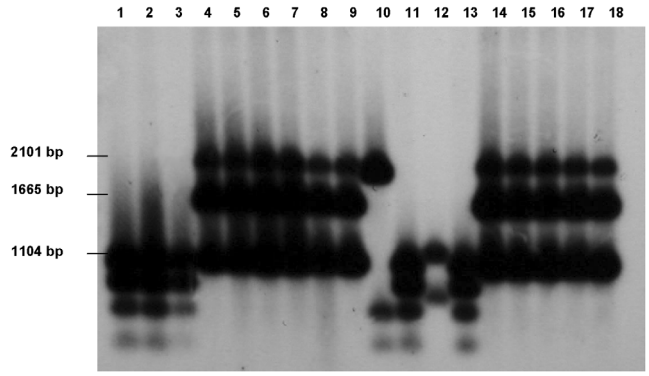

Figure 3. Hybridization of *sucA* probe with 18 *Sac*I digested DNA samples of *B. japanicum* isolates. Lane 4 shows the USDA110 strain.

The hybridization patterns of the 18 totally digested genomic DNA samples of the *B. japonicum* isolates hybridized with the *sucA* and *topA* probes are shown in Figures 3 and 4, respectively. The insensitivity of bands in the case of *sucA* was higher than that when the *topA* probe was used. This was due to production of large fragments after digestion of *sucA with* the *Sac*I enzyme.

DISCUSSION

Alpha-ketoglutarate dehydrogenase (*sucA)* and topoisomerase (*topA*) genes consist of 2955 bp (including

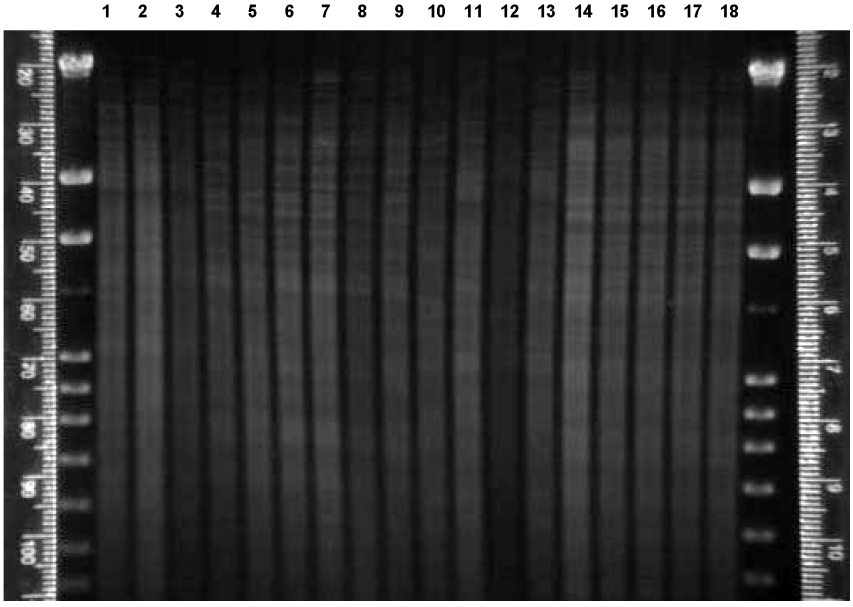

Figure 2. Total digested DNA of 18 strains of *B. japonicum* using the *Sac*I endonuclease enzyme. Lane 4 shows the digested genomic DNA of USDA110 strain. Lambda-PUC mix was used as DNA molecular weight size marker.

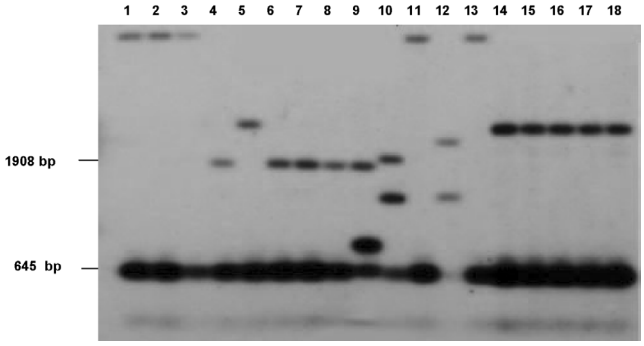

Figure 4. Hybridization of *topA* probe with 18 *Sac*I digested DNA samples of *Bradyrhizobium japanicum* isolates. Lane 4 shows the USDA110 strain.

561A, 1005C, 907G, 482T), and 2484 bp (including 474A, 865C, 785G, 360T), respectively. Molecular weights of their two DNA strands are1822 and 1531 KD respectively. Four primers were designed by the DNAMAN software in order to amplify both genes. The PCR products of the *suc*A and *top*A were 2712 and 1869 bp. All fragments obtained from digestion of the PCR product of the two genes (using *Xho*I and *Sac*I enzymes) were predicted by the DNAMAN software as shown in Figure 5. The *in silico* studies showed that when the *sucA* gene was digested by *Xho*I, 6 fragments were obtained. The sizes of these digested DNA fragments were 765, 691, 486, 338, 261 and 171 bp. When the *sucA* gene was digested by *SacI*, three fragments were produced and the sizes of fragments were 1267, 1104 and 341 bp (Fig. 5).

When the *topA* gene was digested by the *Xho*I enzyme, 4 fragments with sizes of 918, 636, 211 and 125 bp were obtained. When the *topA* gene was digested by *Sac*I, five fragments were with sizes of 212, 636, 266, 654 and 124 bp were generated (Fig. 6).

The data show that digestion of the PCR products of the *sucA* and *topA* genes using *Sac*I and *Xho*I produces three and five fragments (1267, 1104 and 341 bp, Lane 3, 212, 636, 266, 654 and 124 bp, Lane 4), and six and four fragments (765, 691, 486, 338, 261 and 171 bp, Lane 5, 918, 636, 213 and 104 bp, Lane 6), respectively. These results are in agreement with those obtained by the DNAMAN software. The lack of the complete clarity of the small fragments on the agarose gel in Figure 1 is due to the use of a low concentration of agarose gel during electrophoresis. Despite this, all the fragments can still be observed on the gel. Also, the digestion of *topA* gene with the *sac*I enzyme resulted in two fragments (approximately similar in size, 654, 636 bp) which can be seen on the gel as an intensive band.

With respect to the *Sac*I cutting sites on the *sucA* probe, genomic DNA of the USDA110 strain was hybridized to 1.1, 1.7 and 2.1 kb fragments, as predicted by the DNAMAN software (Figs. 3 and 5). Similarly for the *topA* probe, genomic DNA of USADA110 was hybridized to 1.9, 6.4, 6.5 and 0.2 kb fragments (Figs. 4 and 6). In Figure 6, the intense hybridized band belongs to the 6.5 and 6.4 bp fragments. The two other hybridized bands (1.9 and 0.2 bp) display lower intensities.

Based on hybridization patterns of the *sucA* probe, 18 strains of *B. japonicum* can be categorized into 4 different groups. The isolates 1, 2, 3, 11 and 13 belong to group I and the isolates 4, 5, 6, 7, 8, 9, 14, 15, 16, 17 and 18 belong to group II. The isolates 12 and 10 belong to groups III and IV respectively (Fig. 3).

According to the hybridization patterns of the *topA* probe, 18 isolates of *B. japonicum* are categorized into 6 different groups. Group I includes the 1, 2, 3, 11 and 13 isolates. Group II is divided into two subgroups II-I that include 6, 4, 7 and 8 and group II-II that include the 5, 14, 15, 16, 17, and 18 isolates. The isolates 12, 10 and 9 are separately placed in groups III, IV and V, respectively (Fig. 6).

In comparison to the hybridization pattern obtained

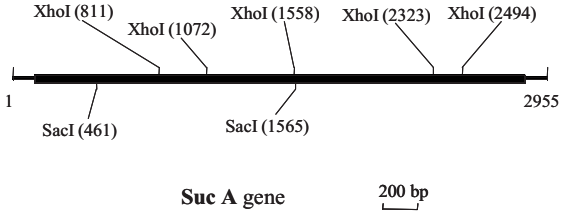

Figure 5. Cutting sites of the *sucA* gene produced with the *Xho*I and *Sac*I endonuclease enzymes. Broad line is a part of the gene which was amplified in PCR.

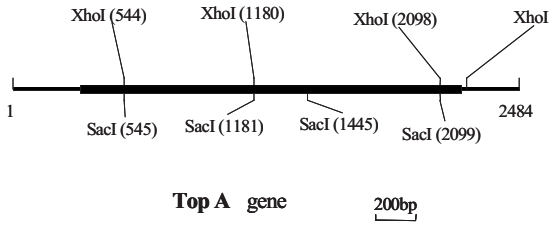

Figure 6. Cutting sites of the *topA* gene produced with the *Xho*I and *Sac*I endonuclease enzymes. Broad line is a part of the gene which was amplified in PCR.

by the *sucA* probe, the pattern of the eighteen isolates of *B. japonicum* was completely different when hybridized by the *topA* probe. It can also be revealed that the diversity of organic base sequence in the *topA* probe among the *Bradyrhizobium* bacterial strains is higher than that of the *sucA* probe. The results of this study also show that designing different probes can be employed to study diversity among different prokaryotic genera, such as the rhizobial bacteria.

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