

## CONSTRUCTION OF A GENOMIC BANK OF *Bradyrhizobium* spp. (RHIZOBIACEAE)

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### ABSTRACT

A wild type strain of *Bradyrhizobium* spp. that associates with *Neonotonia wightii* (a tropical forage legume) was analysed with molecular biology techniques. A genomic bank of the total DNA from this bacteria was constructed by using the high copy number pUC 119 plasmid, as the cloning vector, established in *Escherichia coli* HB101 by transformation. The total DNA of *Bradyrhizobium* was cloned as a collection of Sau 3AI fragments. The hybrid plasmids of the bank contain 10-20kb rhizobia DNA fragments inserted into the *lac z* region. Assuming that the molecular weight of the *Bradyrhizobium* genome is about 4,200kb and the average size of the inserts is 15kb, there is more than 95% probability that a *nod* sequence will be represented in each 10<sup>3</sup> transformants, according to the Clarke and Carbon (*Cel* 9: 91-99, 1976) mathematical expression. Although various chemical and physical methods were used, symbiotic plasmids were not detected in this strain of rhizobia.

### INTRODUCTION

The rhizobia-legume symbiosis has intensively studied (Hirsch, 1992) due to its agricultural importance and as a model system to study plant-microbe interaction mechanisms (Weinstein *et al.*, 1992). Expression of rhizobia is encoded by the nodulation (*nod*) and nitrogen fixation (*nif*) genes (Nap and Bisseling, 1990; Rhijn *et al.*, 1993). Some of these affect host specificity (*hcn*), whereas others (common *nod* genes) perform general functions for nodulation of any host (Long, 1989; Dénarié *et al.*, 1992). A wide

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variety of approaches have been used to identify and characterize plasmid-borne genes and gene fixing functions (Long, 1989), several of which have been mapped (Lamb and Hennecke, 1986; Hahn and Hennecke, 1987; Long, 1989; Hayes, 1990), mostly in *rhizobium* species. *Bradyrhizobium* includes broad-host-range species that associate with most of the tropical forage legumes, however very little is known about the genetic determinants of symbiosis despite its importance in the improvement of tropical pasture.

A number of different strategies can be used to clone a particular DNA fragment or gene. Cloning vector systems have had an important role in DNA amplification and analysis of fragments on Rhizobiaceae (Timmis, 1981; Prakash and Atherly, 1986). Using these techniques, *nif* and *nod* genes of rhizobia have been identified (Ditta *et al.*, 1980; Kahn and Timblin, 1984; Schofield *et al.*, 1987).

## MATERIAL AND METHODS

### *Bacterial strains*

*Escherichia coli* was HB 101 (pro<sup>-</sup> leu<sup>-</sup> thi<sup>-</sup> lacy Sm<sup>r</sup> endoI recA<sup>-</sup> r<sup>-</sup>m<sup>-</sup>) strain; *Bradyrhizobium* spp, a wild-type broad-host-range strain isolated from *Neonotonia wightii* cv. Tinaroo (soja perene) at the Instituto de Zootecnia, Nova Odessa, SP, Brasil.

### *Enzymes*

Restriction endonucleases *Bam*HI, *Sau*3AI and T4 DNA ligase were obtained from BOEHRINGER-MANNHEIN GmbH, BIOCHEMICA. T4 DNA ligase was used at a concentration of 1 *weiss* unit/ml for ligation. Lysozyme was obtained from SIGMA CHEMICAL COMPANY and used at a concentration of 5 mg/ml in 10 mM Tris-HCl (pH 7.6)/1 mM EDTA/10 mM NaCl. Bacterial alkaline phosphatase, was obtained from BOEHRINGER MANNHEIM GmbH, BIOCHEMICA and dialysed into 10 mM glycine, pH 9.8/0.1 mM ZnCl<sub>2</sub> for storage. DNA was treated with this enzyme at 50°C for 60 min in 0.1 M Tris HCl (pH 8.). The reaction was terminated by phenol/chloroform extraction. RNase A was obtained from BOEHRINGER MANNHEIM GmbH, BIOCHEMICA and was used at a concentration of 10 mg/ml in 0.1 M Sodium Acetate/0.3 mM EDTA (pH 4.8).

### *Bacterial transformation*

Transformation was performed by the calcium chloride procedure (Maniatis *et al.*, 1982) by mixing 10<sup>9</sup> cells of the recipient and the DNA from the donor in ligation buffer solution. The suspension was stored on ice for 2-3 hours and transferred to a water

bath (42°C) for 2 min. It was then plated on selective agar plates and incubated at 37°C overnight.

#### *Isolation of Bradyrhizobium DNA*

Total DNA from *Bradyrhizobium* spp was obtained from 100 ml of log-phase cell growth in Yeast-Tryptone broth (Beringer, 1974). Washed cells were resuspended in 4 mM Tris-Acetate/2 mM EDTA, pH 7.9 and lysed with SDS (3%)/50 mM Tris-HCl (pH 12.6) for 30 min at room temperature. The pH was adjusted to 7.0 by the addition of 3 M sodium acetate. Lysis was completed with 0.5 ml of lysozyme solution and 0.3 ml of the mixture of RNase solution and Pronase (500 µg/ml). DNA was isolated and purified by phenol/chloroform/isoamyl alcohol (25:25:1) extraction and by ethanol precipitation.

#### *Size fractionation of Bradyrhizobium DNA*

Total *Bradyrhizobium* spp DNA was partially digested with *Sau3AI* to give fragments in the 10-20 kb range. 50 µg of this DNA was electrophoresed and restriction fragments in this range were purified from the agarose gel using a dialysis bag preheated in 2 mM EDTA. Fractions were monitored for DNA size on 1% agarose gel. The DNA was electroeluted for 60 min at 100V in 4 mM Tris-Acetate (pH 7.9)/2 mM EDTA and, then extracted in phenol/chloroform and precipitated with ethanol. These DNA molecules were used for construction of the genomic bank.

#### *Construction of the Bradyrhizobium spp genomic bank*

pUC 119 DNA was digested with *Bam*HI and treated with bacterial alkaline phosphatase. *Sau3AI* size-fractionated *Bradyrhizobium* DNA, ligated to this vector, was used to transform *E. coli* HB101 cells. Transformants were selected on Ampicillin (50 µg/ml) agar plates. Phenotypic expression time after heat shock (42°C) was kept short (60 min) to avoid generation of siblings.

## RESULTS AND DISCUSSION

In contrast to *Rhizobium*, *Bradyrhizobium* species apparently do not contain symbiotic plasmids and the genes involved in the interaction with host plants are on the chromosome (Lamb and Hennecke, 1986; Ebeling *et al.*, 1987; Nieuwkoop *et al.*, 1987). The total cellular DNA of the strain of *Bradyrhizobium* was cloned into pUC 119, as a collection of *Sau 3AI* restriction enzyme fragments approximately 10-20 kb in size. This enzyme recognizes the 4-bp site, GATC, and generates fragments compatible with several

convenient vectors. The insert size was estimated using the migration pattern of lambda phage *EcoRI*-digested DNA. The resultant hybrid plasmids (Figure 1) were used to transform *E. coli* HB101. The efficiency of transformation was  $2 \times 10^7$  transformants/ $\mu\text{g}$  of DNA (with control pUC 119 DNA), thus yielding sufficient transformants of *E. coli* when 3  $\mu\text{l}$  of the DNA solution were used.

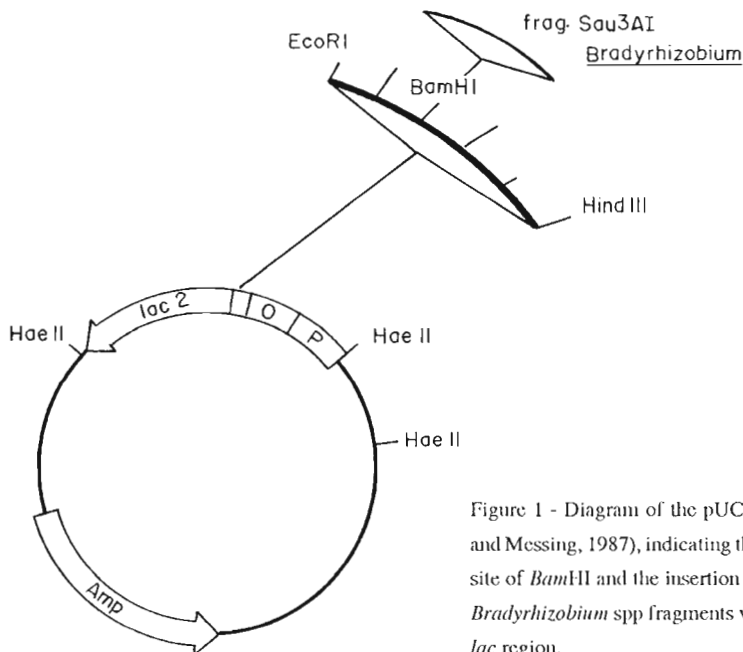


Figure 1 - Diagram of the pUC 119 plasmid (Vicira and Messing, 1987), indicating the restriction enzyme site of *Bam* HI and the insertion of the DNA *Sau* 3AI *Bradyrhizobium* spp fragments within the polyinker *lac* region.

Considerable difficulty has been reported in the genetic transformation of *Bradyrhizobium* (Bullerjahn and Bezinger, 1982). *E. coli* has been used as a rhizobia DNA recipient. Ditta *et al.* (1980) reported no instability of *R. meliloti* DNAs in HB 101 maintained under selective pressure, obtaining reproducible plasmid restriction patterns from cloned DNAs even after prolonged culture on solid medium. However, Weinstein *et al.* (1992) demonstrated that the broad-host-range stabilization loci from RK2 plasmid cause total retention of plasmids in cells of *R. meliloti*, during symbiosis with alfafa.

Assuming that the average size of the inserts was 15 kb, and the molecular weight of the *Bradyrhizobium* genome was similar to that of *E. coli*, approximately 4200 kb, it is possible to calculate based on the Poisson Distribution, that there is more than 95% probability that a *nod* sequence of DNA will be represented in each  $10^3$  recombinants (see Clarke and Carbon, 1976). In the enterobacterium *Klebsiella pneumoniae*, a cluster

of 15 genes located in 24 kb of DNA is required for nitrogen fixation (Reidel *et al.*, 1979; Hahn and Hennecke, 1987). By transcriptional analysis and by using Tn5 mutagenised strains of cowpea rhizobia, Jagadish and Szalay (1984) identified a 12 kb sequence containing several of the genes essential for symbiotic nitrogen fixation. They suggest that these genes are located on the chromosome. In Rhizobiaceae, an even greater number of genes may be necessary for effective symbiosis. Large inserts may reduce the absolute number of clones comprising the genomic bank, increasing the probability of intact gene functions but significantly reducing the efficiency of transformation.

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### RESUMO

Através de bancos genômicos vem sendo possível identificarem-se seqüências genéticas *nif e nod* em diversas espécies da Família Rhizobiaceae. Um isolado natural de *Bradyrhizobium* spp, simbiote da leguminosa forrageira tropical *Neonotonia wightii* Lackey (soja perene), foi submetido a métodos de biologia molecular, visando transferência e clonagem genéticas. Assim, utilizando-se do plasmídeo pUC 119, como veículo de clonagem, um banco genômico de DNA dessa bactéria foi construído e estabelecido por transformação em *Escherichia coli* HIB 101. O DNA celular total do *Bradyrhizobium* foi clonado no pUC como uma coleção de fragmentos de restrição *Sau* 3AI. Fragmentos de 10-20 kb foram ligados ao pUC 119 digerido com *Bam* III. Os plasmídios híbridos do banco contiveram, assim, insertos do DNA do rizóbio dentro da região *lac z*. Assumindo-se que o peso molecular do genoma do rizóbio seja aproximado ao de *E. coli*, 4200 kb, e que o peso médio dos insertos seja 15 kb, infere-se, com 95% de probabilidade, que a cada  $10^3$  recombinantes obtidos, um fragmento da seqüência genética *nod* esteja representado.

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