Mechanisms and Genetics of Mineral and Organic Phosphate Solubilization by Phosphate Solubilizing Bacteria

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Strains from the genera *Pseudomonas, Bacillus* and *Rhizobium* are the common phosphate solubilizers. The key mechanism behind mineral phosphate solubilization is the release of organic acids and mineralization of organic phosphorus is done by phosphatases. Phosphate solubilizing bacteria leads to the growth of plants by enhancing the process of biological nitrogen fixation, providing elements such as iron, zinc and production of phytohormones such as auxin and gibberellic acid. A few genes involved in mineral phosphate solubilization and some phosphatase encoding genes have been isolated in the recent past. Therefore, it is presumed that genetic manipulation of phosphate-solubilizing bacteria will further help to improve their ability to solubilize insoluble phosphorus and thus lead to improvement of plant growth and crop yield.

Seed or soil inoculation with Phosphate Solubilizing Bacteria (PSB) helps in solubilization of insoluble phosphates resulting in higher crop yields. These processes take place in the rhizosphere. The phosphate solubilizing bacteria solubilize phosphates and use these for their growth and metabolism. The surplus amount of phosphorus (P) is absorbed by plants. Application of phosphate solubilizing bacteria such as *Agrobacterium, Bacillus, Enterobacter, Pseudomonas, Rhizobium* around the roots of plants, in soils, and in fertilizers release soluble phosphorus, promote plant growth, and also protect plants from pathogen infection (Rodriguez and Fraga 1999; Chang and Yang 2009). The bacteria in addition to P solubilization also enhance plant growth by increasing biological nitrogen fixation, making available trace elements such as iron, zinc etc. and by production of plant growth promoting substances (Gyaneswar et al. 2002). PSB also produce antibiotics (Hariprasad and Niranjana 2008). Rock phosphate with inoculation of PSB (*Bacillus megaterium*) increased the availability of P and K in soil, the uptake of N, P and K by shoot and root, and the promotion of growth of pepper and cucumber (Han 2006).

*Pseudomonas, Bacillus* and *Rhizobium* are the most powerful phosphate solubilizers (Rodriguez and Fraga 1999). A few species of the genus *Pseudomonas* such as *P. putida, P. aeruginosa, P. corrugate, P. lutea, P. fluorescens, P. rhizosphaerae* and *P. stutzeri* are known to be efficient phosphate solubilizers (Gulati et al. 2007). The free living PSB *Pseudomonas putida* stimulates plant growth by various means. The PSB helps in synthesis of siderophores that helps in solubilizing iron and making it available to plants, production of phytohormones such as auxin and the production of the enzyme 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase which hydrolyzes ACC, the precursor of ethylene (Glick et al. 1997). The bacilli group is comprised of species such as *B. brevis, B. licheniformis, B. megaterium* and *B. sphaericus*. Other rhizobacteria isolates include *Agrobacterium* sp., *Arthrobacter* sp., *Staphylococcus* sp. etc. (de Freitas et al. 1997).

**MINERAL PHOSPHATE SOLUBILIZATION**

In acidic soils, phosphorus is associated with Al and Fe compounds whereas in calcareous soils, calcium phosphates are the main form (Gyaneswar et al. 2002). Due to acidification of soil by natural and anthropogenic processes, aluminium ions get mobilized which are toxic to plants and lead to chemical fixation of P and ultimately lead to the formation of hardly soluble compounds (Illmer and Schinner 1995). Superphosphate contains a sufficient amount of calcium to precipitate half of its own P (Lindsay 1979). Rock phosphate (sedimentary rock containing phosphate minerals, phosphorite) exist as apatite, fluorapatite, and hydroxyapatite (Bashan et al. 2012). The most common mineral complexes in acid agricultural soils are variscite and strengite. The most stable minerals in alkaline soils are calcium phosphates such as brushite, monetite and fluorapatite.

PSB solubilizes mineral phosphate by the production of organic acids, siderophores, protons, humic substances, carbon dioxide and hydrogen sulphide which acidifies the medium and lower down the pH. However, the main mechanism of mineral P solubilization occurs by production of organic acids
by lowering of pH or by chelation of cations bound to P or by forming soluble complexes with the metal ions. The insoluble phosphates are converted into $\text{H}_2\text{PO}_4^-$, $\text{HPO}_{4}^{2-}$, and $\text{PO}_4^{3-}$ (soluble orthophosphates) (Kundu et al. 1995). The organic acids are the metabolism products of microbes by oxidative respiration or by fermentation of carbon sources (Sharma et al. 2013). These organic acids are produced in the periplasm of many Gram-negative bacteria through a direct oxidation pathway of glucose (DOPG, non-phosphorylating oxidation). The enzymes of the DOPG, glucose dehydrogenase (GDH) and gluconate dehydrogenase (GADH) are oriented to the outer face of the cytoplasmic membrane so that they oxidize their substrates in the periplasmic space. The organic acids diffuse outside the cells and solubilize mineral phosphates by supplying protons and metal complexing organic acid anions (Whitelaw 2000; Pérez et al. 2007). PSB can solubilize P in absence of organic acids (Illmer and Schinner 1992).

Bacteria isolated from proteoid roots of *Telopea speciosissima* seedlings solubilized calcium phosphates in culture medium in the presence of ammonium salts. It was proposed that protons were excreted in exchange for ammonium ions so cation exchange between protons and calcium in the medium might be a reason for the solubilization of mineral phosphates (Wenzel et al. 1994). Chelation of $\text{Ca}^{2+}$ ions by organic acids such as lactic acid, citric acid etc. which are the end products of organic substrates might be the reason for solubilization (Kucey 1983). When NaOH was added P solubilizing activity of *Rhizobium* was abolished which depicted that P solubilization activity was due to reduction of pH (Halder and Chakrabarty 1993). In addition to reduction of pH, importance of chelating property of organic acids was shown by Kucey (1988). When 0.05 M EDTA was added to the media it had same solubilizing effect as shown by *Penicillium bilaii*. Citrate solubilizes aluminium phosphates and form complexes with free Al$^{3+}$ ions. Due to this capacity of complex formation free cations would shift the balance of the solubilization reaction towards the primary compounds, toxic effects of free Al would be mitigated by complex formation. Removal of citrate anions by chelation would result in further dissociation of citric acid and release of more $\text{H}^+$ (Illmer et al. 1995).

**ORGANIC PHOSPHATE SOLUBILIZATION**

Organic P solubilization is known as mineralization of organic phosphorus. Organic P exist as phytate (dodecasodium inositol hexaphosphate). Organic P in soils mostly comprises of inositol phosphates, nucleic acids and phospholipids. P can be released from organic compounds by enzymes such as nonspecific phosphatases, phytases, phosphonatases and C-P lyases. The mineralization of organic P is mediated by these enzymes which are produced by plant roots, fungi and bacteria. The mineralization of these P compounds is carried out by several phosphatases (also called phosphoanhydrolases) by the hydrolysis of phosphoester or phosphoanhydride bonds. The phosphoanhydrolases are either acid or alkaline. High concentrations of inorganic P reduce phosphatase activity in soils (Fox and Comerford 1992). *Rhizobium, Enterobacter, Serratia, Citrobacter, Proteus, Klebsiella, Pseudomonas* and *Bacillus* are efficient producers of phosphatases (Rodríguez and Fraga 1999). They can be further classified as specific or nonspecific acid phosphatases, in relation to their substrate specificity. Bacterial nonspecific acid phosphatases (NSAPs) are formed by three molecular families, molecular class A, B, and C (Thaller et al. 1995). Phosphatases are either secreted outside the plasma membrane or retained as membrane-bound proteins. These enzymes act on organic phosphoesters (RNA and DNA) which are then converted to low molecular weight components. At first RNase and DNase convert them to nucleoside monophosphate and then by phosphatases which release P and organic by products (Rodriguez and Fraga 1999). Another type of enzymes are the phytases which release P from phytate. Phytate is the stored form of P of seeds and pollen as inositols (Sharma et al. 2013). Phosphonatases and C-P Lyases cleaves C-P in organophosphonates.

**GENETICS OF MINERAL PHOSPHATE SOLUBILIZATION**

The major mechanism of phosphate solubilization by gram negative bacteria is secretion of gluconic acid. Two genes are involved in gluconic acid production; *PQQ synthase* and *gabY* genes (Igual et al. 2001). Oxidative metabolism of glucose by glucose dehydrogenase produces gluconic acid which requires pyrroloquinolinequione (PQQ) as a co-factor. Two genes involved in PQQ biosynthesis and PQQ transport, encoding PQQ synthase and a PQQ transporter from P-solubilizing *Erwinia herbicola* were cloned by using its genomic DNA library to select *E. coli* transformants for mps phenotype (Gyaneswar et al. 2002). Another gene involved in PQQ transport was similarly cloned (Babukhan et al. 1995). Various genes involved in P...
knowledge about genetics of mineral phosphate solubilization is less and it needs further research.

**GENETICS OF ORGANIC PHOSPHATE SOLUBILIZATION**

In the model system, *E. coli*, P starvation leads to expression of over 400 proteins and this effect is mediated by a two component regulatory system *Pho*R and *Pho*B, in which *Pho*R (sensor) phosphorylates *Pho*B (regulator). *Pho*R binds to specific DNA sequences called PHO box (Willsky and Malamy 1976; Wanner and Chang 1987; Wanner 1996). PHO box sequences are present upstream of the genes regulated by P starvation. *Rhizobium* has a functional homologue of *Pho*B (Wanne 1996), but a *Pho*R counterpart has not yet been detected in *Rhizobium* (Gyaneswar et al. 2002). Phosphatases production is regulated by inorganic phosphate (Pi) concentration. The alkaline phosphatase (gene *pho*A) of *E. coli* is suddenly and fully induced when the Pi concentration decreases from 100 mM to 0.16 mM (Rosenberg et al. 1987). Alkaline phosphatase of *Morganella morganii* similar to that of *E. coli* is also produced under conditions of low-Pi availability (Thaller et al. 1994). Only under conditions of phosphate limitation, the cleavage of the C-P bond from organophosphates by phosphonoacetaldehyde hydrolase and C-P lyases is inducible (Wackett et al. 1987; Kertez et al. 1991).

Regulation of the expression of phosphatase genes of family *Enterobacteriaceae* are similar to the *pho* genes from *E. coli*. This statement is supported by the fact that the sequence in the −35 region of *phoC* (*Zymomonas mobilis*) was similar to that of the ‘pho box’ in *E. coli* (Pond et al. 1989). *Morganella morganii* and *Providencia stuartii* shows HPAP phenotype. HPAP phenotype is high-level phosphate-irrepressible production of acid phosphatase (Pompei et al. 1990; Pompei et al. 1993). There are other regulatory systems for some bacterial phosphatases. In *Pseudomonas fluorescens*, MF3 expression of the *apo* gene (acid phosphatase) was regulated by the growth temperature (Rodriguez and Fraga 1999). In *E. coli*, MG1655 *napA* gene (acid phosphatase) appears to be switched off when cells were grown on glucose and turned on when growth was supported by alternative carbon sources (Rossolini et al. 1994; Thaller et al. 1995). Two enzymes produced by *Salmonella typhimurium*, an acid hexose phosphatase and a cyclic phosphodiesterase was positively regulated by cyclic adenosine monophosphate (cAMP) and the cAMP receptor protein (CRP) (Kier et al. 1977). A negative control by cAMP has also been found for the pH 2.5 acid phosphatase gene (*appA*) from *E. coli* (Rodriguez and Fraga 1999).

Bacterial phosphatase-encoding genes are isolated by expression cloning systems by histochemical screening of genomic libraries (Rodríguez and Fraga).

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Gene or Plasmid</th>
<th>Features</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Erwinia herbicola</em></td>
<td><em>mps</em></td>
<td>Produces gluconic acid</td>
<td>Rodriguez et al. 2006</td>
</tr>
<tr>
<td></td>
<td></td>
<td>solubilizes mineral P in</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td><em>E. coli</em> HB101</td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas cepacia</em></td>
<td><em>gabY</em></td>
<td>Produces gluconic acid</td>
<td>Babu khan et al. 1995</td>
</tr>
<tr>
<td></td>
<td></td>
<td>solubilizes mineral P in</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td><em>E. coli</em> JM109</td>
<td></td>
</tr>
<tr>
<td><em>Enterobacter agglomerans</em></td>
<td><em>pKKY</em></td>
<td>Solubilizes P in <em>E. coli</em> JM109</td>
<td>Rodriguez et al. 2006</td>
</tr>
<tr>
<td><em>Rahnella aquatilis</em></td>
<td><em>Pk1M10</em></td>
<td>Produces gluconic acid</td>
<td>Kim et al. 1998</td>
</tr>
<tr>
<td></td>
<td></td>
<td>solubilizes mineral P in</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td><em>E. coli</em> DH5</td>
<td></td>
</tr>
<tr>
<td><em>Serratia marcescens</em></td>
<td><em>pKG3791</em></td>
<td>Produces gluconic acid</td>
<td>Krishnaraj and Goldstein 2001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>solubilizes mineral P</td>
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A medium containing the phosphatase substrate phenolphthalein diphosphate (PDP) and the stain methyl green (MG) allows identification of the phosphatase positive phenotype (pho1) as green-stained colonies, while the phosphatase negative (pho2) clones grow as unstained colonies (Riccio et al. 1997). Several bacterial phosphatase-encoding genes from different species, such as *Providencia stuartii*, *Providencia rettgeri* and *Morganella morganii* have been isolated (Thaller 1994; Thaller et al. 1995; Riccio et al. 1997; Thaller 1997).

Another method is Luria Agar containing 5-bromo-4-chloro-3-indolyl phosphate (BCIP), which permits the direct selection of dark blue transformant colonies on indicator plates. Acid phosphatase-encoding gene (phoC) from *Zymomonas mobilis* was cloned using this method (Pond et al., 1989). Acid phosphatase gene agp of *E. coli* gene was isolated by shotgun cloning technique to amplify the genes responsible for high level para-nitrophenyl-phosphate (pNPP) hydrolysis (phosphatase activity) (Pradel and Bouquet, 1988). Various genes involved in organic phosphate solubilization has been isolated (Table 2).

### BETTER PSB

Understanding the genetic basis of release of organic acids will help to transfer the mps (mineral phosphate solubilizing) ability to various rhizosphere competent bacteria. PSB can be developed or made better by transfer/ overexpression of genes involved in PQQ synthesis. *Rhizobium* possess apo-GDH genes but not for PQQ factor (Matsushita et al. 1997), so PQQ genes can be transferred to make a better PSB, while overexpression of these genes in *Pseudomonas* will make them a better PSB (Gyaneswar et al. 2002).

Another approach is to screen the *mps* genes directly in the desired bacteria by over/under expression of genes and selection for transformants with *mps* ability. This method was first applied with *Synechosystis PCC 6803* in *E. coli*. Genes for utilization of salicylate were transferred to bacteria, and the recombinant bacterium was able to survive and enhance plant growth (Colbert et al. 1993). Genetic engineering could also increase the survival of the inoculant strain by making them competent enough to utilize certain nutrients better than the rest of the microbial population (Glick and Bashan 1997).

Development of genetically modified bacteria is advantageous than transgenic plants. It is easier to

### GENETIC ENGINEERING TO DEVELOP

#### Table 2: Cloning of genes involved in organic phosphate solubilization

<table>
<thead>
<tr>
<th>Bacteria</th>
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<th>References</th>
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<tbody>
<tr>
<td><em>Franciscella taluensis</em></td>
<td>acpA</td>
<td>Acid phosphatase gene with wide range of substrate specificity</td>
<td>Reilly et al. 1996</td>
</tr>
<tr>
<td><em>Morganella morganii</em></td>
<td>PhoC and NapA</td>
<td>Acid phosphatases. NapA gene was transferred to <em>Burkholderia cepacia</em> IS-16. Phosphatase activity was increased</td>
<td>Thaller et al. 1994; Thaller et al. 1995</td>
</tr>
<tr>
<td><em>Rhizobium meliloti</em></td>
<td>napD</td>
<td>Acid phosphatase gene</td>
<td>Deng et al. 1998</td>
</tr>
<tr>
<td><em>Sinorhizobium meliloti</em></td>
<td>Nape</td>
<td>Acid phosphatase gene</td>
<td>Deng et al. 2001</td>
</tr>
<tr>
<td><em>Bacillus sp. DS11</em></td>
<td>Phy</td>
<td>Phytase gene</td>
<td>Kim et al. 1998</td>
</tr>
<tr>
<td><em>Bacillus subtilis VTT E-68013</em></td>
<td>phyC</td>
<td>Phytase genes</td>
<td>Kerovuo et al. 1998</td>
</tr>
<tr>
<td><em>Bacillus amylojavfaces</em> FZB45</td>
<td>phyA</td>
<td>Phytase gene</td>
<td>Idriss et al. 2002</td>
</tr>
<tr>
<td><em>Zymomonas mobilis</em></td>
<td>phoD</td>
<td>Acid and Alkaline Phosphatase</td>
<td>Reyes and Scopes 1991</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>ushA</td>
<td>5'-nucleotidase</td>
<td>Burns and Beacum 1986</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>Agp</td>
<td>Acid glucose-1-phosphatase</td>
<td>Pradel et al. 1990</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>cpsB</td>
<td>2'-3' cyclic phosphodiesterase</td>
<td>Beacum and Garrett 1980</td>
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</table>
modify a bacterium than complex higher organisms with many growth promoting traits in a single organism and instead of engineering crop by crop, a single engineered inoculant can be used for several crops (Rodríguez et al. 2006).

If phosphate solubilizing genes are inserted into microbes which do not have this capability than in the near future mixing of two populations of bacteria (phosphate solubilizers and nitrogen fixers) can be avoided (Bashan et al. 2000).

**CONCLUSION**

Various research studies in different parts of the world are going on to isolate and identify different phosphate solubilizing microbes which could be used to formulate bio-fertilizers and applied in phosphorus deficient soils. There has also been works regarding the isolation and cloning of genes responsible for solubilization of insoluble phosphates which will help to formulate better PSB strains through genetic engineering. Although PSB has been isolated, knowledge about the genetics of solubilization of mineral phosphates and mineralization of organic phosphorus is less explored. Further studies on these aspects will help in the advancement of knowledge regarding PSB.

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