Molecular Biotyping, Phylogenetic Analysis of Indole Acetic Acid Producing *Rhizobium* Sp. CU8 and Co-inhabitant Non-rhizobial Nodule Associated Bacteria From *Mimosa Pudica* L.

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Research Article

Keywords: Nitrogen fixers, Phylogenetic analysis, Plant growth promoters, Root nodule, 16S rRNA

Posted Date: August 23rd, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-810699/v1>

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Abstract

The interaction between rhizobia and other nodule associated bacteria assist to mitigate nutrient stress in leguminous plants by fixing atmospheric nitrogen and synthesizing plant growth regulators. Beneficial effects of microbial inoculants emphasize the need for further research and their use in modern agriculture. The present work describes the isolation, identification, plant growth promoting properties and phylogenetic analysis of nodule associated bacteria from *Mimosa pudica* L. Isolation and characterization of nodule associated bacteria were done according to standard procedures. Molecular characterization of the isolates was performed using 16S ribosomal RNA. Plant growth promoting ability was analyzed by quantifying the levels of Indole acetic acid. Evolutionary distance and relatedness was analyzed using neighbor joining method. *Rhizobium* sp. CU8 and three other co-resident non-rhizobial nodule associated bacteria (*Bacillus cereus* MY5, *Ralstonia pickettii* MY1 and *Lactococcus lactis* MY3) exhibiting nitrogen fixation, plant growth promotion and other potential microbial activities were characterized. Phylogenetic analysis revealed the genetic relatedness and evolutionary significance of *Rhizobium* sp. CU8 and other co-inhabitant non-rhizobial nodule associated bacteria from *M. pudica*. Present study identified the four isolates as potential biofertilizers due to their nitrogen fixing and growth promoting characteristics. *L. lactis* MY3 is the first co-resident nitrogen fixer and plant growth promoter reported from the root nodules of *M. pudica*.

Introduction

The members of Leguminosae, are associated with endophytic, root nodule associated bacteria (NAB) which ameliorates nutrient stress by fixing atmospheric nitrogen (N_2) and producing plant growth promoters. The genus *Mimosa* received considerable attention in the recent years because of its potential to fix atmospheric nitrogen. Biological nitrogen fixation is ecologically important, contributing ~ 100–290 million tons of nitrogen annually to the natural ecosystem and enhancing the growth of agronomically important forage and crop plants. Biological nitrogen fixation (both symbiotic and non-symbiotic) reduces the use of synthetic nitrogen fertilizers.

Plant growth promoting bacteria (PGPB) enhance plant growth by fixing atmospheric nitrogen and its assimilation to the plant, production of siderophores to chelate iron and its absorption, solubilization of minerals such as phosphorus, synthesis of phytohormones, augmenting plant nutrient uptake (Glick 1995; Kloepper 1992) and the production of substances like antibiotics (Glick 1995). In addition, the PGP microbes express abiotic stress tolerance like extreme temperature, drought, salinity, pH, heavy metal and pesticide pollution (Gopalakrishnan et al. 2015).

PGPB has beneficial effects on legume growth and some strains enhance the nodulation and nitrogen fixation by effective interaction between plant and rhizobia (Parmar and Dadarwal 1999). Most of the nodulating bacteria are free-living rhizobacteria, however, some are intracellular or intercellular endophytes (Sturz et al. 2000) and gain advantage of being protected from environmental stresses and microbial competition (Kobayashi and Palumbo 2000). The endophytes and epiphytes are the two

different types of plant growth promoting rhizobia associated with host tissue. There are many endophytic and epiphytic bacteria which are directly or indirectly involved in plant growth and development. Endophytic bacteria live in plant tissues without affecting the normal metabolism of the host or gaining any benefit other than a noncompetitive environment inside the host. It has been demonstrated that bacterial endophytes have beneficial effects on host plants, such as growth promotion and biological control of pathogens (Downing and Thomson 2000; Ryu et al. 2005; Sturz et al. 1999). Legume root nodules may contain microbes other than rhizobia (Martínez-Hidalgo and Hirsch 2017; Zgadzaj et al. 2015), however, the function of these co-residents in the nodules is yet to be fully elucidated, their main role might be to assist rhizobia during the nodule infection process and to promote plant growth (Chibeba et al. 2020; Peix et al. 2015).

Modern agriculture faces challenges, such as loss of soil fertility, fluctuating climatic factors and increasing pathogen and pest attacks. Sustainability and environmental safety of agricultural production relies on eco-friendly approaches like use of biofertilizers, biopesticides and crop residue recycling. From the agricultural and ecological viewpoints, “how can we increase food quality and quantity, and to improve the sustainable plant productivity, while maintaining environmental quality?” the importance of nitrogen fixing and plant growth promoting bacteria, their gene conservation can contribute better in sustainable agriculture and 16S ribosomal RNA typing is used to identify microorganisms. The 16S rRNA based phylogenetic analysis revealed the distribution of genotypes in *Rhizobium* sp., *B. cereus*, *R. pickettii* and *L. lactis*. Our understanding of microbial interactions in the rhizosphere must be complemented by combining the basic and applied studies.

The beneficial effects of microbial inoculants, particularly nitrogen fixing and plant growth promoters (PGP), from the root nodule of *M. pudica* accentuate the need for research and its application in modern agriculture. The present study is focused on the isolation, identification, characterization, screening and comprehensive evaluation on phylogenetic relationship based on 16S rRNA gene of potential nitrogen fixers and plant growth promoters from root nodule of *M. pudica*.

Materials And Methods

Isolation of nodule associated bacteria

Bacteriae were isolated from the root nodules of *M. pudica* grown in different locations in the University of Calicut campus (geographic coordinates: 11°08'01.0"N, 75°53'19.0"E; 11°08'00.4"N, 75°53'17.5"E). The nodule associated bacteria (NAB), were isolated from healthy pink coloured root nodules, washed thoroughly using running tap water, surface sterilized using 70% (v/v) ethanol for 30s, 0.1% (w/v) HgCl₂ for 2 min and washed thrice with sterile double distilled water under aseptic condition for one min (Rajendran et al. 2012). Nodules were crushed using a sterile glass rod and the extracts were plated on to Yeast Mannitol agar medium (pH 6.8), supplemented with Congo red dye (0.025 g l⁻¹). The cultures were incubated at 28±2°C for 24-48hrs. Single colony-forming units were checked for purity by repeated

transfer on to nutrient agar medium pH-7(Vincent 1970). Pure cultures were maintained on nutrient agar medium with regular subculturing and used for analysis.

CHNS analysis

Soil samples collected from the rhizosphere of *M. pudica* was used for CHNS elemental analysis using a Euro Vector CHNS Analyser Model No: Ea 3000. Instrument parameters were carrier pressure 100kPa, purge flow 80 mL/min, oxygen flow 20 mL and run for 7 min. Temperature of front furnace was 980°C and GC oven temperature was 105°C. Sample (8.5-9.5 mg) was used for the analysis.

Phenotypic characterization

Bacterial isolates grown in nutrient agar medium were subjected to phenotype characterization based on the morphological and biochemical characters examined using Bergey's manual of systematic bacteriology (Bergeys 2001). The morphological characters were analysed using gram staining, motility test by hanging drop method and endospore staining by malachite green method using a phase contrast microscope. Biochemical analysis was performed using indole production, hydrolysis of urea, methyl red (MR) test, voges proskauer (VP), citrate utilization and nitrate reduction test. The Intrinsic antibiotic resistance of the isolates was determined by disc method with Ampicillin (Amp) (10 mcg/disc), Tetracycline (TE) (30 mcg/disc), and Penicillin G (PG) (10 IU/disc) (Cappuccino and Sherman 1983).

Molecular characterization

DNA extraction, 16S ribosomal RNA typing and sequencing

Genomic DNA of the three species was extracted and purified using CTAB method (Ausubel et al. 1995). The purified DNA was quantified using a Nanodrop 2000 spectrometer (UV scanning Thermo scientific). PCR amplification of the 16S rRNA gene fragment was carried out using the universal primers 1-27F (AGAGTTTGATCCTGGCTCAG) and 1495R (CTACGGCTACCTGTTACGA) (Lane 1991) at an annealing temperature of 50°C. The band size was verified using agarose gel electrophoresis. The PCR products were cleaned and sequenced from Agrigenome Lab Pvt Ltd, Cochin, Kerala. Cloned 16S rRNA sequences were minimally edited and manually aligned using Bioedit software. Species identification and homology between the sequences were identified using BLAST (<https://www.ncbi.nlm.nih.gov/BLAST/>). The cloned sequences were submitted to GenBank, NCBI.

Characterization of plant growth promoting potential of bacterial isolates

Plant growth enhancement potential of the four isolates was verified by the production of indole acetic acid, organic acid and capacity to fix atmospheric nitrogen in plants.

(a). Production of indole acetic acid (IAA)

The isolates with IAA production capacity was identified using bacterial culture grown in nutrient broth supplemented with 0.1% L-Tryptophan(w/v) incubated at 30°C for 48hrs. Indole acetic acid (IAA)

production was analysed using colorimetric method of Gordon and Weber (1951). IAA in the culture was quantified using standard calibration curve prepared using gradient concentrations of IAA.

(b). Production of organic acid

Assessed by growing bacterial culture in calcium carbonate agar [CaCO_3 5 gl^{-1} , glucose 50 gl^{-1} , yeast extract 5 gl^{-1} , agar 15 gl^{-1}] medium and the clear zone around the colony confirmed the production of organic acid.

(c). Assessment of nitrogen fixation

The N_2 fixation capacity was evaluated by growing the cultures in nitrogen free malate containing bromothymol blue medium [DL- Malic acid 5 gl^{-1} , KOH 4 gl^{-1} , K_2HPO_4 0.5 gl^{-1} , $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.05 gl^{-1} , $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$ 0.01 gl^{-1} , MgSO_4 0.01 gl^{-1} , NaCl 0.02 gl^{-1} , CaCl_2 0.01 gl^{-1} , Na_2MoO_4 0.002 gl^{-1} , Yeast extract 0.05 gl^{-1} , Bromothymol Blue 2 mL^{-1} for 24- 48hrs at 30°C in 120 rpm on a rotary shaker (Day and Dobereiner 1976). Colour change of the medium from pale green to pale blue indicates the ability to fix atmospheric N_2 .

16S rRNA based phylogenetic analysis

Phylogenetic analysis based on 16S rRNA sequence were performed using the MEGA 7.0 (Kumar et al. 2016) based on neighbor joining statistical method (Felsenstein 1981) and the branching support of 1000 bootstrap (Felsenstein 1985). The phylogenetic tree construction based on, 16S rRNA was initially performed using the cloned sequence along with sequences retrieved from GenBank and aligned with ClustalW. The model selection was performed using MEGA 7(Kumar et al. 2016) based on the lowest Bayesian Information Criterion (BIC) value (Schwarz 1978). To compare the similarity or diversity, the nucleotide sequences of 40 selected *Rhizobium*, *Bacillus*, *Ralstonia*, *Lactococcus* species from different geographical regions were retrieved from NCBI, GenBank (<http://www.ncbi.nlm.nih.gov/>). The list of sequences retrieved from NCBI with strain name, accession number and locations are given in online resource 1.

Statistical analysis

Using the SPSS software (27.0V, SPSS, Chicago, USA), one way ANOVA was performed to analyze the concentration of IAA in the isolates after 48hrs. Statistical analysis was carried out according to Turkey's test ($P \leq 0.05$). The data were an average of 4 separate experiments with three replicates ($n=3$).

Results

Isolation and CHNS elemental analysis

Four nodules associated bacteria were isolated from the healthy nodules of *M. pudica*. The isolates were purified and subcultured on nutrient agar medium (pH-7). CHNS analysis of the rhizosphere soil showed nitrogen (0.274%), carbon (1.534%), hydrogen (2.094%) and sulphur (0%). The soil was acidic to slightly neutral with a mean pH value of 6.49 ± 0.1 .

Phenotypic characterization

Phenotypic characteristics of the four isolates are presented in Table 1.

Table 1

Morphological, biochemical and physiological features of the four isolates extracted from root nodules of *Mimosa pudica*

Phenotypic characterization	<i>Rhizobium</i> sp. CU8	<i>Bacillus cereus</i> MY5	<i>Ralstonia pickettii</i> MY1	<i>Lactococcus lactis</i> MY3
Morphological Features				
Gram staining	gram negative	gram positive	gram negative	gram negative
Motility	motile	motile	motile	non-motile
Spore production	non sporulating	sporulating	non sporulating	non sporulating
Biochemical Features				
Indole production	+	+	+	+
Hydrolysis of urea	+	+	+	+
Methyl red (MR) test	-	-	-	+
Voges proskauer (VP)	+	+	+	-
Citrate utilization	+	+	+	+
Nitrate reduction test	+	+	-	-
Antibiotic susceptibility	TE,PG, Amp	TE, PG, Amp	TE, PG	TE, PG, Amp
(+/– indicates test is positive or negative. TE– tetracycline, PG– penicillin–G, Amp– ampicillin)				

Molecular characterization

DNA extraction, 16S ribosomal RNA typing and sequencing

Genomic DNA was extracted from the four isolates using CTAB method. The 260/280 ratio of the DNA samples were 1.8-2 indicating the purity of the samples. Molecular characterization by 16S rRNA confirmed that the four isolates are *Rhizobium* sp. CU8, *B. cereus* MY5, *R. pickettii* MY1 and *L. lactis* MY3.

The 16S rRNA gene from the *Rhizobium* sp. CU8, *B. cereus* MY5, *R. pickettii* MY1 and *L. lactis* MY3 possessed 99–100% homology with the nucleotide sequence of other species retrieved from NCBI. *L. lactis* MY3 from the root nodules of *M. pudica* are not reported earlier. GenBank accession numbers were provided for the 16S rRNA gene sequence of *Rhizobium* sp. CU8 (MN744368), *B. cereus* MY5 (MH997483), *R. pickettii* MY1 (MH997486) and *L. lactis* MY3 (MW132401).

Characterization of plant growth promoting activities

(a). IAA production potential of the isolates

IAA production during the 48hr of growth was quantified in *Rhizobium* sp. CU8, *B. cereus* MY5, *R. pickettii* MY1 and *L. lactis* MY3 using Salkowski reagent. *R. pickettii* MY1 and *Rhizobium* sp. CU8 developed colour reaction immediately after the addition of reagents indicating the formation of IAA and better IAA production was observed when the cultures were incubated for 25 min in dark. The highest quantity of IAA was produced in *R. pickettii* MY1 ($49.8630 \pm 0.1779 \mu\text{g/mL}$) followed by *B. cereus* MY5 ($13.5159 \pm 0.2416 \mu\text{g/mL}$), *Rhizobium* sp. CU8 ($11.6895 \pm 0.1837 \mu\text{g/mL}$) and *L. lactis* MY3 ($4.9315 \pm 0.0790 \mu\text{g/mL}$) (Fig. 1) after 48hrs of incubation, which was significant at $P < 0.05$.

(b). Production of organic acid

Among the four isolates, *L. lactis* MY3 showed a clear zone after 24hrs of incubation due to the degradation of calcium carbonate leading to the production of organic acid. Other three isolates doesn't show any clear zone around the colony.

(c). Nitrogen fixing potential of the isolates

The four isolates, *Rhizobium* sp. CU8, *B. cereus* MY5, *R. pickettii* MY1 and *L. lactis* MY3 exhibited N_2 fixing ability grown in nitrogen free malate medium containing bromothymol blue as indicator. The *Rhizobium* sp. CU8, *B. cereus* MY5 and *R. pickettii* MY1 showed significant colour change from pale green to pale blue indicating N_2 fixing ability within 24hrs. However, *L. lactis* MY3 developed the colour change only after 48hrs.

Phylogeny based on 16S rRNA gene

The cloned sequence and the sequences retrieved from the GenBank were used to construct the phylogenetic tree using Neighbor Joining (NJ) method with 1000 bootstraps. Models with the lowest BIC scores were considered to describe the best nucleotide substitution pattern. The TN93 + G (Tamura Nei Model) displayed the lowest BIC scores (10125.617- online resource 2) to construct consensus NJ tree from the aligned sequences (Fig. 2). The multiple sequence alignment based phylogram using MEGA 7.0 and TN93 + G model based on bootstrap analysis of 1000 replicates was performed to estimate the confidence of the tree topologies. The phylogenetic position of the *Rhizobium* sp. CU8, *B. cereus* MY5, *R. pickettii* MY1 and *L. lactis* MY3 in relation to other species of this genus is illustrated in Fig. 2; the numbers adjacent to nodes are the statistical frequency of the indicated species.

The genus *Rhizobium* and *Ralstonia* fall under the same phylum proteobacteria classified as subclass α and β proteobacteria. Based on 16S rRNA homology, both genera are placed in separate group originated from a single node. *L. lactis* and *B. cereus* were grouped as a separate clade.

Phylogenetic tree revealed that *Rhizobium* sp. CU8 and *B. cereus* MY5, showed highest relatedness with other members of the genus. *Rhizobium* sp. CU8 shows closest relatedness with Indian *Rhizobium* sp. S19. *B. cereus* MY5 shows highest similarity to *B. subtilis* IMG04 from India among other members of this genus. In the case of *R. pickettii* MY1 and *L. lactis* MY3, the maximum similarity of these two native strains were shown to *R. pickettii* CP12 from China and *L. lactis* KUMS-T18 from Iran.

Group I *Rhizobium* sp. CU8 and Group II *Ralstonia pickettii* MY1 showed 100% bootstrap support within the genus level. *L. lactis* MY3 in Group III is tightly clustered with *L. lactis* KUMS-T18 with bootstrap support 98%. In Group IV, *B. cereus* MY5 clustered with *B. subtilis* IMG04 with bootstrap support (> 50%). However, none of the isolates that clustered in different phylogenetic trees based on 16S rRNA analysis.

Discussion

The interaction between rhizobia and other nodule associated bacteria are of relevance in the improvement of N_2 fixation and plant growth promotion in leguminous plants (Barea et al. 2005; Ryu et al. 2005). The isolation, characterization and phylogenetic relationship of newly identified bacteria from root nodule of *M. pudica* illustrated the N_2 -fixation and PGP activity of these isolates. The isolation procedure consists of nodule surface sterilization specifically aimed to allow the obtention of nodule endophytes (Rajendran et al. 2008) resulting in the isolation of three non-rhizobial bacteria from *M. pudica* nodule. In the past, bacteria isolated from the nodules with dissimilar growth and appearance to that of typical rhizobia were considered as contaminants and discarded, however, recent studies convincingly demonstrated the occurrence of non-rhizobial bacteria in the nodules and their role on the host plants, rhizobial strains or the symbiosis are under investigation (Martínez-Hidalgo and Hirsch 2017). It is now well recognized that non-rhizobial bacteria can promote plant growth by an array of mechanisms including solubilization and mobilization of nutrients (Srivastwa et al. 2014), N_2 -fixation (Castellano-Hinojosa et al. 2016), production of phytohormones (Chinnaswamy et al. 2018), along with microbial processes. Nodule endophytes belonging to the genera *Bacillus*, *Burkholderia*, *Pseudomonas* and *Enterobacter* have been isolated from different legumes (Dudeja et al. 2012; Martínez-Hidalgo and Hirsch 2017).

The morphological and microscopic features of the isolates were in congruence with the earlier reports. All the isolates were sensitive to standard antibiotics suggesting their prevention mechanisms. The isolates, *R. pickettii* MY1, *L. lactis* MY3, *B. cereus* MY5 and *Rhizobium* sp. CU8 exhibited important agrobiotechnological properties, such as nitrate reduction, production of organic acid, indole acetic acid and ability to fix N_2 . Interestingly, urease activity was also observed in the isolates, indicating the importance of consortia with N_2 fixing bacteria as a requirement for survival (Chibeba et al. 2020).

The present result confirms the nitrogen fixation ability of *R. pickettii* MY1, *B. cereus* MY5, *Rhizobium* sp. CU8 and *L. lactis* MY3. Zhao et al. (2011) reported endophytic non-rhizobial *Bacillus cereus* and *Ralstonia* spp. are potent N₂ fixers (Bulut 2013). The genus *Rhizobium* is the first bacteria participating in nitrogen fixation in legumes (Lindström and Mousavi 2020).

L. lactis MY3 is a rare observation from the root nodule of *M. pudica* and can be used as an agent for plant growth promotion (Lamont et al. 2017). According to Higdon et al. (2020), *Lactococcal* bacteria exists as a diazotroph in maize without nifHDKENB homologs, and hypothesized that *L. lactis* isolates from the mucilage microbiota of *Sierra Mixe* maize possess genes enabling BNF activity and elucidated that all the important genes for the BNF trait in *L. lactis* underpinning the ability to fix atmospheric nitrogen present in the mucilage-derived *lactococci*, which supports the hypothesis that *lactococci* can exist as diazotrophs. *L. lactis* MY3 develop organic acid indicating the interactions between PGPR and plants can enhance the secretion of organic acids, which play an important role in the process of the activation and absorption of insoluble nutrients by plants (Pii et al. 2015).

A diverse group of microbes, including free living, epiphytic and tissue colonizing bacteria synthesize IAA (Patten and Glick 1996). The four strains produced considerable quantity of IAA, which is comparable with earlier studies on various bacteria including *Rhizobium* sp., *B. cereus*, *R. pickettii* and *L. lactis* (Kumar and Ram 2012; Kuklinsky-Sobral et al. 2004; Mohite 2013; Strafella et al. 2021). According to Datta and Basu (2000), most of the studies reported that IAA producing organisms are gram negative, however, few *Bacillus* known to produce IAA which is gram positive strains (Wahyudi et al. 2011). Present study showed that *B. cereus* MY5 is IAA producing gram positive bacteria.

The evolutionary history was derived using the neighbor joining method based on the Tamura-Nei model (Tamura and Nei 1993). The bootstrap consensus tree developed from 1000 replicates represented the evolutionary history of the taxa analyzed (Felsenstein 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein 1985). *Rhizobium* sp. CU8 and *B. cereus* MY5 showed highest similarity with native strains.

Conclusions

This study reports the isolation, molecular identification, characterization and phylogenetic relationship of the *Rhizobium* sp. CU8, *R. pickettii* MY1, *L. lactis* MY3, and *B. cereus* MY5 from the root nodule of *M. pudica*. The biochemical analysis confirms the nitrogen fixing potential, plant growth promotion and other potential microbial activities of the obtained isolates. The bacteria with N₂ fixing capacity act as plant growth promoters hence can be used as biofertilizers. *L. lactis* strain MY3 is a new report from the root nodule of *M. pudica* with plant growth promotion and N₂ fixation capacity. Phylogenetic analysis using neighbor joining method showed the relatedness and evolutionary position of the isolates with native strains as well as other geographical locations retrieved from NCBI. The analysis showed that non-

rhizobial bacteria, *L. lactis* MY3 and *B. cereus* MY5 may co-exist with *Rhizobium* sp. CU8 and *R. pickettii* MY1 in the root nodule of *M. pudica*.

However, it requires further studies to assess the role of these isolates in N₂ fixation and plant growth promotion under pot culture as well as in field condition and these can be used as a potential biofertilizer.

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Figures

Figure 1

The quantity of IAA produced in different bacteria spp. isolated during 48hrs of culture. The different letters indicates the significant difference at the P<0.05 level. Values are given as mean±SE for each sample

Figure 2

Neighbor joining tree constructed using 16S rRNA gene sequences of isolates and the homologous gene sequences retrieved from NCBI with homologous sequences obtained from NCBI. The species represented in boxes indicates the isolates from the study

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