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Isolation of endophytic plant growth promoting *Burkholderia* sp. MSSP from root nodules of *Mimosa pudica*

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Endophytic bacteria reside within plant tissues and have often been reported to promote plant growth. Rhizobia are particularly known for their symbiotic relationship with legumes. A bacterial strain MSSP was isolated from surface-sterilized root nodules of *Mimosa pudica*. MSSP was Gram-negative, capsulated, motile, non-endospore forming rod with free nitrogen (N) fixation ability. Unlike N-fixing bacteria forming symbiotic relationship with legumes that largely exist in α-subclass of proteobacteria, MSSP belongs to β-class of proteobacteria. Phylogenetic analysis of 16 S rDNA demonstrated that MSSP belongs to the genus *Burkholderia*. This isolate secretes phytohormone, ACC deaminase, solubilizes phosphate and is antagonistic against phytopathogens.

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PLANT growth promoting endophytic rhizobacteria are involved with host plants in mutual interaction. They promote plant growth directly or indirectly, via production of phytohormones, biocontrol of host plant diseases or improvement of plant nutritional status[1]. Rhizobia are perhaps the best-known beneficial plant-associated bacteria because of their importance in nitrogen fixation[2]. Forthcoming *Bergey's Manual of Systematic Bacteriology* identifies five genera of rhizobia, namely *Rhizobium*, *Sinorhizobium*, *Azorhizobium*, *Bradyrhizobium* and *Mesorhizobium* (www.cme.msu.edu/bergey's, p. 7). They all belong to the α-class of proteobacteria, where they are distributed in four distinct phylogenetic branches. However, during the last few years, bacteria from the β-class of proteobacteria, other than rhizobia have been reported from legumes[3]. Here we report a species of *Burkholderia* from the root nodules of *Mimosa pudica*, which belongs to the β-class of proteobacteria.

Plants of *M. pudica* were uprooted from the campus of Botanical Survey of India, Dehradun. Nodules were collected, washed several times in sterile water, surface-sterilized using 70% ethanol and 0.1% $HgCl_2$, and repeatedly washed with sterile water. Sterile nodules were crushed and the resulting suspension was streaked on yeast extract mannitol (YEM) agar plates[4], which were incubated at 28 ± 1°C. Pure isolates were subjected to phenotypic and biochemical characterization, wherein all isolates appeared similar. Effect of temperature and salinity was observed in 50 ml YEM broth in flasks. The flasks with 0.01, 0.1, 1.0 or 2.0% NaCl were inoculated with log phase culture to a final concentration of 10^3 cfu ml^{-1} and incubated at 28, 35 or 40°C at 150 rpm. Uninoculated broth served as control. Absorbance was measured after different time intervals and growth monitored at 610 nm. Acid production was tested in YEM agar plates supplemented with 25 ppm bromothymol blue indicator dye[5]; change in colour from green to blue indicated alkali reaction, and to yellow, acid production. Carbon source utilization was detected in basal liquid medium[6] (BLM) which contained K_2HPO_4, 1 g; KH_2PO_4,1 g; $FeCl_3.6H_2O$, 0.001 g; $MgSO_4.7H_2O$, 0.2 g; $CaCl_2$, 0.1 g; bromothymol blue indicator dye, 25 mg; DDW, 1000 ml with carbon source at a final concentration of 1 g l^{-1}. This was dispensed in tubes (20 mm) as 5 ml aliquot and inoculated by an exponentially growing bacterial culture (≈ 10^9 cfu ml^{-1}); tubes were incubated at 28 ± 1°C at 150 rpm for 4 days. Change in colour from green to yellow or blue indicated utilization of respective C-source. BLM supplemented with mannitol served as positive control, and that without any C-source as negative control.

Root nodulating ability of MSSP was determined. Surface sterile seeds of *M. pudica*, bacterized[7] with isolate MSSP were sown in pots with sterile soil. After four months,

isolate MSSP formed nodules. Colonies re-isolated from surface-sterilized nodules exhibited phenotypic and biochemical characteristics of strain MSSP verifying Koch's postulate.

Strain MSSP (*M. pudica*) was identified by rDNA analysis. Pure cultures were grown till log phase and genomic DNA was isolated according to Bazzicalupo and Fani[8]. A large fragment of 16S rDNA was amplified by PCR using primers 5′ AGG AGG TGA TCC AAC CGC A 3′ and 5′ AAC TGG AGG AAG GTG GGG AT 3′. A 50 µl reaction mixture included 5–10 ng of DNA as template, 1 µM of each primer, 1 U of Taq DNA polymerase and 100 µM dNTPs. The PCR product was purified and sequenced. Searches in the EMBL/Gen Bank/DDBJ/PDB data libraries were performed using BLAST (blastn) search algorithm[9] in order to establish the identity of the isolate. Sequences of the close relatives were retrieved and aligned with the newly determined sequences. Multiple alignments were performed with CLUSTALX[10]. Phylogenetic analysis used the neighbour-joining method and programs[11] in PHYLIP version 3.6. The 16 S rDNA sequence of the following strains (type strains unless otherwise indicated) were obtained from GenBank (accession numbers in parentheses): *Burkholderia* sp. AB 101 (gb/AF219126), *Burkholderia* sp. AB2 (gb/AF219125), *Burkholderia* sp. CEB01056 (emb/AJ491304), *Burkholderia* sp. m35b (gb/U96937), *Burkholderia pyrrocinia* (dbj/AB021369), *B. cepacia* (gb/U96927), *B. vietnamiensis* (gb/U96929), *B. multivorans* (dbj/AB092606), *B. gladioli* (dbj/AB024491), *B. plantarii* (gb/U96933), *B. glumae* (gb/U96931), *B. mallei* (gb/AY305760), *B. pseudomallei* (gb/AY305818) and *B. phezaninium* (gb/U96936).

A neighbour-joining dendrogram was generated using the sequence from MSSP and representative *Burkholderia* sequences from EMBL/Gen Bank/DDBJ and PDB (Figure 1). Phylogenetic analysis of 16 S rRNA of MSSP strain revealed that it does not belong to any of the four branches of rhizobia known in the α-subclass of Proteobacteria, but belonged to the β-subclass of Proteobacteria. From this analysis, it appeared to belong to a new species of *Burkholderia*. MSSP was separated from other Burkholderiae clusters (except

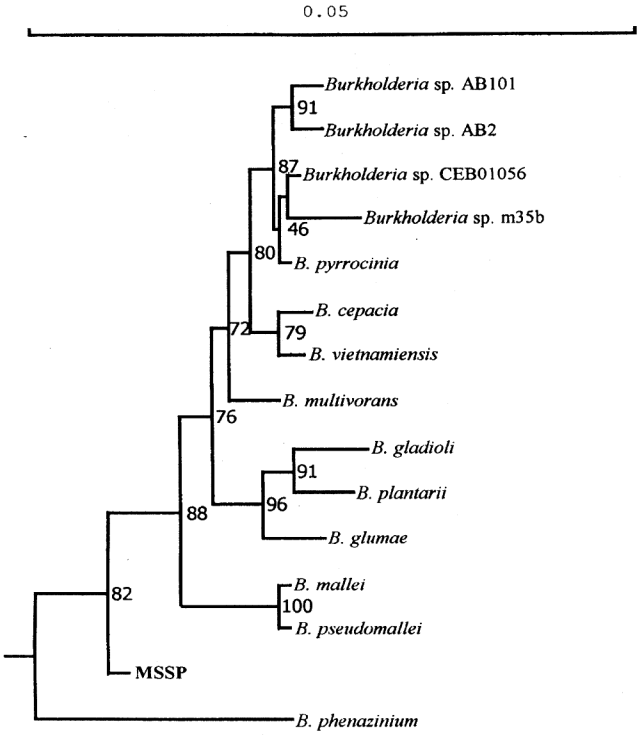

Figure 1. Phylogenetic relationship between MSSP and representative *Burkholderia* species based on full length 16 S rDNA sequences constructed using the neighbour joining method. Bootstrap values (from 1000 replication) are indicated. Accession numbers are reported in text.

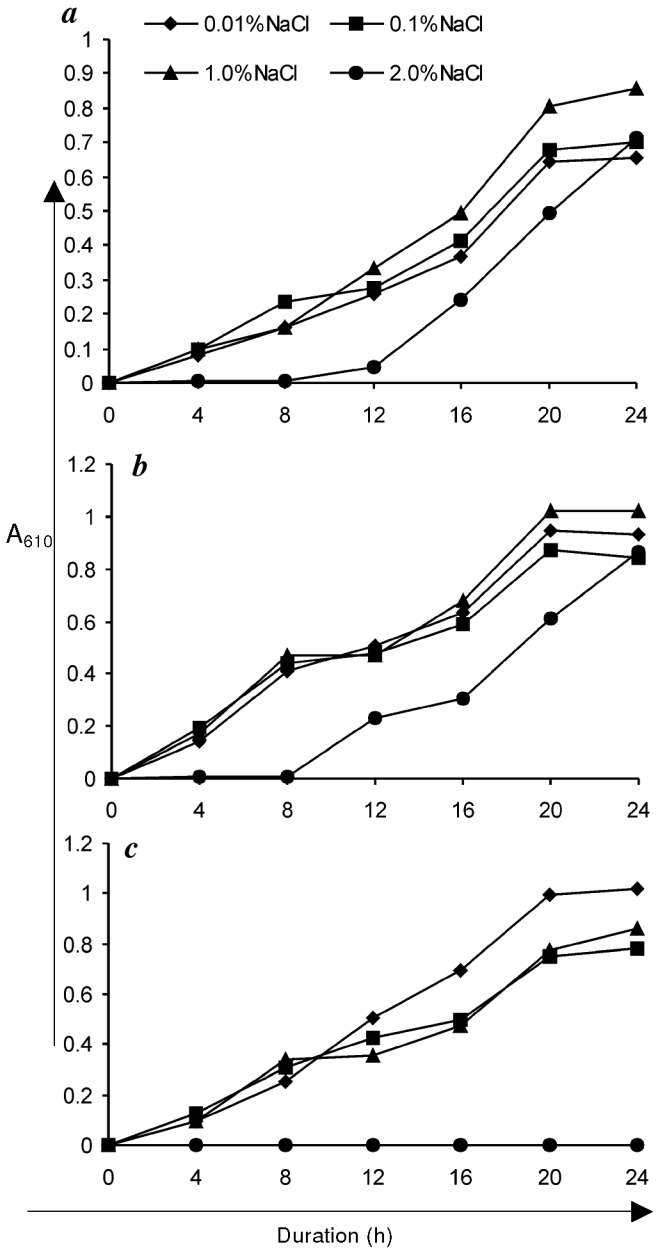

Figure 2. Effect of salinity and temperature on growth of MSSP: (*a*) 28°C, (*b*) 35°C and (*c*) 40°C.

Table 1. Phenotypic and physiological properties of *Burkholderia* sp. MSSP

Isolate		MSSP	
Host		*M. pudica*	
Colony diameter (mm) on YEMA (3 days, 28°C)		2–3	
Growth at	28°C	35°C	40°C
0.01% NaCl	Positive	Positive	Positive
0.10% NaCl	Positive	Positive	Positive
1.00% NaCl	Positive	Positive	Positive
2.00% NaCl	Positive	Positive	No growth
Acid production on YEMA		Positive	
Gram reaction		Negative	
Motility		Positive	
Capsule		Positive	
Endospore		Negative	
Shape		Rod	
Starch hydrolysis		Positive	
Gelatin hydrolysis		Positive	
H_2S production		Negative	
Catalase		Positive	
Oxydase		Positive	
Growth on glucose peptone agar		Positive	
Growth on Hofer's alkaline medium		Negative	
C-source utilization			
Sucrose		Positive ↑	
Galactose		Positive	
Ribose		Negative	
D-xylose		Positive ↑	
Mannitol		Positive	
Maltose		Positive	
Mannose		Negative	
Inositol		Negative	
Arabinose		Positive	
Plant growth promoting activities			
ACC deaminase production	Positive (0.45 IU/ml after 72 h)		
Nitrogen fixation	3.14 *n* mol C_2H_2/min/mg protein		
Phosphate solubilization	Phosphate solubilization index (PSI) = 2.15		
IAA production		Positive	
Siderophore production		Positive	
HCN production		Positive	
Antagonistic activity	*Rhizoctonia solani* – Positive		
	Sclerotinia sclerotiorum – Positive		
	Fusarium oxysporum – Negative		
	Macrophomina phaseolina – Negative		

↑, Gas production; PSI, Clear zone diameter/growth diameter.

B. phezaninium) with bootstrap value of 82%. As per our knowledge, this is the first report of *Burkholderia* sp. isolated from nodules of *M. pudica*. Earlier, *Burkholderia* strain STM 678 and *Burkholderia* strain STM 815 have been reported from the nodules of *Aspalathus carnosa* and *Machaerium lunatum*[12] respectively. However, *Ralstonia taiwanensis*[13] and *Ralstonia eutropha*[14] have earlier been reported from the nodules of *Mimosa*. The nucleotide sequence of 16S rDNA of MSSP is as follows (GenBank accession number: bankit 602753 AY551271):

AGGAGGTGAT CCAACCGCAC CTTCCGATAC GGC
TACCTTG TTACGACTTC
ACCCCAGTCA TGAATCCTAC CGTGGTGACC GTC
CTCCTTG CGGTTAGACT
AGCCACTTCT GGTAAAACCC ACTCCCATGG TGT
GACGGGC GGTGTGTACA
AGACCCGGGA ACGTATTCAG CGCGGCATGC TGA
TCCGCGA TTACTAGCGA
TTCCAGCTTC ATGCACTCGA GTTGCAGAGT GCA
ATCCGGA CTACGATCGG
TTTTCTGGGA TTAGCTCCCC CTCGCGGGTT GGC
AACCCTC TGTTCCGACC
ATTGTATGAC GTGTGAAGCC CTACCCATAA GGG
CCATGAG GACTTGACGT
CATCCCCACC TTCCTCCAGT T

MSSP is Gram-negative, capsulated, motile, non-spore forming rod. Phenotypic and physiological properties of *Burkholderia* sp. MSSP are given in Table 1. At 28 and 35°C,

lower concentrations of NaCl (0.1 and 1.0%) favoured growth of MSSP, while 2% NaCl extended the lag phase. At 40°C, the organism failed to multiply in 2% NaCl, although it grew fairly well at lower concentrations (Figure 2).

Despite the large diversity of legumes (up to 18000 species) and a great number of species forming nodules, rhizobia of ~11,200 nodulating species are uncharacterized. Therefore, current taxonomy and phylogeny of rhizobia are based on isolates from nodules of only 10% of 750 genera. As suggested, India is a mega biodiversity centre and studies of symbionts of the yet unexplored legumes may unravel the presence of new types of bacterial groups in their nodules[14]. The plant growth promoting activity of MSSP was determined by accessing the factors which directly or indirectly benefit the plants[1]. ACC deaminase production[15], nitrogen fixation[16], phosphate solubilization[17], IAA production[18], siderophore production[19], HCN production[20] and antagonistic activity[21] against different phytopathogens. *Burkholderia* sp. MSSP was positive for almost all these characteristics, indicating its role in promotion of growth of host plant while colonizing the roots.

Microbial diversity is considered as one of the most useful resources for bioprospecting. Rhizobia are of particular interest due to their symbiotic nitrogen fixing ability with legumes. As more knowledge is acquired and isolates from unexplored legumes are studied, new species are discovered and former species are split. 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[22] and recently reviewed[3].

Note added in proof: Subsequent to the submission of this paper, the article entitled 'Isolation and anti-fungal activities of 2-hydroxymethyl-chroman-4-one produced by *Burkholderia* sp. MSSP' by J. G. Kang *et al.* was published in *The Journal of Antibiotics* (2004, **57**, 726–731).

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