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Mineral Phosphate Solubilizing Bacteria Isolated from Various Plant Rhizosphere under Different Aluminum Content

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Abstract

The objectives of this study was to isolate and characterize the mineral phosphate solubilizing bacteria from rhizosphere and evaluate their potential as plant growth promoting bacteria in Al-toxic soils. The halo zone formation method was used to isolate PSB using the media containing insoluble phosphates (Ca-P or Al-P) as a source of phosphate. Eight of acid and Al-tolerant PSB isolates that were able to solubilize Ca-P were obtained from rhizosphere of clover, wheat, corn, and sunflower grown in Al-toxic soil. Identification of the isolates based on the 16S rRNA gene sequence analysis demonstrated that the isolates were strains of *Burkholderia* (5 strains), *Pseudomonas* (1 strain), *Ralstonia* (1 strain), and unidentified bacterium (1 strains). All PSB isolates showed the capability to dissolve Ca-P, and only 1 strain (*Ralstonia* strain) was able to dissolve Al-P in agar plate medium. The P-solubilization by these isolates was correlated with pH of medium. Inoculation of the bacterial strains on clover on Al-toxic medium showed that all isolates increased the plant dry weight compared with uninoculated treatment. Our results showed that those PSB isolates have potential to be developed as a biofertilizer to increase the efficiency of P-inorganic fertilizer used in Al-toxic soils.

Introduction

Aluminium (Al) toxicity is a primary factor limiting the growth and yield of the majority of plants grown in mineral acid soils. Numerous studies have demonstrated the marked depressive effects of soluble Al on water and nutrient uptake root and shoot growth and mineral content in different plant species.

Plant roots provide such suitable habitats for the growth of microorganisms that high numbers of many different microbial populations are found on and in the immediate vicinity of the roots. This habitat, the rhizosphere, is defined as the region of soil directly affected by the influence or action of a root (Lynch, 1994).

The rhizosphere develops around each root as it grows, changing the chemical and physical properties of the surrounding soil. Thus, pH in the rhizosphere is usually different from that in the root-free soil, depending on the cation: anion uptake ratio of the roots (Brimecombe *et al*., 2001), and the redox potential is lower than in root-free soil due to high oxygen consumption by roots and microbes (Darrah and Roose, 2001). The environmental soil conditions in turn have an influence on the composition and amount of root exudates. This rhizodeposition includes lysates, liberated through autolysis of sloughed-off cells and tissues, as well as diffused and/or actively secreted root exudates (Neumann and

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Römheld, 2001). The amount of root exudates produced is proportional to the amount of gross photosynthesis and crop dry matter production. Thus, 1-25% of net photosynthesis is assumed to be released into the soil (Kraffczyk *et al*., 1984; Haider *et al*., 1985; Haller and Stolp, 1985).

The number of soil organisms found in the soil is usually much higher in the rhizosphere than in the root-free soil (Curl and Truelove, 1986; Kennedy, 1999). Different rhizosphere bacteria will probably occupy different niches within the rhizosphere depending on e.g. their utilization of root exudates, growth rate and metabolic production (Lynch, 1994). In the rhizosphere, the composition of the bacterial community have been shown to be regulated by the stage of plant growth (Picard *et al*., 2000) and the amount of young and old roots (Liljeroth *et al*., 1991; Duineveld and van Veen, 1999) due to differences in exudates composition.

A number of phosphate solubilizing bacteria (PSB) are known to be present in rhizosphere. They have ability to excrete organic acid that form chelates with Al^{3+}, Fe^{3+}, Ca^{2+}, and Mg^{2+} ions and release phosphate previously fixed by these ions. It is desirable if rhizospheric PSB with Al-P solubilizing ability are obtained. Seed or soil inoculation with mineral PSB is known to improve solubilization of fixed soil phosphorus and applied phosphates, resulting in higher crop yields. However, most of the studies on PSB focused on solubilization of calcium phosphate (Ca-P), which appears in alkaline soils. By contrast, very limited studies are available on aluminum-phosphate (Al-P) solubilizing bacteria, which must be important in Al-toxic soils.

In this study, isolation of PSB from rhizosphere soil of various plants grown in Al acidic soil, and evaluation of their Ca-P and Al-P solubilizing ability under acidic condition were carried out. As basic information, effect of Al on the number of soil microorganisms including PSB in the rhizosphere of various plants was also evaluated.

Materials and Methods

Soil and pot experiments

The subsoil of an Andisols, Japan, was used for making artificial Al-toxic soil due to its properties common to Al-toxic soil such as low organic matter content, low buffering capacity, low available P, and high exchangeable Al. Soil was sieved (2 mm), amended with $Al_2(SO_4)_3$ (0, 0.4, 0.6, 0.75 %, w/w), mixed well, and incubated for 1 month with field water capacity at room temperature. Four plant species that relatively tolerant to the Al toxicity (soybean, corn, rice cultivar Nihon Bare, sunflower) were treated with 0%, 0.4%, and 0.75% $Al_2(SO_4)_3$, while three plant species relatively sensitive to the Al toxicity (clover, wheat, *Sesbania rostrata*) were treated with 0% and 0.6% $Al_2(SO_4)_3$ (data not shown). Seeds were surface sterilized with 70% (v/v) ethanol for 5 min and 6% NaClO for 15 min, and washed several times with sterile distilled water, then germinated for one day in sterile filter paper. Three seedlings each were transferred and grown in 200-ml non-sterilized soils amended with basal fertilizer (50 mg kg^{-1} N-urea; 50 mg kg^{-1} K-KCl, 20 mg kg^{-1} P-CaP, and 0.5 mg kg^{-1} Mo-NH_4MoO_4) just before transplanting. Plants were grown for 2 months in growth chamber with 16 h light and 8 h dark at 25°C. After harvested, the total dry weight of each plant was determined. The rhizosphere soils were sampled as described below for enumeration of PSB (on Ca-P Pikovskaya agar plate), total bacteria (on nutrient agar plate added with 100 µg ml^{-1}cycloheximide), and total fungi (on potato dextrose agar plate, Difco Laboratories, Detroit, MI, USA), and isolation of PSB.

Isolation of PSB from rhizosphere soil and genotypic characterization

The bacteria were initially isolated from the rhizosphere of the plant. The roots of 2-month-old plant were removed from the soils and the 1 gram of fresh root was carefully added to 200 ml Erlenmeyer flask containing 50 ml Ca-P Pikovskaya medium (Pikovskaya, 1948). Serial dilutions were poured on solid (15 g l^{-1} agar) Ca-P Pikovskaya medium. Colonies with different morphology and producing a halo zone as a result of Ca-P solubilization were purified on nutrient agar medium and stored in a slant agar of Picovskaya medium at 4°C. The isolates that have a high ratio halo zone/colony diameter (≥ 1.2) were selected for further experiments.

Amplified rDNA restriction analysis (ARDRA)

The 16S rRNA gene was amplified from each strain by using the forward primer 27f (5'-AGAGTTTGATC[A/C]TGGCTCAG-3') which corresponds to the positions 8-27 of the *E. coli* 16S rRNA and the reverse primer 1492r (5'-TACGG[A/T/C]TACCTTGTTA CGACTT-3') which corresponds to the positions 1492-1513 of the *E. coli* 16S rRNA sequence. Genomic DNAs of *P. putida* strains were prepared using cetyltrimethylammonium bromide for precipitation of proteins, exopolysaccharides, and cell debris (Ausubel *et al*., 1987). Total DNA of each strain was used as a template in PCR, and the reaction condition were those described by Hedlund-Brian *et al*. (1999). PCR products were then digested with the restriction endonucleases, *Hae*III and *Hha*I, and resultant restriction fragments were analyzed by electrophoresis on 2.5% agarose gels.

Repetitive DNA PCR fingerprinting

One hundred nano grams of genomic DNA from each isolate was used as a template for PCR reaction with the primer BOXA1R (5'-CTACGGCAAGGCGACG CTGACG-3'). The PCR reaction and amplification were performed as described previously (De Bruijn, 1992; Versalovic *et al*., 1994). After PCR amplification, PCR reaction mixture was separated by 1.5% agarose gel electrophoresis using Tris-Acetate-EDTA and stained with ethidium bromide.

Phosphate solubilization by PSB isolates at various Al concentration and pH

The ability of isolates to solubilize aluminum-phosphate (Al-P) was evaluated in Al-P Pikovskaya agar medium with $AlPO_4$ (4 g l^{-1}) as phosphate source. The $AlCl_3$ was added to the medium with concentration as follows: 100 µM, 250 µM, and 1000 µM. Because addition of $AlCl_3$ decreases pH of the medium, pH of the control (no $AlCl_3$) was adjusted at 5.5 by adding HCl. The adjustment of pH and addition of $AlCl_3$ were carried out after autoclaving the medium. The phosphate solubilization activity was determined by calculating the ratio of halo zone to colony diameter after 5 days of incubation.

DNA sequencing and sequence analysis

The 16S rRNA genes were sequenced by the chain termination method using a LI-COR Model 4200L-2 auto-DNA sequencer (LI-COR Inc., Lincoln, Neb. USA) according to the manufacturer's instruction. The obtained nucleotide sequences were analyzed with DNASIS-Mac software (version 3.7; Hitachi Software Engineering Co. Ltd., Yokohama, Japan). The BASTN method (Altschul *et al*., 1997) was used for homology searching.

Inoculation of PSB isolates on plant growth under Al-acidic condition

The inoculation effects of PSB isolates on plant growth were evaluated using clover. The clover was germinated in sterile filter paper and transplanted to the acid and low phosphate artificial medium

containing vermiculite. The medium was amended with basal nutrient (g l^{-1}): NH_4NO_3 0.1617; K_2SO_4 0.176; $CaCl_2$ 0.1; $MgSO_4$ 0.062; Fe-EDTA 0.005; $MnSO_4$ 0.0015; H_3BO_3 0.0014; $ZnSO_4$ 0.00028; $CuSO_4$ 0.00025; NH_4MoO_4 0.000124; and $AlPO_4$ 0.271). The medium was irrigated to field capacity with nutrient solution containing 100 µM $AlCl_3$ (pH 4.8) before transplanting, and continued every second day during the growing period. The germinated seed was inoculated with 50 µl of 3 day-old bacterial suspension cultured in Ca-P Pikovskaya medium at a cell density 10^8 CFU ml^{-1}. The uninoculated treatment was served as control. The plant was cultivated in culture chamber at 25°C, light 16 h/dark 8 h. Thirty days after planting, the plant was harvested then total dry weight was determined. The data are means of three replications.

Results and Discussion

Effect of different Al content in soil on total soil microorganisms and PSB

The addition of $AlCl_3$ at 0.4% in all plant, improved the total number of bacteria and PSB. However, generally, the addition of $AlCl_3$ at 0.75% improved the total number of fungi (Figure 1). The number of soil microorganisms (total bacteria, and total fungi) and number of PSB were influenced by the plant cultivation. In planted soils, the number of microorganisms was higher than in non-planted soils. These results suggested that the plant promoted the population of rhizosphere microorganisms.

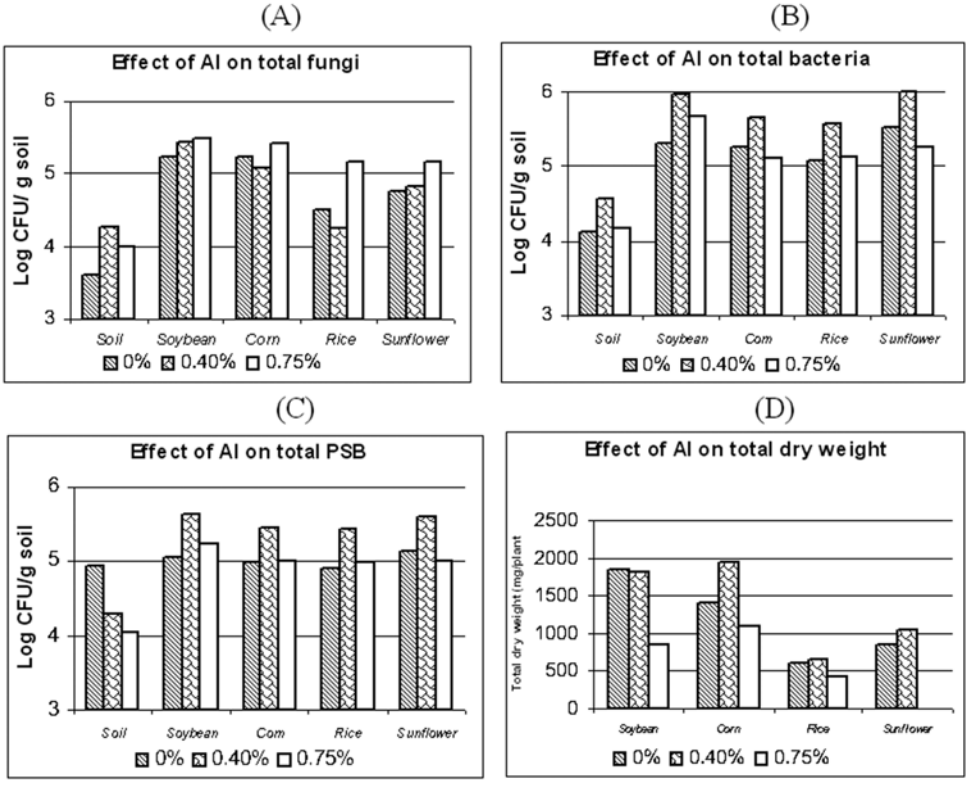

Figure 1. The effect of Al level 0, 0.4%, and 0.75% on total fungi (A), total bacteria (B), total PSB (C), and total dry weight of plant (D)

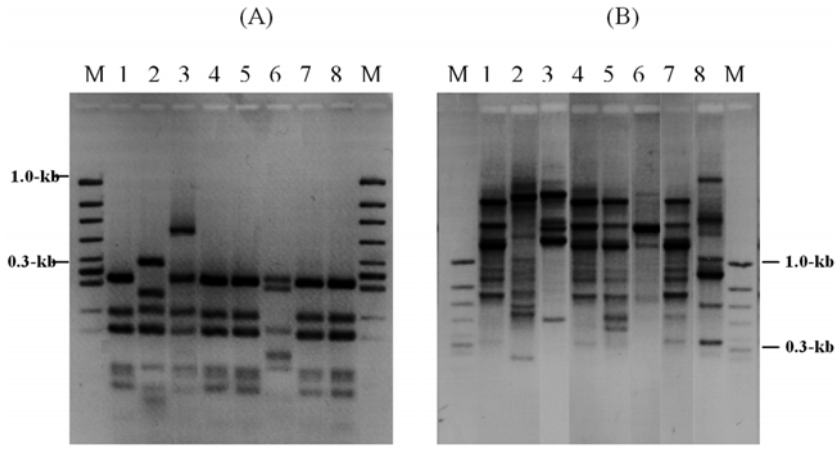

Figure 2. ARDRA profile digested with *Hha*I and *Hae*III restriction enzymes (A) and REP-PCR profile (B) of PSB isolates obtained from rhizospheric mineral acid soils. Lane M: Marker DNA; lane 2-8 were Cl3.6; W1.6; Co.4m; Cl1.0; SF1.6; Co3.0; and Bio respectively.

Isolation and characterization of PSB

Eight bacterial strains able to solubilize Ca-P were isolated from different plant rhizosphere. The genotypic analysis revealed 4 groups discriminated by ARDRA were obtained in this study (Figure 2A). Identification of bacterial isolates by using 16S rRNA gene sequence analysis showed that the isolates belong to *Burkholderia* (5 strains), *Pseudomonas* (1 strain), *Ralstonia* (1 strain), and unidentified bacterium (1 strain) (Table 1). By using REP-PCR analysis, 7 different bacterial groups could be recognized (Figure 2B).

Table 1. PSB bacteria isolated from rhizosphere subsoil of an Andisol

Strain	Origin	Plant added (g/kg)	$AlCl_3$ after harvesting	Soil pH Gram reaction	16S rRNA gene sequence[a]	
					Best match	Identity
Cl3.6	Clover	6	4.1	Negative	*Burkholderia* sp.	98%
W1.6	Wheat	6	4.2	Negative	*Burkholderia* sp.	99%
Co.4m	Corn	4	4.3	Negative	*Unidentified bacterium*	-
Co.4h	Corn	4	4.3	Negative	*Burkholderia* sp.	97%
Cl1.0	Clover	0	5.5	Negative	*B. cepacia*	100%
SF1.6	Sunflower	0	5.9	Negative	*Pseudomonas* sp.	99%
Co3.0	Corn	0	5.8	Negative	*Burkholderia* sp.	98%
Bio	Corn	4	4.3	Negative	*Ralstonia thomasii*	99%

[a]The partial of 16S rRNA gene were amplified by PCR using universal primer. The PCR products were sequenced and compared with GeneBank Database

The effect of Al concentration and pH on Al-P and Ca-P solubilization of PSB isolates

In the Ca-P solubilizing assay in agar plate medium all PSB isolates formed the halo zone surrounding colony. However, at pH 7, only strain Bio produced the halo zone when Al-P was used as a phosphate source. This finding indicated that only strain Bio has an ability to dissolve Al-P. The ability of PSB isolates to solubilize Al-P also was examined under different pH and Al concentration (Table 2). As shown in Table 2, the halo zone formation on Al-P in strains C13.6, C0.4m, C11.0, Co.3.0, and Bio was induced by addition with 250 mM $AlCl_3$ at pH 4.5, while the halo zone formation in strains C13.6, C0.4m, C11.0, Co.3.0 was also induce by decreasing pH at 4.1 without the addition of $AlCl_3$.

Table 2. The ratio of halo zone/colony diameter formation by PSB isolates at various phosphate source, pH, and aluminum concentration, after 14 days incubation at 25^{0}C

Strain	Ca-P	Al-P added with Al (mM)						
		0	0	100	0	250	0	1000
	pH 7.0	pH 7.0	pH 5.5		pH 4.5		pH 4.1	
Cl3.6	1.20	1.00	1.00	1.00	1.18	1.20	1.10	ng
W1.6	1.75	1.00	1.00	1.00	1.00	1.00	1.00	ng
Co.4m	1.44	1.00	1.00	1.00	1.22	1.24	1.13	ng
Co.4h	1.65	1.00	1.00	1.00	1.00	1.00	1.00	ng
Cl1.0	1.43	1.00	1.00	1.00	1.20	1.25	1.10	ng
SF1.6	1.10	1.00	1.00	1.00	1.00	1.00	1.00	ng
Co3.0	1.53	1.00	1.00	1.00	1.20	1.25	1.10	ng
Bio	1.07	1.10	1.36	1.27	1.40	2.33	1.00	ng

ng: no growth

Inoculation effects of PSB isolates on plant growth

Inoculation of PSB isolates on clover with Al-P as a phosphate source showed that all isolates improved the total dry weight of clover (Figure 3). These results indicated that PSB isolates obtained in this study have a potential as plant growth promoting rhizobacteria in aluminum toxic soils.

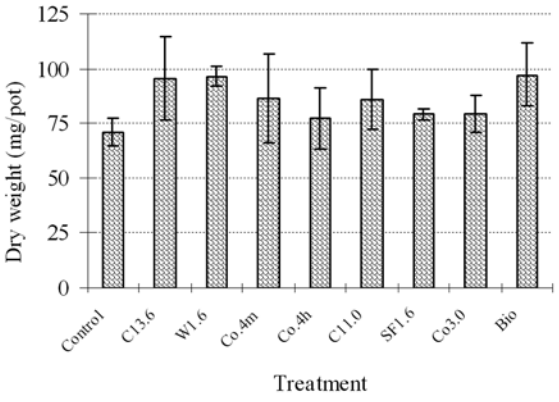

Figure 3. The inoculation effect of PSB isolated from rhizosphere acid soils on dry weight of clover.

The rhizosphere is an ecological niche, which is mainly a black box. Hardly anything is known about the availability of nutrients (organic or inorganic), the dangers, which affect the survival of microorganism (e.g. predation, harmful compounds) or interaction among the rhizosphere organisms (De Weger *et al*., 1995).

In this study, the population of microorganisms in rhizosphere soil was more influenced by aluminum concentration than by the kind of host plant. Surprisingly, in some case, the addition of $AlCl_3$ on subsoil of an Andisol improved the total microorganisms and plant growth used in this study. Moreover, in addition of $AlCl_3$ at 0.6% or 0.7%, the population of fungi in some case is still high. The high population of soil fungi in Al toxic condition may be caused by tolerance of the soil fungi to acidic and Al toxic condition (Alexander, 1977). Relatively little research has been reported about aluminum effects on microorganisms, compared to the plant research. The complex chemistry of Al, which polymerizes, interacts with phosphates and organic acids, and acidifies culture media, frequently complicates the interpretation of

experimental results (Piña and Cervantes, 1996).

The PSB isolates were obtained from rhizosphere of corn (4 strains), clover (2 strains), wheat (1 strain), and sunflower (1 strain), and most of them belong to genus *Burkholderia*. Previously, it has been reported that genus *Burkholderia* is a common plant associate bacteria (Balandreau *et al*., 2001). Several strains of this genus have also been reported as nitrogen fixer rhizobacteria (Estrada-de los Santos *et al*., 2001), phosphatase producing bacteria (Rodriguez *et al*., 2000), and plant growth promoting rhizobacteria (Peix, 2001).

Unfortunately, the Al-P solubilization ability and the Al tolerance of PSB isolates on agar plate medium were quite low. Much more strategies are needed in order to isolate the bacteria that have strong abilities of the Al-P solubilization and the Al tolerance from rhizosphere.

Most of PSB isolates improved the plant growth of clover under acidic and low available phosphate conditions. However, these isolates are poor in Al-P solubilizing ability except strain Bio. Other mechanism than phosphate solubilization, which promote the plant growth will be present in seven PSB isolates, but it is unclear at present. Anyhow, screening of Al-P solubilizing strains under Al acidic condition is necessary.

This result shows that PSB isolates obtained in this study has a potential as plant growth promoting rhizobacteria in aluminum toxic soils. However, the inoculation effect of these isolates on plant growth promotion needs to be further evaluated under different crop and agroclimatic conditions, particularly in the real aluminum toxic soils.

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