Isolation and Molecular Characterization of Endorhizospheric Diazotrophs of Western Ghats

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The acceptance and applicability of plant growth promoting rhizobacteria (PGPR) as potential tools for sustainable agriculture is explicit in their remarkable ability to enhance plant growth by a variety of mechanisms. In the present study, several samplings were conducted in different forest areas of Western Ghats in order to explore the structural and functional diversity of agriculturally important microorganisms. Strains isolated from endorhizosphere microhabitats were grown on N free Malate medium and the amount of Nitrogen was measured by Kjeldahl digestion of extracts. The best performing strains were characterized by 16S rRNA sequencing. Of the 12 efficient strains, seven strains belonged to genus *Pseudomonas*; four belonged to *Rhizobium* and one to *Acinetobacter*. All the strains fixed more than 10mg of NH_4^+/gm of Carbon utilized, the highest being strainMDAZOXXIE249a; 17.5mg of NH_4^+ followed by 15.8 mg NH_4^+ from strains MDAZOXX1E256a and MDAZOXXIE251a individually. The diazotrophs identified in this study belonged to two major groups: alpha and gamma proteobacteria. Strain *Acinetobacter schindleri* could be a novel diazotroph isolated in the study.

Key words: Diazotrophs, Kjeldahl digestion, 16S rRNA sequencing, Phylogenetic analysis.

Plant growth promoting rhizobacteria prove a key to sustainable agriculture; provided the difficult task of maintaining optimum viable and functionally active population within the crucial rhizosphere ecosystem is accomplished. Environmental stresses such as the application of agrochemicals practiced in agriculture have damaging effects in soil such as, decrease in microbial diversity and structural change of microbial communities[1]. Therefore it is important to study how the plant growth promoting rhizobacteria interact with each other in community and in close association with plants and to decipher their structural and functional diversity so as to exploit their potentialities.

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Diazotrophy is a very useful attribute of many of the PGPRs. A wide range of bacterial genera are capable of fixing atmospheric dinitrogen and convert them into forms available to plants[2, 3]. Over the years, researchers have isolated, characterized and studied diazotrophs from diverse environments[4, 5, 6]. The diverse role of diazotrophs in soil including phosphate solubilization, phytohormone production, and as biocontrol agents has been exemplified by Hafeez and Glick *et al*.[7, 8]. These attributes further add to their field applicability as potential agents for bioinoculation[9.] Western Ghats, a region known for its rich biodiversity is chosen in the present study to isolate and characterize the diverse plant growth promoting rhizobacteria with an ability to fix high amount of nitrogen from endorhizosphere microhabitat of various plant species. An attempt was made to analyse their taxonomic affiliation and evolutionary relationship between the isolates.

MATERIALS AND METHODS

Collection of samples and isolation of diazotrophs

Root samples were collected from selected forest areas of Western Ghats of Uttara Kannada district of Karnataka, India, by composite sampling method[10]. The following plant species were selected for the collection of samples: *Lantana camara, Acacia latronum, Oryza sativa, Musa paradisica, Areca catechu, Tectona grandis, Eugenia jambulana, Terminalia tomentosa* and *Terminalia arjuna.*

A total of 200 bacterial cultures were isolated from root samples. The endorhizospheric bacteria were isolated from the washed and surface sterilized root samples of different species of plants. The root samples were macerated in 20 ml of sterile water. One ml suspension from the macerated roots was spread uniformly on the surface of selective agar media and incubated at 30ÚC. After the required incubation period, the representative colonies of different types of bacteria were purified, sub-cultured and stored for further analysis.

The isolates were further tested for free living nitrogen fixation. The petriplates amended with sterilized Norris N-free medium composed of Malic acid 5g/L, KOH 3g/L, K_2HPO_4 0.5g/L, $FeSO_4.H_2O$ 0.1g/L, $MnSO_4$ 0.1g/L, $MgSO_4.7H_2O$ 0.1 g/L, NaCl 0.02g/L, $CaCl_2.2H_2O$ 0.01g/L, Na_2MoO_4 0.002g/L and agar 1.75g/L and 2 ml of Bromothymol blue (0.5% in alcohol), pH 6.5 were spotted with 10 ml of overnight cultures of the isolates and incubated on at 28+2Ú C for 48 h. Morphological and gram nature of the isolates was examined by colony morphology and microscopic observations. Growth characteristics of isolates in nitrogen free broth were studied. The ability of the isolates to form pellicle in semisolid nitrogen free medium confirmed the nitrogen fixation under microaerophilic conditions.

Estimation of Nitrogen fixation

A total of 40 strains able to grow on N free Malate medium were subjected for quantitative estimation of the amount of nitrogen fixed in broth culture by Microkjeldahl method[11] after growing the organisms in 250 ml of N- free Malate medium at 28 + 2Ú C for 10 days. Ten ml of this culture was used for estimation of nitrogen.

To the 10 ml of broth culture, 5 ml of concentrated H_2SO_4 and 200 mg catalyst mixture (potassium sulphate, copper sulphate and selenium in the ratio of 10 :1:0.1) were added and allowed for digestion in block digestor for 2 h to get clear digest. The clear digest was cooled and diluted with distilled water up to 10 ml. This was distilled in a distillation unit after addition of 20 ml of 40 per cent sodium hydroxide solution to make the digest alkaline, in a Parnas- Wayner type distillation unit. The evolved ammonia was absorbed in four per cent boric acid with mixed indicator and finally titrated with 0.05 N H_2SO_4 for colour change from green to red. From the volume of acid consumed, total Nitrogen content was calculated and expressed as mg of NH_4^+ / gm of Carbon utilized.

Statistical analysis

Statistical analysis was done using completely randomized design with ANOVA at 1% significance.

Molecular characterization of potent strains

The genomic DNA was extracted from twelve best performing strains by following the protocol[12]. 16S rRNA gene was amplified[13] by using universal forward and reverse primers (16S F: 5'-AGAAGTTTGATCMTGGCTCAG-3' and 16S R : 5'-TACGGYTACCTTGTTACGAC-3'). The single sharp amplicon of 1.45 Kb size was eluted using the Qiagen's MiniElute gel extraction kit, Germany. This purified amplicon was ligated to PTZ57R/T cloning vector using InsT/Aclone™ cloning kit (MBI, Fermentas, USA). Competent *E. coli* DH5a cells were prepared following the protocol described by Sambrook and Russell [14], which were then used for transformation of ligated mixture. The transformed clones were identified by blue/ white assay.

The recombinant clones were screened for the presence of cloned fragment through colony PCR using the M13 primers as well as restriction confirmation using XbaI and BamHI. Recombinant plasmids containing the insert (1.45 Kb) were sent for sequencing to Eurofins Private Ltd., Bangalore.

Phylogenetic analysis

Both forward and reverse 16S rRNA sequences obtained for the individual strains were joined using CAP contig assembly program in BioEdit version 7.1.3.0 and vector contamination if any were removed and submitted in BLAST Program[15] in order to retrieve the sequences of

closest NCBI strain which is hitting maximum homology with the query sequence. Phylogenetic and molecular evolutionary analyses were conducted using *MEGA* version 4.0[16]. The sequences determined together with closely related sequences were then aligned using Clustal_W [17] and phylogenetic tree was constructed using neighbor-joining method [18]. Reliability of the topology of tree was verified by 500 bootstrap resamplings [19] in order to get a best estimated tree.

The taxonomic specific 16S rRNA sequences of all diazotrophic isolates were also analyzed by using Ribosomal Database Project[20] by sequence matching with related unique homologous sequences in order to affiliate the taxonomic ranks for the isolates.

RESULTS AND DISCUSSION

Out of a total of 200 rhizobacteria isolated in 27 different samplings, we could obtain 40 strains able to grow on N free malate medium. Strains fixing more than 10mg of NH_4^+ / gm of Carbon utilized were selected for further molecular characterization and phylogenetic analysis. Table 1 summarizes amount of nitrogen fixed by the strains, blast analysis results and Genbank accession numbers procured for eight isolates. Twelve diazotrophs representative of three genera have been characterized in this study. According to identification by means of 16S rRNA sequencing, the Table1 majority of the efficient isolates belonged to members of the genus *Pseudomonas.* Other isolates effective in this study have been identified as *Rhizobium* and *Acinetobacter*. It is known that the diazotrophic trait is scattered among wide range of bacterial genera. Predominance of species of *Pseudomonas* as diazotrophs in our results emphasizes the ubiquitous occurrence and capacity of these heterotrophs to grow on a wide range of carbon sources and diverse environmental conditions. Similar results were obtained by Dorris *et al.*[21] when diazotrophs were isolated from diverse environmental samples. Also, as stated by Bowen *et al.*[22], the abundance of Pseudomonads could be due to the conducive environmental conditions in the rhizosphere. Endorhizospheric Pseudomonads were also obtained in maize [23] and from tomatoes[24], which showed plant growth promotional activity. However, the approaches used for the characterization of the diazotrophs were different from the present study.

A phylogenetic tree depicting the evolutionary relationship between the strains under study is illustrated in Figure 1. According to taxonomic affiliation as determined by RDP analysis, the isolates MDAZOXVIE199c,

Table 1. Identification of isolates by 16SrRNA sequencing and amount of nitrogen fixed by the isolates

Strain	Closest NCBI strain	Percentage homology	Accession Number	Amount of nitrogen fixed (mg of NH_4^+ / gm of Carbon utilized)
MDAZOXVIE199c	*Pseudomonas sp.* ECU1011	100%	KJ535392	12.40
MDAZOXVIE200a	*Rhizobium miluonense* strain CC-B-L1	99%	KJ535393	13.37
MDAZOXVIIE211c	*Pseudomonas sp.* IM4	100%	—	13.30
MDAZOXVIIE208b	*Pseudomonas putida* BD18-S22	99%	--	12.23
MDAZOXXIIE211b	*Pseudomonas putida* strain ppnb1	98%	KJ535398	12.20
MDAZOXXIE243a	*Pseudomonas alcaligenes* strain R1-96	100%	KJ535394	12.10
MDAZOXXIIE249a	*Rhizobium sp.* CoE	99%	—	17.15
MDAZOXXIIE251a	*Pseudomonas plecoglossicida* strain FPC951	97%	KJ535395	15.50
MDAZOXXIIE252b	*Acinetobacter schindleri* strain: MTCC 9827	96%	KJ535396	13.02
MDAZOXXIE256a	*Pseudomonas putida*	99%	KJ535397	15.10
MDdiazo375	*Rhizobium pusense* strain BJ	97%	KJ460990	13.20
MDAZOXXIIE261c	*Rhizobium sp.*HGR4	98%	—	13.90
			SeM	0.25
			CD@1%	0.90

MDAZOXVIIE208b, MDAZOXXIE243a, MDAZOXXIE256a, MDAZOXVIIE211c, MDAZOXXIE251a, MDAZOXXIE252b and MDAZOXXIIE211b belonged to order Pseudomonadales of class gamma proteobacteria. As apparent in the phylogenetic tree they all form a single clade indicating they are a monophyletic group. Also we can see that there is separation of two main taxa in the tree, i.e., alpha proteobacteria (Order: Rhizobiales) and gamma proteobacteria (Order: Pseudomonadales). Class gamma proteobacteria exhibits enormous variety in terms of their phenotype and metabolic capabilities[25] and in phylogenetic trees they are distantly related to alpha proteobacteria[26, 27]. Our results are consistent with this as the estimate of overall average distance determined by nucleotide pair wise distance analysis was found to be 0.085; which is the estimate of the evolutionary divergence between the alpha and gamma proteobacteria under study. The mean diversity for the entire population is 0.159.

Another diazotrophic isolate obtained in this study showed maximum homology of 96% with *Acinetobacter schindleri*. This isolate although belonged to the same order Pseudomonadales but diverged into a different family Moraxellaceae. It can be observed in the reconstructed phylogenetic tree that this genus *Acinetobacter* formed an individual clade and the many *Pseudomonas* species isolated in the current study have descended from this clade and are excluded. Strain MDAZOXVIE199c formed an out group and is distantly related to other strains isolated in this study.

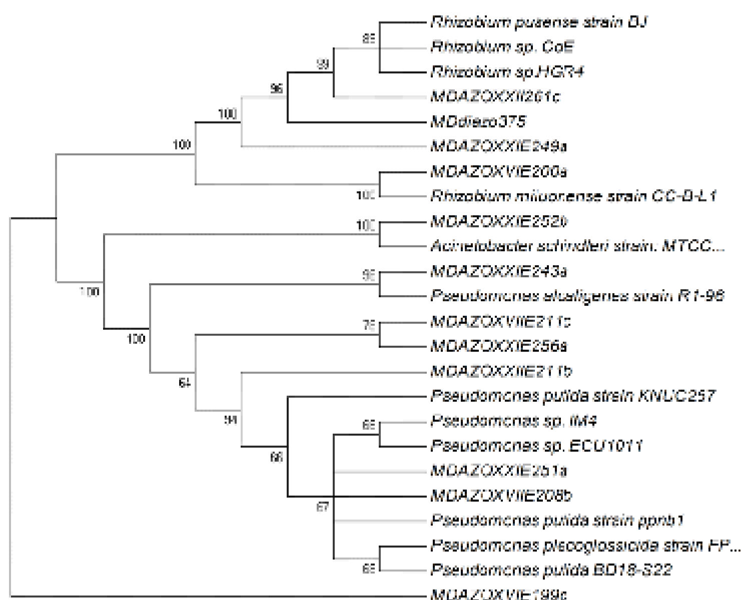

Fig. 1. Phylogenetic tree based on 16S rRNA sequencing constructed using neighbour-joining method showing relationships of the isolated diazotrophs and their closest NCBI strains. Bootstrap values greater than 50% are indicated at nodes. Scale bar 0.05 substitutions /nucleotide positions

Several diazotrophic species of *Acinetobacter* have been isolated by many researchers [28, 29]. The importance and plant growth promoting potential of these endorhizospheric inhabitants [30] cannot be undermined as long as they are not implicated in human pathogenesis.

Moreover several strains of *Rhizobium* species and *P. putida* have been shown to induce systemic disease resistance in plants [31, 32]. It has also been proved that these PGPR strains, *Pseudomonas* and *Acinetobacter* promote Fe, Zn, Mg, Ca, K and P uptake by crop plants [33].

CONCLUSION

Our results indicate the usefulness of the strains as bioinoculants for field application. Extensive research is needed in this context of

diversity analysis of diazotrophs, effective nitrogenase systems and an insight in to the novel genes imparting this important trait for these isolates. Since our experiments are carried out *in vitro* and overcoming the problem of optimizing the biological activity and viability of these PGPRs until field application remains as one of the challenges, further evaluation of the putative isolates under field conditions is warranted in order to ascertain their full potential as bioinoculants. The predominant occurrence of genera from Gammaprotebacteria indicates the conserved diazotrophic trait among most of them. *Acinetobacter schindleri* strain MDAZOXXIIE 252b could be a novel isolate whose diazotrophic potential has not been tested so far for field applications. These efforts are exercised with intent to develop competent biofertilizing PGPR from endorhizospheric diazotrophs associated with some of the important plant species of the 'Biodiversity hotspot of Karnataka'. Studies of these kinds could unravel the multitude of efficient plant growth promoting rhizobacteria, which naturally improves soil fertility and thus obviate the need to add synthetic nitrogen fertilizers in to the soil which would have negative environmental effects.

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