ISOLATION OF PSEUDOMONAS SP. FROM WRIGHTIA TINCTORIA LEAF AND ITS 16S rRNA STUDY

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ABSTRACT

The 16S rRNA gene sequencing study covers the whole prokaryotic community, acting as an important tool in identification incorporating computational advances, most prominent in bacterial identification. The objective of this study was to isolate and to identify Endophytic bacteria associated with Wrightia tinctoria. In the current study, an endophytic bacteria was isolated from Wrightia tinctoria leaf and was found to be Pseudomonas sp. Isolate was identified using the polymerase chain reaction-amplified 16S ribosomal RNA (rRNA) sequence similarity based method and was sequenced by Sangers method. The obtained sequence was compared through online databases to identify the resembling library sequences.

KEYWORDS: Wrightia tinctoria, Pseudomonas, 16s rRNA, BLAST, EZ Taxon

Bacterial Endophytes have been defined as bacteria that live inside plant tissues without harming the plant or gaining benefit other than securing residency (Kado; 1992). Bacteria that can be isolated from plant tissue surface or extracted from within the plant and do not visibly cause any harm to the plant (Hallmann et al; 1997). The bacteria have been found in most plants, inhabit the internal tissues and create varied relationships with their host plants (Ding et al; 2013). They encourage plant development, yield and can act as biocontrol agents. They are also beneficial to their host by producing a variety of natural products that could be exploited for potential use in medicine, agriculture or industry. They have the potential to remove soil contaminants by promoting phytoremediation and could play a role in soil fertility through phosphate solubilization and nitrogen fixation (Ryan et al; 2008).

Prokaryotic organisms are known to possess the small and short 16S subunit rRNA, which is said to contain 1,542 nucleotide bases, it is cost effective and easily sequencable. Plays an important role to characterize bacteria in a diversified ecological niches along with host associated communities, especially endogenous human microbiome (Arimugam et al; 2011; Costello et al; 2009; Turnbaugh et al; 2009), and host-free communities, such as soil and ocean environments (Gilbert et al; 2009; Hackl et al; 2004). Several aspects of the 16S rRNA gene make it optimal as a marker for these types of studies. It is omnipresent, size and high degree of functional conservation result in precise mutation rates throughout prokaryotic evolution (Woese; 1987). The 16S rRNA gene includes both conserved regions, which can be put in use to design amplification primers beyond taxa, along with nine hypervariable regions (V1-V9), which can be effectively used to distinguish between taxa (Clarridge, 2004). Helpful in Studying bacterial phylogeny and taxonomy playing a role of housekeeping genetic marker, due to its presence in the entire bacterial group, its existance as a multigene family, or operons, the function of the 16S rRNA gene over time has not changed, suggesting that random sequence changes are a more accurate measure of time (evolution) and the 16S rRNA gene (1,500 bp) is large enough forinformatics purposes (Patel; 2001). Studies shows that 16S rRNA gene sequencing provides genus identification in most cases (90%) but less so with regard to species (65 to 83%), with from 1 to 14% of the isolates remaining unidentified after testing (Drancourt et al; 2000, Mignard & Flandrois; 2006, Woo et al; 2003). Hence the aim of present study was to isolate the bacteria from Wrightia tinctoria leaves and identify the bacteria using 16S rRNA.

MATERIALS AND METHODS

Collection of Plant Materials

The leaf of Wrightia tinctoria (Roxb.) R.Br. was collected in the month of February from Ramakrishna Mission Vivekananda College, Mylapore, Chennai – 4 and was taxonomically identified. Leaf sample was immediately processed (Fisher & Petrim; 1987).

Isolation of Bacteria

The surface sterilized leaf segments of Wrightia tinctoria were placed with equal spaces in Petri dishes containing Nutrient Agar medium. The pure bacterial culture was obtained and utilized for subsequent studies.

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Isolation of Genomic DNA

1.5ml of bacterial culture was transferred to a micro centrifuge tube and spun at 10000 rpm for 2 minutes at 4° C. The supernatant was decanted and the pellet was re-suspended in 467µl of TE buffer by repeated pipetting. 30µl of 10% SDS and 3µl of 20mg/ml of Proteinase K was added to the sample and incubated for 1 hr at 37° C. Equal volumes of Phenol: Chloroform (24:1) was added and mixed by inverting the tubes until the phase was completely mixed. The tubes were spun at 12,000 rpm for 10 minutes at 4° C. The upper aqueous layer was transferred to a new tube and an equal volume of Chloroform was added. The samples were mixed by gently inverting the tubes and spun at 12,000 rpm for 10 minutes at 4° C. The upper aqueous phase was transferred to a new tube and 1/10th volume of 3M sodium acetate was added. Double the volume of 95% ice cold ethanol was added and mixed by inversion until the DNA was precipitated. The tube was spun for 10 min at 12,000 rpm at 4° C and the supernatant was discarded. The pellet was washed with 0.2 ml of 70% ethanol and tube was spun as before. 70% ethanol was discarded and the pellet was air dried. The DNA was then suspended in TE buffer and run on 0.8% Agarose gel (Sambrook & Rusell; 2001).

Agarose Gel Electrophoresis

Agarose was weighed and transferred to a conical flask. 50 ml of 1X TAE was added and Agarose was melted to a clear solution by heating. It was allowed to cool until it reached bearable temperature. 2.5µl of ethidium bromide stock solution was added. A gel casting tray was placed in a leveling table and the melted agarose was poured. After the gel solidified, the comb was taken out carefully. The casted gel was placed in an electrophoresis tank and 1X TAE buffer was added until the gel was completely submerged. DNA sample was mixed with the gel loading buffer and loaded into the well. The samples were then electrophoresed at 50V until the gel loading buffer reached 2/3rd of the gel. This gel was then viewed under UV Trans-illuminator.

Qualitative and Quantitative Determination of DNA by Spectrophotomeric Method

The UV spectrophotometer wavelength was set at 260nm and 280nm. The instrument was set at zero absorbance with T.E buffer or sterile water as blank. 5 or 7ul of the sample was taken in a quartz cuvette and made up to 3ml with TE buffer or sterile water. Absorbance of the solution with the sample was read. The concentration of DNA in the sample was calculated using the given formula: $A_{260} \times 50 \mu g \times$ dilution factor.

Polymerase Chain Reaction (PCR)

100mg of DNA was used for molecular identification of respective sample. The PCR reaction was performed for 20µl. PCR reaction was performed for 16S rRNA gene. The PCR tubes were placed in thermo-cycler and the reaction was carried out.

RESULTS

The Bacteria thriving inside the *Wrightia tinctoria* leaf was isolated using Nutrient agar. Pure colonies were obtained. Isolation of DNA was carried out (Fig-1). The qualitative and quantitative determination of DNA revealed that DNA was pure with the value of 1.8. The DNA was later amplified for 16S rRNA region using Polymerase chain reaction and ladder comprising of 1500 base pairs were obtained (Fig-2). The amplified 16S rRNA gene fragments were about 377 bp in length. The 16S rRNA genes were sequenced. The sequence thus obtained was compared with BLAST (NCBI) and EZ taxon database to acquire similar sequences pertaining to genus present in the database. The partial 16S rRNA gene sequences were deposited in the GenBank with accession number KJ923924.
DISCUSSION

The application of 16S rRNA gene sequencing in studying closely related organisms has led to increase in 16S rRNA databases. Computational approach has also enhanced the application of 16S rRNA gene sequence analysis to bacterial identification (Patel; 2001; Amann et al; 1995: Rantakokko-Jalava et al; 2000). The endophytic bacterial isolates were identified using 16S rRNA based method of bacterial identification. We isolated an endophytic bacteria from the leaf of Wrightia tinctoria and performed the 16s rRNA study. In recent years, the study of endophytic bacteria often has utilized culture-independent methods, mostly based on the PCR amplification of bacterial 16S rDNA. Some important studies of root endophytic bacteria were focused on single crop species, including maize and rice, because of their importance to food supply and safety (Sturz et al; 1997; Chelius & Triplet; 2001; Sun et al; 2007). Evaluation of endophytic bacteria by Terminal Restriction Fragment Length Polymorphism (T-RFLP) based on 16S rDNA PCR had been carried out by several researchers (Liu et al; 1997). In our study, the isolated bacteria when subjected to 16S rRNA study showed 98% similarity with that of Pseudomonas sp. present in the database. Further studies will be carried out to obtain bioactive compounds from the bacteria for drug discovery.

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