Plant growth promotion abilities and microscale bacterial dynamics in the rhizosphere of Lupin analysed by phytate utilization ability.

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Summary

In the rhizosphere, phosphorus (P) levels are low due to P uptake into the roots. Rhizobacteria live on carbon (C) exuded from roots, and may contribute to plant nutrition by liberating P from organic compounds such as phytates. We isolated over 300 phytate (Na-inositol hexa-phosphate; Na-IHP)-utilizing bacterial strains from the rhizosheath and the rhizoplane of *Lupinus albus* (L.). Almost all of the isolates were classified as *Burkholderia* based on 16S rDNA sequence analysis. Rhizosheath isolates cultured with Na-IHP as the only source of C and P showed lower P uptake at the same extracellular phytase activity than rhizoplane strains, suggesting that bacteria from the rhizosheath utilized phytate as a C source. Many isolates also utilized insoluble phytate (Al-IHP and/or Fe-IHP). In co-culture with *Lotus japonicus* seedlings, some isolates promoted plant growth significantly.

Introduction

Organic phosphorus (P) is generally estimated to contribute at least 50 % of total-soil P. To become available for plants, organic P must be dephosphorylated by phosphatases, because plants acquire P as inorganic P (Richardson, 2001a). The rhizosphere, that is, the soil in the immediate vicinity of roots, is characterized by high microbial densities (Hiltner, 1904); the stimulation of microbial growth by roots is commonly known as the rhizosphere effect. In the rhizosphere, organic substances (sugars, organic acids, polysaccharides, etc.) are exuded from roots to the soil, where they are utilized by microorganisms as readily available sources of carbon (C) and energy for growth and reproduction (Whipps, 1990). However, rhizosphere microorganisms have to compete with plant roots for most other elements such as P ~~(Fig. 1)~~. Phosphatase activity is high within the rhizosphere, turning it into a zone of organic P depletion (Tarafdar and Jungk, 1987). Major components of soil organic P are the soil phytates, mainly consisting of inositol penta- and hexa- phosphates (IHP; Dalal, 1977). Phytase is one of the phosphatases responsible for the dephosphorylation of phytate, which is thought to play an important role in plant nutrition (Tarafdar and Claassen, 1988; Findenegg and Nelemans, 1993).

In general, many plant species do not use soil phytate as a P source,

because they lack extracellular phytase activity. However, when exogenous phytase was added (Idriss *et al.,* 2002), or when the phytase gene from *Aspergillus niger* was expressed in transgenic plants (Richardson *et al.,* 2001b), the plants became able to uptake phytate P. Thus, the presence of phytase in the rhizosphere may enhance plant growth under field conditions. Noteworthily, phytase activity derived from soil microorganisms such as *Pseudomonas sp.* (Richardson *et al.,* 2000, 2001c) or *Bucillus amyloliquefaciens* (Idriss *et al.,* 2002) has been shown to contribute to plant P nutrition (Hayes *et al.,* 2000; Richardson *et al.,* 2000, 2001c; Idriss *et al.,* 2002). Plant-growth-promoting rhizobacteria (PGPR) are a class of free living bacteria in the rhizosphere that have beneficial effects on plants (Kloepper *et al.,* 1980; Bloemberg and Lugtenberg, 2001). Several mechanisms have been proposed to explain PGPR effects, including the production of plant-growth-regulating substances (Barea *et al.,* 1976; Steenhoudt and Vanderleyden, 2000) and the enhancement of nutrients availability (Nautiyal *et al.,* 2000).

Though Na-IHP-utilizing bacteria are ubiquitous and can be found in cultivated soils as well as in wetland, grassland, and forest soils (Richardson and Hadobas, 1997), the utilization of phytate by crops and microbes is generally limited under field conditions. Presumably, IHP utilization is inhibited by IHP adsorption to soil particles and the formation of insoluble salts (Hayes *et al.,* 2000). This hypothesis is supported by the fact that soil phytase activity could be stimulated by application of organic acids, especially citrate (Hens *et al.,* 2003). Exudation of organic acids from roots enhances P availability by chelating cations such as Fe, Al, or Ca, which form practically insoluble phosphates. White lupin (*Lupinus albus* L.) possesses a particularly high organic acid releasing ability and shows more vigorous growth than other species under low P conditions, and Na-IHP as the only P source condition (Adams and Pate, 1992). Therefore, white lupin appears an adequate model to study phytate decomposition in the rhizosphere. The aim of this study was to elucidate mechanisms of the interactions between plants and bacteria which make phytate P available to plants, as a basis for increasing the efficiency of phytate utilization by crops.

Results

Isolation and identification of Na-IHP utilizing bacteria

In total, more than 300 Na-IHP-utilizing bacterial strains were isolated from edaphosphere soil, rhizosheath, and rhizoplane (Table 1). These isolates were able to grow on Na-IHP as the only C and P source. As all isolates developed colonies in upper layers of the semi-solid phytate specific medium, they appeared to be aerobe or micro-aerobe.

Table. 1

For further analysis, we chose the isolates obtained at the flowering stage 58 days after sowing (Table 2), as the rhizosphere effects could be expected to be most clearly pronounced at this stage (Smalla *et al.,* 2001). Sequence analysis of 16S rDNA was performed on 73 isolates (36 strains from the rhizosheath and 37 strains from the rhizoplane). The results indicated that all isolates fell into the beta-subdivision of the *proteobacteria*; 70 strains showed high similarity with the *Burkholderia* subgroup (Table 3a, b). No clear differences regarding cultivable Na-IHP-utilizing bacteria were observed between the plots that had been subjected to different long-term chemical fertilizer treatments since 1914 (Tables 3a, b).

Table. 2

Table. 3a

Table. 3b

Utilization of different type IHP by Na-IHP utilizing bacteria

Growth rates, the amounts of phosphate accumulated in the media, and extracellular phytase activities varied widely between the Na-IHP-utilizing bacterial strains that were isolated from the root space of *Lupin* at the flowering stage (Tables 3a, b). In Na-IHP utilizing test, we used limiting phytate specific broth which was prepared by replacing the 10 g Na-IHP in the phytate specific broth by 1 mM Na-IHP, some strains did not show the ability of growth in limiting phytate specific broth. In none of these parameters, however, there were any clear differences between the groups of isolates from the rhizosheath and the rhizoplane. Interestingly, there were 2 rhizosheath isolates (PpRsG5 and FpRsG6) whose media contained extremely high amounts of phosphate and showed particularly high phytase activities (Table 3a). The dependence of the phosphate release rate on extracellular phytase activity differed between strains isolated from the rhizosheath and from the rhizoplane, respectively (Fig. 2). All isolates did grow on Al-IHP as the only carbon and phosphorus source, but only 46 isolates were able to grow on Fe-IHP (Tables 3a, b). All cultivated Al-IHP utilizing bacteria decreased the medium pH.

Fig. 1

Plant growth promotion by Na-IHP-utilizing bacteria

Growth of lotus seedlings in gel culture with or without Na-IHP-utilizing bacteria and with phytate as the sole phosphorus source in the soil showed characteristic differences. While shoot length was enhanced by 6 bacterial strains from the rhizosheath and by 9 strains from the rhizoplane, root growth was not significantly promoted ($p=0.05$; Tables 3a, b). The dry weight and total P of bacteria-free controls and 16 PGPR candidates are shown in Fig.2. Among these candidate strains, FpRpG4 induced 3-fold increases in *Lotus* dry weight and total P content, and a more than 6-fold increase in shoot length (Fig. 3).

Fig. 2

Fig. 3

Discussion

We have isolated potential PGPRs living in experimental fields with different long-term fertilization patterns and the isolates were identified by screening for the ability to grow on Na-IHP as the only source of C and P. As there was no correlation between bacterial growth and the amount of phosphate released into the Na-IHP medium, the rate of decomposition of Na-IHP in the medium did not seem to be regulated only by the P requirement for growth. The dependence of the phosphate release rate on extracellular phytase activity differed between strains isolated from the rhizosheath and from the rhizoplane, respectively (Fig. 1). Rhizosheath bacteria seemed to take up a smaller fraction of the P released. Similar to other extracellular depolymerases, extracellular phytase activity was suggested to appear after the exhaustion of easily accessible nutrient sources (Idriss *et al.,* 2002). Consequently, the production of phytase in the rhizosheath might be influenced by environmental conditions. Bacterial growth depends on soil micro-environmental parameters (water content, particle size, organic matter content, etc.), which interact in a complex manner. As the physicochemical properties of soil are strongly affected by the activity of roots, roots induce physical and chemical micro-scale gradients in the soil, which in turn determine spatial patterns of microorganism diversity around the roots. Our results suggest that Na-IHP-utilizing bacteria may adapt to their habitat on a spatial gradient of C and P availability in the rhizosphere. Bacteria obtained from the rhizoplane live very close to plant roots, which provide large amounts of C. These bacteria may utilize phytate as a P source because inorganic P is depleted close to the roots. On the other hand, bacterial growth in soil is generally limited by carbon availability, so that bacteria in the

rhizosheath may utilize phytate as a C rather than a P source. But this study can only be considered as a starting point in the micro-scale bacterial dynamics in the rhizosphere of lupin analyzed by phytate utilization ability, so the trial to understand this phenomenon in the natural environment by localization analysis is progressed.

The genus *Burkholderia* comprises of 29 species including soil and rhizosphere bacteria as well as plant and human pathogens (Yabuuchi *et al.,* 1993; Gillis *et al.,* 1995). *Burkholderia* also includes PGPR and rhizobia (Moulin *et al.,* 2001), and a significant proportion of maize-, wheat-, and particularly lupin-associated bacteria belong to *B. cepacia* genomovar III (Balandreau *et al.*, 2001). The ability to utilize phytate has not been reported as a common feature of *Burkholderia* yet, but most of the isolates in this study belonged to this genus. So our results indicate that some *Burkholderia* have the ability to grow on Na-IHP as the only source of C and P and exist in the rhizosphere of lupin. *Burkholderia* has an unusually complex genome that consists of three circular chromosomes (>600kb) with a total size of >7 Mb. The genomes of some strains undergo frequent large-scale gene rearrangements, causing a high ability of environmental adaptation (Rodley *et al.,* 1995; Lassie *et al.,* 1996). For the same reason, misidentification on the species level is relatively common (LiPuma, 1998). Besides, in the rhizosphere and especially the rhizoplane, horizontal gene transfer occurs frequently (Elsas *et al.,* 1998), as particularly high microbial population densities develop in this region. For example, *Burkholderia* was shown to be a recipient of large plasmids carrying genes necessary for the ability to degrade the herbicide 2,4-D (Newby *et al.,* 2000). The rapid migration of genetic information between different bacteria in the rhizosphere and rhizoplane, and the rapid gene rearrangement might have favored the distribution of the ability to utilize phytate across the strains isolated in this study.

Our results indicate that plants and bacteria interact via phytate utilization. Under phosphate-deficient conditions, plants and microorganisms mutually support each other by the C made available by plants (organic acid exudation) and P supplied by bacteria (decomposition of phytate). Our attempts to demonstrate PGPR effects in Lotus showed both positive and negative results (Tables 3a, b). Though a significant positive linear relationship between total P content and dry weight was observed in plants in which growth was promoted by some of the PGPR candidates isolated from the rhizosheath (Fig. 2). Our isolates such as FpRpG4 show strong PGPR effect accompanied by increasing P uptake of inoculated plant, but some other isolates promoted plant growth without increasing P uptake. So our results suggest that not

only phytase activities, but also the other effects besides phytase are contributed for the plant growth promoting effects of these strains. Although another experiments such as creating a phytase mutant are needed to prove the contribution, the production of plant-growth-regulating substances by the bacteria might be responsible for this finding (Barea *et al.,* 1976).

One method to enhance the efficiency of applied P fertilizers and/or phosphorus containing organic compounds is the inoculation with P-solubilizing PGPR. Attempts to use such bacteria as biofertilizers have been made since the 1930s (Cooper, 1959), but field experiments were not successful. Free phosphate binds strongly to Al and Fe ions in the soil, preventing its uptake by plant roots. Consequently, the availability for plant growth of IHP-derived P is determined by the concentration of Al and Fe ions and by the pH of the soil, because IHP binds to these ions in a pH-dependent manner (Shang *et al.,* 1992). Therefore, bacteria that have the ability to utilize Al and/or Fe-IHP are promising candidates for efficient biofertilizers. However, such strains are rare. In this study, all the isolates which could utilize Na-IHP as a C and P source, were also able to utilize Al-IHP, but only 62 isolates were found to utilize Fe-IHP (Table 3a, b). In addition, all isolates decreased the culture medium pH in Al-IHP utilization tests, but only 13 isolates including two colony-forming strains showed this ability when growing on Fe-IHP. As root-derived organic acids do not only provide a carbohydrate substrate for microorganisms but also release phosphate from scarcely soluble phosphorus compounds in the soil, bacterial strains living close to the ability to excrete organic acids themselves. In this respect, the physiological characteristics of our isolated strains reflect the environmental conditions of their specific micro-habitat.

These experiments provide the first evidence that *Burkholderia* have phytase activity and show plant growth promoting effect with phytate as the sole phosphorus source. One strain, FpRpG4, evoked particularly strong effects such as 3-fold increases in plant dry weight and total P content, and a more than 6-fold increase in shoot length. These strong responses point at a close interdependence of plant and bacterial growth. Future attempts to elucidate the interaction between phytate utilizing bacteria and higher plants in the field will benefit from the characterization of such remarkably efficient strains.

Experimental procedures

Field design and sampling

White lupin (*Lupinus albus* L. cv. kievskij) was cultivated from May to September 2002 in long-term experimental fields (fertilization patterns were +NPKS, +NPK, +NPS, +NKS, +PKS, or –NPKS on different plots ever since 1914) of Hokkaido University, Sapporo, Japan. The soil was classified as brown lowland soil (pH (H_20) 6.2) according to the USDA soil taxonomy. Samples were collected from each site before and at 12, 40, 58, and 78 days after sowing. Before cultivation, a soil-microorganism suspension was prepared from 10 g of edaphosphere soil suspended in 100 mL of sterilized 0.9 % NaCl solution by shaking for 2 hour at 120 rpm; afterwards, the suspension was allowed to set for 20 min. After sowing, rhizosheath and rhizoplane samples were taken from 6 randomly selected plants in each field, and microorganism suspensions were prepared by a modified water fractionation method originally described by Ishizawa *et al.* (1957). Roots were cautiously removed from the field and immersed in bottles filled with sterile water. After 20 min, roots were transferred to new bottles which were gently shaken to remove the remaining soil; this suspension of soil that had adhered to the roots represented the rhizosheath. To prevent endophyte contamination of the solution, cross sections of roots were covered with paraffin, and roots were put into a third set of bottles with sterile water for 4 hours. This last root medium represented the rhizoplane (Fig. 1). From each sample, step-wise ten-fold dilution series were prepared using 0.9% NaCl solution. For further analysis, the 3rd and 4th dilutions of the suspensions from edaphosphere soil, and the 2nd and 3rd dilutions of the suspensions from rhizosheath and rhizoplane samples were used. The preparation and handling of microorganism suspensions was performed at room temperature.

Isolation of Na-IHP utilizing soil bacteria

Two types of phytate-specific media were used to isolate and culture bacteria that utilize Na-IHP (phytic acid: Inositol hexaphosphoric acid dodecasodium salt from rice, Sigma, St. Louis, USA) as the only source of C and P. These media were based on modified phytate specific broth (Richardson and Hadobas, 1997) which contained (per liter) 10 g Na-IHP, 1.0g $(NH_4)_2SO_4$, 0.1 g $MgSO_4$ $7H_2O$, 7.0 g KCl, 0.1 g $CaCl_2$ $2H_2O$, 1.0 mL 0.1 M FeNa-EDTA, 1.0 mL of a complete trace element solution (per liter: 15.0 g Na_2EDTA $2H_2O$, 0.43 g $ZnSO_4$ $7H_2O$, 0.24 g $CoCl_2$ $6H_2O$, 0.99 g $MnCl_2$ $4H_2O$, 0.22

g Na_2MoO_4 $2H_2O$, 0.19 g $NiCl_2$ $6H_2O$, 0.08 g Na_2SeO_3 $6H_2O$, 0.15 g H_3BO_4). The first of the PS media, solid PS medium, contained 1.5% agar (Wako, Osaka, Japan) and 0.01 g/L bromocresol green as a pH indicator. The second one, semi-solid phytate specific medium, resembled the first but the agar was replaced by 0.2 % Gelrite (Wako, Osaka, Japan), allowing to discriminate bacteria of different respiratory types (Hashidoko *et al.,* 2002). To isolate Na-IHP-utilizing bacteria, different amounts of diluted sample solutions were incubated with the semi-solid phytate specific medium to obtain colonies. Colonies were then plated on solid phytate specific medium, and transferred to new plates repeatedly to create pure colonies. Different strains were characterized by their respiratory type as expressed in the semi-solid phytate specific medium, by their colony morphology, and by the pH changes they induced in the solid phytate specific medium. All steps of the cultivation of bacteria were performed at 26°C.

Identification of Na-IHP utilizing soil bacteria

To identify bacteria**,** 16S rDNA sequence-based identification was performed. Total DNA was isolated as described by Hiraishi (1992). The primers used for partial 16S rDNA fragment amplification were 27f: 5'-AGAGTTTGATCCTGGCTCAG-3' (positions 8 to 27 of *E. coli* 16S rDNA) and 1492r: 5'-GGTTACCTTGTTACGACTT-3' (positions 1,510 to 1,492 of *E. coli* 16S rDNA). The fragment was amplified in 20 µL reaction volumes containing 1 µL of DNA template using T-Gradient (Biometra, Göttingen, Germany) as a thermal cycler and KOD DASH (Toyobo, Osaka, Japan) as thermostable DNA polymerase.

The cycling profile was: 2 min pre-denaturing at 98°C, and 33 cycles of PCR (98°C for 15 sec, 64°C for 2 sec, 72°C for 45 sec) followed by 72°C for 2 min. The PCR products were mixed with 80 µL TE and 60 µL 20% PEG/2.5 M NaCl, kept on ice for 1 hour and then centrifuged at 18,000 *g* for 20 min. DNA concentrations were determined by UV spectrophotometry (UV-1600, Shimadzu, Kyoto, Japan) at 260 nm. Primers used for the sequencing reaction were r2L': 5'-GACTACCAGGGTATCTAATC-3' (positions 805 to 786 of *E. coli* 16S rRNA) and r3L: 5'-TTGCGCTCGTTGCGGGACT-3' (positions 1,111 to 1,093 of *E. coli* 16S rRNA). The sequencing reactions were performed using a DTCS Quick Start kit (Beckman Coulter, California, USA). The 20 µL reaction volume contained 100 fmol DNA template and 2 µmol sequencing primer. Thirty cycles of sequencing reactions (96°C for 20 sec, 50°C

for 20 sec, 60°C for 4 min) were run. The reaction products were analyzed by a DNA sequencer (CEQ8000, Beckman Coulter) after purification by ethanol precipitation. The sequences determined were evaluated using the BLASTn program (Altschul *et al.,* 1997) from DDBJ (DNA Databank of Japan, Shizuoka, Japan).

Utilization of Na-IHP

The bacteria's ability to utilize Na-IHP was estimated by their growth rate, P release ability, and extracellular phytase activity in limiting phytate specific broth. Limiting phytate specific broth was prepared by replacing the 10 g Na-IHP in the phytate specific broth by 1 mM Na-IHP. Test bacteria were proliferated in phytate specific broth shaken at 200 rpm for 2 days. Cells were pelleted by centrifugation (10000 *g*, 1 min), washed twice with distilled water, and resuspended in sterile physiological water (0.9% w/v, NaCl). The bacterial density in the suspensions was adjusted to an OD610 of 0.01. Twenty µL of the suspensions were further cultivated in 2 mL limiting phytate specific broth with horizontal shaking (200 rpm) for 1 week. The bacterial growth rate was determined by the change in the OD610 during this period. The P release ability was estimated from the change of P concentration in the broth during the cultivation period; P was determined by the molybdate blue method (Murphy and Riley, 1962). Extracellular phytase activity at the end of the cultivation period was determined by the method of Richardson and Hadobas (1997); one unit (U) of activity was defined as 1 µmol P_i released from Na-IHP (which was present in excess in these tests) per minute.

Utilization of Al- and Fe-IHP by Na-IHP-utilizing isolates

Al- and Fe-IHP were prepared from Ca-Phytate (Wako, Osaka, Japan) by the method of Jackman and Black (1951). The Al-IHP and Fe-IHP preparations contained 0.2% Ca-IHP and 0.6% free P_i, and 1.7% Ca-IHP and 5.5% free P_i, respectively. For utilization analysis, Na-IHP in the semi-solid PS medium was replaced with 0.1% Al- or Fe-IHP, respectively. After 4 weeks of cultivation at 26°C, the extent of utilization of Al-IHP and Fe-IHP was judged by the progress of colony formation, and pH changes induced in the media were estimated from the discoloration of bromocresol green.

Inoculation tests

To investigate the interaction between plants and Na-IHP utilizing bacteria, inoculation tests using lotus (*Lotus japonicus* B-129 “Gifu”; Seed Center Forage of the National Agricultural Research Center for the Hokkaido Region, Hokkaido, Japan) seedlings were performed. Lotus was selected as the model plant for the inoculation tests, because lupin seeds are rather large, making it difficult to induce P deficient conditions in young plants. For inoculation tests, bacteria were proliferated in phytate specific broth shaken at 200 rpm for 2 days and collected by centrifugation at 10,000 *g* for 1 min. The pellet obtained was washed twice with physiological water (0.9% NaCl, w:v) before resuspension in water to adjust the OD610 to 0.01. Seeds were sterilized by immersion in concentrated sulfuric acid for 20 min and in 50% Na-hypochlorite containing 2% Tween 20 for 20 min, and then rinsed with sterilized water several times (Imaizumi *et al.,* 1997). Germination took place on 0.6% agar solid medium containing 0.1 mM $CaCl_2$. After 1 week, seedlings were transferred to 100 mL test tubes containing 50 mL of a modified, semi-solid Murashige and Skoog medium (Murashige and Skoog, 1962), in which KH_2PO_4 was replaced by 1 mM Na-IHP, and which also contained 5 mM MES and 0.07% Gelrite. Fifty µL of the bacterial suspensions to be tested were added, and the culture tubes were kept at 16 h light (66 µE m^{-1} s^{-2} at top of the tubes)/8 h dark conditions at 23°C under gnotobiotic conditions. Each treatment comprised of three replicates, and two sets of control plants were kept under aseptic condition. After 11 weeks, plants were harvested, and plant height, dry weight, and P concentration were determined. Changes in medium pH were estimated from the discoloration of bromocresol green. Total P was measured by the molybdate blue method after digestion as described by Murphy and Riley (1962).

Statistical analysis

Data were analyzed by one-way analysis of variance and multiple range Duncan test (threshold $P = 0.05$) using SPSS 10.0 Software (SPSS Chicago, IL, USA).

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Table and Figure legends

Table 1. Numbers of strains isolated at different stages of cultivation. Samples were collected before and 12, 40, 58, and 78 days after sowing.

Table 2. Taxonomic identification of 73 isolated strains based on 16S rRNA sequences.

Table 3a. Characterization of 36 strains of Na-IHP-utilizing bacteria isolated from the rhizosheath of Lupinus albus.
Table 3b. Characterization of 37 strains of Na-IHP-utilizing bacteria isolated from the rhizoplane of Lupinus albus.
a. The first letter indicates the long-term fertilization pattern of the plot from which the strain was originally isolated; C stands for full fertilization including nitrogen (N), phosphorus (P), potassium (K), and sulphur (S); N, P, K, or S indicate that the element was not applied, and F indicates the absence of fertilization. Bold font strains were selected for fig. 3.
b. Data shown are the results of the taxonomic classification based on 16S rRNA sequences.
c. The respiratory type as determined in cultures on semi-solid medium (A, aerobic; MA, micro-aerobic).
d. OD610 increment over the cultivation period as a measure of biomass increase.
e. Phosphate release from Na-IHP during a 7-day cultivation period.
f. Extracellular phytase activity; one unit, U, of activity was defined as 1 µmol Pi released from Na-IHP per minute
g. Shoot and root length, respectively, of inoculated Lotus japonicus in cm; mean values ± SD are given, followed by different letters which indicate significant differences in Duncan's test, P = 0.05. Two sets of control experiments with plants without Na-IHP-utilizing bacteria were performed, yielding shoot lengths of 1.1 ± 0.19 and 1.2 ± 0.17 cm, and root lengths of 1.9 ± 0.45 and 1.4 ± 0.28 cm.
h. Changes of medium pH after 11 weeks of cultivation as indicated by the discoloration of bromocresol green; -, +, and ++ indicate no (blue), little (green) and

large (yellow) pH decreases, respectively.

Fig. 1. Correlation between phosphate release into the medium and extracellular phytase activity in bacterial strains cultured in Na-IHP utilization tests.

Fig. 2. Correlation between dry weight and total P content in Lotus japonica plants grown in the absence (non-inoculated) or presence of bacterial strains isolated from the rhizosheath or rhizoplane, as indicated. Using strains in this figure were written by bold font in table 3. Solid line: Dry weight =9.7+0.15 (Total P), R^2 = 0.37, t = 3.079, P = 0.01).

Fig. 3. Lotus seedlings at 11 weeks after germination. Two sets of plants from control experiments without Na-IHP-utilizing bacteria, and three sets of plants grown in the presence of the bacterial strains indicated are shown.

Table 1. Numbers of strains isolated at different stages of cultivation. Samples were collected before and 12, 40, 58, and 78 days after sowing.

	before	12 days	40 days	58 days	78 days	total
Edaphosphere soil	26					26
Rhizosheath		32	40	36	43	151
Rhizoplane		22	28	37	36	123

Table 2. Taxonomic identification of 73 isolated strains based on 16S rRNA sequences.

	Rhizosheath	Rhizoplane
Burkholderia spp.	35	35
Stenotrophomonas maltophilia	1	
Agrobacterium rhizogenes		1
Uncultured gamma proteobacterium		1

Table 3A. Characterization of 36 strains of Na-IHP-utilizing bacteria isolated from the rhizosheath of *Lupinus albus*.

				The results of Utilization of Na-IHP test			The results of inoculation test[g]	
Strain name[a]	Identification result[b]	Identity(%)	Respiratory type[c]	Biomass (OD 610)[d]	Phosphate release(umol/mL)[e]	Phytase activity(uU/min)[f]	Shoot length (cm)	Root length (cm)
NpRsG1	*Burkholderia cepacia*	90	MA	0.256	0.647	11.510	0.8 ± 0.20 cd	1.1 ± 0.28 bc
NpRsG2	*B. anthina*	96	MA	0.013	0.787	2.380	0.8 ± 0.28 cd	1.4 ± 0.80 bc
NpRsG3	*B. vietnamiensis*	99	MA	0.144	0.814	7.743	1.0 ± 0.28 cd	1.3 ± 0.37 bc
NpRsG4	*B. terricola*	96	MA	0.261	0.703	3.475	0.8 ± 0.57 cd	1.3 ± 0.43 bc
NpRsG5	*B. cepacia*	93	MA	0.255	0.569	4.571	1.0 ± 0.40 cd	1.1 ± 0.48 bc
NpRsG6	*B. terricola*	96	MA	0.168	0.966	2.380	0.8 ± 0.40 cd	2.0 ± 0.40 ab
NpRsG7	*B. vietnamiensis*	98	MA	0.017	0.190	3.097	1.4 ± 0.60 bcd	1.4 ± 0.45 bc
NpRsG8	*B. graminis*	97	A	0.225	0.776	2.380	1.6 ± 0.73 bcd	1.4 ± 0.48 bc
NpRsG9	*Stenotrophomonas maltophilia*	97	A	0.128	0.004	0.000	1.3 ± 0.28 bcd	1.4 ± 0.63 bc
NpRsG10	*B. caribiensis*	95	A	0.316	0.054	0.000	2.6 ± 0.50 a	1.3 ± 0.35 bc
PpRsG1	*B. cepacia*	98	A	0.251	1.134	3.841	0.7 ± 0.39 cd	1.0 ± 0.25 c
PpRsG2	*B. cepacia*	98	MA	0.250	0.692	2.745	2.8 ± 0.51 a	1.3 ± 0.51 bc
PpRsG3	*B. cepacia*	97	MA	0.233	1.000	2.745	1.1 ± 0.30 cd	1.6 ± 0.49 abc
PpRsG4	*B. graminis*	96	MA	0.189	0.793	2.014	1.1 ± 0.10 cd	2.0 ± 0.06 abc
PpRsG5	*B.* sp. JRB1	96	A	0.291	3.663	19.545	2.2 ± 1.08 a	2.5 ± 0.49 a
PpRsG6	*Burkholderia* sp. JRB1	96	MA	0.283	1.861	8.588	2.6 ± 0.54 a	2.0 ± 0.06 abc
PpRsG7	*B. terricola*	99	MA	0.194	0.496	0.919	0.8 ± 0.40 cd	1.2 ± 0.40 bc
PpRsG8	*B. terricola*	95	MA	0.310	0.384	0.553	1.2 ± 0.00 cd	1.4 ± 0.40 bc
PpRsG9	*B. terricola*	95	A	0.243	1.145	5.301	1.1 ± 0.35 cd	1.4 ± 0.15 bc
FpRsG1	*B.* sp. SOD-7	95	A	0.248	0.737	3.841	1.1 ± 0.52 cd	1.2 ± 0.44 bc
FpRsG2	*B.* sp. ON1	91	A	0.292	0.821	1.649	1.4 ± 0.10 bcd	2.1 ± 0.06 ab
FpRsG3	*B. graminis*	96	A	0.182	0.513	0.553	2.6 ± 0.49 a	1.1 ± 0.11 bc
FpRsG4	*B. cepacia*	96	MA	0.228	0.927	2.745	0.9 ± 0.10 cd	1.4 ± 0.67 bc
FpRsG5	*B. pyrrocinia*	97	MA	0.354	0.641	0.919	1.6 ± 0.90 bc	1.7 ± 0.15 abc
FpRsG6	*B.* sp. TNFYE-5	96	MA	0.454	0.530	1.649	1.0 ± 0.20 cd	1.6 ± 0.84 abc
FpRsG7	*B. cepacia*	97	MA	0.205	0.809	3.110	0.9 ± 0.23 ab	1.3 ± 0.72 bc
FpRsG8	*B. graminis*	93	MA	0.258	0.496	4.571	1.1 ± 0.65 cd	1.5 ± 0.72 abc
KpRsG1	*B. cepacia*	99	MA	0.056	0.076	0.000	1.6 ± 0.73 bc	2.1 ± 0.06 ab
KpRsG2	*B. pyrrocinia*	100	A	0.193	1.117	3.110	1.1 ± 0.95 cd	1.5 ± 0.25 abc
KpRsG3	*B. terricola*	95	MA	0.176	0.216	2.355	0.9 ± 0.45 cd	1.3 ± 0.87 bc
SpRsG1	*B. anthina*	99	A	0.009	0.088	2.355	1.2 ± 0.64 cd	1.5 ± 0.82 abc
SpRsG2	*B. pyrrocinia*	97	A	0.003	1.095	7.419	0.7 ± 0.24 cd	1.2 ± 0.42 bc
SpRsG3	*B. vietnamiensis*	99	MA	0.356	0.298	3.872	1.1 ± 0.35 cd	1.4 ± 0.25 bc
CpRsG1	*B. anthina*	99	MA	0.009	0.146	2.355	0.5 ± 0.00 d	1.7 ± 0.67 abc
CpRsG2	*B. anthina*	98	MA	0.003	0.152	1.631	1.3 ± 0.80 bcd	1.7 ± 0.72 abc
CpRsG3	*B. anthina*	99	MA	0.009	0.125	3.802	1.0 ± 0.48 cd	1.3 ± 0.98 bc

[a] The first letter indicates the long-term fertilization pattern of the plot from which the strain was originally isolated; C stands for full fertilization including nitrogen (N), phosphorus (P), potassium (K), anc the element was not applied, and F indicates the absence of fertilization.

[b] Data shown are the results of the taxonomic classification based on 16S rRNA sequences.

[c] The respiratory type as determined in cultures on semi-solid medium (A, aerobic; MA, micro-aerobic).

[d] OD610 increment over the cultivation period as a measure of biomass increase.

[e] Phosphate release from Na-phytate during a 7-day cultivation period.

[f] Extracellular phytase activity; one unit, U, of activity was defined as 1 µmol P_i released from Na-IHP per minute

[g] Shoot and root length, respectively, of inoculated Lotus japonicus in cm; mean values ± SD are given, followed by different letters which indicate significant differences in Duncan's test, P = 0.05. Two se without Na-IHP-utilizing bacteria were performed, yielding shoot lengths of 1.1 ± 0.19 and 1.2 ± 0.17 cm, and root lengths of 1.89 ± 0.45 and 1.39 ± 0.28 cm.

[h] Changes of medium pH after 11 weeks of cultivation as indicated by the discoloration of bromocresol green; -, +, and ++ indicate no (blue), little (green) and large (yellow) pH decreases.

Table 3B. Characterization of 37 strains of Na-IHP-utilizing bacteria isolated from the rhizoplane of Lupinus albus.

				The results of Utilization of Na-IHP test			The results of inoculation test[g]		
Strain name[a]	Identification result[b]	Identity(%)	Respiratory type[c]	Biomass (OD 610)[d]	Phosphate release(umol/mL)[e]	Phytase activity(uU/min)[f]	Shoot length (cm)	Root length (cm)	pH[h]
NpRpG1	*Burkholderia terricola*	98	MA	0.375	0.282	2.716	0.8 ± 0.26 gh	1.1 ± 0.40 bcdefg	+
NpRpG2	*B. terricola*	99	MA	0.437	-0.093	5.453	0.8 ± 0.25 gh	0.6 ± 0.32 fg	-
NpRpG3	*B. caribiensis*	99	A	0.250	0.066	1.993	3.7 ± 1.28 b	0.8 ± 0.29 efg	++
NpRpG4	*B. vietnamiensis*	96	A	0.012	-0.038	1.631	3.2 ± 1.16 bc	1.0 ± 0.82 cdefg	++
NpRpG5	*B. terricola*	95	MA	0.305	0.094	1.631	1.4 ± 0.13 efgh	1.7 ± 0.47 abcd	+
NpRpG6	*B. vietnamiensis*	99	MA	0.021	-0.011	1.631	4.2 ± 0.55 b	1.3 ± 0.21 abcdef	++
NpRpG7	*B. cepacia*	99	A	0.007	-0.016	1.631	1.1 ± 0.13 fgh	1.6 ± 0.47 abd	++
NpRpG8	*B. terricola*	95	MA	0.372	0.146	2.355	0.8 ± 0.10 gh	1.2 ± 0.10 bcdefg	-
NpRpG9	*B. vietnamiensis*	99	MA	0.008	-0.005	1.631	1.4 ± 0.29 efgh	1.3 ± 0.38 abcdef	+
NpRpG10	Uncultured γ−proteobacterium pIF16S1-2	96	MA	0.047	0.000	0.546	0.8 ± 0.20 fgh	1.4 ± 0.48 abcdef	-
PpRpG1	*B. cepacia*	98	MA	0.258	0.602	5.610	1.2 ± 0.10 fgh	0.9 ± 0.10 defg	++
PpRpG2	*B. cepacia*	98	MA	0.251	0.591	4.525	2.4 ± 0.15 cde	1.1 ± 0.10 bcdefg	++
PpRpG3	*B. graminis*	96	MA	0.261	0.255	4.163	4.1 ± 0.10 b	1.1 ± 0.10 bcdefg	++
PpRpG4	*Agrobacterium rhizogenes*	95	A	0.081	0.005	1.631	2.6 ± 1.80 cd	0.7 ± 0.28 efg	+
PpRpG5	*B. pyrrocinia*	98	A	0.021	-0.016	1.270	0.9 ± 0.06 fgh	1.1 ± 0.06 bcdefg	++
PpRpG6	*B. vietnamiensis*	98	A	0.227	0.732	6.333	0.7 ± 0.16 gh	0.4 ± 0.10 g	++
PpRpG7	*B.* sp AB2	98	A	0.248	0.830	6.695	0.7 ± 0.28 gh	1.0 ± 0.22 cdefg	++
PpRpG8	*B. cepacia*	98	MA	0.297	0.743	5.248	1.6 ± 0.41 fgh	1.7 ± 0.20 abcd	-
PpRpG9	*B. pyrrocinia*	98	A	0.277	0.673	6.695	2.6 ± 1.32 cd	1.8 ± 0.30 abc	-
PpRpG10	*B. anthina*	99	MA	0.241	0.586	6.695	0.8 ± 0.15 fgh	2.0 ± 0.34 a	++
FpRpG1	*B. caribiensis*	99	MA	0.269	0.071	1.993	0.5 ± 0.11 h	1.4 ± 0.32 abcdef	++
FpRpG2	*B. caribiensis*	96	MA	0.245	0.087	1.270	3.9 ± 0.52 b	1.4 ± 0.30 abcdef	++
FpRpG3	*B. caribiensis*	96	MA	0.310	0.548	4.887	2.0 ± 0.32 def	1.0 ± 0.08 cdefg	-
FpRpG4	*B. caribiensis*	98	MA	0.013	0.049	2.355	7.5 ± 1.45 a	1.8 ± 0.41 abc	++
FpRpG5	*B. pyrrocinia*	97	A	0.268	0.705	7.157	0.9 ± 0.23 fgh	1.3 ± 0.61 abcdef	-
FpRpG6	*B. anthina*	98	MA	0.255	0.835	7.419	0.8 ± 0.12 gh	1.3 ± 0.62 abcdef	++
FpRpG7	*B. cepacia*	98	MA	0.218	0.759	7.057	0.8 ± 0.32 fgh	1.6 ± 0.17 abcde	++
KpRpG1	*B.* sp. BPC2	98	MA	0.216	0.689	6.333	0.9 ± 0.46 defg	1.3 ± 0.67 abcdef	++
KpRpG2	*B. pyrrocinia*	95	MA	0.276	0.732	5.610	1.3 ± 0.05 efgh	1.3 ± 0.54 abcdef	++
KpRpG3	*B. cepacia*	96	A	0.246	1.014	7.419	0.6 ± 0.29 gh	1.1 ± 0.59 bcdefg	++
KpRpG4	*B. pyrrocinia*	97	MA	0.363	0.510	4.887	1.7 ± 0.65 defg	1.4 ± 0.57 abcdef	-
SpRpG1	*B. anthina*	97	MA	0.018	0.732	5.610	2.0 ± 1.09 def	1.1 ± 0.68 bcdefg	++
SpRpG2	*B. pyrrocinia*	98	A	0.212	0.786	5.972	1.1 ± 0.49 fgh	1.2 ± 0.18 abcdefg	++
SpRpG3	*B. anthina*	98	A	0.011	0.081	1.631	1.6 ± 0.45 defg	1.1 ± 0.05 bcdefg	++
CpRpG1	*B. pyrrocinia*	94	MA	0.376	0.776	5.610	1.4 ± 0.18 efgh	2.0 ± 0.92 a	++
CpRpG2	*B. pyrrocinia*	98	A	0.232	0.510	3.802	1.7 ± 0.13 defg	1.3 ± 0.28 abcdef	-
CpRpG3	*B. pyrrocinia*	96	MA	0.323	0.786	5.972	0.8 ± 0.37 fgh	1.3 ± 0.35 abcdef	++

[a] The first letter indicates the long-term fertilization pattern of the plot from which the strain was originally isolated; C stands for full fertilization including nitrogen (N), phosphorus (P), potassium (K), and sulphur (S); N, P, the element was not applied, and F indicates the absence of fertilization.

[b] Data shown are the results of the taxonomic classification based on 16S rRNA sequences.

[c] The respiratory type as determined in cultures on semi-solid medium (A, aerobic; MA, micro-aerobic).

[d] OD610 increment over the cultivation period as a measure of biomass increase.

[e] Phosphate release from Na-phytate during a 7-day cultivation period.

[f] Extracellular phytase activity; one unit, U, of activity was defined as 1 µmol P_i released from Na-IHP per minute

[g] Shoot and root length, respectively, of inoculated Lotus japonicus in cm; mean values ± SD are given, followed by different letters which indicate significant differences in Duncan's test, P = 0.05. Two sets of control exper without Na-IHP-utilizing bacteria were performed, yielding shoot lengths of 1.1 ± 0.19 and 1.2 ± 0.17 cm, and root lengths of 1.89 ± 0.45 and 1.39 ± 0.28 cm.

[h] Changes of medium pH after 11 weeks of cultivation as indicated by the discoloration of bromocresol green; -, +, and ++ indicate no (blue), little (green) and large (yellow) pH decreases.

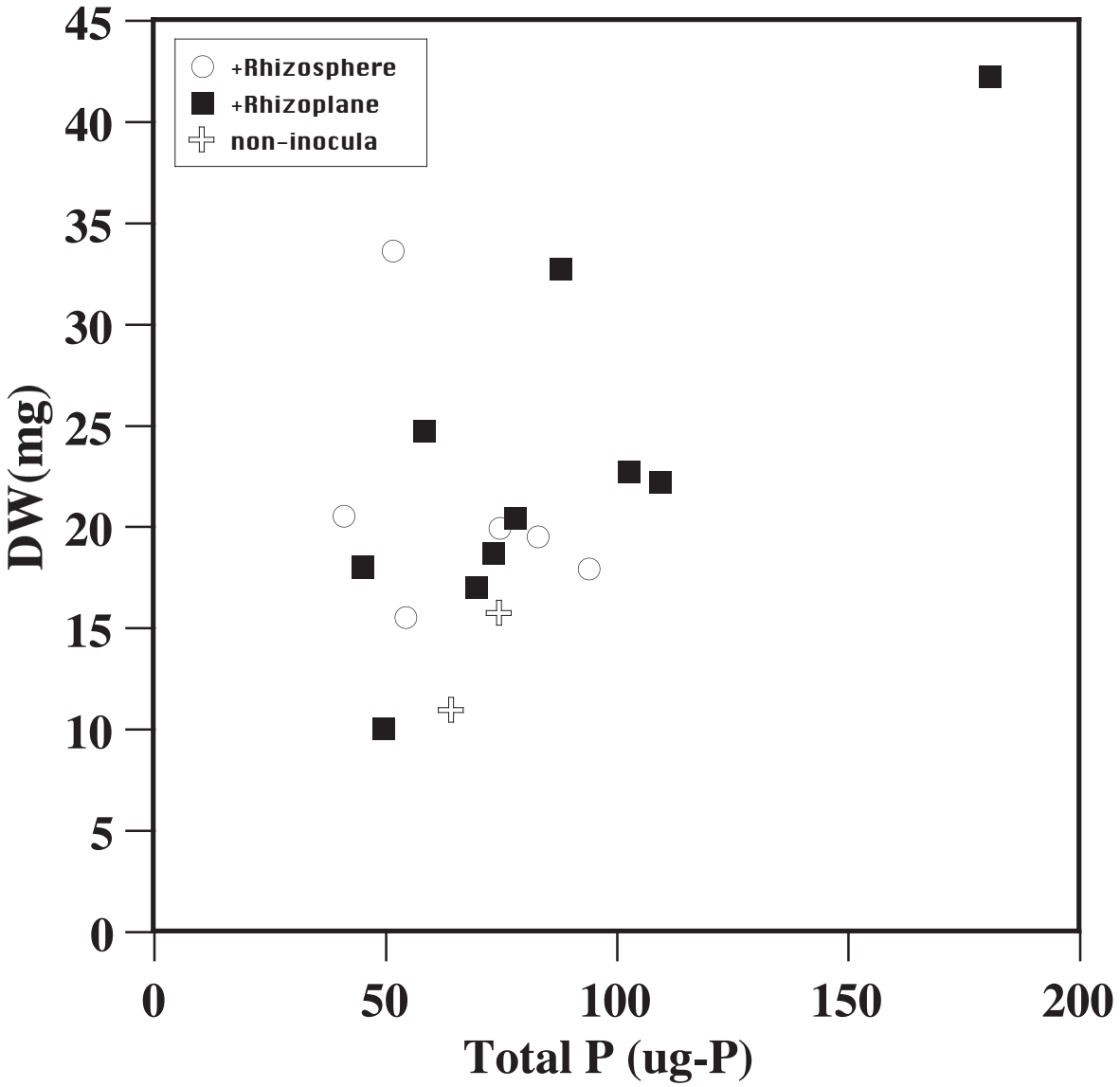

Fig.1. Inoculation test using lotus: Correlation between dry weight (DW) and total P was shown about 16 inoculated and 2 non-inoculated samples.

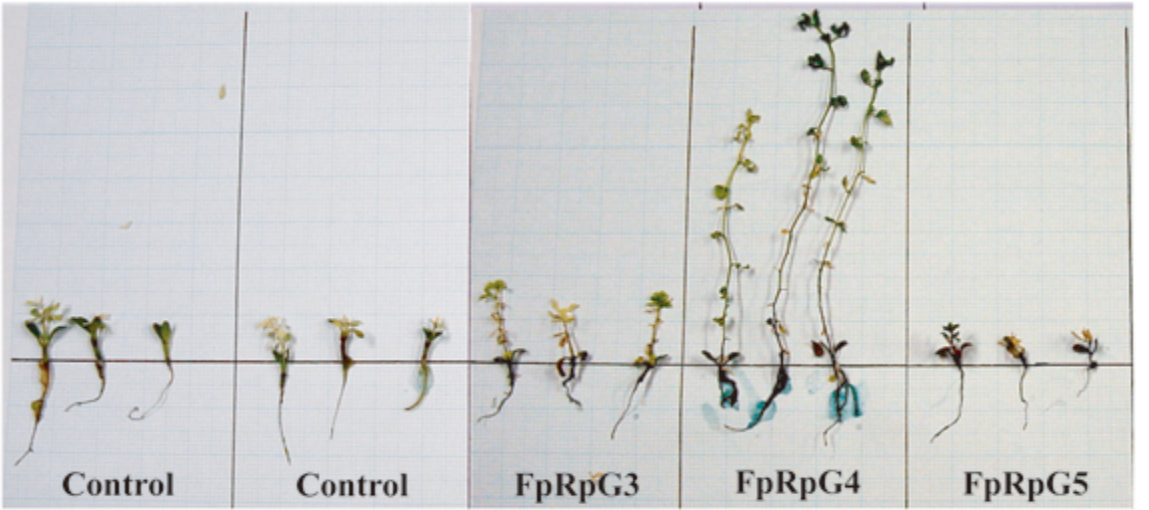

Fig. 2. Lotus seedlings at 11 weeks after germination. Two sets of plants from control experiments without Na-IHP-utilizing bacteria, and three sets of plants grown in the presence of the bacterial strains indicated are shown.

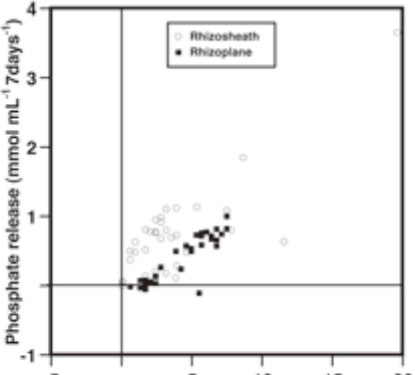

Fig. 3. Correlation between phosphate release into the medium and extracellular phytase activity in bacterial strains cultured in Na-IHP utilization tests.