Isolation and characterization of phosphate solubilizing bacteria from Chinese cabbage

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Abstract
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.

Key Words
Phosphate solubilizing bacteria, *Pseudomonas*, 1-aminocyclopropane-1-carboxylate, indole-3-acetic acid.

Introduction

Phosphorus (P), after nitrogen is the major plant growth-limiting nutrient despite being abundant in soils in both inorganic and organic forms. Chemical fertilizers added to the soils to circumvent the problem of P deficiency, further compound the situation by the fact that almost 75-90% of added P fertilizer is precipitated by Fe, Al and Ca complexes present in the soils (Gyaneshwar *et al*. 2002). Phosphorus biofertilizers in the form of micro-organisms can help in increasing the availability of accumulated phosphates for plant growth by solubilization (Ryu, J.H *et al*. 2006). 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 (Poonguzhali, S *et al*. 2005). To isolate and characterize the phosphate solubilizing bacteria (PSB) associated with the rhizosphere of Chinese cabbage (*Brassica campestris* sub sp. *pekinses*), using tri-calcium phosphate (TCP) as the insoluble phosphate source. Identification of the most promising PSB by 16S rDNA sequencing and studying their PGP characteristics like production of indolic compounds, 1-amino-1cyclopropance carboxylate (ACC) deaminase, siderophores and zinc (Zn) solubilization.

Methods

Collection of plant samples and isolation of phosphate solubilizing bacteria
Chinese cabbage plants sampled from the research plots of the experimental field at Cheongwon, Chungbuk, Republic of Korea, were immediately transferred to the laboratory and processed within 24h. The PSB from the rhizosphere and root interior were isolated on Pikovskaya agar medium with 0.5% TCP as the inorganic phosphate source using dilution and plating method.

Determination of P solubilization in plate and broth assays
Sixteen PSB strains were checked for solubilization halos in Pikovskaya and NBRIP media with 0.5% TCP as the phosphate source. The soluble P present in the culture supernatant of 48 h cultures grown with TCP was estimated by Murphey and Riley (1962) method.

Characterization of selected PSB by substrate utilization using BIOLOG plates
Pure cultures grown on Biolog Universal Growth agar for 24h and suspended in sterile saline (0.85% NaCl) were inoculated to the BIOLOG plates at 150 µl per well and incubated at 28°C for 48h. The plates were read with the multi-well plate reader at 595nm. Cluster analysis and dendogram was constructed using the UPGMA software.

Determination of plant growth-promoting characteristics of PSB isolates
Zinc solubilization was determined on plates supplemented with 0.1% Zn in the form of Zinc oxide. Presence of indolic compounds was determined spectrophotometrically at 530 nm using indole-3-acetic acid (IAA) as a standard with Salkowski reagent. Presence of ACC deaminase was checked in plates containing

DF salts minimal media supplemented with 3 mM ACC as the nitrogen source. Siderophores presence was checked using Chrome azurol-S (CAS) assay.

Identification of the selected PSB by 16S rDNA sequencing
The strains were identified by the analysis of their 16S rRNA gene sequences and sequence homologies were determined using BLAST. The identified gene sequences were submitted to GenBank/NCBI under the mentioned accession numbers

Results
Plant growth promotion by PSB included mechanisms other than solubilization of insoluble phosphates. 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). A recent study showed that the endophytic *Pseudomonas rhodesiae* from red pepper promoted plant growth and induced systemic resistance of plants against *Xanthomonas*. The PSB strains in this study produced ACC deaminase, which stimulates plant root elongation through lowering the ethylene concentration in plants. 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).

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).

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, except for the strain CBPE30 showing 100% sequence similarity with *Rhizobium radiobacter* (Figure. 1).

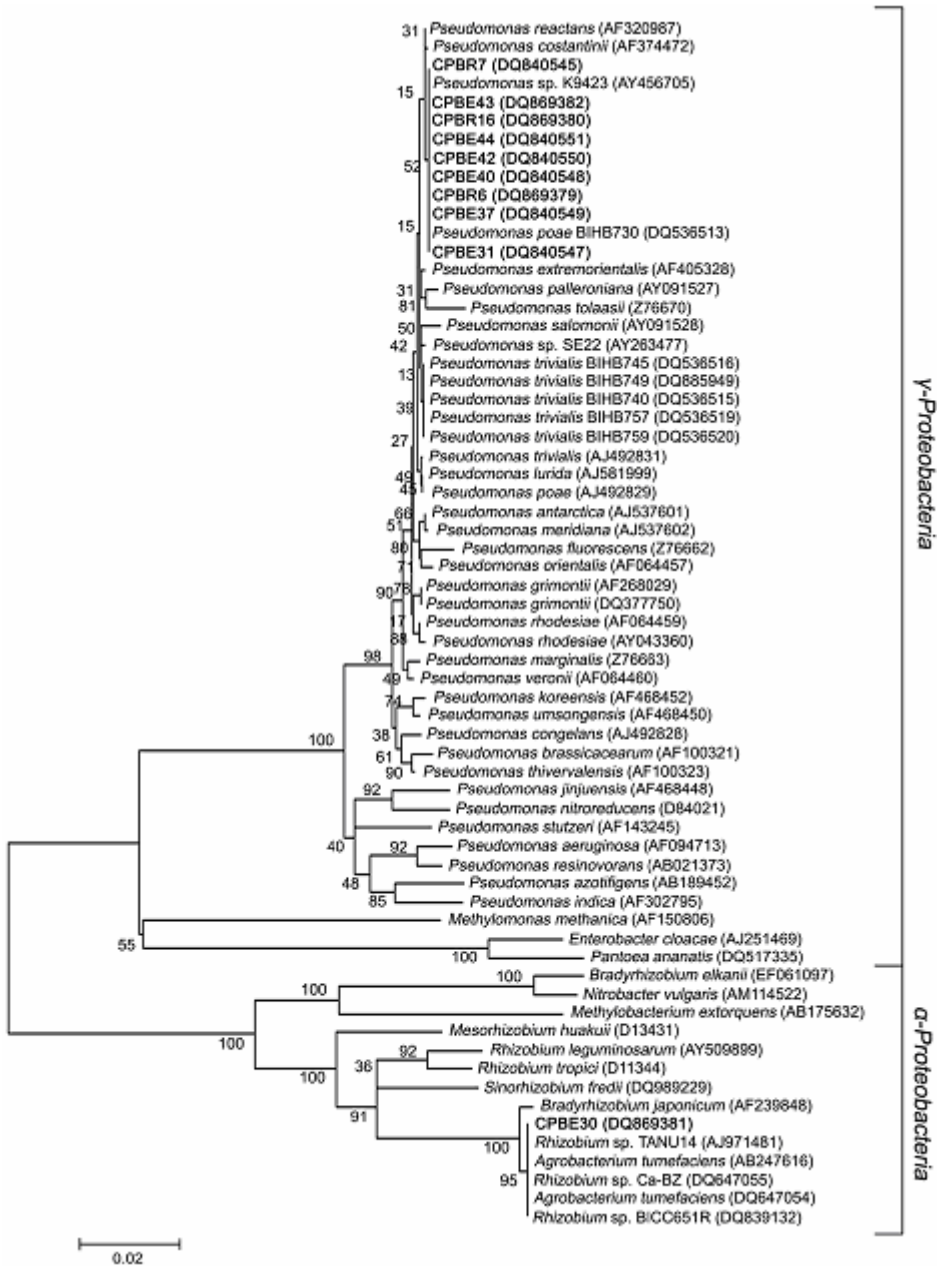

Figure 1. Phylogenetic tree based on 16S rDNA gene sequence comparison showing the position of the phosphate solubilizing bacterial strains isolated from Chinese cabbage and other related species of the genus.

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 represents0.02 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.

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. 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. 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, 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). 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.

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

Strain	Dry weight (mg plant^{-1})	Soluble P		Acid Phosphomono-esterase*
		Plant (µg g^{-1})	External solution (µg P ml^{-1})	
Pseudomonas poae CPBR6	18.0±1.73e	333.3±27.9e	0.56±0.06f	634.3±25.6a
Pseudomonas poae CPBR7	24.0±2.89d	495.1±31.8k	8.64±0.83b	430.9±17.9d
Pseudomonas poae CPBR16	76.0±4.62ba	435.0±26.0b	4.77±0.39d	455.7±32.2c
Rhizobium radiobacter CPBE30	73.0±3.46b	259.5±22.8f	9.95±1.07a	440.3±29.0dc
Pseudomonas trivalis CPBE31	30.0±2.31c	317.7±17.7e	5.87±0.56c	403.9±31.1e
Pseudomonas poae CPBE37	79.0±6.35a	269.6±14.8f	5.24±0.43dc	525.7±26.4b
Pseudomonas trivalis CPBE40	13.0±2.31fe	642.5±36.1a	8.55±0.89b	267.0±32.9g
Pseudomonas poae CPBE42	13.0±1.73fe	642.5±30.3a	1.83±0.08e	509.7±14.2b
Pseudomonas poae CPBE43	26.0±3.46dc	49.8±6.22h	6.03±0.31c	252.6±30.4g
Pseudomonas trivalis CPBE44	8.0±0.58f	161.8±10.3g	ND	403.6±19.4e
Uninoculated control	9.0±0.58f	536.0±21.9b	2.84±0.37e	358.9±28.2f
LSD (P≤0.05)	5.06	27.6	1.05	18.5

Values are the 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^{-1} mg protein^{-1} enzyme activity of the root extracts.

Conclusion

The PSB isolates from Chinese cabbage apart from solubilization, also possessed other characteristics that may promote plant growth thus making them as a promising inoculant for crops. Further studies on the genetics of P solubilization and rhizospheric competence will help to develop them as successive bioinoculants.

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