Plant and Soil (2006) 287:15–21
DOI 10.1007/s11104-006-9056-9

Genetics of phosphate solubilization and its potential applications for improving plant growth-promoting bacteria

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Received: 31 May 2006

Key words: genetically modified microorganisms, organic acids, phosphatases, phosphorus solubilization, phytases, plant growth promoting bacteria

Abstract

Plant growth-promoting bacteria (PGPB) are soil and rhizosphere bacteria that can benefit plant growth by different mechanisms. The ability of some microorganisms to convert insoluble phosphorus (P) to an accessible form, like orthophosphate, is an important trait in a PGPB for increasing plant yields. In this mini-review, the isolation and characterization of genes involved in mineralization of organic P sources (by the action of enzymes acid phosphatases and phytases), as well as mineral phosphate solubilization, is reviewed. Preliminary results achieved in the engineering of bacterial strains for improving capacity for phosphate solubilization are presented, and application of this knowledge to improving agricultural inoculants is discussed.

Introduction

Plant growth-promoting bacteria (PGPB) are soil and rhizosphere bacteria that can benefit plant growth by different mechanisms (Glick, 1995). Given the negative environmental impact of chemical fertilizers and their increasing costs, the use of PGPB as natural fertilizers is advantageous for the development of sustainable agriculture.

There are two components of P in soil, organic and inorganic phosphates. A large proportion is present in insoluble forms, and therefore, not available for plant nutrition. Inorganic P occurs in soil, mostly in insoluble mineral complexes, some of them appearing after the application of chemical fertilizers. These precipitated forms cannot be absorbed by plants. Organic matter, on the other hand, is an important reservoir of immobilized P that accounts for 20–80% of soil P (Richardson, 1994). To convert insoluble phosphates (both organic and inorganic) to a form accessible to the plants, like orthophosphate, is an important trait for a PGPB for increasing plant yields.

Molecular biology techniques are an advantageous approach for obtaining and characterizing improved PGPB strains (Igual et al., 2001; Rodríguez and Fraga, 1999). Release of genetically modified organisms is controversial. While some countries encourage it, others prohibit the use of the technology and require labeling of products containing genetically modified food ingredients. However, studies carried out so far have shown that following appropriate regulations, genetically modified microorganisms can be applied safely in agriculture (Armarger, 2002; Morrissey et al., 2002). Chromosomal insertion of the genes is one of the tools to avoid horizontal transfer of the introduced genes within the rhizosphere.

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Some barriers should be overcome first to achieve successful gene insertions using this approach, such as the dissimilarity of metabolic machinery and different regulating mechanism between the donor and recipient strains. Despite the difficulties, significant progress has been made in obtaining genetically engineering microorganisms for agricultural use (Armarger, 2002).

There are several advantages of developing genetically-modified PGPB over transgenic plants for improving plant performance: (1) With current technologies, it is far easier to modify a bacterium than complex higher organisms, (2) Several plant growth-promoting traits can be combined in a single organism, and (3) Instead of engineering crop by crop, a single, engineered inoculant can be used for several crops, especially when using a nonspecific genus like *Azospirillum*.

Introduction or over-expression of genes involved in soil phosphate solubilization (both organic and inorganic) in natural rhizosphere bacteria is a very attractive approach for improving the capacity of microorganisms to work as inoculants. Insertion of phosphate-solubilizing genes into microorganisms that do not have this capability may avoid the current need of mixing two populations of bacteria, when used as inoculants (nitrogen fixers and phosphate-solubilizers) (Bashan et al., 2000). We report on recent advances in the manipulation of genes related to microbial phosphate-solubilization and its relationship to rhizobacteria, as improved inoculants.

Organic phosphate solubilization

Phosphorus can be released from organic compounds in soil by three groups of enzymes: (1) Nonspecific phosphatases, which perform dephosphorylation of phospho-ester or phosphoanhydride bonds in organic matter, (2) Phytases, which specifically cause P release from phytic acid, and (3) Phosphonatases and C–P Lyases, enzymes that perform C–P cleavage in organophosphonates. The main activity apparently corresponds to the work of acid phosphatases and phytases because of the predominant presence of their substrates in soil.

Availability of organic phosphate compounds for plant nutrition could be a limitation in some soils resulting from precipitation with soil particle ions. Therefore, the capability of enzymes to perform the desired function in the rhizosphere is a crucial aspect for their effectiveness in plant nutrition. Nevertheless, the efficiency of plant and microbial phosphatases on organic P depletion in the rhizosphere and P uptake by plants has been well documented (Tarafdar and Jungk, 1987; Tarafdar and Claassen, 1988).

Nonspecific acid phosphatases

Bacterial nonspecific acid phosphatases (phosphohydrolases) (NSAPs) are formed by three molecular families, which have been designated as molecular class A, B, and C (Thaller et al., 1995a). From their cellular location, these enzymes seem to function as organic phospho-ester scavengers, providing the cell with essential nutrients (releasing inorganic phosphates from nucleotides and sugar phosphates, for example, while the organic by-products are incorporated into the cell) (Beacham, 1980; Wanner, 1996).

Interest in these enzymes has increased during the last decade because of their potential biotechnological applications. Macaskie et al. (1997) reported on the successful use of Class A NSAPs as tools for environmental bioremediation of uranium-bearing wastewater, and Baskanova and Macaskie (1997) and Bonthrone et al. (1996) on heavy metal biomineralization (particularly Ni^{2+}). A new biotechnological application for NSAPs would be to transfer and express these genes in PGPB to obtain improved phosphate-solubilizing strains using recombinant DNA technology.

Several acid phosphatase genes from Gram-negative bacteria have been isolated and characterized (Rossolini et al., 1998). These cloned genes represent an important source of material for the genetic transfer of this trait to PGPB strains. Some of them code for acid phosphatase enzymes that are capable of performing well in soil. For example, the *acpA* gene isolated from *Francisella tularensis* expresses an acid phosphatase with optimum action at pH 6, with a wide range of substrate specificity (Reilly et al., 1996). Also, genes encoding nonspecific acid phosphatases class A (PhoC) and class B (NapA) isolated from *Morganella morganii* are very promising, since the biophysical and functional properties of the encoded enzymes were extensively studied

(Thaller et al., 1994; Thaller et al., 1995b). Besides, they are P-irrepressible enzymes showing broad substrate action and high activity around pH 6 and 30°C.

Among rhizobacteria, a gene from *Burkholderia cepacia* that facilitates phosphatase activity was isolated (Rodríguez et al., 2000a). This gene codes for an outer membrane protein that enhances synthesis in the absence of soluble phosphates in the medium, and could be involved in P transport to the cell. Besides, cloning of two nonspecific periplasmic acid phosphatase genes (*nap*D and *nap*E) from *Rhizobium (Sinorhizobium) meliloti* was accomplished (Deng, et al., 1998, 2001).

Heterologous expression of these genes in agriculturally important bacterial strains would be the next step in programs of improving organic phosphate mineralization in PGPB. The *napA* phosphatase gene from the soil bacterium *Morganella morganii* was transferred to *Burkholderia cepacia* IS-16, a strain used as a biofertilizer, using the broad-host range vector pRK293 (Fraga et al, 2001). An increase in extracellular phosphatase activity of the recombinant strain was achieved.

Insertion of the transferred genes into the bacterial chromosome is advantageous for stability and ecological safety. In our lab, a plasmid for the stable chromosomal insertion of the *phoC* phosphatase gene from *Morganella morganii* was constructed, based on the delivery system developed by de Lorenzo et al. (1990). This plasmid was transferred to *Azospirillum* spp. Preliminary results indicate that strains with increased phosphatase activity were obtained.

Phytases

Most phytases (myo-inositol hexakisphosphate phosphohydrolases) belong to high molecular weight acid phosphatases. In its basic form, phytate is the primary source of inositol and the major stored form of phosphate in plant seeds and pollen. Nevertheless, monogastric animals are incapable of using the P bound in phytate because their gastrointestinal tracts have low levels of phytase activity. Thus, nearly all the dietary phytate phosphorus ingested by these species is excreted, resulting in phosphorus pollution in areas of intensive animal production, and why phytases have emerged as very attractive enzymes for industrial and environmental applications. Genetic studies of phytases began in 1984, and the first commercial phytase, produced by genetically modified microorganisms, appeared on the market in the mid 1990s (Yanming et al., 1999).

Most genetic engineering studies have focused on the search for phytases that are optimal for improving animal nutrition. Another attractive application of these enzymes that is not currently exploited is solubilization of soil organic phosphorus through phytate degradation. Phytate is the major component of organics forms of P in soil (Richardson, 1994). The ability of plants to obtain phosphorus directly from phytate is very limited. However, the growth and phosphorus nutrition of *Arabidopsis* plants supplied with phytate was improved significantly when they were genetically transformed with the phytase gene (*phyA*) from *Aspergillus niger* (Richardson et al., 2001a). This resulted in improved P nutrition, such that the growth and P content of the plant was equivalent to control plants supplied with inorganic phosphate.

The enhanced utilization of inositol phosphate by plants by the presence of soil microorganisms has also been reported (Richardson et al., 2001b). Therefore, developing agricultural inoculants with high phytase production would be of great interest for improving plant nutrition and reducing P pollution in soil. Although phytase genes have been cloned from fungi, plants, and bacteria (Lei and Stahl, 2001), we will discuss only bacteria because they are the most feasible for the genetic improvement of rhizobacteria.

Thermally stable phytase genes (*phy*) from *Bacillus* sp. DS11 (Kim et al., 1998a) and from *B. subtilis* VTT E-68013 (Kerovuo et al., 1998) has been cloned. Acid phosphatase/phytase genes from *E. coli* (*appA* and *appA2 genes)* have also been isolated and characterized (Golovan et al., 2000; Rodríguez et al., 1999). The bi-functionality of these enzymes makes them attractive for solubilization of organic P in soil. Also, neutral phytases have great potential for genetic improvement of PGPB. Neutral phytase genes have been recently cloned from *B. subtilis* and *B. licheniformis* (Tye et al., 2002), A *phyA* gene has been cloned from the FZB45 strain of *B. amyloliquefaciens*. This strain was isolated from a

group of several *Bacillus* having plant-growth-promoting activity (Idriss et al., 2002). It showed the highest extracellular phytase activity, and diluted culture filtrates of these strains stimulated growth of maize seedlings under limited phosphate in the presence of phytate. Culture filtrates obtained from a phytase-negative mutant strain, whose *phyA* gene was disrupted, did not stimulate plant growth. In addition, growth of maize seedlings was enhanced in the presence of purified phytase and the absence of culture filtrate.

These experiments provide strong evidence that phytase activity can be important for stimulating growth under limited P in soil, and supports the potential of using phytase genes to improve or transfer the P-solubilizing trait to PGPB strains used as agricultural inoculants.

Inorganic phosphate solubilization

Isolation of mineral phosphate-solubilizing (mps) genes

In most bacteria, mineral phosphate-dissolving capacity has been shown to be related to the production of organic acid (Rodríguez and Fraga, 1999). Goldstein (1996) proposed direct glucose oxidation to gluconic acid (GA) as a major mechanism for mineral phosphate solubilization (MPS) in Gram-negative bacteria. GA biosynthesis is carried out by the glucose dehydrogenase (GDH) enzyme and the co-factor, pyrroloquinoline quinone (PQQ). Some genes involved in MPS in different species have been isolated (Table 1).

Goldstein and Liu (1987) were the first to clone a gene involved in MPS from the Gram-negative bacteria *Erwinia herbicola*. Expression of this gene allowed production of GA in *E. coli* HB101 and conferred the ability to solubilize hydroxyapatite. *E. coli* can synthesize GDH, but not PQQ, thus it does not produce GA. The cloned 1.8 kb locus encodes a protein similar to the gene III product of a *pqq* synthesis gene complex from *Acinetobacter calcoaceticus*, and to *pqqE* of *Klebsiella neumonie* (Liu et al., 1992). These authors suggested that the *E. herbicola* DNA fragment functions as a PQQ synthase gene, and that probably, some *E. coli* strains contain some cryptic PQQ synthase genes that could be complemented by this single open reading frame (ORF) isolated by them.

Coincidentally, nucleotide sequence analysis of a 7.0 kb fragment from *Rhanella aquatilis* genomic DNA that induced hydroxyapatite solubilization in *E. coli*, showed two complete ORFs and a partial ORF. One of the cloned proteins showed similarity to *pqq E* of *E. herbicola, K. neumoniae*, and *A. calcoaceticus* (Kim et al.,

Table 1. Cloning of genes involved in mineral phosphate solubilization (MPS)

Microorganism	Gene or plasmid	Features	Reference
Erwinia herbicola	*mps*	Produces gluconic acid and solubilizes mineral P in *E. coli* HB101 Probably involved in PQQ[1] synthesis	Goldstein and Liu (1987)
Pseudomonas cepacia	*gab*Y	Produces gluconic acid and solubilizes mineral P in *E. coli* JM109 No homology with PQQ genes	Babu-Khan et al. (1995)
Enterobacter agglomerans	pKKY	Solubilizes P in *E. coli* JM109 Does not lower pH	Kim et al. (1997)
Rahnella aquatilis	pK1M10	Solubilizes P and produces gluconic acid in *E. coli* DH5∝ Probably related to PQQ synthesis	Kim et al. (1998b)
Serratia marcescens	pKG3791	Produces gluconic acid and solubilizes mineral P	Krishnaraj and Goldstein (2001)
Synechococcus PCC 7942	*pcc* gene	Synthesizes phosphoenol pyruvate carboxylase	N. Kumar (pers. comm.)

PQQ: pyrroloquinoline quinone.

1998b), while the partial ORF is similar to the *pqq C* of *K. neumoniae*. These authors also report that these genes complement cryptic *pqq E. coli* genes, thus allowing GA production.

Another type of gene (*gabY*) involved in GA production and MPS was cloned from *Pseudomonas cepacia* (Babu-Khan et al., 1995). The deduced amino acid sequence showed no homology with previously cloned direct oxidation pathway (GA synthesis) genes, but was similar to histidine permease membrane-bound components. In the presence of *gabY*, GA is produced only if the *E. coli* strain expresses a functional glucose dehydrogenase (*gcd*) gene. The authors (Babu-Khan et al., 1995), speculated that this ORF could be related to the synthesis of PQQ by an alternative pathway, or the synthesis of a *gcd* co-factor different from PQQ. The reported synergistic effect of exogenous PQQ and this gene supports this alternative, in our opinion. Also, a DNA fragment from *Serratia marcenses* induces GA synthesis in *E. coli*, but showed no homology to *pqq* or *gcd* genes (Krishnaraj and Goldstein, 2001). They suggested that this gene acted by regulating GA production under cell-signal effects.

Other isolated genes involved in the MPS phenotype seem not to be related with *pqq* DNA or *gcd* biosynthetic genes. A genomic DNA fragment from *Enterobacter agglomerans* showed MPS activity in *E. coli* JM109, although the pH of the medium was not altered (Kim et al., 1997). These results indicate that acid production is an important way, but not the only mechanism, of phosphate solubilization by bacteria (Illmer and Shinnera, 1995). More recently, a phosphoenol pyruvate carboxylase (*pcc*) gene from *Synechococcus PCC* 7942 appears to be involved in MPS (Kumar Naresh, pers. comm.). All these findings demonstrate the complexity of MPS in different bacterial strains, but at the same time, offer a basis for better understanding of this process.

Manipulation of mps genes for PGPB improvement

Expression in *E. coli* of the *mps* genes from *Ranella aquatilis* supported a much higher GA production and hydroxyapatite dissolution in comparison with the donor strain (Kim et al., 1998b). The authors suggested that different genetic regulation of the *mps* genes might occur in both species. MPS mutants of *Pseudomonas* spp. showed pleiotropic effects, with apparent involvement of regulatory *mps* loci in some of them (Krishnaraj et al., 1999). This suggests a complex regulation and various metabolic events related to this trait. Expression of a *mps* gene in a different host could be influenced by the genetic background of the recipient strain, the copy number of plasmids present, and metabolic interactions. Thus, genetic transfer of any isolated gene involved in MPS to induce or improve phosphate-dissolving capacity in PGPB strains, is an interesting approach.

An attempt to improve MPS in PGPB strains, using this approach, was carried out (Rodríguez et al., 2000b) with a PQQ synthetase gene from *Erwinia herbicola*. This gene, isolated by Goldstein and Liu (1987), was subcloned in a broad-host range vector (pKT230). The recombinant plasmid was expressed in *E. coli*, and transferred to PGPB strains of *Burkholderia cepacia* and *Pseudomonas aeruginosa*, using tri-parental conjugation. Several of the exconjugants that were recovered in the selection medium showed a larger clearing halo in medium with tricalcium phosphate as the sole P source. This indicates the heterologous expression of this gene in the recombinant strains, which gave rise to improved MPS ability of these PGPBs. More recently, a genomic integration of the *pcc gene of Synechococcus PCC* in *P. fluorescent* 7942 allowed phosphate solubilization in the recipient strain (Kumar Naresh, pers. comm.).

In other work, a bacterial citrate synthase gene was reported to increase exudation of organic acids and P availability to the plant when expressed in tobacco roots (López-Bucio et al., 2000). Citrate overproducing plants yielded more leaf and fruit biomass when grown under P-limiting conditions, and required less P-fertilizer to achieve optimal growth. This shows the putative role of organic acid synthesis genes in P uptake in plants.

Concluding remarks

Although knowledge of the genetics of phosphate solubilization is still scanty, some genes involved in mineral and organic phosphate solubilization have been isolated and characterized.

Initial achievements in the manipulation of these genes open a promising perspective for obtaining PGPB strains with enhanced phosphate solubilizing capacity, and thus, a more effective use of these microbes as agricultural inoculants.

Acknowledgements

H.R. received support from Consejo Nacional de Ciencia y Tecnología of Mexico (CONACyT Catedra Patrimonial de Excelencia grant EX-000580). We thank Ira Fogel at CIB for editing the English text.

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