The Journal of Microbiology, April 2009, p. 147-155 Vol. 47, No. 2
DOI 10.1007/s12275-008-0201-4
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Characterization of Plant-Growth Promoting Diazotrophic Bacteria Isolated from Field Grown Chinese Cabbage under Different Fertilization Conditions

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(Received August 11, 2008 / Accepted January 19, 2009)

Diazotrophic bacteria isolated from the rhizosphere of Chinese cabbage were assessed for other plant growth promoting characteristics *viz*., production of IAA, ethylene, ACC deaminase, phosphate solubilization, and gnotobiotic root elongation. Their effect on inoculation to Chinese cabbage was also observed under growth chamber conditions. A total of 19 strains that showed higher nitrogenase activity identified by 16S rRNA gene sequence analysis were found to be the members of the genera *Pseudomonas* and *Agrobacterium* belonging to α- and γ-*Proteobacteria* groups. These strains were also efficient in producing IAA and ACC deaminase though they produced low levels of ethylene and no phosphate solubilization. In addition, inoculation of selected diazotrophic bacterial strains significantly increased seedling length, dry weight, and total nitrogen when compared to uninoculated control. The colonization of crop plants by diazotrophic bacteria can be affected by many biotic and abiotic factors, and further studies are oriented towards investigating the factors that could influence the establishment of a selected bacterial community.

Keywords: diazotrophs, *nifH* gene, IAA production, acetylene reduction, ethylene, *Agrobacterium*, *Pseudomonas*

Nitrogen (N) an important plant nutrient has been a common factor limiting plant growth, sustained by the use of chemical fertilizers. Surplus use of chemical fertilizers to maintain higher yields runs counter to the principle of sustainable agriculture that demands low input with a high output, and is also an environmental concern (Shantharam and Matoo, 1997). Despite the benefits of chemical fertilizers that have realized in terms of crop productivity, the full genetic potential for maximum crop yield on farms is rarely realized (Kennedy *et al*., 2004). Biological nitrogen fixation (BNF), a process of harnessing atmospheric elemental nitrogen involving intricate interactions between associative diazotrophic bacteria and higher plants can help ensure that the supply of nutrients contributing to optimized yield is maintained.

Diazotrophic bacteria convert atmospheric N (N_2) to ammonia, which can be used by the plant. Thus, diazotrophic bacteria promote plant growth because of their competitive advantage in a carbon-rich and N-poor environment. Diazotrophic bacteria can be considered as plant growth promoting rhizobacteria (PGPR), a group of bacteria that display beneficial effects on plant growth and yield (Vande Broek and Vanderleyden, 1995; Dobbelaere *et al*., 2003) since, besides fixing N, the bacteria also promote plant growth by other mechanisms. In addition to reducing the need for N-supplemented fertilizer (Dawe, 2000), diazotrophic bacterial associations with plants also increases the efficiency of the applied fertilizer (Okon and Labandera-Gonzolez, 1994; Kennedy *et al*., 1997; Gunarto *et al*., 1999). Although the magnitude of BNF from biofertilizer may account for a fraction of total crop N requirement, the effects of reducing losses from an ecosystem may be very significantly contribute to the N economy of crop production (Kennedy *et al*., 2004).

To exploit the full potential of the diazotrophic PGP bacterial strains, it is important to isolate indigenous bacteria that are well-adapted to the environmental conditions to utilize them as inoculant strains (Soares *et al*., 2006). Chinese cabbage is an important vegetable in Asia; indeed, in South Korea it is the national food. It is widely cultivated throughout Korea because of its rapid growth and is an economically important cash crop as well as an efficient food producer. In the current study, attempts were made to isolate the diazotrophic PGPR strains from Chinese cabbage grown under different fertilized conditions. Rhizospheric and endophytic isolates were characterized and taxonomically identified irrespective of their source. To detect the N_2 fixation, a standard acetylene reduction assay (ARA) was used and the presence of *nifH* genes responsible for the production of nitrogenase iron (Fe) protein subunit was also detected by polymerase chain reaction (PCR) using specific primers. In addition, the effect on N-uptake and plant growth by diazotrophic bacterial inoculation was assessed under gnotobiotic and pot culture conditions.

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Materials and Methods

Isolation and characterization of diazotrophs from the rhizosphere of Chinese cabbage

Chinese cabbage plants were sampled from the research plots of an experimental field at Cheongwon (36° 58' 0N and 127° 57' 0E), Chungbuk, Republic of Korea. Soil properties included pH - 5.03, organic matter - 9.31 g/kg, soil type - sandy loam and coarser texture, total nitrogen - 1.11 g/kg, available P_2O_5 - 0.29 g/kg, available Ca - 2.15 cmol/kg, available K - 1.11 cmol/kg, and available Mg - 0.63 cmol/kg. Sampling was done on November 9, 2004 at harvest (i.e. when cabbage heads attained full maturity). Samples were collected from an untreated (control) plot, a plot treated with organic fertilizer, and a plot treated with chemical fertilizer that were arranged in a completely randomized block design. Chemical fertilizers were applied at 320, 78, 198 kg/ha N, P_2O_5, K_2O respectively, and N and K were applied in split doses, as a basal dressing and as a topdressing 12, 24, and 38 days after planting. Organic fertilizer treatment included application of a commercially available organic fertilizer (Chammatna gold, KG Chemicals, Seoul, Korea) that contained 50% organic matter and 5% N, 1% P, and 1% K. About 9.1 tonnes of the organic fertilizer was applied as basal dressing before planting and no further fertilizer amendment was made. The samples were immediately transferred to the laboratory under aseptic conditions and processed within 24 h. Roots with adhering soil (5 g) were placed in 450 ml of sterile phosphate buffered saline (PBS; 1.2 g/L Na_2HPO_4, 0.18 g/L NaH_2PO_4, 8.5 g/L NaCl, pH 7.6) and agitated at 120 rpm for 20 min to extract rhizosphere bacteria. To recover the endophytic bacteria, root tissues (5 g) were surface disinfected using 2% NaOCl applied for 10 min, cut, triturated in 50 ml flasks containing 10 ml sterile PBS, and incubated at 28°C with agitation (200 rpm) for 30 min. The disinfection process was checked by plating aliquots of the final rinse onto 10% tryptic soy agar (TSA) consisting of 3 g/L tryptic soy broth (Difco Laboratories, USA) and 15 g/L Bacto agar (Difco). Appropriate dilutions from the root samples (10^{-3} to 10^{-5} for rhizosphere bacteria, and 10^{-1} to 10^{-3} for endophytic bacteria) were inoculated into three tubes containing N-free semi-solid malate medium (NFb) (Baldani and Dobereiner, 1980) and the population was estimated using the most-probable number method (Alexander, 1965). The tubes which showed positive pellicle formation were sub-cultured in fresh semisolid media and streaked onto solid NFb media with ammonium sulfate and incubated at 30°C for 3 days. The colonies were further purified and analyzed by phenotypic and taxonomic identity.

Each isolate was streaked onto NFb media and Congo red agar (Rodríguez Cáceres, 1982). To determine the ARA, vials containing 40 ml of NFb were inoculated with 0.4 ml of bacterial suspension grown in NFb to an optical density at 600 nm of 1.0 and incubated at 30°C till exponential phase. The vials were again incubated for 24 h after replacing the air in the headspace with acetylene (10%, v/v). One milliliter of the headspace sample was injected to a DS 6200 Gas Chromatography (Donam Instruments, Korea) fitted with flame ionization detector and a Porapak-Q column (Hardy *et al.*, 1968) to measure the ARA of the cultures. Sterile NFb medium served as control. The protein concentration was determined by the method of Lowry *et al.* (1951) with bovine serum albumin (BSA) as standard. The presence of *nifH* gene in the isolates was determined according to Ueda *et al.* (1995) by the amplification of 390 bp fragment of *nifH* through PCR using specific primers 19F; 5'-GCI WTY TAY GGI AAR GGI GG-3' and 407R; 5'-AAI CCR CCR CAI ACI ACR TC-3'. The PCR reaction conditions were as follows; 0.5 min at 94°C, 1 min at 50°C, and 0.5 min at 72°C for 40 cycles. Sterile distilled water used as a negative control instead of extracted DNA samples.

Taxonomical identification of the bacterial strains

Genomic DNA of the bacterial strains grown in NFb medium was extracted according to Sambrook *et al.* (1989). The 16S rRNA nucleotide sequences were identified by PCR direct sequencing by the fluorescent dye terminator method (ABI Prism^{TM} Bigdye^{TM} Terminator cycle sequencing ready reaction kit v.3.1; Applied Biosystems, USA) using the primers 27F; 5'-AGA GTT TGA TCC TGG CTC AG-3' and 1492R; 5'-GGT TAC CTT GTT ACG ACT T-3'. The products were purified by Millipore-Montage dye removal kit and were run in an ABI3730XL 50 cm capillary DNA sequencer (Applied Biosystems 50 cm capillary). Nearly complete 16S rRNA gene sequences were aligned and bacterial identities were deduced by BLAST search to ascertain their closest relatives. Phylogenetic analyses were performed using MEGA version 3.1 (Kumar *et al.*, 2004) after multiple alignments of the data by CLUSTAL W (Thompson *et al.*, 1994). Distances were obtained using options according to the Kimura two-parameter model (Kimura, 1980) and clustering was performed using the neighbor-joining method (Saitou and Nei, 1987).

Determination of other plant-growth promoting traits

Aliquots (100 μl) of the strains grown in NFb broth were transferred to 50 ml of LB medium containing 500 μg/ml of L-tryptophan (Sigma-Aldrich, USA) and grown at 28°C for 7 days for the estimation of indole-3-acetic acid (IAA). The production of IAA in the cell free suspension was determined spectrophotometrically at 530 nm with Salkowski's reagent (1 ml of 0.5M $FeCl_3 \cdot 6H_2O$ in 50 ml of 35% $HClO_4$) according to Bric *et al.* (1991). Pure IAA (Sigma-Aldrich) was used for standards and uninoculated media served as a control.

To test for ethylene production, the bacterial strains were grown for 9 days at 30°C without agitation in 40 ml of NFb supplemented with 1.32 g/L ammonium sulfate and 0.4 g/L methionine (NFb_{AS}+met) (Arshad and Frankenberger, 1998) contained in 120 ml vials each fitted with a rubber septum. One milliliter of aerial phase was with drawn from each culture vial and analyzed in a Gas Chromatograph fitted with flame ionization detector and a Porapak-Q column to measure ethylene production. For measuring 1-aminocyclopropane-1-carboxylate (ACC) deaminase, cells from cultures grown to stationary phase in NFb broth (15 ml at 30°C) were collected by centrifugation (10,000 rpm, 10 min, 4°C) and resuspension in 7.5 ml NFb supplemented with 3 mM ACC and incubated at 30°C (48 h, 120 rpm) to induce ACC deaminase activity. The α-ketobutyrate produced from

the enzymatic cleavage of ACC was measured spectrophotometrically at 540 nm, as described previously (Shah *et al*., 1998). The enzyme activity was estimated using a standard curve of α-ketobutyrate (Sigma-Aldrich). A stock solution of α-ketobutyrate was prepared in 0.1 M Tris-HCl (pH 8.5) and stored at 4°C, from which a series of standards ranging from 0.1~1.0 μmol were prepared.

Gnotobiotic assay using growth pouches was performed to measure the root elongation induced by ACC deaminase. Canola seeds (*Brassica campestris*) obtained from Seminis (Korea) were used for the root elongation assay, since it is a better representative of ethylene sensitive plants (Glick *et al*., 1994; Li *et al*., 2000; Penrose and Glick, 2001; Ghosh *et al*., 2003; Madhaiyan *et al*., 2006, 2007). The bacterial strains grown with ACC to induce ACC deaminase activity were used as inocula. The seeds were surface sterilized using sequential treatments with 70% ethanol for 1 min and 1% NaOCl for 10 min. After thorough rinsing 10 times with sterile distilled water the seeds were kept immersed in the bacterial suspension at room temperature for 4 h. The seeds were then transferred aseptically to 12.5×15.0 cm CYG seed germination pouch (Mega International, USA) that were sterilized at 121°C for 20 min along with 20 ml of distilled water. The growth pouches were incubated in a growth chamber maintained at 20±1°C with a cycle beginning with 12 h of dark followed by 12 h of light (Penrose and Glick, 2003). Ten pouches were used for each treatment. The root lengths of canola seedlings were measured afteron 15 days of growth and the data were analyzed.

Table 1. Diazotrophic population from tissues and rhizosphere of Chinese cabbage sampled from different sites

Treatment	Population (log CFU/g of sample)	
	Rhizosphere	Endophytic
Control	4.57±0.33 c	3.28±0.10 b
Organic fertilizer	4.81±0.29 b	3.34±0.20 b
Chemical fertilizer	5.03±0.31 a	3.54±0.17 a

Each value represents mean of four replicates per treatment. Within each vertical column, values followed by the same letter are not statistically different according to Fischer's protected LSD ($P \leq 0.05$).

Effects of bacterial inoculation on plant growth

Surface sterilized Chinese cabbage seeds (*Brassica campestris* L. ssp. *Peckinensis*, cv winter pride; Seminis) were sown in plastic pots (top diameter, 90 mm; bottom diameter, 60 mm; height, 85 mm) filled with approximately 200 g air-dried Wonjo-Mix bed soil (Nong-Kyung, Chungbuk, Korea). The soil, which displayed 40±5% moisture content and 30±5 kPa water holding capacity, contained 65%~75% cocoa peat, 15%~20% zeolite, 10%~15% perlite, and the following macronutrients: 150~200 mg/L NH_4-N, 100~150 mg/L NO_3-N, 200~300 mg/L available P_2O_5. The pots were arranged in a completely randomized design and were maintained in a growth chamber operating with a temperature of 25°C, relative humidity of 70%, and light intensity of 90~110 μmol/m^2/sec to study the effects of bacterial inoculation on its growth and nutrient uptake. For the preparation of bacterial inocula, the culture grown to mid-exponential growth phase (OD_{600}=1.2) was centrifuged and the cell pellets were washed and resuspended in sterile distilled water adjusted to a cell density of 10^8 CFU/ml (OD_{600}=1.0). Bacterial application to the soil was carried out at 10 and 20 days after sowing (DAS) by inoculating 1 ml of the bacterial suspension (10^8

Table 2. Details of diazotrophic bacterial strains including both rhizospheric and endophytic from Chinese cabbage and their identification by 16S rDNA sequences

Strain	Treatment, origin	ARA[a]	*nifH* gene (PCR amplification)	GenBank no. (sequence length, bp)	Homologous microorganism (% identity)
CBCEN2	Unfertilized, root interior	561.1±35.28 a	+	EF427845 (1365)	*Agrobacterium tumefaciens* (99)
CBCEN3	Unfertilized, root interior	566.6±50.00 a	+	EF427846 (1360)	*Agrobacterium tumefaciens* (99)
CBCEN4	Unfertilized, root interior	211.7±17.15 h	+	EF427847 (1362)	*Agrobacterium tumefaciens* (99)
CBCEN7	Unfertilized, root interior	326.4±24.48 f	-	EF427848 (1264)	*Agrobacterium tumefaciens* (100)
CBCEN8	Unfertilized, root interior	178.9±16.69 i	+	EF427849 (1412)	*Pseudomonas* sp. (99)
CBCEN9	Unfertilized, root interior	310.5±14.15 f	+	EF427850 (1414)	*Pseudomonas* sp. (99)
CBCEN14	Organic, root interior	246.8±27.02 g	+	EF427851 (1361)	*Agrobacterium tumefaciens* (99)
CBCEN17	Organic, root interior	494.2±25.52 b	+	EF427852 (1361)	*Agrobacterium tumefaciens* (99)
CBCEN20	Organic, root interior	363.5±25.12 e	-	EF427853 (1358)	*Agrobacterium tumefaciens* (100)
CBCEN21	Organic, root interior	132.9±13.22 j	+	EF427854 (1409)	*Pseudomonas* sp. (99)
CBCEN25	Chemical, root interior	327.6±27.48 f	-	EF427855 (1363)	*Agrobacterium tumefaciens* (99)
CBCEN26	Chemical, root interior	434.4±19.86 dc	+	EF427856 (1361)	*Agrobacterium tumefaciens* (100)
CBCN5	Unfertilized, rhizospheric	366.4±38.34 e	+	EF427857 (1309)	*Agrobacterium tumefaciens* (99)
CBCN8	Unfertilized, rhizospheric	156.1±9.30 ji	+	EF427858 (1416)	*Pseudomonas* sp. (99)
CBCN13	Unfertilized, rhizospheric	447.3±27.31 c	+	EF427859 (1363)	*Agrobacterium tumefaciens* (100)
CBCN15	Unfertilized, rhizospheric	365.9±38.05 e	-	EF427861 (1361)	*Agrobacterium tumefaciens* (99)
CBCN23	Organic, rhizospheric	439.8±21.82 dc	+	EF427860 (1362)	*Agrobacterium tumefaciens* (99)
CBCN27	Organic, rhizospheric	415.0±17.32 d	+	EF427862 (1265)	*Agrobacterium tumefaciens* (99)
CBCN33	Chemical, rhizospheric	149.4±11.20 ji	+	EF427863 (1417)	*Pseudomonas* sp. (99)

Each value represents the average of two replicates per treatment. In the same column, significant differences according to LSD at $P \leq 0.05$ levels are indicated by different letters (LSD value for ARA is 30.25). [a]ARA activity expressed in pmol C_2H_4 /mg of protein/ h.

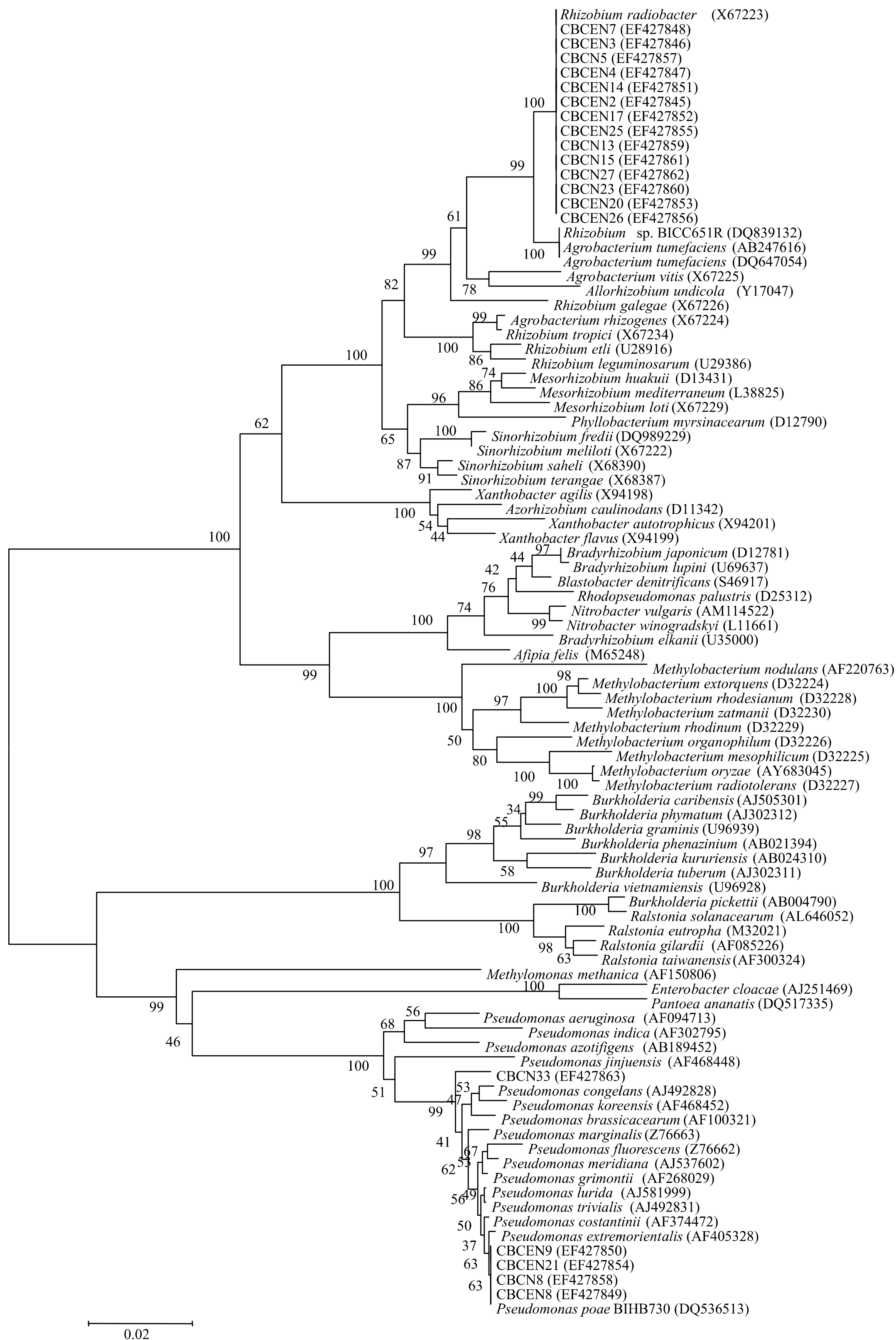

Fig. 1. Phylogenetic relationships of nitrogen fixing bacteria isolated from Chinese cabbage based on 16S rDNA sequence and related sequences. The tree was constructed using closely related sequences based on Euclidean distance (neighbour joining algorithm with Kimura parameter 2). The numbers at the nodes indicate the levels of bootstrap support based on data for 1,000 replicates. The scale bar indicates 0.02 fixed substitutions per nucleotide position. Sequence accession numbers are indicated in parantheses.

CFU/ml) near the root zone. Uninoculated control plants were treated with sterile distilled water at the time of inoculation.

The crops were uprooted 30 DAS for biological and chemical analysis. Seedlings (10 g) were placed in a 120 ml vial and after replacing the air in the headspace with acetylene (10%, v/v), the vials were incubated at 30°C for 4 h. ARA *in planta* was measured by gas chromatography as described earlier. The viable bacterial counts on roots were determined according to Miles and Misra (1938). The remaining plant samples were oven dried at 70°C for 4 days. N concentration in the plant samples was measured in a Kjeldahl Auto1030 analyzer after digestion with sulphuric acid and potassium sulphate (Jackson, 1973).

Statistical analysis

Data were evaluated by analysis of variance (ANOVA) using the general linear model version 9.1 (SAS Institute, USA). Means were compared using the least significant difference (LSD). Significance levels were within confidence limits of ≤0.05 (SAS, 2004).

Results

Isolation, characterization, and taxonomical identification of diazotrophic bacteria

Enumeration of diazotrophic population from the rhizosphere of Chinese cabbage indicated that the culturable N-fixing bacterial numbers varied between fertilizer treatments. Both rhizosphere and endophytic bacterial numbers were dominant in the plants grown in soil treated with chemical fertilizer. The N-fixing bacterial population was less in plants grown without fertilizer (Table 1). The isolates were grouped based on the single colony morphology and growth pattern on Nfb media and a representative isolate from each group irrespective of the source was selected for further investigation. A total of 53 isolates including 19 endophytic isolates were taken for ARA to measure the nitrogenase activity. Bacterial isolates that had higher nitrogenase activity, ranging from 132.9-566.7 C_2H_4/mg protein/h for endophytes and 149.4~447.4 C_2H_4/mg protein/h for rhizospheric isolates were selected for further investigation. In general, the endophytic isolates recorded higher ARA than those from the rhizosphere and the origin; ARA activities of the 19 selected isolates are summarized in Table 2. These isolates were further identified by 16S rRNA gene sequencing analysis to ascertain their taxonomical position and their physiological PGP characteristics were measured.

The selected isolates formed small deep red and scarlet colonies in Congo red medium and the presence of *nifH* gene analyzed through PCR amplification using specific primers showed successful amplification of a 390 bp product in 15 isolates. The 16S rRNA gene sequencing analysis of 1,265 and 1,365 bp revealed two clusters among these isolates. The first group formed a cluster under γ-*Proteobacteria* that had close sequence similarity with *Pseudomonas* sp. and the other group of α-*Proteobacteria* had close sequence similarity with *Agrobacterium tumefaciens*. However, the clustering was not based on their origin and both groups included members of rhizospheric and endophytic origin. The gene sequences of the strains have been submitted to the GenBank under the accession numbers EF427845-EF427863 (Table 2). A

Table 3. Plant-growth promoting characteristics of diazotrophic bacterial strains isolated from Chinese cabbage

Strain	IAA (μg/ml)	Ethylene production (nmol/mg protein)	ACC deaminase[a]	Root elongation (cm)[b]
CBCEN2	21.6±2.08 bc	1.00±0.06 ba	48.6±2.08 g	8.83 ± 0.09 gh (26.9)
CBCEN3	21.7±2.71 bc	1.05±0.14 a	46.9±3.98 hg	8.83 ± 0.24 gh (26.9)
CBCEN4	21.1±1.79 dc	0.86±0.09 dc	42.3±2.48 jl	8.55 ± 0.18 i (22.8)
CBCEN7	22.2±1.27 bac	0.83±0.05 dc	44.2±4.16 hl	10.15 ± 0.22 a (45.8)
CBCEN8	9.6±0.92 f	0.01±0.00 h	47.4±4.27 hg	9.88 ± 0.19 b (41.9)
CBCEN9	10.3±1.91 f	0.02±0.00 h	47.1±4.68 hg	8.92 ± 0.20 f (28.1)
CBCEN14	18.87±2.23 e	0.35±0.03 g	38.6±4.97 jk	8.79 ± 0.11 gh (26.2)
CBCEN17	23.6±2.08 a	0.61±0.05 f	38.2±4.73 k	8.63 ± 0.01 hi (24.0)
CBCEN20	22.5±1.44 bac	1.04±0.02 a	54.8±2.19 f	9.32 ± 0.16 de (33.8)
CBCEN21	8.7±0.98 f	0.01±0.00 h	49.9±4.56 g	8.65 ± 0.06 hi (24.2)
CBCEN25	22.9±2.83 a	0.37±0.05 g	62.2±5.89 d	9.87 ± 0.07 b (41.8)
CBCEN26	22.0±2.31 bac	0.90±0.06 bc	75.5±3.18 b	9.90 ± 0.03 b (42.2)
CBCN5	21.2±1.85 bdc	0.90±0.06 bc	56.7±3.29 ef	10.24 ± 0.15 a (47.1)
CBCN8	6.7±0.98 g	0.02±0.00 h	61.6±2.08 d	9.31 ± 0.08 de (33.7)
CBCN13	22.1±1.21 bac	0.70±0.06 fe	61.4±6.00 d	9.49 ± 0.04 cd (36.3)
CBCN15	21.5±2.02 bdc	0.78±0.09 de	59.8±4.50 ed	9.11 ± 0.29 ef (30.8)
CBCN23	22.8±1.62 bac	0.99±0.08 ba	69.5±5.48 c	9.62 ± 0.5 c (38.2)
CBCN27	19.8±1.04 ed	0.29±0.06 g	43.7±2.14 hl	9.22 ± 0.5 e (32.4)
CBCN33	4.3±0.75 h	0.01±0.00 h	88.0±5.20 a	9.55 ± 0.15 c (37.2)
LSD (P≤0.05)	1.774	0.111	3.79	0.231

Each value represents the average of two replicates per treatment. In the same column, significant differences according to LSD at $P \leq 0.05$ levels are indicated by different letters.

[a] ACC deaminase activity expressed in nmol α-KB /mg protein/ min;

[b] the root length is given in cm; the value corresponding to uninoculated control is 6.96 cm. In parenthesis, is given % increase over control.

Table 4. Effect of inoculation of diazotrophic isolates on seedling growth, nitrogenase activity and total nitrogen in Chinese cabbage

Treatments	Seedling length (cm)	Dry weight (g)	Seedlings ARA activity (pmol C_2H_4/g DW/h)	Total N (%)
CBCEN2	9.53±0.88 c	1.08±0.10 d	150.01±2.89 d	7.16±0.42 a
CBCEN3	9.33±1.06 c	1.02±0.06 ed	131.71±6.18 ed	6.98±0.51 ba
CBCEN4	11.33±1.06 a	1.94±0.22 a	114.65±8.46 e	6.48±0.34 d
CBCEN8	9.03±0.31 c	0.58±0.04 g	239.45±17.00 b	6.53±0.62 dc
CBCEN9	9.30±0.69 c	0.87±0.07 ef	227.74±24.68 cb	7.19±0.43 a
CBCN5	10.58±1.49 ba	1.36±0.15 c	142.88±6.28 d	6.48±0.35 d
CBCN8	9.60±1.21 bc	0.86±0.09 ef	207.27±12.86 c	6.77±0.50 bc
CBCN13	9.63±1.17 bc	0.76±0.06 f	327.05±17.93 a	6.52±0.42 dc
CBCN33	10.85±1.13 a	1.53±0.16 b	238.79±22.40 b	7.03±0.33 ba
Control	4.38±0.68 d	0.53±0.06 g	61.91±6.15 f	6.29±0.34 d
LSD ($P\leq0.05$)	0.989	0.169	22.66	0.28

Each value represents mean of four replicates per treatment. Within each vertical column, values followed by the same letter are not statistically different according to Fischer's protected LSD ($P\leq0.05$).

phenogram reflecting the relationship among the strains and candidate sequences of various N_2-fixing strains obtained from the NCBI database is presented in Fig. 1.

Plant-growth promoting traits of diazotrophic bacteria

The *Pseudomonas* and *Agrobacterium* strains were further investigated for the PGP traits that include production of IAA, ethylene, and ACC deaminase (Table 3). Qunatitative assay revealed that all the selected strains from Chinese cabbage could produce considerable amounts of IAA with five strains producing more than 18.0 μg/ml of IAA. Strains CBCEN17 and CBCEN25 produced higher amounts of IAA (23.6 and 22.9 μg/ml, respectively). Production of ethylene could be detected in the presence of methionine for all the cultures tested, ranging from 0.01∼1.05 nM in Nfb media. The highest ethylene production was observed with *Agrobacterium* spp. strains CBCEN3 (1.05 nmol) and CBCEN20 (1.04 nmol). The diazotrophic isolates varied in their ability to utilize ACC and significant differences could be observed in the ACC deaminase activity of cell free extracts. ACC deaminase activity in the cell free extracts of endophytic and rhizosphere isolates ranged between 38.1∼75.5 and 43.7∼88.0 nmol α-KB/mg protein/min, respectively. The activity was higher in the cell free extracts obtained from CBCN33 and CBCEN26 (Table 3). None of the strains were able to solubilize the insoluble tri-calcium phosphate (0.5%) supplemented in the Pikovskaya agar plates even after incubating for 7 days when the cultures were spotted to the centre of the plates.

Bacterial inoculations increased the root length of canola from 22.8%∼47.1% compared with uninoculated control. Strains CBCN5 and CBCEN7 produced higher root length accounting for 10.24 cm and 10.15 cm, respectively, representing increases of 45.8% and 47.1%, respectively, over the control (Table 3). Of the 19 strains analyzed, nine strains that had higher root elongations were further selected for pot experiments.

Effects of bacterial inoculation on plant growth

A pot experiment was carried out with Chinese cabbage to evaluate the effects of bacterial inoculation. Inoculation of diazotrophic strains had a considerable impact on the growth and nutrient uptake of canola (Table 4). Nitrogenase activity of the seedlings also increased significantly with the bacterial inoculation. Strain CBCN13 recorded the highest ARA for the seedlings (327.1 pmol C_2H_4/g DW/h) followed by strains CBCN33 and CBCEN8. Inoculation of the daizotrophic bacterial strains significantly increased the seedling length and dry weight when compared to control with the endophytic strain CBCEN4 recording the highest seedling length (11.3 cm) and dry weight (1.94 g). Besides increasing the biomass of plants, inoculation of the bacterial strains had a significant impact on the total N in plant tissues when compared to uninoculated control. With the exception of the endophytic strains CBCEN4 and CBCEN8, and the rhizospheric strains CBCN5 and CBCN13, all other strains increased the total N content in the plants significantly compared to control. The endophytes CBCEN2 and CBCEN9 recorded higher N concentration in plants (Table 4).

Discussion

Isolation, screening, and selection of bacterial inoculants that efficiently promote the growth of plants are important steps to optimize high crop yields and improve sustainability of crop systems (Roesch *et al*., 2007). Although in this study diazotrophic isolates were isolated from Chinese cabbage from different fertilizer treatments, the aim was to screen and identify the most efficient plant growth promoting diazotrophic bacteria. Hence, the effects of fertilizers on the diazotrophic population and their significance were not considered. A selective media and ARA measurement was used for their isolation and selection. Previous investigations have proven these techniques to be simple, easy, and economical (Döbereiner and Day, 1976; Dobbelaere *et al*., 2003). PCR revealed the presence of *nifH* gene in 47.3% of the endophytic isolates and 17.6% of rhizospheric isolates, when the total isolates were considered. Poly *et al*. (2001) designed a number of *nifH* PCR primer sets, although most of them target the same or overlapping sites and differ primarily in their use of degenerate oligonucleotides or modified nucleotides. Hence, the absence of amplifications for

nifH gene in the bacterial isolates tested, though they were ARA positive, may be explained by the variability of the *nifH* gene (Zehr *et al*., 2003).

Based on 16S rDNA sequence analysis, the 19 selected strains were found to be members of the genera *Pseudomonas* and *Agrobacterium* showing 99%~100% sequence similarity. Nitrogen fixation in *Pseudomonas* sp. associated with plants is probably more common than initially thought, and the presence of *nif* genes and regulation of both nitrogenase synthesis and activity have been characterized in *Pseudomonas* (Desnoues *et al*., 2003). To date, the *Agrobacterium* genus is considered to be phytopathogenic, although N_2-fixation by wild *Agrobacterium* species has been reported (Kanvinde and Sastry, 1990; Keramat *et al*., 2002). The presence of *nifH* gene encoding the ferroprotein monomer of nitrogenase that is highly conserved among the diazotrophs was confirmed in this study, although the presence of *virA* (a regulatory gene conserved among the pathogenic *A. tumefaciens* or *A. rhizogenes*) was not examined. A previous investigation (Young *et al*., 2001) reclassified the genus *Agrobacterium* under *Rhizobium*, although this has been questioned (Farrand *et al*., 2003). However, according to a recent report, *Rhizobium rhizogenes* strains can be pathogenic and can induce tumors or hairy roots in plants while nodulating *Phaseolus vulgaris* (Velázquez *et al*., 2005). These investigations together with the results from this study provide evidence for the reclassification of the phytopathogen *Agrobacterium* and their potential use as a bioinoculant.

Increasing evidence indicates that besides increased nutrient uptake, synthesis, and export of phytohormones by microorganisms may also play an important role in plant growth promotion (Ryu *et al*., 2006), which is also true for diazotrophs (Dobbelaere *et al*., 2003; Roesch *et al*., 2007). The presently selected diazotrophs from Chinese cabbage were equally efficient in IAA production as some of the diazotrophic bacteria isolated from maize collected from five regions within the southern state of Rio Grande do Sul in Brazil (Roesch *et al*., 2007). A single PGPR does not need to possess all PGP characteristics; plant growth can result from even a single mechanism. The strains presently tested were not able to solubilize the insoluble phosphates in the culture medium and produced low levels of ethylene. Ethylene is a gaseous plant growth hormone that plays a key role in multifarious physiological changes in plants at molecular level (Vogel *et al*., 1998), while elevated levels of ethylene are harmful to plants. Our results confirm the wide distribution of ACC deaminase activity in different bacterial genera that have been previously reported (Belimov *et al*., 2001; Dell'Amico *et al*., 2005; Poonguzhali *et al*., 2006, 2008; Shaharoona *et al*., 2007). We postulate that the low level of ethylene produced by the strains contribute in some extent to the stimulation of plant growth at an early stage, while the ACC deaminase enzyme produced will protect the deleterious effect of any negative consequences of rise in ethylene levels (Li *et al*., 2000). The presence of ACC deaminase is likely a positive factor for *Agrobacterium* strains, given the recent finding that ACC deaminase of the phytopathogen *P. brassicacearum* Am3 promotes plant growth in tomato by masking the phytopathogenic properties of this bacterium (Belimov *et al*., 2007).

Because of their ability to transform atmospheric N_2 into a form (ammonia) that can be used by the plant, researchers were originally very optimistic about the potential of associative diazotrophic bacteria to promote the growth of many cereals and grasses (Vlassak *et al*., 1992). Many of the diazotrophs, for example *Azospirillum*, which were originally discovered, based on their N-fixing capability, were later found to promote growth through other mechanisms such as production of phytohormones. Similarly, in this study we have proven that, apart from diazotrophy, the strains may be better exploited as bioinoculants since they possess other plant growth promoting characteristics, in particular IAA and ACC deaminase. Further elucidation of their role in plant growth under pot culture and field levels will clarify the potential of these associative diazotrophs as potential bioinoculants and a valuable partner in future agriculture.

Acknowledgements

The authors thank the BrainKorea21 (BK-21), Republic of Korea for their financial grant and Agricultural Research Promotion Centre (ARPC), Republic of Korea for their financial assistance.

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