

Isolation and Characterisation of Diazotrophic Bacteria from Rhizosphere of different Rice Cultivars of South Assam, India

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ABSTRACT

Free living heterotrophic bacteria were isolated from the rhizosphere of 10 local and cultivated varieties of rice grown in Karimganj district of South Assam. Among the 25 isolates, 11 isolates with plant growth promoting activity were identified based on phenotypic and 16S rDNA sequence analysis. The strains were identified as *Shingomonasa zotifigens*, *Pseudomonas putida*, *Stenotrophomonas maltophila*, *Acinetobacter radioresistance*, *Alkaligenes faecalis*, *Enterobacter cloaceae* subsp. *dissolvens*, *Pantoea agglomerans*, *Klebsiella pneumoneae*, *Achromobacter xyloxidans*, *Herbispirillum rubrisubalbicans* and *Herbispirillum sp.* The efficient strains are isolated from the local varieties of rice plant. The isolate KR-23 (*Sphingomonas azotifigens*) was a novel bacteria reported for the first time as nitrogen fixing bacteria from India. The nitrogen fixing ability along with IAA production, ACC deaminase activity and P-solubilisation by the bacteria has shown their potential for plant-growth-promoting rhizobacteria. KR-6 (*Stenotrophomonas maltophila*) and KR-7 (*Herbispirillum rubrisubalbicans*) have been reported earlier as plant pathogens but they have shown a high potential for nitrogen fixing and auxin producing activity in the present study.

Key words: Nitrogen fixation; Plant growth promoting rhizobacteria; 16S rDNA sequence; Indole-3-acetic acid; ACC deaminase activity.

INTRODUCTION

Free living nitrogen fixing bacteria have been considered as an alternative for inorganic nitrogen fertiliser for promoting plant growth (Ladha and Reddy, 2000; Park *et al.*, 2004). In spite of the fact that a variety of nitrogen fixing bacteria have been isolated from the rhizosphere of various crops, interest in isolating more beneficial plant growth promoting bacteria has increased recently due to their potential use as bio fertiliser (Vessey, 2003). Cultivated rice (*Oryzasativa*) originated from species of wild rice and was domesticated several thousand years ago (Hoshikawa, 1989; Oka, 1988; Kennedy *et al.*, 2001). Wild rice species are likely to harbour unique populations of nitrogen-fixing bacteria that differ from those in extensively bred modern varieties of cultivated rice (Hurek *et al.*, 2000). Production of phytohormones (Tienet *al.*, 1979; Haahtelaet *al.*,

1990) and competitive suppression of plant phytopathogens (Glick, 1995; Park *et al.*, 2004) are the beneficial effects of these microbes on plant. The agricultural soil of South Assam experience heavy annual rainfall, resulting in leaching of nutrients causing economic loss to the farmers (Anonymous, 2007). The use of bio-fertilisers may be an alternative to the chemical fertiliser for achieving sustainable rice farming while improving productivity in rice-agro ecosystem.

The objective of the present study was to isolate, identify and molecular characterisation of diazotrophic bacteria from the rhizosphere of native local varieties of rice from different microhabitats of South Assam, India and characterise them for nitrogen fixation and plant growth promoting activities.

MATERIALS AND METHODS

Bacterial growth media

Burk's N-free medium comprising : 10g glucose, 0.41g KH_2PO_4 , 0.52g Na_2SO_4 , 0.2g CaCl_2 , 0.1g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.005g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.0025g $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 1.8g agar (all per litre distilled water) for semi solid and 15g agar for solid medium was used throughout the study (Wilson and Knight, 1952). The pH of the medium was adjusted to 7 ± 0.1 before autoclaving at 121°C for 15mins

Soil sample collection and isolation of diazotrophs

Ten native rice cultivars grown under different microhabitats in Karimganj districts of South Assam were randomly selected. Riceplant were uprooted from a depth of 0-5cm for rhizospheric soil sampling. The samples were then immediately placed in sterilized plastic packs and sealed brought to the laboratory and stored at a temperature of 4°C . Ten grams of the rhizospheric soil sample was transferred to a 250ml of Erlenmeyer flask containing 90ml of sterile distilled water and shaken (120rpm) for 30mins. Serial dilution was made and 0.1ml aliquots (10^3 - 10^5) were spread on plates containing Burk's N-free medium. The plates were incubated for 7 days at 30°C . Morphologically different colonies appearing on the medium were isolated and sub-cultured for further analysis.

Phenotypic characterisation of the bacterial isolates

Physiological and biochemical characters of the bacterial isolates were examined according to the methods described in Bergey's Manual of Systematic Bacteriology (Holt *et al.*, 1994). Colour, pigment, form, diameter, elevation, margin, surface, opacity and texture of the colonies of respective isolates were observed. Motility and morphology were examined with the help of phase contrast microscopy. The gram reaction was performed as per standard protocol. Plate assays were performed for determining the starch hydrolysis, carbon source utilisation of the isolates. MR-VP test, indole test, urea hydrolysis test were also performed.

16S rDNA gene amplification and sequencing

Genomic DNA was obtained by using

standard bacterial procedure (Sambrook *et al.*, 1989; Park *et al.*, 2004). Extraction of the lysate was done two times with chloroform to remove residual phenol. The primers used for PCR amplification of the 16s ribosomal DNA are 27F ($5'$ -AGAGTTTGATC CTGGCTCAG- $3'$) and 1492R ($5'$ -TACGGTTACCT TGTTACGACTT- $3'$) (Weisberg *et al.*, 1999). Each PCR mixture (50 μl) contained primers (at a concentration of 20pmol) a mixture of dNTP (Promega Co., Southampton, England) (at a concentration of 200 μM), Taq polymerase buffer and chromosomal DNA (ca 100ng) and enzyme (4 μl) were added to the reaction mixture. The thermo cycling condition consisted of an initial denaturation step at 94°C for 4min, 35 amplification cycles of 94°C for 1 min, 58°C for 1 min and 72°C for 3 min and a final extension step of 72°C for 10 min with Gene-Amp PCR system (Perkin-Elmer Co., Norwalk, Conn.). PCR products were run and visualised on a 0.7% agarose gel. The 16S rDNA nucleotide sequences was determined by PCR direct sequencing using ABI PRISM 310 Genetic Analyser (PE Applied Biosystems, Foster City, CA) and Big Dye Terminator cycle system (PE Applied Biosystems). Related sequences were obtained from Genebank Database, National Centre for Biotechnology Information (NCBI) using BLAST, Version 2 (Altschul *et al.*, 1990; Park *et al.*, 2004). The sequences were aligned and the consensus sequences was computed using Clustal W software. The evolutionary history was inferred using the Neighbor-Joining method (Saitou N *et al.*, 1987) and the evolutionary distances were computed using the Maximum Composite Likelihood method (Kumar S *et al.*, 2004) MEGA version 5 (Kumar *et al.*, 1993).

Acetylene reduction assay (ARA)

Nitrogenase activity of bacterial strains was determined in semisolid nitrogen free malate medium (by using acetylene reduction assay (ARA; HARDY *et al.* 1973). Pure bacterial colonies were inoculated on to NFM medium in McCartney vials of 10 ml capacity and incubated at $28 \pm 2^\circ\text{C}$ for 48 h. Acetylene gas (10% vol/vol) was injected to the vials. After incubation for 16 h at $28 \pm 2^\circ\text{C}$, gas samples (100 μl) were analyzed on a Hewlett Packard gas chromatograph (Model HP 6890, USA) using Porapak-N column and H₂ flame ionization detector. After completion of ARA the cell were

predigested by adding 10% SDS and sonicated briefly. Protein concentration in the resulting mixture of suspension was determined by Lowry's method. (Lowry *et al.*, 1951).

Indole-3-acetic acid production

IAA production by the isolates was determined by growing the isolates in Burk's medium supplemented with L-tryptophan (100mg/l) at 30°C. The supernatant of the culture fluid was obtained by centrifuging the stationary phase cultures at 10,000rpm for 15min and the pH was adjusted to 2.8 with 1N HCL. The auxins from the acidified cultures extracted with the equal volumes of ethyl acetate (Park *et al.*, 2004; Tienet *et al.*, 1979) was evaporated to dryness and re-suspended in 4ml of ethanol. Analysis of the samples was done by HPLC (Water series 474, USA) using UV detector on a Nova-Pak 5-ODS C-18 column. Methanol: acetic acid: water (30:1:70 v/v/v) was used as mobile phase at a rate of 0.6mlmin⁻¹ (Rasulet *et al.*, 1998). Pure indole-3-acetic acid (Sigma USA) was used as standard. The IAA of the samples was quantified by comparing the retention times, peak areas, and UV absorbance spectra with those of the standard using a Millenium 32 Login software with an interface (Waters, USA) attached with computer.

Phosphorous solubilisation

Test for P-solubilisation was done following Goldstein (1986). The appearance of clearing zone around bacterial colonies after 96h of growth at 30°C was used as indicator for P-solubilisation. Plates inoculated with heat killed cells served as control.

ACC deaminase activity

ACC deaminase activity was determined following the method described by Glick *et al.*, 1995. For this 1 µl of each LB pure bacterial culture was inoculated into agar plates containing NFb or NFb-ACC modified by addition of 1-aminocyclopropane-1-carboxylate (5.0 gl⁻¹) as unique nitrogen source. Plates were incubated at 28°C and observed daily for colony formation for upto 4 days. Colonies were re-inoculated and incubated in the same experimental conditions. Newly colonies formed in NFb with addition of ACC were considered positive for ACC deaminase activity.

Statistical analysis

Results of the measurements were subjected to analysis of variance (ANOVA) using SAS package, Version 8.2 (SAS, 2001).

RESULTS

The isolation and identification of diverse groups of diazotrophs has paved the way to decrease the costs and use of inorganic N-fertiliser as well as minimises the risk of pollution of agroecosystem from the application of chemical fertilisers. Previous researches on isolation of nitrogen fixing bacteria have revealed a broad diversity of diazotrophs associated with different crop rhizosphere (Vessey, 2003). Diverse free living or associative N₂-fixing microorganisms (aerobes, facultative anaerobes, heterotrophs, phototrophs) grew in wetland rice fields and contributed to soil N. The location, variety of rice plant, soil type and ARA, IAA activity and ACC deaminase activity of different isolates from rhizosphere soil has been shown in Table 1. Total 25 free living nitrogen fixing bacterial isolates were isolated on Burk's nitrogen free media. The isolates were then purified and sub-cultured on solid nitrogen free medium.

The ability to reduce acetylene was an indicator of nitrogen fixing potential and was specific for monitoring functional nitrogen fixing potential (Andrade *et al.*, 1997). Out of 25 isolates, 11 were selected for their efficiency in nitrogen fixing, having ARA activity, IAA activity and ACC deaminase activity. Among all the strains KR-23 exhibited highest nitrogenase activity (300 nmolC₂H₄ h⁻¹mg⁻¹protein).

Biochemical characterisation of the strains showed that all the strains were gram -ive, motile rods and having varying metabolic activities. The biochemical characteristics of all the isolates are given in Table 2.

From 16S rDNA sequence analysis of the strains it was observed that ninety nine percent sequence identity was observed between the 16S rDNA sequence of KR-4 with *Pseudomonas putida*; KR-23 and KR-5 with *Spingomonas azotifigens*; KR-6 with *Stenotrophomonas maltophilia*; KR-16 with *Herbispirillum sp.* KR-7 with *Herbispirillum*

Table 1: Plant source, isolate number, location and acetylene reduction assay of different isolates from rhizosphere of rice plant

Native rice cultivars	Bacterial Isolates	Microhabitats	Nitrogenase activity (ethylene/h/mg protein)	IAA activity (mg/ml)	ACC deaminase activity
Ranjit	K1	Akbarpur	19.85±2.35	-	-
	K22	Nilambajar	42.83±3.45	-	-
	K16	Kanisail	205±2.11	1.22±1.09	+
	K2	Nilambajar	11.43±3.45	-	-
Badah	K18	Ghoramara	39.89±4.23	1.04±1.22	+
	K9	Akbarpur	32.11±3.22	0.09±1.89	-
	K3	Kanisail	89.12±4.22	-	-
Jaya	K4	Laxmibajar	280±2.11	2.23±2.21	+
	K5	Chorgola	300.21±3.45	1.45±1.22	+
Biroin	K7	Akbarpur	180±3.45	1.01±1.21	+
	K11	Ghoramara	56.77±2.11	-	-
	K12	Asimganj	90.89±3.24	0.01±1.01	-
	K15	Patharkandi	106.6±4.54	0.89±1.45	+
Kalijeera	K17	Patharkandi	45.67±2.44	-	-
	K6	Asimganj	150±2.32	0.98±1.25	+
	K8	Chorgola	115.66±2.56	1.09±2.21	+
Latma	K23	Ghoramara	290.22±3.45	1.45±1.22	+
	K34	Kanisail	210±2.34	1.87±1.50	+
	K33	Nilambajar	140.77±3.23	0.03±1.22	-
Latiali	K76	Akbarpur	112±1.23	0.11±1.23	-
	K28	Chorgola	68.88±2.55	0.89±1.22	+
	K20	Laxmibajar	54.32±3.23	0.98±1.44	+
	K61	Nilambajar	75.77±3.11	-	-
Pankaj	K81	Chorgola	67.76±2.13	-	-
	K19	Asimganj	56.66±3.24	-	-

Each value represents mean of three replicates. The difference between the results is significant at a level of 5% significance. ($P \leq 0.05$).

Table 2: Biochemical characteristics of diazotrophic isolates

Biochemical tests	K23	K4	K6	K16	K7	K28	K18	K8	K34	K15	K20
Indole test	-	-	-	-	-	-	-	-	-	-	-
Voges-Proskauer test	-	-	-	-	-	+	+	-	-	+	-
Gas production from glucose	+	-	+	-	-	+	+	+	+	+	-
Starch hydrolysis	+	-	-	-	-	-	-	-	-	-	-
Urea hydrolysis	-	-	-	+	+	+	+	-	-	-	-
Nitrate reduction	-	-	+	-	-	-	+	-	+	+	+
Catalase test	+	+	+	+	+	+	+	+	+	+	+
Oxidase test	+	+	-	+	+	-	-	+	-	-	-
Gelatin hydrolysis	-	-	+	-	-	+	+	-	-	-	-



Fig. 1: Phylogenetic tree derived from analysis of the 16S rDNA sequences of Strain KR-23, KR-4, KR-6, KR-16 and KR-7 and related sequences obtained from NCBI. Scale bar, 0.02 substitutions per nucleotide position

rubrisubalbicans, KR-28 with *Klebsiella pneumoniae*, K15 with *Pantoea agglomerans*, KR-34 with *Acinetobacter radioresistance*, KR-18 with *Enterobacter cloaceae* sub sp *dissolvens*, KR-8 with *Alcaligenes faecalis* and KR-20 with *Achromobacter xyloxidans*. Isolation of all the above bacteria were already reported from different crops by many workers (Bhromsiri *et al.*, 2010; Sa *et al.* 2009; Ureta *et al.*, 1995; Gholami *et al.*, 2009; Xie *et al.*, 2006). A phenogram reflecting the relationship among the strains and candidate sequences of various nitrogen fixing strains obtained from database of NCBI has been presented in Fig 1. The sequences obtained were submitted to NCBI with accession numbers KR-4: JN222977, KR-23: JN085438, KR-5: JN085437, KR-6: JN085439, KR-16: JF990839, KR-7: JF906701, KR-28: JN162393, KR-8: JN162397, KR-20: JN162396, KR-15: JN162392; KR-34: JN162392, KR-18: JN162395.

DISCUSSION

Plant growth promoting rhizobacteria offer an environmentally sustainable approach to increase crop production and health. The IAA production and ACC deaminase activity of the microbes help in the stimulation of growth and pathogenesis of the plants. In this study all the 11 strains produced considerable amount of IAA and are also capable of ACC deaminase activity which can be comparable to the earlier studies on various bacteria by different workers (Malik *et al.*, 1997; Sucksstorf and Berg, 2003; Park *et al.*, 2004; Xie *et al.*, 2006). The amount of ARA and IAA activity and the capability of ACC deaminase activity is shown in Table 1. Among all the isolates *Sphingomonas azotifigens*, *Pseudomonas putida*, *Pantoea agglomerans*, *Enterobacter cloaceae* sub sp. *dissolvens*,

Klebsiella pneumoneae and *Herbispirillum* sp can solubilise phosphorous.

This study reports the isolation and characterisation of the strains of *S.azotifigens*, *S. maltophila*, *P. putida*, *Herbispirillum* sp., *H. rubrisubalbicans*, *P. agglomerans*, *A. xyloxidans*, *A. radioresistance*, *A. faecalis*, *E. cloaceae* subsp. *dissolvens* and *K. Pneumoneae* from the inorganic fertiliser rich rhizosphere soil of rice agro-ecosystem of South Assam confirming their nitrogen fixing potential.

Sphingomonas azotifigens is reported from the rice fields of South Assam for the first time in India in our study. All the 11 isolates are capable of IAA production and ACC deaminase activity, among them some are capable of P-solubilisation. The isolates *Pseudomonas putida*, *Sphingomonas azotifigens*, *Herbispirillum* sp and has been regarded as PGPR in earlier studies (Gholami, 2009; Park *et al.*, 2004; Baldani *et al.*, 2009), but *Stenotrophomonas maltophila* and *Herbispirillum rubrisubalbicans*, as producer of IAA and capable of ACC deaminase is also reported first time in our study. These two bacteria are reported as pathogenic and endophytes in many earlier study (Reinhardt *et al.*, 2008; Denton *et al.* 1999; Hale *et al.*, 1972; Gillis *et al.*, 1990). An increased knowledge of the role of these isolates in plant growth promotion under pot culture as well as field condition is required to prove them as plant growth promoting rhizobacteria.

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