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Isolation of Plant-Growth-Promoting Endophytic Bacteria from Bean Nodules

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Abstract: Plant growth promoting rhizobacteria (PGPR) was isolated from bean (*Phaseolus vulgaris* L.) nodules. DLA strain was found to increase bean yield and growth when plant were co-inoculated with *Rhizobium leguminosarum* bv. phaseoli under nitrogen-free conditions, compared to plant inoculated with *R. leguminosarum* bv. phaseoli alone. DLA strain was Gram-positive spore-forming rods. Biolog tests indicated that the DLA strain belonged to the genus *Bacillus*. Phylogenetic analysis of 16S rRNA gene hypervariant region sequences demonstrated that DLA strain is *Bacillus subtilis* strain.

Key words: bean, nodule, *Bacillus* sp., plant-growth-promoting bacteria

INTRODUCTION

Plant growth promoting rhizobacteria (PGPR) can promote plant growth directly or indirectly[1]. Indirect promotion of plant growth is through antibiosis via antibiotic type compounds[2], through enhanced resistance to pathogenic diseases and/or abiotic stresses such as frost damage[3]. It is in the understanding of these mechanisms that promising alternatives for new inoculants are being discovered. Direct methods of increasing plant growth are through phytohormones, such as auxin, cytokinin and gibberellin. Screening PGPR isolates for quantity of auxin production has been proposed as a mechanism for selecting PGPR species for wheat inoculation[4]. Gibberellin is produced by many PGPR strains[5]. Included within the PGPR grouping is endophytes[6] as well as the nitrogen fixing rhizobia[7]. Endophytes are sheltered from environmental stresses and microbial competition by the host plant, and they seem to be ubiquitous in plant tissues, having been isolated from flowers, fruits, leaves, stems, roots, and seeds of various plant species[8]. Bai *et al*. [9] reported the isolation of 3 nonrhizobial species from soybean root nodules. Endophytic PGPB have the potential to be used as agricultural inoculants[6]. In this paper, we describe isolation and identification of a plant growth promoting endophytic bacteria from a fields-grown bean plant, and an additional objective was to determine the importance of combining *Bacillus* sp. and *Rhizobium* sp. for plant growth promotion of bean plant.

MATERIALS AND METHODS

Isolation of endophytic bacteria: Bean (*Phaseolus vulgaris* L.) seedlings at the R3 stage[10] were selected at the Ste-Anne-de-Bellevue, Quebec, Canada. The roots were washed thoroughly, and 20 healthy nodules were detached along with a portion of the root. The nodules were placed in sterilized petridish and were surface sterilized by rinsing with 95% ethanol for 20 sec, and then with acidified 0.1% $HgCl_2$ solution for 5 min. The nodules were then rinsed 5 times by sterile H_2O. Twenty nodules were placed into separate sterile Eppendorf tubes with 1 mL of sterile H_2O. Immediately following surface sterilization, the nodules were crushed aseptically, nodule contents were streaked onto PBY plates[11], and the plates were incubated at 30 °C for 2 days. Non-*Bradyrhizobium* colonies were chosen on the basis of colony morphology and growth rate. Colonies were picked and then were purified by single-colony streaking on LB plates. The putative nodule endophyte strains were designated as DLA.

Plant material and cultivation conditions: Seeds of bean (*Phaseolus vulgaris* L.) was surface-sterilized in 2% sodium hypochlorite for 3 min and then rinsed 5 times with distilled water[12]. The seeds were germinated and grown in sterilized vermiculite in trays. After 5-8 days after

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seedling, one seedling was transplanted into each ø11 cm pot, containing 1,000 mL of sterilized sand and turface (2:1, v:v) m ixture. At three d a ys after transplanting, the healthy seedlings were inoculated with induced *R. leguminosarum* bv. phaseoli 127K105 (Nitragin Inc., Milwakee, WI, USA) as described below. Plants were watered daily with a half strength modified nitrogen-free Hoagland's solutions[13], in which the $Ca(NO_3)_2$ and KNO_3 were replaced with 0.5 mM $CaCl_2$, 0.5 mM K_2HPO_4, and 0.5 mM KH_2PO_4 to provide nitrogen free nutrient solution. The temperature in the greenhouse was maintained at 25±2 °C with a relative humidity of 75% and a 16 hr photoperiod created by using supplemental lighting from high-pressure sodium lamps. All plants were harvested 30 days for bean after transplanting. The experiment was structured following a randomized complete block design (RCBD) with four replications. *Rhizobium leguminosarum* bv. phaseoli and DLA strain was cultured in LB medium and incubated on an orbital shaker at 150 rpm for 48 h at 27ºC. The cells in cultured bacterial broth were collected by centrifugation at 2,822 x g for 15 min at 4 °C and washed with sterilized tap water. The pelleted cells was resuspended with sterilized tap water and then the cells were adjusted to about 10^8 cells mL^{-1}, based on an optical density $OD_{620} = 0.08$[12]. One mL of inoculum was applied to each seedling. Plant growth and nodulation measurements included plant height, leaf greenness (SPAD-502, Minolta, Japan), leaf area (Delta-T Devices, Cambridge, UK), nodule number and nodule dry weight, shoot and root dry weight (Zhang *et al.*, 1995). The photosynthesis of plants was measured using Li-Cor 6400 (Li-Cor Inc, USA), respectively. All data were analyzed statistically by analysis of variance using CoStat software (CoHort softwate, Monterey, USA). Means comparisons were conducted using an ANOVA protected least significant difference (LSD) ($P<0.05$) test. Treatments were compared using a randomized complete block model with four replications of each treatment.

Phenotypic characterization, extraction of plasmid and genomic DNA of DLA strain: The cultured DLA cells were found to be Gram positive and were assayed for carbon utilization using Biolog GP Microplates (Biolog Inc., Hayward, Calif.), following the manufacturer's instructions. *Bacillus cereus* was used as controls. DLA strain was identified by Biolog test, and named as *Bacillus* sp. DLA. All identification process of this strain is based to Bai *et al*.[9].

Genomic DNA was extracted from DLA strain grown in LB broth at 30 °C using the standard lysozyme-SDS-Pronase protocol[14]. Plasmid DNA was isolated from cultures grown in LB plus ampicillin (50 ug mL^{-1}) at 37 °C, using QIAprep spin miniprep kits (Qiagen) according to the manufacturer's instructions. The DNA was purified using DNeasy Tissue kits (Qiagen, Mississauga, Ont.). PCR products, and plasmid DNA to be used as template in DNA sequencing reactions, were excised from agarose gels and purified using QIAEX II gel extraction kits (Qiagen).

PCR amplification and DNA sequencing: The complete 1.6-kb 16S rDNA region was amplified using the universal bacterial 16 rDNA primers 27f (5'-AGA GTT TGA TCM TGG CTC AG) and 1492r (5'-TAC GGY TAC CTT GTT ACG ACT T)[9]. The PCR amplification of the target sequence was carried out in a total volume of 50 µl of the following reaction mixture: 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 1.5 mM $MgCl_2$, 200 µM dNTPs, 25pmol of each primer, 2 µl of the template, and 2.5 U of Taq DNA polymerase (Takara Shuzo, Otsu, Japan). The PCR was performed in a GeneAmp PCR system 2400 (Perkin-Elmer, Norwalk, CT, U.S.A.) using the following protocol: initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 sec, annealing at 58 °C for 30 sec, and an extension at 72 °C for 30 sec, followed by an additional extension at 72 °C for 5 min.

The DNA sequencing was performed using an ABI prism 377XL DNA sequencer (Applied Biosystems, Foster City, CA, U.S.A.). The sequences were compared with databases available at the National Center for Biotechnology Information (NCBI) using the BLAST program.

Phylogenetic analysis: Phylogenetic trees were reconstructed by the neighbor-joining method[15], using the distance matrix from the alignment. 16S rDNA sequence of the following strains (type strains, unless otherwise indicated) were obtained from GenBank (accession numbers in parentheses): *B. weihenstephanensis* (AB021199); *B. mycoides* (AB021192); *B. thuringiensis* WS2625 (Z84587); *B. mojavensis* (AB021191); *B. vallismortis* (AB021198); *B. atrophaeus* (AB021181); *B. s ubtilis* (X60646); *B. carboniphilus* (AB021182); *B. psychrosaccharolyticus* (AB021195); *B. marinus* (AB021190); *B. flexus* (AB021185); *B. niacini* (AB021194); *B. megaterium* (D16273).

RESULTS AND DISCUSSIONS

Plant growth: We isolated a plant growth promoting endophytic bacteria from a fields-grown bean plant, and co-inoculated *R. leguminosarum* bv. phaseoli with DLA

Table 1: DLA strain effects on yield, photosynthetic rate and growth of bean seedlings grown for 4 weeks under greenhouse conditions

Strain	Leaf greenness (SPAD)	Leaf area (cm^2 $plant^{-1}$)	Photosynthetic rate (umol m^{-2} s^{-1})	Nodule number ($plant^{-1}$)	Nodule dry weight ($plant^{-1}$)	Dry weight (g $plant^{-1}$)		
						Leaf	Root	Total
Control	31.2b	232	10.8b	216b	0.160b	0.871b	0.152	1.023b
DLA	35.5a	256	12.1a	278a	0.192a	0.960a	0.169	1.129a

* LSD at the 5% level.

Table 2: Utilization of various carbon sources by the isolate *Bacillus* sp. DLA

Carbon sources	Results	Carbon sources	Results	Carbon sources	Results
D-trehalose	-[a]	D-mannitol	+	D-alanine	+
Methyl pyruvate	+	D-mannose	-	L-alanine	+
Glycerol	-	D-melibiose	-	L-asparagine	-
Dextrin	+	D-raffinose	+	L-glutamic acid	-
Glycogen	+	L-rhamnose	-	L-histidine	-
Tween 20	+	D-sorbitol	+	Hydroxy-L-proline	-
L-arabinose	+	Sucrose	-	L-leucine	-
L-arabitol	+	Acetic acid	-	L-ornithine	-
D-fructose	-	cis-aconitic acid	-	L-phenyl alanine	+
L-fucose	-	Citric acid	+	L-proline	+
D-galactose	+	Formic acid	+	D-serine	-
"-D-glucose	-	D-galactonic acid	+	L-serine	-
m-inositiol	+	"-keto butyric acid	+	(-amino butyric acid	+
cellobiose	-	"-keto glutaric acid	-	Thymidine	+
i-erythritol	+	DL-lactic acid	+	Phenyl ethylamine	+
"-lactose	-	Propionic acid	-	2,3-butanediol	-
Maltose	-	Succinic acid	-	L-aspartic acid	+

[a]Symbols; +, positive; -, negative

strain. Results of the measurement of growth response and photosynthetic rate are given in Table 1. Shoot and total dry weight under greenhouse conditions were significantly increased by DLA strain treatment. Total dry weight in treatment containing DLA strain was increased by 10.4%. Leaf greenness under DLA strain treatment was significantly increased but leaf area was not increased, compared to control. Nodule number and nodule dry weight was increased by 28.7 and 20.0%, compared to the control treatment. Photosynthetic rate in DLA strain treatment was increased by 12.0%, compared to control. In this study, inoculation *R. leguminosarum* bv. phaseoli with DLA strain could increase plant growth and photosynthetic rate, compared to control treatment.

Phenotypic characterization: DLA strain was determined to be Gram-positive spore-forming rods. This strain showed the growth curve by cultivation time in Fig. 1, and optical density (A_{600}) was continuously increased after 38 hr. This strain did not identified at the species level using the Biolog system, because of a very high percentage of false-positive results. Despite numerous attempts, the SIM values for the DLA strains, and the *B. cereus* control (0.421), were below the threshold of 0.5 acceptable for species identification. The Biolog database matches with the highest SIM values were to *B. subtilis* for DLA strain (0.401) (Table 2). Therefore, these test indicated that the DLA strain was *Bacillus* sp.

Analysis of 16S rDNA sequences: Phylogenetic analysis of the hypervariant (HV) sequences of the DLA strain and key *Bacillus* species (Fig. 2) demonstrated that DLA sequence was strain of *B. subtilis*. Single PCR products of the expected size (1.6 kb) were amplified from DLA using

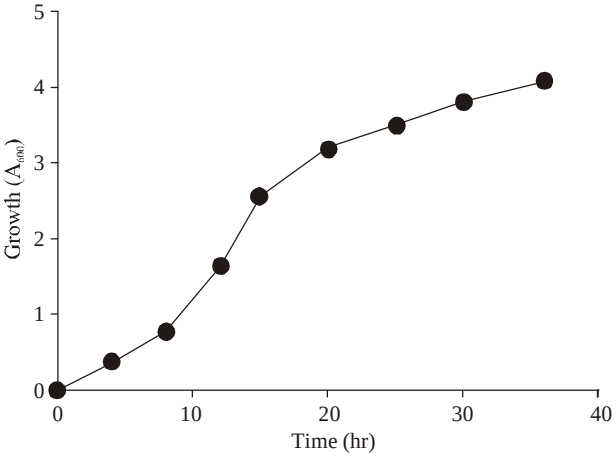

Fig. 1: Effect of culture time on the growth of *B. subtilis* DLA

bacterial 16S rDNA primers. The PCR products were cloned, and single strand sequences of 400-450 nucleotides from both ends of each clone were determined. The DLA strain sequence was identical with *B. subtilis* and/or *B. subtilis* NEB4. BLAST comparisons revealed that the DLA sequences have very high homology to the 5' and 3' ends of *B. subtilis* 16S rRNA genes. The clusters into which the DLA HV sequences fit were the same as those reported by Goto *et al*.[16] and had high bootstrap support. In addition, the sequences obtained for this strain was 100% identical to previously characterized *Bacillus* strains. Therefore, we have designated the nodule isolates as *B. subtilis* DLA. The oxidation of reserves by germinating spores is thought to be at least one reason why these species frequently yield false-positive results in Biolog tests. As preliminary analysis of 16S rDNA sequences also indicated that the

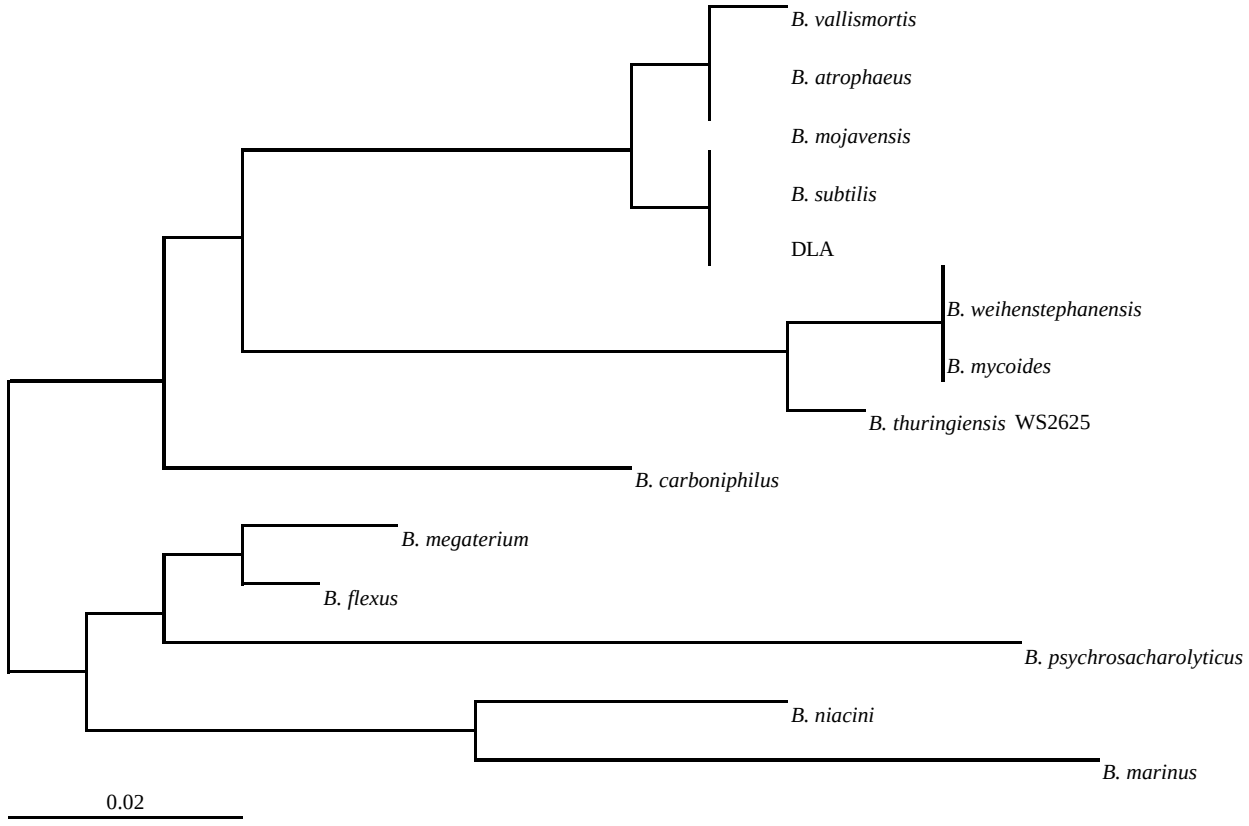

Fig. 2: Phlogenetic tree based on 16S rRNA sequences showing the position of *B. subtilis* DLA. The scale bar represents 0.02 substitutions per base positions

DLA strain was *Bacillus* species, it was possible to perform phylogenetic analysis using the approximately 275 nucleotides from the HV region of the 16S rDNA. Goto *et al*.[16] demonstrated that many *Bacillus* strains may be reliably classified to the species level on the basis of the sequences of a minimum of 219 nucleotides from

the HV region and validated this for numerous strains in the *B. subtilis* cluster (*B. subtilis-B. mojavensis-B. atrophaeus-B. vallismortis*).

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