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16S rDNA-RFLP analysis of phylogenetic tree of *Rhizobium* bacteria

KEYWORDS: Rhizobium spp, % G+C, 16S rDNA, RFLP-PCR and Molecular Biotyping.

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ABSTRACT *Soil microorganisms play important role in soil formation, fertility and so also productivity. Nitrogen is an important element to support plant growth. Plants depend on soil microorganisms for fixed nitrogen. Among the nitrogen fixing microorganisms, the role of Rhizobium is quite significant. The aim of this study was to study diversity of Rhizobium spp. in agricultural lands of Madhya Pradesh. Physicochemical properties of soil was studied using standard methods while molecular methods used to study diversity within Rhizobium species. Further, population analysis of Rhizobium species in relation to genetic diversity was carried out using 16S rDNA-RFLP PCR. Rhizobium were identified and genetically by determining the %Guanine plus Cytosine content of the whole genome, followed by Restriction enzyme (Mbo I, Hap II, Taq I) treatment of Polymerase Chain Reaction (PCR) amplified product of 16S rDNA segment was performed. The sequences recognized by the restriction enzymes are distributed at variable intervals in the genome of an organism and also vary in number. The separation carried out by electrophoresis (1.6-2% agarose gel) resulted in specific banding pattern differing within as well as among different species. The technique used was helpful in characterizing Rhizobium isolates to be used as inoculants for improving agricultural land quality of Madhya Pradesh (India).*

Introduction

Members of the genus Rhizobium are symbiotic nitrogen-fixing bacteria which are able to invade and form nodules on the roots of leguminous plants. The most dramatic progress in the construction of microbial phylogeny is based on sequencing analysis of the ribosomal genes. The 16S or small subunit ribosomal RNA gene is useful for estimating evolutionary relationships among bacteria because it is slowly evolving and the gene product is both universally essential and functionally conserved (van Berkum and Eardly, 1998). Direct sequencing of genes coding for 16S rRNA (16S rDNA) has been used to establish genetic relationships and to characterize strains at the species or higher level (Laguerre et al., 1996). The full-length sequence analysis of 16S rDNA is one of the most important methods to estimate the phylogeny of rhizobia (Young and Haukka, 1996), while the 900 bp partial 16S rDNA sequencing correlated well with full-length 16S rDNA sequencing (Terefework et al., 1998) and has been used for rapid screening of the phylogenetic relationships among a large number of rhizobia. Sequences of 16S rDNA are known to be highly conserved among eubacteria (Woose, 1987) and analysis of genetic variations in this region is not appropriate to differentiate strains within species (Laguerre et al., 1996). However, it is very useful for identification of species. Pairs of universal primers, forward and reverse primers, were design for amplification of 16S rDNA regions in most eubacteria. Pairs of universal primers were used to amplify 16S rDNA (Lane, 1991; van Berkum and Fuhrmann, 2000) to ascertain the non-symbiotic isolates belonging to the genus Bradyrhizobium (Pongsilp et al., 2002). Novel nitrogen-fixing symbionts in genera Methylobacterium, Blastobacter, Burkholderia, Ralstonia, Ochrobactrum, Devosia, Phyllobacterium and Herbaspirillum have been discovered by 16S rDNA sequence analysis (Chen et al., 2001; Sy et al., 2001; Rivas et al., 2002; van Berkum and Eardly, 2002; Valverde et al., 2003; Ngom et al., 2004; Chen et al., 2005; Valverde et al., 2005; Barrett and Parker, 2006; Chen et al., 2006). These findings suggest that the gene responsible for symbiosis with legumes is transmissible horizontally and functions in a relatively wide range of bacterial taxa (Fuentes et al., 2002; Rivas et al., 2002). Phylogenetic analysis of the 16S rDNA has been constructed in many previous studies. According to Ngom et al. (2004), the clusters in the phylogenetic tree, which was constructed based on nearly the full length of 16S rDNA, correlated well with the taxonomy of strains: i) a first cluster contains Bradyrhizobium and Blastobacter in Bradyrhizobiaceae; ii) a second cluster contains Ochrobactrum in Brucellaceae; iii) a third cluster consists of two genera Phyllobacterium and Mesorhizobium in Phyllobacteriaceae; iv) a fourth cluster consists of genera Sinorhizobium, Allorhizobium and Rhizobium in Rhizobiaceae. Besides 16S rDNA, sequence analysis of 23S or large subunit ribosomal RNA gene has been also studied. However, the 23S rRNA gene has not been extensively used to estimate the genetic relationships among the Rhizobiaceae, but there are several dramatic differences which may be helpful for classification and identification purposes (van Berkum and Eardly, 1998). Terefework et al. (1998) reported that the 23S dendrogram showed deeper branching than the 16S dendrogram and more genotypes were resolved, although in some cases the sequence divergence is not particularly high.

The bacterial species from soils of Madhya Pradesh at ten different locations were examined following known biochemical tests and DNA purified from 20 strains of Rhizobium was studied. Furthermore, the pattern of similarities as represented by genetic hierarchy was studied using molecular biotyping methods.

METHODS AND MATERIALS

Soil samples (0-30cm depth) were collected from 10 agricultural sites of Neemuch, Hoshangabad, Betul- Multai, Sehore, Bhopal, Tikamgarh, Chindwara, Raisen Vidhisha- Sanchi and Ujjain districts of Madhya Pradesh. These districts fall in central part of the province. Surface litter was scrapped away and soil samples stored in pre-sterilized high-density polythene (HDPE) bags (Forster, J., 1995). Samples were passed through 2 mm sieve to have homogenous particles for further analysis. The Rhizobium species confirmed from soil samples were named as R1- R20.

The dilutions (10^{-1} to 10^{-8}) were inoculated on YEMA (Yeast Extract Mannitol Agar) plates and incubated at 28 2 2 C for 24 to 72 h. Fast growing Rhizobium species appeared

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within 24 hours and the slow growing needed cells further incubation of 72-96 h. The glistening white Rhizobium, like colonies were picked up and purified by continuous streaking on YEMA and CRYEMA plates (Subba Rao, NS., 1984). The composition of media was mannitol-10g, K_2HPO_4-0.5g, $MgSO_47H_2O$-0.2g, NaCl-0.1g,yeast extracts-0.4g, agar-15.0g, distilled water-1 L and Congo red solution (10.0ml). pH of the medium was adjusted to 6.8 and sterilized at 15 psi (15 min).Yeast extract mannitol agar had the following ingredient: mannitol-10 g, K_2HPO_4-0.5g, $MgSO_47H_2O$-0.2g, NaCl-0.1g,yeast extracts-0.4g, agar-15.0g, distilled water-1 L, pH of the medium was adjusted to 6.8 and sterilized at 15 psi (15 min).

DNA isolation

Isolation of DNA was done by Marmur's method (1961), with slight modifications. The Rhizobium inoculums (2.0 O.D) was added to 50ml YEM broth and put at 28 2C (24 h) in a shaking incubator, the cell pellet obtained by centrifugation (10,000 rpm). Cells were suspended in 25ml saline EDTA solution in Erlenmeyer flasks. Lysis of cells as done by addition of 1ml of lysozyme (37C, 30 min) followed by 25% of 2ml SDS at 60C (10min). The suspension was allowed to cool at room temperature, and 5ml of 3M sodium acetate was gently mixed in 50ml of 24:1 Chloroform-isoamyl alcohol followed by centrifugation (10,000 rpm, 30min). Out of the three layers obtained following centrifugation, the uppermost one bearing nucleic acid was pipetted out in 100 ml beaker and two volumes of chilled ethanol added (Helms, C., 1985; Tracy, S., 1981). The white fibrous precipitate at the interface was gently pooled out with the help of glass rod.

Determination of Tm value

The Tm of each DNA sample was determined as described by Mandel and Marmur (Mandel, et al., 1968; Marmur, J., 1961). The % G+C content of the samples was determined by using the equation %G+C = 2.44 (Tm – 69.4) as suggested by De Ley, J. (1970).

PCR-RFLP of amplicon

Polymerase chain reaction (PCR) is most useful widely used genetic tool in study of molecular biology of organisms. It is widely applied on cloning, sequencing and phylogenetic study. The efficiency of PCR technique is based on "master mix" preparation consisting buffer, dNTP's mix (2mM), Primer 1, Primer 2, Taq polymerase, sterile water except the DNA template. The reproducibility and reliability of results depended upon proper pipetting of all the components of "master mix" and their further distribution. After addition of template DNA, it was exposed to temperature cycles in a thermal cycler (PTC-1148, MJ Mini Thermal cycler, BIORAD, USA). The conserved sequence in DNA i.e., the 16S rDNA was amplified using the reverse primer (5' ACGGCTACCTTCTTAGCACTT3') and the forward primer (5' AGAGTTTGATCCTGGCTCAG-3') at 55C (annealing) for 30 cycles in PCR unit (PTC-1148, MJ Mini Thermal cycler, BIO-RAD, USA). Amplified DNA was subjected to RFLP analysis using restriction enzymes (Taq I, Hpa II, Mbo I,) after amplification. The amplified 16S rDNA was then digested separately with 3 different restriction enzymes, by incubating overnight at 37°C. Enzyme activity was stopped by low temperature (4°C) and by adding 2µl of 6x loading buffer. Further, the enzyme digested PCR product along with 1kb DNA ladder (Bangalore Genie, India) in a separate well was estimated by electrophoresis (Walker, et al., 1998) at 55mV on (2% agarose, 2h).The Bio-Rad Gel Doc™ XR and ChemiDoc™ XRS gel documentation system are easy-to-use, high-performance systems. They use a CCD camera to capture image in real time, which allows you to more accurately position and focus the image (Molecular Imager Gel Doc XR System 170-8170, 170-8171, BIO-RAD, USA).

Data analysis:

All restriction patterns were coded in binary form and analysed using NTSYS software (Rohlf, 1990). A simple matching coefficient was calculated to construct a similarity matrix and the UPGMA algorithm was used to perform hierarchical cluster analysis and to construct a dendrogram.

Result and Discussion

Present study, however, concentrated on the nitrogen fixing strains. Hofer's alkaline broth test conducted is because Agrobacterium grows at higher pH levels and not rhizobia. The isolates strains failed to utilize peptone when were grown on glucose peptone agar medium. Rhizobium respond negatively ketolactose test. Microscopic observations on pure culture cells confirmed the gram-negative nature. In addition, gelatin was not liquefied by cells grow on gelatin medium. Bacterial cells once inoculated on pre-sterilized yeast extract Mannitol Agar (YEMA) produced white, translucent glistering colonies with entire margin soil samples from Neemuch, Hoshangabad, Betul-Multai, Ujjain, Sehore, Bhopal, Tikamgarh, Chindwara, Sanchi-Vidisha, Raisen etc were subjected to the above mentioned biochemical parameters.

The composition of DNA in bacterial genome is similar as it shows presence of all the four defined bases. The helix of DNA with double stranded structure shows pairing between A+T and G+C, thus (A+T)/ (G+C) ratio or (G+C) content reflects compatibility of microbial strain in relation to evolutionary stress. The G+C content is examined in the form of temperature of melting (Tm). The bases are joined by hydrogen bonds and show regular pairing. It is obvious that the DNA with higher G+C content will stand higher melting temperature as more energy is needed to separate the double stranded DNA. The melting temperature thus is calculated by observing midpoint of the rising curve. The optical density of DNA shows further use in the presence of greater amount of G+C content. In all the organisms ranging between eukaryotes to prokaryotes, the highest degree of variation is observed in case of microbes (between 25-85%). The composition of G+C content with slight variation shows similar base sequences thus giving emphasis on relatedness among species in contrast to dissimilarity as observed in PCR-based observations. Samples analyzed presently, amongst 20 strains i.e., R1 to R20, the Tm ranged between 94.3 to 95.6°C. However, % G+C content of isolated strains ranges between 60.7 to 63.9%. All the bacteria tested and examined for Tm values and G+C content, were similar with narrow range of difference with respect to % G+C. Thus present observation as listed in provides most similarity amongst microbial isolates in relation to DNA as a parameter.

Furthermore, the pattern of genetic diversity was studied using known and established molecular biotyping methods. Genotyping of the isolates was done by using molecular methods. Taq I restriction enzyme when used to have 16S rRNA digestion the group of strains showed variation in pattern on DNA profiling studies with 2.0 % agarose (Fig. 1& 2). In the presence of Taq I the digestion of 16 S rDNA showed bands between 200 bp and 500bp. DNA profile once put on computation data on NTSYS using unweight pair group method with arithmetic averaging (UPGMA) over Taq I digested rDNA, Rhizobium isolates. The dendrogram shows divergence at 100% similarity into two broader groups and S16, S15, S12 and S2 strain shows 65% dissimilarity with all the strains isolated from agricultural soil of Madhya Pradesh.

Figure 1: Restriction enzyme Taq I treated 16s rDNA segments of isolated strains of *Rhizobium spp.* on 2% agarose gel.

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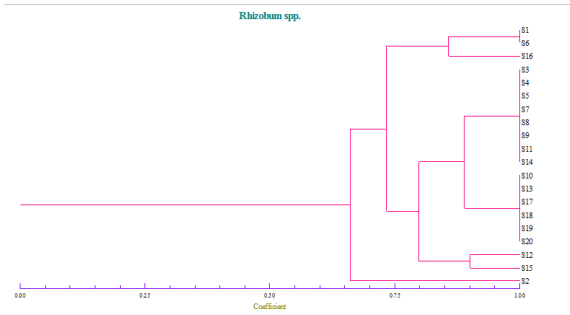

Fig.: 2. Dendrogram based on analysis of 16SrDNA segment with restriction enzymes Taq I of *Rhizobium* using Jaccord's Coefficient and UPGMA cluster analysis method.

Conclusions

On the basis of different physiological and biochemical parameter studied, it seems all the strain of Rhizobium sp. are same. Findings on the isolated Rhizobium strains from agricultural soils of M.P. with regard to the level of gene sequences will help establish the improved strains as biofertilizers. The utility of present observation falls with relative similarity between patterns of genome i.e., alignment of bases of DNA, although preference was given to the pattern of base-sequences in NTSYS based genomic analysis. Thus, the present observations give an insight on molecular orientation of Rhizobium species occurring naturally in the agricultural soils of Madhya Pradesh.

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