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Phenetic and Functional Characterization of Endophytic Root-nodule Bacteria Isolated from Chickpea (*Cicer arietinum* L.) and Mothbean (*Vigna aconitifolia* L.) of Arid-and Semi-arid Regions of Rajasthan, India

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Abstract: In the present study we recovered endophytic root-nodule bacteria from chickpea (*Cicer arietinumi* L.) and mothbean (*Vigna aconitifolia* L.). Phenotypic and genotypic characterization of isolates was performed by employing biochemical and genetic approaches. Sequencing data showed that most isolates belonged to genus, *Pseudomonas* spp. being a dominant species. They also showed similarity with *Rhizobium*, *Agrobacterium* and *Erwinia* spp. Isolates were screened functionally for indole-3-acetic acid, siderophore production and inorganic phosphorus (Pi) solubilization. All isolates showed Pi solubilization except CJS-2. Nine isolates (CSS-1, CBS-1, CLS-3, CCS-1, CHS-1, VS-1, VL-1, VN-1, VN-2) were found positive for IAA production and eight isolates (CBS-1, CCS-1, CHS-2, CKS-2, CNS-2, VS-1, VJ-1) exhibited positive results for siderophore production. An understanding of the phonetic and functional diversity of these microbes that interact with plants will be worthwhile to fully achieve the biotechnological potential of efficient plant-microbe partnerships for a range of applications.

Key words: Plant-growth promotion, γ-proteobacteria, *Pseudomonas*, *Rhizobium*

INTRODUCTION

To have insight on linked belowground functioning the one has to explore microbial diversity that makes rhizosphere functional. Plant productivity is often limited by soil nutrient availability and relies on the interface, the rhizosphere, between living roots and soils. This is the central area of exchange involving the organic C flux from root fuels and, microbial decomposers that make nutrients available to roots. Rhizosphere action profoundly affects the physico-chemical and structural properties of soils, including their development (Beerling and Berner, 2005). The global necessity to increase agricultural productivity from steadily decreasing land resources base has placed significant strain on the fragile agro-ecosystems. Therefore, it has become necessary to adopt strategies to maintain and improve agricultural productivity through the employment of high input practices. Improvement in agricultural sustainability requires optimal use and management of soil fertility and soil physical properties and relies on soil biological processes and soil biodiversity (Tilak *et al*., 2005). Genetic diversity of bacteria is being analyzed increasingly by PCR-based genomic fingerprinting methods. As more knowledge is acquired and isolates from unexplored legumes are studied, new species are discovered and former species rectified. Molecular tools for the identification of bacteria were used and 16S rRNA gene analysis was intensively used to understand the phylogenetic relationships. Bacterial phylogenetic classification is based on sequence analysis of the SSU 16S rRNA molecule or its genes. Homology tree based on sequence alignment of 16S rDNA of bacterial isolates permitted rapid phylogenetic analysis. However, strains isolated from different geographic location shared similar DNA homology. Phylogenetic analysis on the basis of 16S rDNA sequences provided better understanding in evaluation of genetic diversity of rhizobacteria isolated from same and different ecological niche; phylogenetic analysis of 500 bp of terminal region of 16S rDNA from cultivated strain has been found to show existence of large bacterial diversity. The legume biodiversity is concentrated in tropical regions, while most studies are on cultivated leguminous plants from temperate region. In the past ten years, several symbionts capable of forming nodules and fixing nitrogen in legume roots have been documented and grouped under α and β subclass and few γ-proteobacteria (Benhizia *et al*., 2004), *Ralstonia taiwanensis* (renamed as *Cupriavidus taiwanensis*) (Chen *et al*., 2001) and *Agrobacterium* like strains (Mhamdi *et al*., 2005).

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The legume host preferred by these non-rhizobial proteobacteria possesses high diversity (Balachandar *et al*., 2007).

In view of the limited information on the microbial diversity of arid-and semi-arid regions of Rajasthan, the objectives of this present research was to assess *in vitro* phonetic and functional characterization of endophytic root-nodule bacteria isolated from chickpea (*Cicer arietinum* L.) and mothbean (*Vigna aconitifolia* L.) of Indian Thar desert, Rajasthan.

MATERIALS AND METHODS

Collection of nodule samples: For the recovery of bacterial isolates, the nodules were collected from cultivated legumes chickpea (*Cicer arietinum* L.) and mothbean (*Vigna aconitifolia* L.) that grown in the arid regions of Rajasthan.

Isolation and maintenance of bacterial strains: Nodules were surface-sterilized using 70% ethanol and 0.1% $HgCl_2$ and repeatedly washed with sterile water. Sterile nodules were crushed in a sterilized Petri dish with the help of sterilized glass rod in 1 mL sterilized distilled water and the resulting suspension was streaked on Yeast Extract Mannitol (YEM) agar plates amended with Congo red (Vincent, 1970) and plates were incubated in an incubator at 28±2°C for 24 to 36 h. After 24-36 h, the translucent, glistening and elevated colonies were picked and purified by single colony streaking on the YEMA slants. A total of 22 bacterial strains with distinct colony morphologies were kept for further studies. To obtain pure culture, a pure colony of isolate was inoculated in YEM broth and placed in orbital shaker (120 rpm) at 28±2°C for 24 to 36 h and maintained in refrigerator. A 2.5 mL, autoclaved mixture of glycerol+YEM broth (1:1 v/v) was added to 5 mL of overnight grown culture in the same medium. One milliliter of this mixture was suspended in cryovial and preserved at -80°C.

Biochemical characteristics of isolates: Several biochemical tests, viz., lysine, ornithine, phenylalanine deamination, adonitol, sorbitol, starch and gelatin hydrolysis, catalase, oxidase, citrate utilization and urease were performed.

PCR amplification, sequencing and analysis of 16S rDNA: Total DNA from bacterial isolates was prepared according to the procedure of Bazzicalupo and Fani (1994). The amplified 16S rRNA gene was obtained from each bacterial isolate by PCR amplification employing the eubacterial universal primers (Weisburg *et al*., 1991); fDI (5'-AGAGTTTGATCCTGG-3') and rP2 (5'-TACCTTGTTACGACTT-3') which was targeted at universally conserved regions and permitted amplification of approximately 1,500-bp fragment. PCR amplification was carried out in a TPersonal Thermocycler (Biometra, Germany). Reaction tubes contained 25 ng (5 μL) of DNA extract, 1 U of *Taq* polymerase (Genei, India), 1 X buffer (10 mM Tris-Chloride [pH 9.0], 1.5 mM $MgCl_2$, 500 mM KCl) (Genei), 10 mM dNTPs (Genei) and 0.25 mM of each primer (Genei). Initial DNA-denaturation and enzyme activation steps were performed at 95°C for 7 min, followed by 25 cycles of denaturation at 94°C for 1 min, annealing at 51°C for 1 min and extension at 72°C for 1 min and a final extension at 72°C for 10 min. The presence and yield of specific PCR product (16S rRNA gene) was monitored on 0.8% agarose (wt/vol); gel electrophoresis was carried out at 100 V for 30 min in 1 X Tris-acetate-EDTA buffer and visualization by ethidium bromide staining and viewing on a UV transilluminator. PCR products obtained from 22 bacterial strains were purified with an EXO-SAP. Components were supplemented with gold buffer (Applied Biosystem) and sequenced on an Applied Biosystem 310 Genetic analyzer (ABI Prism 310 Genetic analyzer), using big dye terminator cycle sequencing Ready Kit (Lab India). The partial sequences amplified by the fDI primer were used to determine the similarities. All the sequences were subjected to phylogenetic analysis. The homology of partial sequences were compared with the sequences from the DNA databases and similar sequences showing above 95% were retrieved by nucleotide Basic Local Alignment Search Tool (BLAST) program at the National Center for Biotechnology Information (NCBI) BLAST server. Multiple sequence alignment of retrieved sequences was done by EBI ClustalW server. Phylogenetic tree was constructed by using MEGA 4.0.2 software. Phylogenetic tree was obtained with bootstrap values in cluster algorithm, phylip format and topological algorithm. The nucleotide sequences obtained in this study have been submitted to the GenBank and assigned accession numbers JF699681 to JF699702.

Functional characteristics of isolates: Indole acetic acid (IAA) production was detected according to Gordon and Weber (1951). Quantitative analysis of IAA was performed at different concentrations of tryptophan (0, 50, 150, 300, 400 and 500 ppm). Bacterial cultures were grown for 48 h in YEM broth media at 28±2°C. Fully grown cultures were centrifuged at 3000 rpm for 30 min. The

supernatant (2 mL) was mixed with two drops of orthophosphoric acid and 4 mL of the Salkowski reagent (50 mL, 35% of perchloric acid, 1 mL 0.5 M $FeCl_3$ solution). Development of pink colour was predictive IAA production. Optical density was taken at λ = 530 nm with the help of spectrophotometer. Concentration of IAA produced by cultures was measured with the help of standard graph of IAA obtained in the range of 10-100 ppm. For siderophore assay a method described by Schwyn and Neilands (1987) was employed with modification. All isolates were screened for phosphate solubilization on Pikovskaya's agar plates containing (per litre) 0.50 g Yeast extract, 10.0 g Dextrose, 5.0 g Calcium phosphate, 0.50 g Ammonium sulphate, 0.20 g Pottasium chloride, 0.10 g Magnesium sulphate, 0.0001 g Mangnese sulphate, 0.001 g Ferrous sulphate and 15.0 g Agar, pH 7.0±0.2. Plates were inoculated and incubated at 28±2°C, analyzed for zone of clearance up to 7 days.

RESULTS

Collection of plant samples: The leguminous plants mothbean (*Vigna aconitifolia* L.) with root nodules were collected from different localities of Rajasthan during summer and winter season of year 2008-2009.

Biochemical characterization for recovered isolates: Out of nineteen biochemical tests six (lysine, ornithine, phenylalanine deamination, adonitol, sorbitol, starch and gelatin hydrolysis) were found negative whereas four tests (catalase, oxidase, citrate utilization and urease) found positive in all isolates. Nine isolates (CSS-1, CBS-1, CCS-2, CKS-2, CNS-2, VS-1, VL-1, VJ-1, VN-2) were found positive for nitrate reduction test. Only two isolates (CLS-3, VJ-1) found negative in ammonia production test. Thirteen (CLS-1, CLS-2, CCS-1, CHS-1, CHS-2, CJS-2, VS-1, VS-2, VS-3, VL-1, VJ-1, VJ-2, VN-1) isolates were found positive for lipid hydrolysis test. Five isolates (VL-1, VN-1, CJS-2, CLS-3, CLS-2) were found positive for H_2S production. Among twenty two isolates eleven found positive for casein hydrolysis (Table 1).

Partial 16S rRNA characterization: The 16S rRNA gene sequencing was performed so as to retrieve sequence similarity and bacterial identity from sequence databases. The sequences obtained were compared with sequences from the Gene Bank database of different bacterial strains, through the NCBI world Wide Website, at www.ncbi.nlm.nih.gov/BLAST. Sequencing of 16S rDNA fragments was performed and the obtained sequences of

Table 1: Biochemical characteristics for chickpea isolates

	Biochemical test									
Isolate	Cit	Lys	Orn	Ure	Cat	Oxi	Phe. Dea.	Nit. Red.	Amm. Pro.	Lip. Hyd.
CSS-1	+	-	-	+	+	+	-	+	+	-
CBS-1	+	-	-	+	+	+	-	+	+	-
CLS-1	+	-	-	+	+	+	-	-	+	+
CLS-2	+	-	-	+	+	+	-	-	+	+
CLS-3	+	-	-	+	+	+	-	-	-	-
CCS-1	+	-	-	+	+	+	-	-	+	+
CCS-2	+	-	-	+	+	+	-	+	+	-
CHS-1	+	-	-	+	+	+	-	-	+	+
CHS-2	+	-	-	+	+	+	-	-	+	+
CKS-2	+	-	-	+	+	+	-	+	+	-
CJS-2	+	-	-	+	+	+	-	-	+	+
CNS-2	+	-	-	+	+	+	-	+	+	-

	Biochemical test								
Isolate	H_2S	Sta	Gel	Cas	Glu	Ado	Lac	Ara	Sor
CSS-1	-	-	-	-	+	-	-	-	-
CBS-1	-	-	-	+	+	-	-	-	-
CLS-1	-	-	-	+	+	-	-	-	-
CLS-2	+	-	-	-	+	-	-	-	-
CLS-3	+	-	-	+	+	-	-	-	-
CCS-1	-	-	-	+	+	-	-	-	-
CCS-2	-	-	-	-	+	-	-	-	-
CHS-1	-	-	+	-	+	-	-	-	-
CHS-2	-	-	-	-	+	-	-	-	-
CKS-2	-	-	-	+	+	-	-	-	-
CJS-2	+	-	-	+	+	-	-	-	-
CNS-2	-	-	-	-	+	-	-	-	-

	Biochemical test									
Isolate	Cit	Lys	Orn	Ure	Cat	Oxi	Phe. Dea.	Nit. Red.	Amm. Pro.	Lip. Hyd.
VS-1	+	-	-	+	+	+	-	+	+	+
VS-2	+	-	-	+	+	+	-	-	+	+
VS-3	+	-	-	+	+	+	-	-	+	+
VL-1	+	-	-	+	+	+	-	+	+	+
VL-2	+	-	-	+	+	+	-	-	+	-
VL-3	+	-	-	+	+	+	-	-	+	-
VJ-1	+	-	-	+	+	+	-	+	-	+
VJ-2	+	-	-	+	+	+	-	-	+	+
VN-1	+	-	-	+	+	+	-	-	+	+
VN-2	+	-	-	+	+	+	-	+	+	-

	Biochemical test								
Isolate	H_2S	Sta	Gel	Cas	Glu	Ado	Lac	Ara	Sor
VS-1	-	-	-	+	+	-	-	-	-
VS-2	-	-	-	-	+	-	-	-	-
VS-3	-	-	-	-	+	-	-	-	-
VL-1	+	-	-	-	+	-	-	-	-
VL-2	-	-	-	+	+	-	-	-	-
VL-3	-	-	-	-	+	-	-	-	-
VJ-1	-	-	-	+	+	-	-	-	-
VJ-2	-	-	-	+	+	-	-	-	-
VN-1	+	-	-	+	+	-	-	-	-
VN-2	-	-	-	-	+	-	-	-	-

Cit: Citrate, Lys: Lysine, Orn: Ornithine, Ure: Urease, Cat: Catalase, Oxd: Oxidase, Phe. Dea.: Phenylalanine deamination, Nit. Red.: Nitrate reduction, Amm. Prod.: Ammonia production, Lip. Hyd.: Lipid hydrolysis, Sta: Starch hydrolysis, Gel: Gelatin hydrolysis, Cas: Casein hydrolysis, Glu: Glucose, Ado: Adonitol, Lac: Lactose, Ara: Arabinose, Sor: Sorbitol, +: Positive, -: Negative

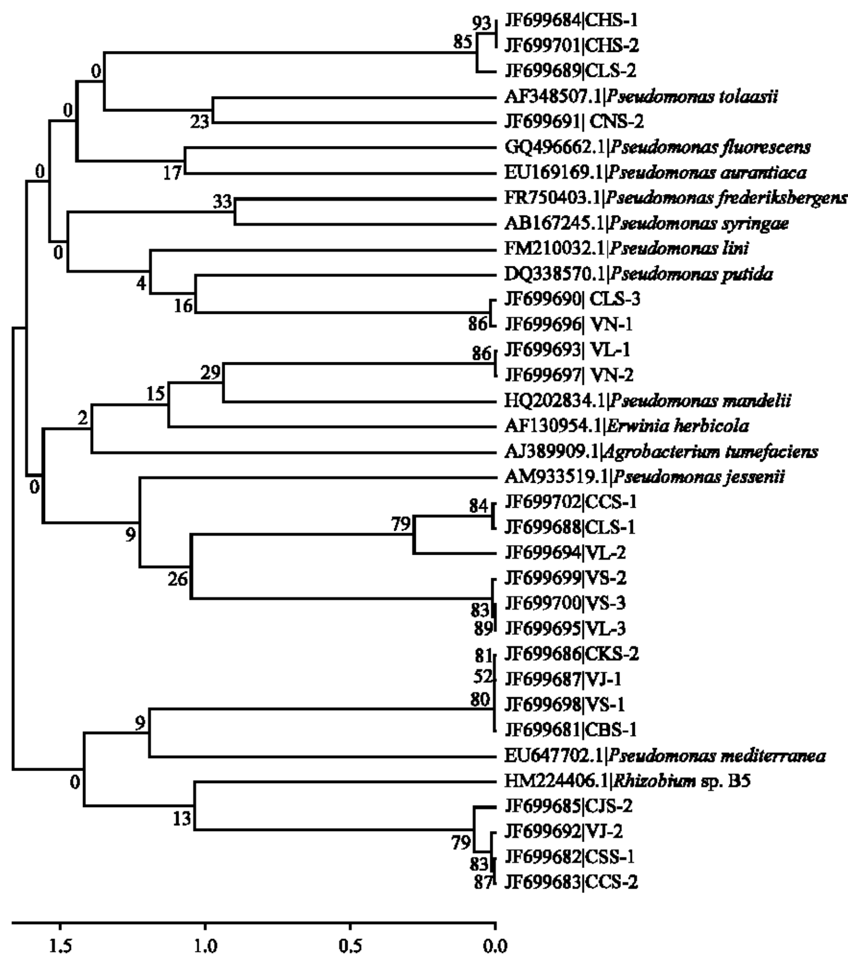

Fig. 1: Combined phylogenetic tree for chickpea and mothbean strains with related sequences based on a distance analysis by employing MEGA 4.0.2 software

all isolates were submitted to NCBI Gen Bank under accession No. JF699681 to JF699702. All the sequences obtained from twenty two isolates were aligned with each other to determine genetic diversity amongst the endophytic root-nodule bacteria. A comparison of 16S rDNA sequence with the reference strain, to which they matched, was performed. A consensus tree was drawn from these aligned sequences using MEGA version 4.0.2 (Fig. 1). Sequencing data showed that most isolates belonged to genus, *Pseudomonas* spp. being a dominant species. They also showed similarity with *Rhizobium*, *Agrobacterium* and *Erwinia* spp. A large group was made by isolates VS-1, VJ-1, VJ-2, CBS-1, CKS-2, CJS-2, CSS-1 and CCS-2 with the reference strains, *P. fluorescens* and *P. aurantiaca*. These isolates also exhibited significant relatedness with *P. lini*, *P. putida*, *P. mediterranea* and *Rhizobium* sp. *B5* reference strains. Another group includes isolates VL-2, VL-3, VS-2, VS-3, CCS-1 and CLS-1 which showed similarity with reference strain *P. jessenii*. Third group showed relatedness with *P. syringae* and *P. frederiksbergens* this group was made up of isolates VN-1, CLS-2, CLS-3, CHS-1 and CHS-2. One bacterial isolate, showed distinct group from others and exhibited similarity with reference strains, *P. tolaasii*. Two isolates VL-1 and VN-2 found similar with *P. mendelii*, *E. herbicola* and *A. tumefaciens*.

Functional attributes of PGPBs for PGP traits: The results of PGP traits of rhizobacteria are described in Table 2. All bacterial isolates showed +ve results for phosphate solubilisation except one isolate (CJS-2). Nine isolates (CSS-1, CBS-1, CLS-3, CCS-1, CHS-1, VS-1, VL-1, VN-1, VN-2) were found positive for IAA production and eight isolates (CBS-1, CCS-1, CHS-2, CKS-2, CNS-2, VS-1, VJ-1) gave positive results for siderophore production.

Table 2: Plant growth promoting traits for chickpea Isolates

Isolates	IAA production	Siderophore production	Phosphate solubilization
CSS-1	+	-	+
CBS-1	+	+	+
CLS-1	-	-	+
CLS-2	-	-	+
CLS-3	+	-	+
CCS-1	+	+	+
CCS-2	-	-	+
CHS-1	+	-	+
CHS-2	-	+	+
CKS-2	-	+	+
CJS-2	-	-	-
CNS-2	-	+	+
VS-1	+	+	+
VS-2	-	-	+
VS-3	-	-	+
VL-1	+	-	+
VL-2	-	-	+
VL-3	-	-	+
VJ-1	-	+	+
VJ-2	-	-	+
VN-1	+	-	+
VN-2	+	+	+

+: Presence, -: Absence

DISCUSSION

In the present study phonetic and functional characterization of root-nodule associated bacteria was determined. Phenetic diversity of bacteria is being analyzed increasingly by PCR-based genomic fingerprinting methods. As more knowledge is acquired and isolates from unexplored legumes are studied, new species are discovered and former species rectified. Due to improved methods of characterization, the classification of rhizobia has undergone drastic changes and the phylogenetic analysis of the family Rhizobiaceae and related genera has been upgraded (Chen *et al*., 2005). The legume biodiversity is concentrated in tropical regions, while most studies are on cultivated leguminous plants from temperate region (Moulin *et al*., 2001). In the past ten years, several symbionts capable of forming nodules and fixing nitrogen in legume roots have been documented and grouped under α and β subclass of Proteobacteria, which include *Methylobacterium nodulans* (Sy *et al*., 2001), *Devosia* sp. (Rivas *et al*., 2002), *Ochrobactrum lupini* (Trujillo *et al*., 2005), *Agrobacterium* like strains (Mhamdi *et al*., 2005) and few γ-proteobacteria (Benhizia *et al*., 2004). The legume host preferred by these non-rhizobial proteobacteria possesses high diversity (Balachandar *et al*., 2007). Researchers extensively applied the Restriction Fragment Length Polymorphism (RFLP) analysis of PCR amplified 16S rRNA gene for identification of rhizobia and thereby several novel species have been reported during the last decade (Chen *et al*., 2001). Phylogenetic analysis based on the 16S rRNA gene sequences showed that the novel strains formed a subclade in the genus *Rhizobium* together with *Rhizobium galegae*, *Rhizobium huautlense* and *Rhizobium alkalisoli*, with 99.8% gene sequence similarity between the strains. The improvement of molecular biology-based approaches will be fundamental for analysing microbial diversity and community structure and to predict responses to microbial inoculation/processes in the environment (‘ecological engineering’).

Our results indicate that the number of bacteria present was independent of the legumes localities, although the physiological diversity of the isolates seems to be related to the cultivars from which the isolate was obtained. This is interesting because IAA is a plant hormone with no apparent function in bacterial cells and it could be speculated that IAA production may improve the fitness of the plant-bacterium interaction (Patten and Glick, 2002). The interaction between plant roots and organisms within rhizosphere assists in acquiring essential mineral nutrients and prevents the accumulation of toxic elements. An essential mineral element that most frequently limit plant growth is phosphorus (P); it is taken up in the form of inorganic phosphate (P_i, $H_2PO_4^-$) from the soil solution. The concentration of P_i in the soil solution is very low (2-10 μm) which limits P_i diffusion to the root system with the resultant P_i depletion in the rhizosphere. In addition, P deficiency increases the abundance of P_i transporter proteins and promotes the exudation of organic acids, ribonucleases (Rnases) and phosphatases to mobilize P from organic/insoluble compounds (Roesti *et al*., 2006). Phosphorus is one of the most important plant nutrients and a large portion of inorganic phosphates applied to soil as fertilizer is rapidly immobilized after application and becomes unavailable to plants (Rodriguez and Fraga, 1999). Previous experiments have shown that endophytic bacteria possess the capacity to solubilize immobilized mineral phosphates (Verma *et al*., 2001), suggesting that during initial colonization, endophytic bacteria could enhance phosphate availability to the host plant.

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