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Characterization of plant growth promoting rhizobia from root nodule of *Crotolaria pallida* grown in Assam

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From five different sites of Assam, 22 bacterial strains were isolated from the root nodules of *Crotolaria pallida*. The isolates were subjected to morphological and physiological characterization, and compared with reference strains *Rhizobium leguminoserum* MTCC-99, *Bradyrhizobium japonicum* MTCC-120 and *Mesorhizobium thiogangeticum* MTCC-7001. Although isolates showed close similarity in their morphological features, they had wide variation in their physiological features. All the 10 selected isolates were found to be potent phosphate solubilizer and the isolate DCP1 showed the highest phosphate solubilization efficiency (PSE; 187%). IAA production was detected in 8 isolates with the highest production (66 µg/mL) by MKCP1. The isolates also showed wide variation in their pH and salt tolerance ability. *NifH* gene analysis revealed the presence of *nifH* gene in 6 isolates. The result of PCR-RFLP grouped the isolates into three different 16S rDNA types; 2 isolates were related to *B. japonicum* MTCC-120, 6 isolates were related to *R. leguminoserum* MTCC-99 and 2 isolates were related to *M. thiogangeticum* MTCC-7001.

Keywords: *Crotolaria pallida*, IAA, *nifH*, PSE, rhizobia,16S rDNA

Introduction

Rhizobia are soil bacteria capable of forming root nodules on leguminous plants, in which atmospheric nitrogen is reduced to ammonia. They contribute 65% of total atmospheric nitrogen fixed in an environment and thus reduce the requirement for nitrogenous fertilizers, which in turn reduce adverse environmental effects[1]. Due to their considerable importance in agriculture and environment, rhizobial diversity associated with different leguminous plants has been extensively studied. Rhizobia are important members of plant growth promoting rhizobacteria (PGPR)[2]. They enhance the growth of plants by employing different mechanisms. The beneficial mechanisms includes biological nitrogen fixation, solubilization of phosphorous, production of phytohormones like indole acetic acid (IAA), cytokinin, gibberellin, production of antibiotics, lytic enzymes, hydrogen cyanide and siderophores[3]. Soil pH plays a major role in legume growth as it affects the symbiotic efficiency of rhizobia. Soil pH of Northeast region of India, particularly Assam, is comparatively lower than other parts of India and it mostly ranges from pH 4-6[4]. So the native rhizobial strains can serve as an important source for acid tolerant rhizobia. Salinity also adversely affects the survival and proliferation of *Rhizobium* sp. Isolation and inoculation of salt tolerant rhizobia on legumes strains could enhance the nitrogen fixing ability of the plants growing under saline conditions.

Microbial biodiversity is considered to be one of the most valuable resources and, in recent years, a keen interest has been taken in isolation and selection of environment friendly microorganisms including rhizobia[5]. Earlier it was believed that rhizobia are the members of only α-Protobacteria. However, recent works on rhizobial diversity has reported the isolation of many β-Protobacteria form different leguminous plants[6]. Further, in recent years, rhizobial diversity has been studied using more advanced PCR based genotyping methods in place of earlier traditional phenotypic and biochemical characteristics. Hence, enabling the differentiation among closely related rhizobial strains more accurately than previously considered. 16S rDNA, which codes for 16S rRNA gene, is considered the most authenticated marker for bacterial identification. RFLP analysis of PCR amplified 16S rDNA is a successful technique for studying the diversity of rhizobia[7]. Detection and analysis of *nod* (nodulation) genes that codes for nod

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factor and *nif* (nitrogen fixing) genes that codes for nitrogenase enzyme are also successfully employed for rhizobial characterization[8].

The genus *Crotolaria* belongs to the family *Fabaceae* with more than 500 species. *C. pallida* is a annual shrub, useful in agriculture as fodder crop, as cover plant in plantations, and as green manure in intercropping system[9]. Assam is well known for its highly diverse flora and fauna. Till date no significant work has been reported regarding the characterization of *Rhizobia* associated with *C. pallida* from Assam. The aim of the present investigation is to study rhizobial diversity and to characterize rhizobia associated with root-nodules of *C. pallida* collected from different sites of Assam. Their ability for plant growth promotion activity and to tolerate low acidity and high salt concentration was also assessed in order to select potential PGPR strains for use as a biofertilizer.

Materials and Methods

Collection of Sample

Fresh root nodules of *Crotolaria pallida* plant were collected from five different sites, namely, Cachar, Hailakandi, Karimganj, NC Hills and Tezpur, of Assam. The collected nodules were washed with tap water and surface sterilized with 75% ethanol for 3 min and 0.1% $HgCl_2$ for 5 min. Then they were further washed 5-6 times with sterile distilled water. The surface-sterilized nodules were crushed with the help of sterile glass rod and streaked on yeast extract mannitol agar (YEMA) plate. The plates were incubated at 28±2°C for 3-5 d[10]. Single colonies were picked up and were streaked on YEMA plates for obtaining pure cultures. Pure cultures of three reference strains *Rhizobium leguminoserum* MTCC-99, *Bradyrhizobium japonicum* MTCC-120 and *Mesorhizobium thiogangeticum* MTCC-7001 were also obtained from Institute of Microbial Technology (IMTECH), Chandigarh and maintained as per instruction.

Morphological Characterization

Morphological and microscopic characteristics of all the isolates were investigated. After an incubation of 3 to 7 d at 28°C on YEMA plate, individual colonies were characterized based on their size, colour, shape and elevation[10]. Microscopic features of the isolates were studied by Gram staining technique[1].

Physiological Characterization

The isolates were also investigated for eight different physiological characteristics, namely catalase test, oxidase test, nitrate reduction test, starch hydrolysis test, urease test, ketolactose test, citrate utilization test and gelatin liquefaction test, following standard procedure[11].

Plant Growth Promoting Ability Test of Isolates

For the determination of plant growth promoting ability of the isolates, phosphate solubilization and indole acetic acid (IAA) production were checked.

Phosphate Solubilisation

Ability of the isolates to solubilize inorganic phosphate was detected by spotting the isolates on Pikovskya's agar plates[12]. The solubilization ability of the isolates was expressed in terms of PSE (phosphate solubilisation efficiency).

IAA production

Production of IAA by the isolates was observed on YEM broth medium supplemented with L-tryptophan[13]. The amount of IAA produced by the isolates was determined by comparing the data with a standard graph prepared by measuring the OD with different concentration (10-100 µg/mL) of IAA.

Salt and pH Tolerance

The isolates were streaked and incubated on three different YEMA plates adjusted with three different (1.0, 2.0 & 3.0%) concentration of NaCl[14]. Similarly, ability of the isolates to grow in acidic pH was tested by incubating the isolates on three different YEMA plates adjusted at three different pH (4.0, 5.0 & 6.0)[15]. Growth of the isolates on standard YEMA media (pH 7.0; NaCl 0.1%) was used as control.

Genomic DNA Extraction

Genomic DNA extraction of the isolates was performed using the standard phenol-chloroform extraction procedure[16].

PCR Amplification of *nifH* Gene

PCR amplification of *nifH* gene was carried out in isolates to determine its presence. Primers used for *nifH* gene amplification reaction were zehrf-TGCGACCCAAAAGCAGA and zehr- AAAGCC ATCATCTCACC[17]. Amplification was performed with a total volume of 50 µL containing 1 µL of 30 ng of genomic DNA, 2.5 µL of 2.5 mM dNTP, 1 µL of 100 pmol of each primer, 5 µL of 10× buffer,

0.5 µL of 1.5 U Taq polymerase and 40 µL of nuclease free water. The reaction conditions were: initial denaturation at 95°C for 5 min, denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, extension at 72°C for 45 sec (for 30 cycles) and then final extension at 72°C for 7 min. Amplification products were resolved on 1.5% agarose gel.

PCR-RFLP Analysis of 16S rDNA

In order to study the genetic diversity of the isolates, PCR-RFLP analysis of 16S rDNA was carried out. Universal primers 27f-AGAGTTTGATCATGGCTCAG and 1492r-ACG GATACCTTGTTTACGACTT were used for amplification reaction[18]. Amplification was performed with a total volume of 50 µL containing 1 µL of 30 ng of genomic DNA, 2.5 µL of 2.5 mM dNTP, 1 µL of 100 pmol of each primer, 5 µL of 10× buffer, 0.5 µL of 1.5 U Taq polymerase and 40 µL of nuclease free water. Amplifications were carried out with the following temperature profile: 5 min at 95°C, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 2 min and a final extension for 7 min at 72°C. Amplified products were visualized after electrophoresis on 1% agarose gel.

The PCR products of 16S rDNA of the isolates and the reference strains were digested with restriction enzyme *Hinf*I. The resulting fragments were resolved with electrophoresis on 2% agarose gel. The RFLP patterns obtained from the digestion were observed with a Gel Documentation System. Similarity matrices were constructed and analyzed by UPGMA (Unweighted Pair Grouping with Mathematic Average) cluster analysis using biostatistical analysis software NTSYS[19].

Results

A total of 22 bacterial strains were isolated from the well separated colonies appeared on YEMA plates inoculated with the samples of root nodule of *C. pallida* plant from five different sites of Assam. Among them, 6 isolates, namely, RCP1, RCP2, ICP1, ICP2, KACP1 and KACP2 were from Cachar; 4 isolates, namely, BCP1, BCP2, MKCP1 and MKCP2, were from Karimganj; 4 isolates, namely, GCP1, GCP2, SICP1 and SICP2, were from Hailakandi; 4 isolates, namely, DCP1, DCP2, NCP1 and NCP2, were from NC Hills; and 4 isolates, namely, SCP1, SCP2, TECP1 and TECP2, were from Sivsagar of Assam. Along with the isolates, 3 reference strains, namely, *R. leguminoserum* MTCC-99, *B. japonicum* MTCC-120 and *M. thiogangeticum* MTCC-7001, were simultaneously cultured and compared for their characteristics with the isolated strains.

Most of the isolates showed similar colony morphology and produced white, creamy and raised colonies when grown on YEMA plates. The colony size of the isolates reached 1-3 mm in diameter after 4-5 d of incubation at 28°C. Microscopic examination revealed that the isolated strains were Gram negative and rod shaped. The isolates showed wide variation in their physiological features indicating large diversity among them (Table 1). Based on the results of morphological and physiological characterization and their comparison with reference strains, 10 isolates were selected for further study. Of the 10 isolates, 5 isolates, namely, RCP2, KACP2, SICP2, NCP1 and NCP2, showed similarity with the reference strain *R. leguminoserum* MTCC-99; 3 isolates, namely, GCP1, SCP1 and TECP1, showed similarity with the reference strain *B. japonicum* MTCC-120; and 2 isolates, namely, MKCP1 and DCP1, showed close similarity with the reference strain *M. thiogangeticum* MTCC-7001 in their phenotypic features.

Plant Growth Promoting Attributes

The 10 rhizobial isolates selected after primary characterization were checked for their plant growth promoting abilities. The result of the tests revealed that 8 isolates were capable of producing IAA; however, the amount varied greatly among the strains (Table 2). The highest IAA production of 66 µg/mL was shown by the isolate MKCP1 from Karimganj. Two isolates, KACP2 from Cachar and GCP1 from Hailakandi, were unable to produce IAA. All the isolates were capable of phosphate solubilization as confirmed by the formation of clear zone around the colonies. The highest phosphate solubilization efficiency (PSE) of 187% was shown by DCP1 from NC Hills.

Salt and pH Tolerance

All the isolates grew well at pH 6.0. However, the isolates showed variability in growth at pH 4.0 and 5.0. Of the 10 selected isolates, 2 isolates, namely, KACP2 from Cachar and GCP1 from Hailakandi, grew well at all the pH range studied. Two isolates, RCP2 from Cachar and NCP1 from NC Hills grew well at pH 5.0 and pH 6.0, but unable to grow at pH 4.0. The remaining 6 isolates, MKCP1, SICP2, DCP1, NCP2, SCP1 and TECP1, grew well at pH 6.0 but

Table 1—Collection site of root nodules from *C. pallida* plants and physiological characteristics of rhizobial isolates

Isolates	Sample collection site	Physiological characteristicss							
		Ketolactose	Catalase	Oxidase	Nitrate	Starch hydrolysis test	Urease	Citrate	Gelatin hydrolysis test
RCP1	Cachar (25.08^0N -82.91^0E)	-	+	+	+	-	+	-	-
RCP2	Cachar	-	+	+	-	-	+	-	-
ICP1	Cachar	-	+	+	-	-	+	-	-
ICP2	Cachar	-	+	+	+	-	+	-	-
KACP1	Cachar	-	+	+	+	-	+	-	-
KACP2	Cachar	-	+	+	-	-	+	-	-
BCP1	Karimganj (24.87^0N -92.35^0E)	-	+	+	+	-	+	+	-
BCP2	Karimganj	-	+	+	+	-	+	-	-
MKCP1	Karimganj	-	+	+	-	-	+	-	-
MKCP2	Karimganj	-	+	+	+	-	+	+	-
GCP1	Hailakandi (24.68^0N -92.57^0E)	-	+	+	+	-	+	+	-
GCP2	Hailakandi	-	+	+	+	-	+	-	-
SICP1	Hailakandi	-	+	+	+	-	+	+	-
SICP2	Hailakandi	-	+	+	-	-	+	-	-
DCP1	N.C.Hills (25.18^0N -93.03^0E)	-	+	+	-	-	+	-	-
DCP2	NC Hills	-	+	+	+	-	+	-	-
NCP1	NC Hills	-	+	+	-	-	+	-	-
NCP2	NC Hills	-	+	+	-	-	+	-	-
SCP1	Tezpur (26.63^0N -92.8^0E)	-	+	+	+	-	+	+	-
SCP2	Tezpur	-	+	+	-	-	+	-	-
TECP1	Tezpur	-	+	+	+	-	+	+	-
TECP2	Tezpur	-	+	+	-	-	+	-	-
R. leuminoserum MTCC-99	Reference	-	+	+	-	-	+	-	-
B. japonicum MTCC-120	Reference	-	+	+	+	-	+	+	-
M. thiogangeticum MTCC-7001	Reference	-	+	+	-	-	+	-	-

+ = Positive, - = Negative.

unable to grow at pH 4.0 and 5.0. In the salt test, only 3 isolates, namely, RCP2, GCP1 and SICP2, were able to grow the test concentrations and 4 isolates, namely, DCP1, NCP1, NCP2 and TECP1, grew well at 1% and 2% salt but unable to tolerate 3% salt. The remaining 3 isolates, namely, KACP2, MKCP1 and SCP1, were unable to tolerate 2% and 3% NaCl but grew well at 1% NaCl. The results are presented in Table 2.

nifH Gene Amplification

To screen the ability of isolates to produce nitrogenase enzyme, PCR amplification of *nifH* gene was performed. The results revealed the presence of *nifH* gene in 6 isolates, namely, MKCP1, RCP2, NCP1, DCP1, SICP1 and KACP1, out of the 10 isolates screened (Fig 1). A band at 360 bp was amplified in *nifH* gene positive isolates. In remaining 4 isolates, namely, NCP2, SCP1, GCP1

Table 2—IAA production, phosphate solubilization(PSE), pH tolerance, salt tolerance and presence of *nifH* gene

Isolate	IAA (µg/mL)	PSE (%)	pH tolerance			Salt tolerance			Presence of *nifH* gene
			pH4	pH5	pH6	1%	2%	3%	
RCP2	45	175	-	+	+	+	+	+	P
KACP2	00	143	+	+	+	+	-	-	P
MKCP1	66	122	-	-	+	+	-	-	P
GCP1	00	125	+	+	+	+	+	+	A
SICP2	55	140	-	-	+	+	+	+	P
DCP1	58	187	-	-	+	+	+	-	P
NCP1	40	155	-	+	+	+	+	-	P
NCP2	43	146	-	-	+	+	+	-	A
SCP1	62	163	-	-	+	+	-	-	A
TECP1	36	127	-	-	+	+	+	-	A

+ = Growth, - = No Growth; P = Presence, A = Absence

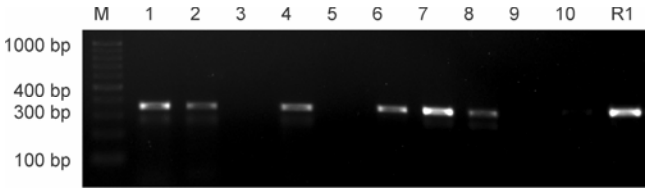

Fig 1—*nifH* gene amplification at 360 bp on 2% agarose gel. [Lanes: M, Marker; 1, DCP1; 2, NCP1; 3, NCP2; 4, MKCP1; 5, SCP1; 6, RCP2; 7, SICP1; 8, KACP1; 9, GCP1; 10, TECP1; & R1, *R. leguminoserum* MTCC-99.]

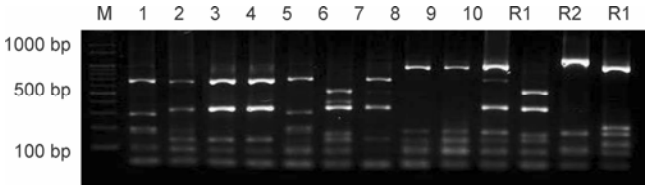

Fig 2—PCR-RFLP profile of isolates on 2% agarose gel. [Lanes: M, Marker; 1, MKCP1; 2, RCP2; 3, NCP1; 4, NCP2; 5, DCP1; 6, SCP1; 7, SICP1; 8, KACP1; 9, GCP1; 10, TECP1; & R1, *R. leguminoserum* MTCC-99. R2. *B. japonicum* MTCC-120. R3. *M. thioganheticum* MTCC-7001.]

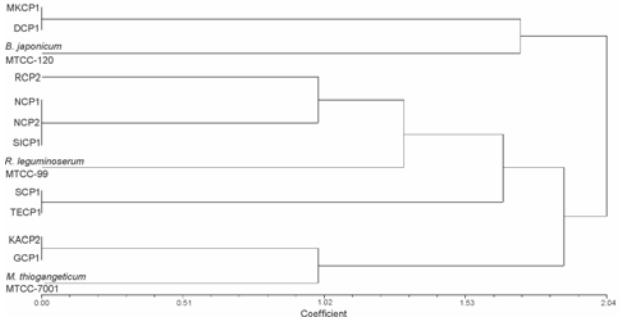

Fig 3—UPGMA dendrogram showing similarity between RFLP of PCR amplified 16S rDNA sequences of rhizobial isolates by restriction enzyme *Hinf*I.

and TECP1, no band amplification of *nifH* gene was observed.

PCR-RFLP Analysis of 16S rDNA

16S rDNA gene that codes for 16S rRNA was amplified. The RFLP pattern obtained from the analysis was heterogeneous, indicating a high genetic diversity among the isolates (Fig. 2). According to the UPGMA dendrogram, the isolates were grouped into three different rDNA types (Fig. 3). The rDNA type I consist of 2 isolates and clustered with reference strain *B. japonicum* MTCC-120. The rDNA type II consists of 6 isolates and clustered with reference strain *R. leguminoserum* MTCC 99. The rDNA type III consists of 2 isolates and clustered with reference strain *M. thiogangeticum* MTCC-7001. The result indicated that the isolates were dispersed in different rDNA types irrespective of their collection sites. However, in contrast to isolates from other sites, the two isolates from NC Hills clustered in a single group rDNA type II.

Discussion

C. pallida is widely found in Assam as a wild legume and it is also grown as a flowering plant by the native peoples of this region since ancient times. In the present study, 22 rhizobial strains were isolated from root nodules of *C. pallida*. The morphological and microscopic features of the isolates were very much similar to the features of reference strains. However, they showed a wide variation in their physiological features. IAA is an important plant hormone and it plays a significant role in plant growth. Many indigenous rhizobial strains with plant

growth promoting ability have already been identified and characterized[20]. Production of IAA by rhizobial isolates of *Crotolaria* spp. was previously reported by Sridevi *et al*[21]. In the present study, of 10 isolates investigated, 8 isolates showed positive result for IAA production. The isolate MKCP1 from Karimganj produced highest amount of IAA and PCR-RFLP analysis revealed that MKCP1 is closely related to *Bradyrhizobium japonicum* MTCC-120. Phosphorus is also an important plant nutrient. The inorganic phosphorus, which is unavailable to the plants, is solubilized by many microorganisms including rhizobia. In the present study, all the isolates were capable of phosphate solubilisation but there was a wide variation in their phosphate solubilisation efficiency. The isolate DCP1 from NC Hills, which is closely related to *Bradyrhizobium japonicum* MTCC-120, showed highest PSE (187%).

Soil pH and salinity plays an important role in limiting the growth of microorganisms. In the present study, those isolates grew well at pH 6 showed variable growth at pH 5 but were unable to grow at pH 4. Similarly, Choudhury *et al*[4] reported the presence of acidic pH tolerant *Rhizobium* strains from different cultivated legumes growing in lower Brahmaputra valley of Assam. In salinity tolerance test, isolates exhibited a wide variation in their salt tolerance, even among isolates from the same site. Most of the strains appear to be sensitive to high salt concentration, except 3 isolates, RCP2, GCP1 and SICP2, that showed growth in all the three salt concentration (1, 2 & 3%). Studying such features (salt & pH tolerance) can be useful for future improvement of rhizobial inoculants by employing suitable genetic engineering techniques[22]. Rhizobia require several classes of specific genes for forming an effective symbiosis. These include *nod* genes, which encode Nod factors, and *nif* genes, which produce the nitrogen-fixing nitrogenase enzyme[8]. The present study reveals the presence of *nifH* gene in 6 isolates confirming their ability to produce nitrogenase enzyme; while 4 isolates showed no amplification of *nifH* gene, which may be explained in several ways including the presence of unique *nif* elements that required to be characterized. Alternatively, these endophytes may lacks nitrogen fixing ability and possibly are only parasitic in nature.

A novel technique for studying rhizobial diversity is restriction digestion of 16S rDNA gene that codes for 16S rRNA. Characterization of rhizobial isolates by 16S rDNA PCR-RFLP was previously reported by many researchers[23]. In the present study, the results of 16S rDNA PCR-RFLP analysis revealed that the isolates are genetically diverse and can be divided into 3 different rDNA types. The results obtained from PCR-RFLP analysis regarding the identity of isolates was in full agreement with that obtained from biochemical analysis. In many earlier studies, most of the rhizobial strains associated with the genus *Crotolaria* were assigned to *Bradyrhizobium*[24]. However, in contrast to the earlier studies, the PCR-RFLP analysis of the present study suggests that most of the isolates are closely related to *Rhizobium* genera. Isolation and characterization of plant growth promoting and stress tolerant rhizobial strains can play a significant role for sustainable agricultural system. The present study increases our knowledge regarding the diversity of nitrogen fixing rhizobial strains associated with *C. pallida* plants growing in Assam. In addition, the present study results in the characterization of rhizobial strains with high plant growth promoting abilities (MKCP1, DCP1) and environmental stress tolerant abilities (RCP2, GCP1, SICP2). Such superior rhizobial strains can be applicable directly as biofertilizers or can be genetically modified for their use in sustainable agricultural systems.

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