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Molecular Characterization of *Azospirillum halopraeferens*

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Abstract: ***In the present investigation, rhizosphere soil samples were collected and screened Azospirillum isolates. Totally 09 isolates were identified. Molecular characterization of the A. halopraeferens TPS05 was evaluated by PCR amplification of 16S rRNA gene. The phylogenetic analysis of A. halopraferens TPS05 strain was observed 100% homology with the strain existing A. melinis (DQ022958) and A.zeae (HE977584). Phylogenetic relatedness of the A. halopraferens TPS05 analysed by neighbour joining method. The 16S rRNA secondary structure and the restriction sites of A. halopraeferens TPS05 were predicted using Genbee online software.***

Keywords: *A. halopraeferens*, 16S rRNA, Secondary structure, phylogenetic tree.

1. Introduction

Application of PGPR as biofertilizer has resulted improved growth and grain yield of various crops such as wheat, rice, maize and sugarcane (Moutia *et al*., 2010). Now-a-days a variety of molecular based methods are used for characterization and identification of bacteria. These include amplified ribosomal DNA restriction analysis (ARDRA) (Zhang *et al*., 2011), random amplified polymorphic DNA (RAPD), repetitive extragenic palindromic elements (REP) and 16-23S intergenic spacer (Li *et al*., 2011).

The genus-specific PCR primer pair Azo494-F/Azo756-R was designed and used for rapid identification of the genus *Azospirillum* (Lin *et al*., 2011). *A. brasilense* is reported as a biofilm producer (Guerrero-Molina *et al*., 2012), and this feature helps *Azospirillum* cells anchor and colonize the root surface. Capsular polysaccharides (CPS) of the *A. brasilense* Sp7, a glycosylated lectin with a molecular mass of 36 kDa was identified with specificity to L-fucose and D-galactose (Sigida *et al*., 2013). Root exudates secreted by plants, the microorganism plant association may improve the plant growth and health by synthesis of vitamins, antibiotics, enzymes and phytohormones (Cohen *et al*., 2015). The objective of the current study, identification of the paddy field soil isolate of *A. halopraeferens* TPS05 by sequencing their 16S rRNA gene. This study to clarify the taxonomic position of this strain based on phylogenetic analysis of its 16S rRNA gene sequence together with these of other representatives of the genus *Azospirillum.*

2. Materials and Methods

a) Isolation of *Azospirillum*

The soil samples were collected from nine villages of Thanjavur district, Tamilnadu, South India for isolation of *Azospirillum.* From the collected soil samples, 1 g was taken and serially diluted using sterile distilled water from 10^{-1} to10^{-8} dilutions. One ml of diluted sample from 10^{-6} to 10^{-8} dilutions was taken and 0.1ml of aliquot was inoculated in test tube containing Nfb (Nitrogen free bromothymol blue) semisolid media. All the tubes were incubated at 32ºC for 48 h and observed the growth by the formation of pellicles. The pellicles were streaked on Nfb solid media and incubated at 32ºC for 24 h. Morphologically divergent *Azospirillum* colonies (white, yellow and pink) were picked from the plates and streaked on basal minimal salt agar medium and incubated at 32ºC for 24 h. After attained sufficient growth, all the isolates were preserved and used for further investigation.

b) Molecular characterization of *Azospirillum*

DNA Isolation

Total genomic DNA was extracted by standard methods of Boudjella *et al*. (2006). All the isolates were grown at 30°C for 5 days in shake flasks containing 100 ml of Nfb liquid medium. Pellet was obtained by centrifugation and washed twice with distilled water. Approximately 200 mg of pellet were used for genomic DNA extraction. The pellet was suspended in 500 µl of the lysis solution [100 mM Tris-HCl (pH 8.0), 20 mM EDTA (pH 8.0), 250 mM NaCl and 2% SDS]. Lysozyme was added to obtain a final concentration of 1 mg/ml, and the solution was incubated at 37°C for 60 min. After the addition of 10 µl of proteinase K (10 mg/ml), the mixture was incubated at 65°C for 30 min. The solution was chilled on ice and extracted with an equal volume of phenol-chloroform-isoamylalcohol (25:24:1). The organic extraction was repeated, and the supernatant was taken and along with 4M Ammonium acetate and two volumes of isopropanol. Total genomic DNA was precipitated by centrifugation for 10 min at room temperature. The pellet was washed with 70% ethanol, dissolved in Tris EDTA buffer (pH 8.0) and stored at -20°C.

Oligonucleotide primers and PCR conditions

The sequences of the oligonucleotide primers used for PCR are listed.

Primer Name	Sequence Details	Number of Base
8F	AGAGTTTGATCCTGGCTCAG	20
1541R	AAGGAGGTGATCCAGCCGCA	20

The design and synthesis of the 16S rRNA oligonucleotide primers have already been described by Heuer *et al*. (1997)

was used in the present study. The random 10-mer oligonucleotide primers (OP-07) used for RAPD-PCR fingerprinting. All the primers were obtained from XDT Technologies (Germany).

All the PCR reactions were carried out in a 50 µl volume containing 1 µl (50 pmol) of each primer, 1 µl of 1.25 mM of dNTPs, 1 µl (50 mg) of template DNA and 1U of DyNAzyme DNA polymerase (Finnzymes, Finland). The buffer supplied with the enzyme was used according to the manufacturer's directions. The amplifications were performed with DNA thermal cycler (Eppendorf Mastercycler Gradient, Germany). The cycling conditions for 16S rRNA and RAPD-PCR differ in annealing temperature. The PCR cycles as follows: one cycle at 95°C for 7 min, 30 cycles at 94°C for 1 min, 62°C for 1 min (16S rRNA) and 42°C for 1 min (RAPD), followed by a single final extension step at 72°C for 7 min. After the reaction completed, 10 µl of amplified DNA was separated on 1.2% low melting agarose (Sigma, USA). Then the agarose gel was stained with ethidium bromide and recorded using a CCD camera in UVP gel documentation system (UVP, England). Standard molecular marker and supplied with DyNAzyme^{TM} II DNA polymerase kit (Finnzymes, Finland) included into all the gels.

DNA fingerprint analysis

The DNA fingerprints of isolates obtained by PCR fingerprinting methods detected by visual examination of the banding pattern. The presence or absence of a band at any position on the gel was used to construct a two-dimensional binary matrix for *Azospirillum* isolates tested. Genetic distances between isolates were calculated using the algorithm of Nei and Li (1979) as provided in the RAP Distance Software package (Armstrong *et al.,* 1994). A pair wise comparison of genetic distances for all *Azospirillum* patterns was used to create a phenogram based on the neighbour-joining method.

Sequencing and phylogenetic analysis of 16S rRNA gene

The amplified PCR products were purified using a QIAquick PCR purification kit (Qiagcn, GmBh, Germany) as recommended by the manufacturer. The sequences of the PCR products were determined by using the BigDye Terminator Cycle Sequencing v2.0 kit on an ABI 310 automatic DNA sequencer (Applied Biosystems, CA, USA) according to the manufacturer's instructions. The 16S rRNA gene sequences determined for the selected *Azospirillum* strains were deposited in the GenBank database and got their accession. The 16S rDNA sequences reported in this study was multiple-aligned using CLUSTAL W, version 1.7 (Thompson *et al.,* 1994) with a selection of *Azospirillum* reference sequences obtained from GenBank. The alignment was corrected manually and converted to a distance matrix. The distance matrix was converted to a phylogenetic tree using the neighbor-joining (NJ) algorithm (Saitou and Nei, 1987) using MEGA 3.1 (Kumar *et al.,* 2004) with multiple substitutions corrected and positions with gaps excluded. The statistical significance of the tree branches was assessed by bootstrap analysis involving the construction of 1,000 trees from the resampled data (Felsenstein, 1985).

In silico restriction endonuclease digestions

Restriction endonucleases, the commercially available online software used for this study for in silico digestion. For each 16S rRNA gene sequence, in silico digestions were performed using NEB cutter v2.0. The in silico analysis allowed for the selection of specific restriction endonucleases for distinguish of *Azospirillum* genera.

Secondary structure determination

The 16S rRNA gene sequences of *Azospirillum* isolates were used for the construction of secondary structure models and were folded using Genbee-NEB Cutter (Brodsky *et al.,* 1995). These secondary structures were used to assess the significance of observed differences in 16S rRNA gene sequence data.

Bioinformatics protocol:

1) The 16S r RNA sequence was blast using NCBI blast similarity search tool. The phylogeny analysis of query sequence with the closely related sequence of blast results was performed followed by multiple sequence alignment.
2) The program MUSCLE 3.7 was used for multiple alignments of sequences (Edgar 2004). The resulting aligned sequences were cured using the program Gblocks 0.91b.This Gblocks eliminates poorly aligned positions and divergent regions (removes alignment noise) (Talavera and Castresana 2007). Finally, the program PhyML 3.0 aLRT was used for phylogeny analysis and HKY85 as Substitution model.
3) PhyML was shown to be at least as accurate as other existing phylogeny programs using simulated data, while being one order of magnitude faster. PhyML was shown to be at least as accurate as other existing phylogeny programs using simulated data, while being one order of magnitude faster. The program Tree Dyn 198.3 was used for tree rendering (Dereeper *et al.,* 2008).

3. Results and Discussion

Molecular characterization

In the present study, among the 9 isolates, *A. halopraferens* TPS05 was selected for the 16S rRNA sequencing and phylogenetic relatedness studies.

The genetic DNA was isolated from the selected *A. halopraferens* isolates and they were partially sequenced using specific 16S rRNA sequencing primer. The sequence was deposited in Genebank (NCBI) and got the accession number (Bankit 2018121). The phylogenetic analysis of *A. halopraferens* TPS05 strain was observed 100% homology with the strain existing *A. melinis* (DQ022958) and *A. zeae* (HE977584). Phylogenetic relatedness of the *A. halopraferens* TPS05 analysed by neighbour joining method (Fig- 1).

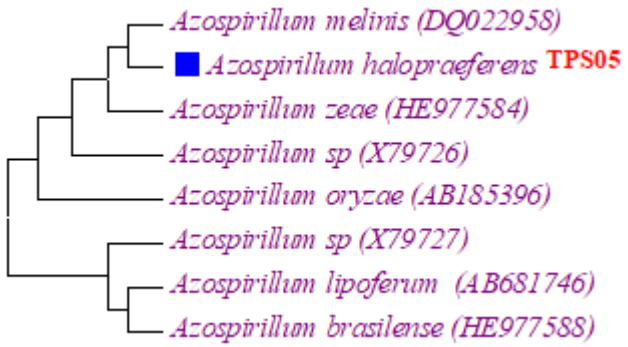

Figure 1: Phylogenetic analysis of A. *halopraferens* TPS05

Nosin Ilyas *et al*., (2012) reported that the molecular weight of amplified products was determined by electophoresis on 1.2% (w/v) agarose gel. A band of 1.5 kb corresponding to 16S rRNA gene for each isolate was obtained. On the basis of colony morphology, gram staining, and carbon/nitrogen utilization pattern (QTS-24), the isolated strains were identified as members of genus *Azospirillum* (Bergey's Manual of Bacteriology). On the basis of unweighted pair group method with arithmetic means (UPGMA) cluster analysis of *Azospirillum* strains isolated from rhizosphere soil and roots of maize plants using OP -01 and OP-06 primer, isolates were grouped into two clusters. UPGMA cluster analysis with OP-01 primer revealed that group I included isolates from well watered plants (at tillering and anthesis stage), whereas group II included isolates from water-stressed plants. UPGMA cluster analysis for OP-06 primer gave similar clusters as that of OP-01.

Ki-Yoon Kim *et al.*, (2010) reported that twelve strains showed characteristics of *A. brasilense* Sp7 and one strain (CW1503) showed characteristics of *A. lipoferum* 687. Among them, *A. brasilense* was found to be the dominant species on plant roots. There was wide variation in nitrogenase activity among the different isolates. *A. brasilense* CW301 and *A. brasilense* CW903, which are associated with wheat roots and taro, had the highest ARA activity. The strains that showed the highest nitrogenase activity in wheat roots viz., *A. brasilense* CW301, *A. brasilense* CW903 and *A. lipoferum* CW1503 were selected and their identifications were confirmed by 16S rDNA sequencing. 16S rDNA sequencing confirmed 98 to 99% homology with *A. brasilense, A. lipoferum*, respectively, and were assigned the Gen Bank accession numbers AY518780, AY518777 and AY518779, respectively. Further colonization studies with *A. brasilense* CW301, *A. brasilense* CW903 and *A. lipoferum* CW1503 showed considerable β-galactosidase activity under aerobic growth. Transconjugant of *A. brasilense* CW301 exhibited the highest activity followed by *A. lipoferum* CW1503.

A large number of restriction enzymes sites were found in the *A. halopraferens* TPS05. The total number of restriction enzymes sites of *A. halopraferens* TPS05 was 46. The GC content of *A. halopraferens* TPS05 was 56%. The AT content of *A. halopraferens* TPS05 was 44% using NEB cutter programme V.20 in www.neb.com/neb cutter 2/index. php (Fig-2).

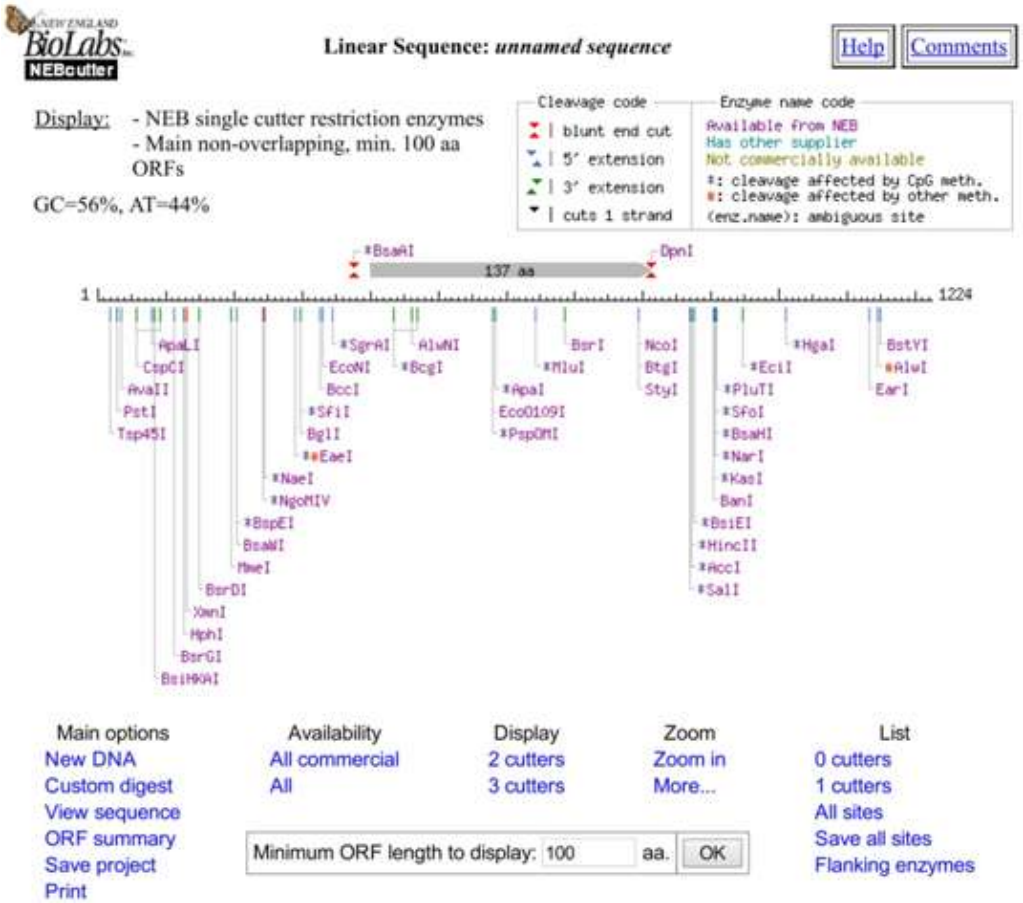

Figure 2: Restriction site analysis of *A. halopraferens* TPS05

Rasool (2015) investigated that the PCR amplifications of 16S rDNA and sequencing of the amplified products has been carried out. AzoLR3 and *Azospirillum* reference sequences which were obtained from Gene Bank were aligned using CLUSTALX and a phylogenetic tree was constructed using the neighbor joining (NJ) method (Saitou and Nei, 1987). The phylogenetic analysis and comparison of isolates with other members of genus *Azospirillum* showed evolutionary relationship among them. AzoLR3 isolate showed 96% similarity with *Azospirillum* genus. Therefore, the bacterial strain AzoLR3 was identified as *Azospirillum* sp. On the basis of 3 % difference in 16S rDNA sequences, novel bacterial species has been proposed (Stackebrandt and Goebel, 1994; Xie and Yokota, 2005).

In the present study, the isolate *A. halopraferens* TPS05 showed 19 stems, 10 hairpins loops and 12 loops in their RNA secondary structure. The free energy structure of 16S rRNA secondary structure of *A. halopraferens* TPS05 was – 115.1 k kal/mol (Fig-3).

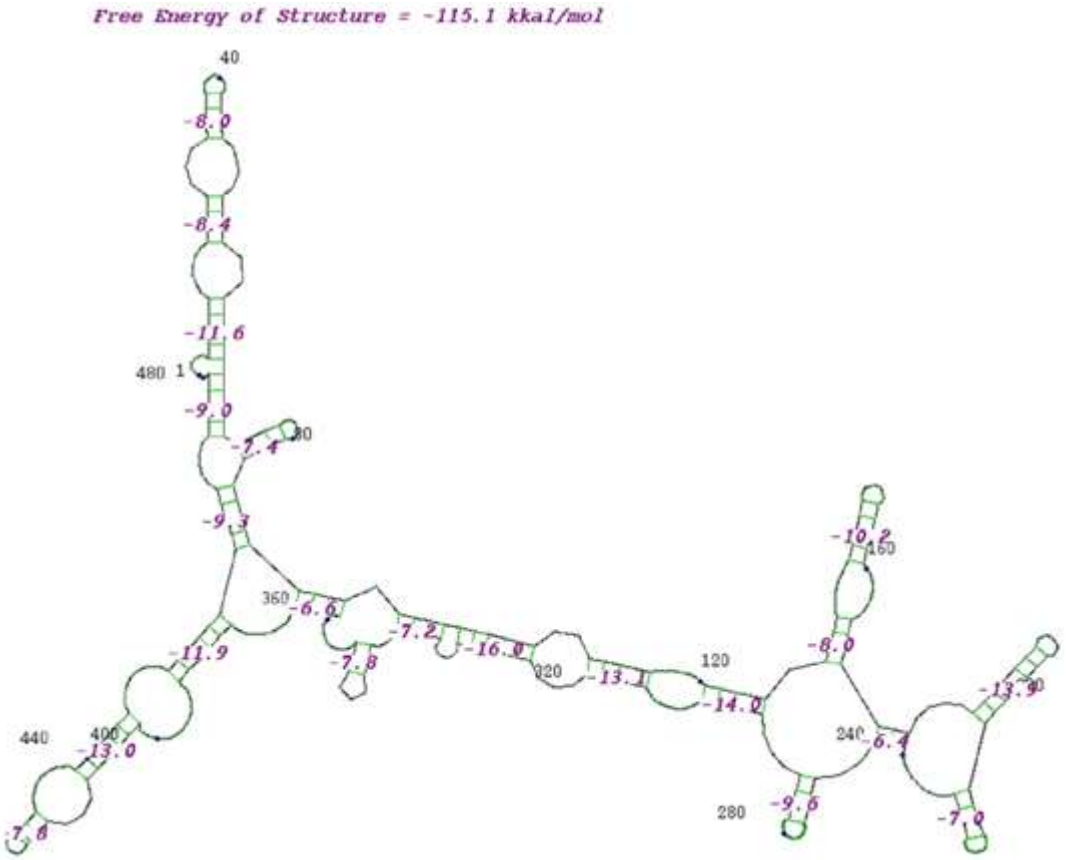

Figure 3: Secondary structure prediction of *A. halopraferens* TPS05

Kanimozhi and Panneerselvam (2010) analysed the variation in the 16S rDNA sequences of three different *Azospirillum* isolates were observed. The phylogenetic analysis of partial 16S rDNA sequences showed that the *A. brasilense* PA03 – HM217758 was closely similar (84%) to the existing species of *Azospirillum* sp. TSH51 AB508854 whereas *A. amazonense* PTA03 – HM217759 was closely similar (78%) to existing species of uncultured *Azospirillum* sp. DQ312–25 EU050699. *A. halopraferens* PA04 – HM217760 was closely similar (93%) to existing species of uncultured *Azospirillum* species DQ312–25 EU050699. In the present study, distinct variation in the secondary structure, GC composition, presence of restriction enzymes in 16S rRNA sequence of three different *Azospirillum* isolates showed molecular level specificity of each and every individual isolates for the development of universal identification system of not only *Azospirillum* but all microorganisms, a polyphasic taxonomic approach utilizing morphological, biochemical, physiological cultural, ecological and molecular characteristic will help taxonomists for the developments of meaningful taxonomic identification system.

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