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Isolation and Identification of Phosphate Solubilizing Bacteria from Chinese Cabbage and Their Effect on Growth and Phosphorus Utilization of Plants

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Phosphate solubilizing bacteria (PSB) were isolated from the rhizosphere of Chinese cabbage and screened on the basis of their solubilization of inorganic tricalcium phosphate in liquid cultures. Ten strains that had higher solubilization potential were selected, and they also produced indole-3-acetic acid, 1-aminocyclopropane-1-carboxylate (ACC) deaminase, and siderophores. The strains were identified to be members of *Pseudomonas*, by 16S rDNA sequence analysis. Seed bacterization with PSB strains increased the root elongation and biomass of Chinese cabbage in seedling culture, although they had no effect on phosphorus uptake of plants. The plant growth promotion by PSB in this study could be due to the production of phytohormones or mechanisms other than phosphate solubilization, since they had no effect on P nutrition.

Keywords: Phosphate solubilizing bacteria, *Pseudomonas*, 1-aminocyclopropane-1-carboxylate, indole-3-acetic acid, acid phosphatase

Although abundant in soils, phosphorus (P) is one of the essential macronutrients required for plant growth and development, and is by far the least available factor for plants. Phosphate solubilization by soil bacteria that makes the P available in soil solution for plant growth is considered to be an important attribute of plant growth promoting rhizobacteria (PGPR) [3]. Numerous investigations document the presence of phosphate solubilizing bacteria (PSB) and fungi from rhizosphere of different crops [10, 22]. In recent decades, increasing evidences indicate that, besides increased nutrient uptake, the synthesis and export of phytohormones by microorganisms may also play an important role in plant growth promotion [20]. Individual or co-inoculation of PSB with other groups of microorganisms enhanced the plant growth by increasing the efficiency of biological nitrogen fixation or the availability of other trace elements and by the production of plant growth promoting (PGP) substances [3, 15]. This study assessed the natural population of PSB associated with Chinese cabbage (*Brassica campestris* L. ssp. *pekinensis*), grown under field conditions in Korea, and their use as PGPR inoculants.

PSB from the rhizosphere and root interior of Chinese cabbage (cultivar Norangchusurk) plants, sampled at maturity from the experimental field (Cheongwon, Chungbuk, Republic of Korea), were isolated on Pikovskaya agar [14] with 0.5% tricalcium phosphate [$Ca_3(PO_4)_2$] by the serial dilution and plating method. The PSB population in the rhizoplane and root interior, identified by clear halo zones around their colonies, accounted to about 10^7 and 10^5 CFU/g fresh weight, respectively. The colonies that had higher solubilization zones were purified and screened based either on the solubilization index on Pikovskaya and NBRIP agar [13] with 0.5% $Ca_3(PO_4)_2$ in plate assays, or through estimation of soluble P in their culture supernatants at 48 h of growth in Pikovskaya broth with 0.5% $Ca_3(PO_4)_2$ [12]. Sterile medium served as a control. A total of ten cultures that had higher solubilization in plates and broth assay were selected and tested for the presence of other PGP characteristics. The production of indole-3-acetic acid (IAA), siderophores, and 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity were tested according to standard procedures mentioned elsewhere [18] and solubilization of Zn was determined by plate assays with 0.1% Zn. Furthermore, the ability to interfere with the quorum-sensing mechanism was assessed using the indicator strain *Chromobacterium violaceum* CV026 with supplementation of 50 nmol of *N*-hexanoyl homoserine lactone or *Agrobacterium tumefaciens* A136 (pCF218) (pCF372) in plate assays [9]. The bacterial strains were further characterized using BIOLOG GN2 plates (BIOLOG Inc., Hayward, CA, U.S.A.) [16] and identified by 16S rRNA gene sequencing analysis using the primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). Total genomic DNA was extracted from cells [20] cultured on nutrient agar, and the 16S rDNA sequence was determined by the

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fluorescent dye terminator method using the sequencing kit (ABI Prism Big dye terminator cycle sequencing ready reaction kit v.3.1). Products were run on a ABI3730XL capillary DNA sequencer (ABI Prism 310 Genetic Analyzer, Tokyo, Japan). The aligned sequences were computed using ClustalV software [4], and sequence homologies were determined using BLASTn search to create an evolutionary distance matrix.

Gnotobiotic plant assays were carried out to measure the plant root elongation promotion (PREP) activity and to assess the impact on the P uptake of plants by the inoculation of bacterial strains. The bacteria were proliferated in nutrient broth for 2 days, and cell pellet collected by centrifugation (8,000 ×*g*, 10 min) was washed twice with sterile 30 mM $MgSO_4$ before resuspending. Surface disinfected Chinese cabbage seeds (70% ethanol for 1 min, 1% NaOCl for 10 min), after three sterile distilled water rinses, were kept immersed in the bacterial suspension for 6 h under shaking conditions. Seeds treated with 30 mM $MgSO_4$ served as the control. The plants were grown on sterile nutrient agar, pH 5.5 [19], in vertically positioned Petri dishes (120×120×10 mm; 12 plants per plate) to measure root elongation, or in tubes to determine the concentration of P or enzyme activities. The plants were incubated in a growth chamber maintained at 20±1°C with a 12/12 h light/dark period and the root length was measured on day 7.

For measuring P concentration or enzyme activities, germinated seedlings with radicles of 0.5 cm length were transferred aseptically to tubes containing 40 ml of sterile agar [19]. The media contained either no added P or P as inorganic insoluble $Ca_3(PO_4)_2$ at an equivalent of 1 mM (*i.e.*, 1,239 μg P per seedling). Ten replicates per treatment were arranged in a completely randomized design, and tubes were discarded if contamination was visible. An uninoculated control was also included and the plants were collected on day 19 or 20 for analysis [19]. The availability of P in agar media was determined by extracting 20-ml agar plugs with 20 ml of sterile distilled water in a shaker at 30 rpm for 16 h. Extracts were then filtered through Whatman 42 paper, and available P was determined [12]. Concentrations of P in plant samples were measured by the method described by Jackson [6]. The acid phosphatase activity of the roots was determined according to Tabatabai and Bremner [24] using modified universal buffer (pH 6.5). Treatment effects were determined by analysis of variance (ANOVA), using the Duncan's multiple range test ($P<0.05$) to test the significance (SAS, Version 9.1; 2004, SAS Institute Inc., Cary, NC, U.S.A.).

Plant growth promotion by PSB included mechanisms other than solubilization of insoluble phosphates [3]. Concurrent to this, the selected PSB strains from Chinese cabbage also efficiently solubilized insoluble ZnO and produced IAA. Except for strains CPBE30, CPBE43, and CPBE44, other strains produced siderophores. ACC deaminase activity of the strains ranged from 33.45 to 129.49 nmol of α-ketobutyrate released per min per mg protein (Table 1). The selected strains also inhibited violacein production in

Table 1. Solubilization of insoluble $Ca_3(PO_4)_2$ in plate and broth assays and other plant-growth promoting characteristics of selected bacterial isolates.

PSB isolate	P solubilization[a]			Zn[b]	IAA (μg/ml)[c]	Siderophore	ACC deaminase*	Root elongation**
	Solubilization index (%)	P (μg/ml)	pH					
CPBR6	183.3±28.9	326.0±2.90	5.17	1.37±0.05	1.95±0.68	+	77.2±7.04	5.22±0.4ba
CPBR7	155.6±9.6	305.1±11.0	5.24	1.33±0.06	1.88±0.04	+	108.3±7.70	4.45±0.2b
CPBR16	150.0±50.0	324.1±6.42	4.88	1.30±0.00	1.85±0.20	+	56.8±3.92	4.73±0.4ba
CPBE30	233.3±28.9	326.4±2.07	5.12	1.30±0.10	1.85±0.13	–	61.4±3.67	5.50±0.4ba
CPBE31	433.3±115.5	247.9±3.52	5.50	1.07±0.21	1.85±0.49	+	47.3±4.22	5.09±0ba
CPBE37	233.3±28.3	301.3±1.45	5.14	1.27±0.06	4.15±3.91	+	84.3±7.12	5.35±1.1ba
CPBE40	111.1±19.3	276.9±3.11	5.14	1.37±0.41	2.25±0.43	+	74.4±4.25	5.23±0.4ba
CPBE42	177.8±38.5	299.0±0.00	4.99	1.10±0.00	1.53±0.26	+	33.5±3.15	5.33±0.1ba
CPBE43	283.3±14.4	440.9±6.83	4.16	1.57±0.06	23.38±0.98	–	129.5±17.03	6.08±0.2a
CPBE44	163.3±4.72	282.1±0.83	5.44	1.23±0.25	2.35±1.24	–	79.4±11.20	5.43±0.5ba

[a]The solubilization index, determined as the proportion of the solubilization halo to the colony diameter on NBRIP agar and soluble P present in the culture supernatant at 48 h of growth in Pikovskaya broth with the corresponding reduction in pH.
[b]The diameter of the halo zone formed on Bunt and Rovira medium supplemented with 0.1% Zn.
[c]The amount of IAA in the culture supernatants supplemented with 100 μg/ml L-tryptophan in the growth media; + and – indicate the presence or absence of siderophores as determined by CAS assay.
*nmol α-ketobutyrate released/min/mg protein; Each value represents a mean±standard deviation (SD) of three replications.
**The root length is given in cm; the value corresponding to uninoculated control is 4.0 cm (the average data of two replications with 10 plates per replication and 12 seedlings per plate). Within each vertical column, values followed by the same letter are not statistically different according to Fisher's protected LSD ($P<0.05$).

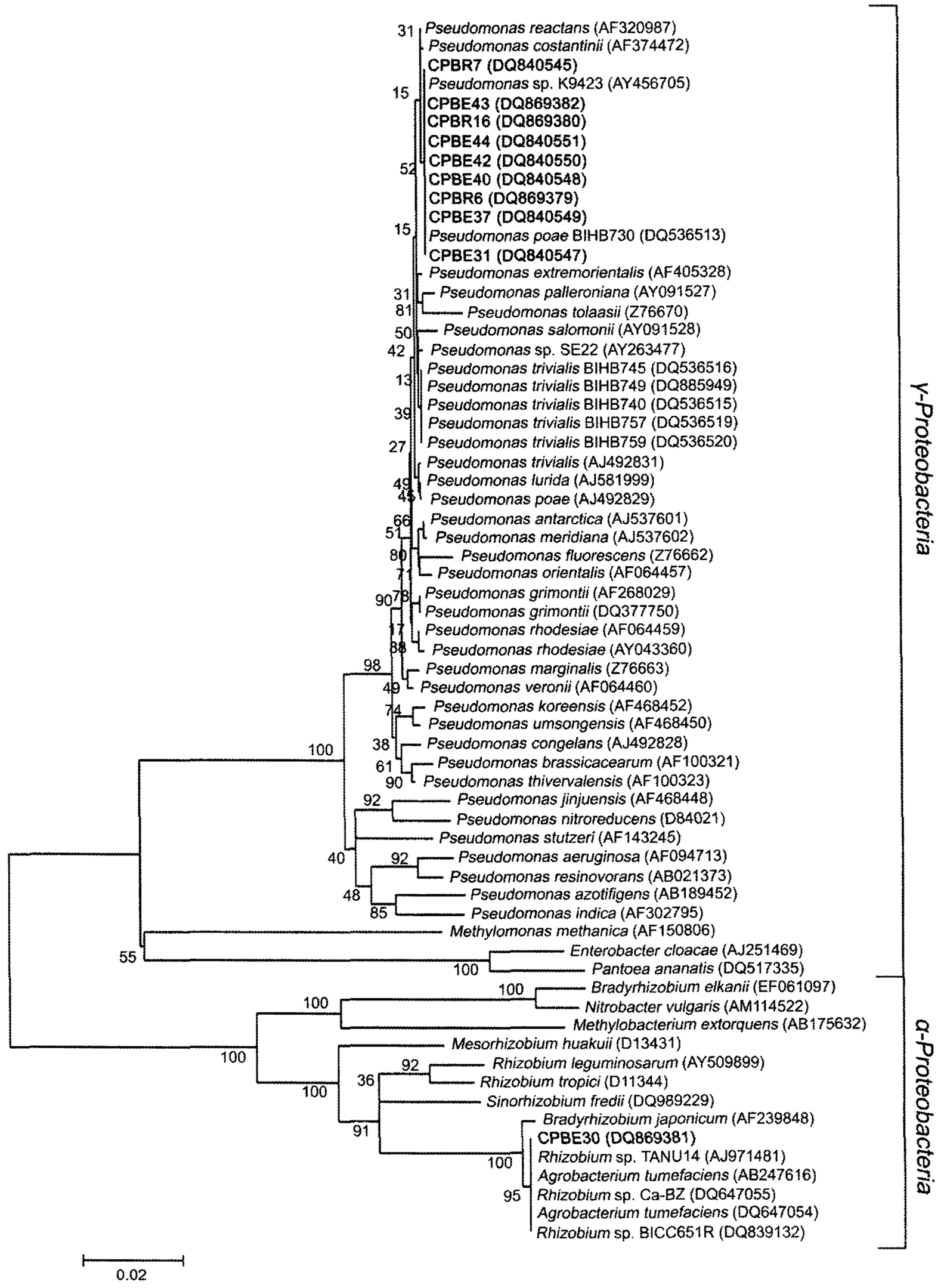

Fig. 1. Phylogenetic tree based on 16S rDNA gene sequence comparison showing the position of the phosphate solubilizing bacterial strains isolated from Chinese cabbage and other related species of the genus.

The numbers at the nodes indicate the levels of the bootstrap support based on a neighbor-joining analysis of 1,000 resampled data sets. The bar represents 0.02 substitutions per site. The strains of rhizosphere origin are designated as CPBR and those of endophytic origin as CPBE. The GenBank accession numbers are indicated in parentheses.

C. violaceum and produced no long-chain acyl homoserine lactone compounds when tested using *A. tumefaciens* (results not shown), indicating an additional advantage for their use in the biocontrol of pathogenic bacteria [7, 17]. The metabolic profiling by BIOLOG plate assay revealed that the PSB strains differed in their carbon source utilization and are functionally dissimilar. The 16S rDNA sequencing identified the strains to be *Pseudomonas*, showing close proximity with *Pseudomonas poae* (99.8–99.9%) and *Pseudomonas trivalis* (99.4–99.6%) reported from the phyllosphere of grasses [1], except for the strain CBPE30 showing 100% sequence similarity with *Rhizobium*

Table 2. Soluble P content of root extracts and external root solutions, and phosphomonoesterase activity of root extracts, from PSB-inoculated 19-day-old seedlings of Chinese cabbage.

Strain	Dry weight (mg/plant)	Soluble P		Acid Phosphomonoesterase*
		Plant (μg/g)	External solution (μg P/ml)	
Pseudomonas poae CPBR6	18.0±1.73 e	333.3±27.9 e	0.56±0.06 f	634.3±25.6 a
Pseudomonas poae CPBR7	24.0±2.89 d	495.1±31.8 k	8.64±0.83 b	430.9±17.9 d
Pseudomonas poae CPBR16	76.0±4.62 ba	435.0±26.0 b	4.77±0.39 d	455.7±32.2 c
Rhizobium radiobacter CPBE30	73.0±3.46 b	259.5±22.8 f	9.95±1.07 a	440.3±29.0 dc
Pseudomonas trivalis CPBE31	30.0±2.31 c	317.7±17.7 e	5.87±0.56 c	403.9±31.1 e
Pseudomonas poae CPBE37	79.0±6.35 a	269.6±14.8 f	5.24±0.43 dc	525.7±26.4 b
Pseudomonas trivalis CPBE40	13.0±2.31 fe	642.5±36.1 a	8.55±0.89 b	267.0±32.9 g
Pseudomonas poae CPBE42	13.0±1.73 fe	642.5±30.3 a	1.83±0.08 e	509.7±14.2 b
Pseudomonas poae CPBE43	26.0±3.46 dc	49.8±6.22 h	6.03±0.31 c	252.6±30.4 g
Pseudomonas trivalis CPBE44	8.0±0.58 f	161.8±10.3 g	ND	403.6±19.4 e
Uninoculated control	9.0±0.58 f	536.0±21.9 b	2.84 ± 0.37 e	358.9±28.2 f
LSD (P=0.05)	5.06	27.6	1.05	18.5

Values are mean±SE of three replicates. Within each vertical column, values followed by the same letter are not statistically different, according to Fisher's protected LSD (P<0.05).
*μg (PNP)/h/mg protein; enzyme activity of the root extracts.

radiobacter (Fig. 1). Although some of the endobacterial isolates associated with the ectomycorrhiza of scots pine have been shown to have close relationship with *P. poae* and *P. trivalis* [5], we report here for the first time the phosphate solubilizing ability by *P. poae* and *P. trivalis*. A recent study showed that the endophytic *Pseudomonas rhodesiae* from red pepper promoted plant growth and induced systemic resistance of plants against *Xanthomonas* [8].

The PSB strains in this study produced ACC deaminase, which stimulates plant root elongation through lowering the ethylene concentration in plants [2]. The PREP activity (calculated as the percent increase of root length on bacterial inoculation over the uninoculated control) of the strains ranged from 10.30 to 53.0%, and the strain CPBE43 possessed the highest values for PREP and ACC deaminase activity. All the strains increased the root length of Chinese cabbage when compared with uninoculated control, although the values remained significant only when the PREP activity was greater than 50%. However, ACC deaminase activity alone could not be responsible for the PREP activity, since the isolate CPBR7 with higher ACC deaminase activity exhibited the least root length and similar results were also observed with a few other strains (Table 1).

Application of PSB resulted in about 25% of reduction in P fertilizer, and increased the available P in soil and the sheath P status in sugarcane [23]. However, inoculation of PSB strain had no effect on the P nutrition of plants, although the presence of metabolized root exudates by bacterial actions enhanced plant growth [11]. Concurrent to this study, inoculation of bacterial strains to Chinese cabbage had no effect on the P concentration of plants. Soluble P in the extracts of inoculated plants remained less than that of the control, except for two strains, CPBE40 and CPBE42. However, bacterial inoculations through seed treatment increased the dry weight of plants, with an exception of strain CPBE44, and increased the available P in the external root solutions with an exception of strain CPBR6, in seedling cultures (Table 2). Hence, it is quite possible that the quantities of soluble P released from the insoluble phosphate source were too small or some other sources may prevent their uptake by plants. Although acid phosphatases have nothing to do with the solubilization of inorganic phosphates, their synthesis is stimulated when the level of inorganic P in the growth medium is limited [19], thus making the apparent relationship between them co-incidental. The acid phosphomonoesterase activity of the root extracts showed higher values in bacterial inoculations, except for strains CPBE40 and CPBE43 that recorded lower values, 267.0 and 252.6 μg (PNP)/h/mg protein, respectively, than the control (Table 2). However, no relationship between the enzyme activities and P content in plants could be obtained in this study.

The present results revealed that the plant growth promotion by PSB strains from Chinese cabbage might possibly be due to the production of phytohormones or other mechanisms, since they had no effect on the P nutrition of plants. These indigenous PSB can potentially be exploited as PGPR for Chinese cabbage with further pot and field experiments because of its increased seeded acreage and commercial crop value in Korea.

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