Phosphate Solubilization: Their Mechanism Genetics And Application
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Citation

Abstract
The global necessity to increase agricultural production from a steadily decreasing and degrading land resource base has placed considerable strain on agro ecosystems (Tilak, 2005). Current strategy is to maintain and improve agricultural productivity exclusively via the use of chemical fertilizers. Although the use of chemical fertilizers is credited with nearly fifty percent increase in agricultural production but they are closely associated with environmental pollution and health hazards (Gaur and Gaind, 1999). Many synthetic fertilizers contain acids, such as sulfuric acid and hydrochloric acid, which tend to increase the acidity of the soil, reduce the soil's beneficial organism population and interfere with plant growth. Generally, healthy soil contains enough nitrogen-fixing bacteria to fix sufficient atmospheric nitrogen to supply the needs of growing plants. However, continued use of chemical fertilizers may destroy these nitrogen-fixing bacteria. Furthermore, chemical fertilizers may affect plant health. For example, citrus trees tend to yield fruits that are lower in vitamin C when treated with synthetic fertilizer. Lack of trace elements in soil regularly dosed with chemical fertilizers is not uncommon. This lack of vital micronutrients can generally be attributed to the use of chemical fertilizers. On the other hand Biofertilizer adds nutrients to soil. Environmentally friendly biotechnological approaches offer alternatives to chemical fertilizers (Dobbelaere et al., 2003). Given the negative environmental impacts of chemical fertilizers and their increasing costs, the use of PGPB is thus being considered as an alternative or a supplemental way of reducing the use of chemicals in agriculture (De Weger et al., 1995; Gerhardson, 2002, Postma, et al., 2003; Welbaum, 2004)

INTRODUCTION
The global necessity to increase agricultural production from a steadily decreasing and degrading land resource base has placed considerable strain on agro ecosystems (Tilak, 2005). Current strategy is to maintain and improve agricultural productivity exclusively via the use of chemical fertilizers. Although the use of chemical fertilizers is credited with nearly fifty percent increase in agricultural production but they are closely associated with environmental pollution and health hazards (Gaur and Gaind, 1999). Many synthetic fertilizers contain acids, such as sulfuric acid and hydrochloric acid, which tend to increase the acidity of the soil, reduce the soil's beneficial organism population and interfere with plant growth. Generally, healthy soil contains enough nitrogen-fixing bacteria to fix sufficient atmospheric nitrogen to supply the needs of growing plants. However, continued use of chemical fertilizers may destroy these nitrogen-fixing bacteria. Furthermore, chemical fertilizers may affect plant health. For example, citrus trees tend to yield fruits that are lower in vitamin C when treated with synthetic fertilizer. Lack of trace elements in soil regularly dosed with chemical fertilizers is not uncommon. This lack of vital micronutrients can generally be attributed to the use of chemical fertilizers. On the other hand Biofertilizer adds nutrients to soil. Environmentally friendly biotechnological approaches offer alternatives to chemical fertilizers (Dobbelaere et al., 2003). Given the negative environmental impacts of chemical fertilizers and their increasing costs, the use of PGPB is thus being considered as an alternative or a supplemental way of reducing the use of chemicals in agriculture (De Weger et al., 1995; Gerhardson, 2002, Postma, et al., 2003; Welbaum, 2004)

It has been estimated that in some soil up to 75% of applied phosphate fertilizer may become unavailable to the plant because of mineral phase reprecipitation (Goldstein, 1986; Sundara et al., 2002). Phosphate-solubilizing bacteria (PSB) are able to convert insoluble phosphates into soluble forms (Illner and Schinner, 1995; Hilda et al., 2000ab; Peix et al., 2001ab; Viverk and Singh, 2001; Sudhakara et al., 2002) and have therefore been used to enhance the solubilization of
reprecipitated soil P for crop improvement (Shekhar et al., 2000; Young et al., 1986; Young, 1990).

**PHOSPHATE AVAILABILITY IN SOIL**

Phosphorus (P) is one of the major essential macronutrients for biological growth and development (Ehrlich, 1990). It is present at levels of 400–1200 mg/kg of soil (Fernandez, 1988). The concentration of soluble P in soil is usually very low, normally at levels of 1 ppm or less than 1 ppm (Goldstein, 1994). The cell might take up several P forms but the greatest part is absorbed in the forms of Phosphate (Beever and Burns, 1980).

**Figure 1**
Figure 1: Phosphate cycle (Figure showing complexity of average soil

Mineral forms of phosphorus are represented in soil by primary minerals, such as apatite, hydroxyapatite, and oxyapatite. They are found as part of the stratum rock and their principal characteristic is their insolubility. In spite of that, they constitute the biggest reservoirs of this element in soil because, under appropriate conditions, they can be solubilized and become available for plants and microorganisms. Mineral phosphate can be also found associated with the surface of hydrated oxides of Fe, Al, and Mn, which are poorly soluble and assimilable. This is characteristic of ferrallitic soils, in which hydration and accumulation of hydrated oxides and hydroxides of Fe takes place, producing an increase of phosphorus fixation capacity (Fernandez, 1988).

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 these 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).

**ORGANIC PHOSPHATE**

A second major component of soil P is organic matter. Organic forms of P may constitute 30–50% of the total phosphorus in most soils, although it may range from as low as 5% to as high as 95% (Paul and Clark, 1988). Organic P in soil is largely in the form of inositol phosphate (soil phytate). It is synthesized by microorganisms and plants and is the most stable of the organic forms of phosphorus in soil, accounting for up to 50% of the total organic P (Dalal, 1977; Anderson 1980; Harley and Smith, 1983). Other organic P compounds in soil are in the form of phosphomonoesters, phosphodiesters including phospholipids and nucleic acids, and phosphotriesters. Of the total organic phosphorus in soil, only approximately 1% can be identified as nucleic acids or their derivatives (Paul and Clark, 1988). Various studies have shown that only approximately 1–5 ppm of phospholipids phosphorus occurs in soil, although values as high as 34 ppm have been detected (Paul and Clark, 1988). Large quantities of xenobiotic phosphonates, which are used as pesticides, detergent additives, antibiotics, and flame retardants, are released into the environment. These C-P compounds are generally resistant to chemical hydrolysis and biodegradation, but several reports have documented microbial P release from these sources (Ohtake, 1996; McGrath, 1998).

**Figure 2**
Figure 2: Figure showing complexity of average soil

(http://www.physicalgeography.net/fundamentals/10t.html)

**ORGANIC PHOSPHATE SOLUBILIZATION**

Organic phosphate solubilization is also called mineralization of organic phosphorus, and it occurs in soil at the expense of plant and animal remains, which contain a large amount of organic phosphorus compounds. The decomposition of organic matter in soil is carried out by the action of numerous saprophytes, which produce the release
of radical orthophosphate from the carbon structure of the molecule. The organophosphonates can equally suffer a process of mineralization when they are victims of biodegradation (McGrath, 1995). The microbial mineralization of organic phosphorus is strongly influenced by environmental parameters; in fact, moderate alkalinity favors the mineralization of organic phosphorus (Paul and Clark, 1988). The degradability of organic phosphorous compounds depends mainly on the physicochemical and biochemical properties of their molecules, e.g. nucleic acids, phospholipids, and sugar phosphates are easily broken down, but phytic acid, polyphosphates, and phosphonates are decomposed more slowly (Ohtake, 1996; McGrath, 1995; McGrath 1998).

Phosphorus can be released from organic compounds in soil by three groups of enzymes:

- Nonspecific phosphatases, which perform dephosphorylation of phospho-ester or phosphoanhydride bonds in organic matter
- Phytases, which specifically cause P release from phytic acid
- 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.

**INORGANIC PHOSPHATE MINERALIZATION**

Several reports have suggested the ability of different bacterial species to solubilize insoluble inorganic phosphate compounds, such as tricalcium phosphate, dicalcium phosphate, hydroxyapatite, and rock phosphate (Goldstein, 1986). In two thirds of all arable soils, the pH is above 7.0, so that most mineral P is in the form of poorly soluble calcium phosphates (CaPs). Microorganisms must assimilate P via membrane transport, so dissolution of CaPs to Pi (H₂PO₄⁻) is considered essential to the global P cycle. Evaluation of samples from soils throughout the world has shown that, in general, the direct oxidation pathway provides the biochemical basis for highly efficacious phosphate solubilization in Gram-negative bacteria via diffusion of the strong organic acids produced in the periplasm into the adjacent environment. Therefore, the quinoprotein glucose dehydrogenase (PQQGDH) may play a key role in the nutritional ecophysiology of soil bacteria. MPS bacteria may be used for industrial bioprocessing of rock phosphate ore (a substituted fluoroapatite) or even for direct inoculation of soils as a ‘biofertilizer’ analogous to nitrogen-fixing bacteria. Both the agronomic and ecological aspects of the direct oxidation mediated MPS trait. (Goldstein et al., 2003)

Among the bacterial genera with this capacity are Pseudomonas, Bacillus, Rhizobium, Burkholderia, Achromobacter, Agrobacterium, Micrococcus, Aereobacter, Flavobacterium and Erwinia (Babu-khan et al 1995; Goldstein, 1987; Sperber 1958; Rodríguez and Fraga, 1999).

**MECHANISM OF PHOSPHATE SOLUBILIZATION**

A number of theories have been proposed to explain the mechanism of phosphate solubilization. Important among them are:

- Acid production theory
- Proton and enzyme theory

**ACID PRODUCTION THEORY**

According to this theory, the process of phosphate solubilization by PSM is due to the production of organic acids which is accompanied by the acidification of the medium (Puente et al., 2004). A decrease in the pH of the filtrate from the initial value of 7.0 to a final value of 2.0 was recorded by many workers (Gaur and Sachar 1980; Gaind and Gaur 1990, 1991; Illmer and Schinner, 1992). The analysis of culture filtrates of PSMs has shown the presence
The amount and type of the organic acid produced varied with the microorganism. The organic acids released in the culture filtrates react with the insoluble phosphate. The amount of soluble phosphate released depends on the strength and type of acid. Aliphatic acids are found to be more effective in P solubilization then phenolic acids and citric acids. Fumaric acid has highest P solubilizing ability. Tribasic and dibasic acids are also more effective than monobasic acids. In the presence of tribasic acids and dibasic acids, a secondary effect appears due to ability of these acids to form unionized association compounds with calcium thereby removing calcium from the solution and increasing soluble phosphate concentration (Gaur and Gaind, 1999).

Organic acids contribute to the lowering of solution pH as they dissociate in a pH dependent equilibrium, into their respective anion(s) and proton(s). Organic acids buffer solution pH and will continue to dissociate as protons are consumed by the dissolution reaction (Welch et al., 2002). Similarly, microorganisms often export organic acids as anions (Duro and Serrano 1981, Konings 1985, Netik et al., 1997).

Besides organic acids, inorganic acids such as nitric and sulphuric acids are also produced by the nitrifying bacteria and thiobacillus during the oxidation of nitrogenous or inorganic compounds of sulphur which react with calcium phosphate and convert them into soluble forms (Gaur and Gaind, 1999).

The most efficient mineral phosphate solubilization (MPS) phenotype in Gram negative bacteria results from extracellular oxidation of glucose via the quinoprotein glucose dehydrogenase to gluconic acid (Kpomblekou and Tabatabai, 1994; Hilda and Fraga, 1999; Hilda et al., 2000). The resulting pH change and reduction potential are thought to be responsible for the dissolution of phosphate in the culture medium.
solubilization in these strains is mediated by glucose or gluconic acid metabolism. As solubilization of phosphate preceded detection of gluconic acid in the medium, perhaps even low levels of the acid (below the detection level of HPLC) started to dissolve the sparingly soluble phosphate. Alternatively, consumption of gluconic acid by growing cells could also take place. In A. brasilense, reduction in the quantity of soluble phosphate after incubation for 48 h can be explained as auto consumption of soluble phosphate by the growing bacterial population (Rodriguez et al., 2000).

The latter may result from production of gluconic acid and NH\textsubscript{4}+ uptake, which may release protons to the medium. In the faster growing A. brasilense strains, perhaps the cells used more NO-3 at the end of the incubation time, thereby releasing OH-, which may account for the higher pH after 48 h. The metabolic mechanism by which gluconic acid was produced was not explored (Rodríguez et al., 2004).

The P-solubilizing capability of gluconic acid was much higher as compared to 2-keto-gluconic acid in the filtrate from strain CC-Al74 culture. The process of acidification and chelation by gluconic acid and 2-keto gluconic acid dissolved tri calcium phosphate (TCP) in cultural medium. The chelation property of gluconic acid enables it to form insoluble complex. Insoluble metal forms may be solubilized by protons, with Ca\textsuperscript{2+} liberating phosphates (Kpomblekou and Tabatabai, 1994; Reyes et al., 1999; Shekhar et al., 2000).

Protons can be pumped into the external medium by various membrane associated pumps which set up ionic gradients for the acquisition of nutrients (Jones and Gadd, 1990; Sigler, and Hofer, 1991; Gadd, 1993). In addition, protons arise from produced organic acids which also possess an organic acid anion which is usually capable of forming a complex with metal cation (Burgstaller,. and Schinner, 1993; Hughes and Poole, 1991).

The production of citric or gluconic acid and the extrusion of H\textsuperscript{+} result from membrane transport mechanisms was described as possible mechanism for dissolving rock-phosphate from hydroxy apatite, iron phosphate, and aluminum phosphate by Penicillium rugulosum (Reyes et al., 1999). These processes are influenced by the sources of the nitrogen, phosphate, and carbon. Citric acid production and the resulting amount of phosphate dissolution are increased if nitrate is the only nitrogen source. Because citric acid is involved not only in the dissolution of phosphate but also in dissolution of iron and other metals from minerals, the process of nitrate accumulation in soils might play an important role for the weathering of rock in general.

The nature and type of acid production is mainly dependent on the carbon source (Reyes et al., 1999). In general, oxalic, citric, and gluconic acid, are strong solubilizing agents of feldspar, biotite, and phyllosilicates, (Torre et al., 1993)

### Figure 5

Table 1: Gluconic acid production by various bacterial strains

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Features</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>Produces gluconic acid and solubilizes mineral phosphate in E. coli JM109 cultured with PQQ.</td>
<td>Bhat and Srinivasan (1995)</td>
</tr>
<tr>
<td><em>Enterobacter agglomerans</em></td>
<td>Solubilizes phosphate in E. coli JM109.</td>
<td>Kim et al. (1997)</td>
</tr>
<tr>
<td><em>Rhodobacter aquatilis</em></td>
<td>Solubilizes phosphate and produces acid in E. coli D15-4. Possibly related to PQQ.</td>
<td>Kim et al. (1998)</td>
</tr>
<tr>
<td><em>Sarcina lutea</em></td>
<td>Produces gluconic acid and solubilizes mineral phosphate.</td>
<td>Krishna et al. (2001)</td>
</tr>
<tr>
<td><em>Pyrococcus vocans</em></td>
<td>Solubilizes acid and solubilizes mineral phosphate.</td>
<td>Komar</td>
</tr>
</tbody>
</table>

### PROTON AND ENZYME THEORY

Esterase type enzymes are known to be involved in liberating phosphorus from organic phosphatic compounds. PSMs (phosphate solubilizing microorganisms) are also known to produce phosphatase enzyme along with acids which cause the solubilization of P in aquatic environment (Alghazali et al., 1986). Illmer and Schinner (1995) reported that out of the four efficient phosphates solubilizing microbes, Penicillium aurathiogriseum, Penicillium simplicissimum, Aspergillus niger and Pseudomonas sp only A. niger could produce organic acid. Two most probable explanations for this are:

Solubilization without acid production is due to the release of protons accompanying respiration or ammonium assimilation (Taha et al., 1969; Kucey 1983; Dighton and Boddy 1989; Parks et al., 1990).

More solubilization occurs with ammonium salts than with nitrate salts as the nitrogen source in the media (Gaur and Gaind, 1999).

Besides these two mechanisms the production of chelating substances (Luo et al., 1993) H\textsubscript{2}S, CO\textsubscript{2} (Kapoor et al., 1989) mineral acids, siderophores (Bossier et al., 1988) biologically active substances like indole acetic acids,
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Gibberellins and cytokinins (Kucey et al., 1988) are also correlated with phosphate solubilization. Chelation involves the formation of two or more coordinate bonds between an anionic or polar molecule and a cation, resulting in a ring structure complex (Whitelaw, 2000). Organic acid anions, with oxygen containing hydroxyl and carboxyl groups, have the ability to form stable complexes with cations such as Ca$^{2+}$, Fe$^{2+}$, Fe$^{3+}$, and Al$^{3+}$ that are often bound with phosphate in poorly forms (Jones, 1998; Kucey, 1988).

Dissolution of phosphate in soil is a very important process for plant growth. Several studies have shown that the phosphate uptake by plants can be markedly increased by either mycorrhizal fungi (Azcon-Aguilar et al., 1986) or inoculation of soil with species capable of solubilizing free phosphate, such as P. Bilaii (Cunningham and Kuiack, 1992; Uzair et al., 2006).

**PHOSPHATE – PLANT INTERACTION**

Phosphorus is one of the major plant nutrient limiting plant growth. It plays a key role in nutrition of plants as it promotes development of deeper roots. The average soil is rich in phosphorus as it contains about 0.05% (w/w) phosphorus (Barber, 1984) but only one tenth of this is available to plants approximately 95–99% is present in the form of insoluble phosphates and hence cannot be utilized by the plants and due to its poor solubility and chemical fixation in the soil (Gaurand Gaind, 1999) causing a low efficiency of soluble P fertilizers.

To increase the availability of phosphorus for plants, large amounts of fertilizer is used on a regular basis. But after application, a large proportion of fertilizer phosphorus is quickly transferred to the insoluble forms. Therefore, very little percentage of the applied phosphorus is used, making continuous application necessary (Abd Alla, 1994).

Soils microorganisms are involved in a range of processes that affect phosphate transformation and thus influence the subsequent availability of phosphate to plant roots (Richardson, 2001). Free living phosphate solubilizing microorganisms (PSM) are always present in soils. The populations of inorganic phosphate solubilizing microorganisms are sometimes very low, less than $10^5$ CFU g$^{-1}$ of soil as observed in a soil in Northern Spain (Peix et al., 2001). In four Quebec soils the number of root free PSM ranged from 2.5-to $3 \times 10^6$ CFU g$^{-1}$ of soil and they represented from 26-46% of the total soil microflora (Chabot et al., 1993). As observed with other soil microbes the number of PSM is more important in the rhizosphere than in non rhizosphere soil (Kucey et al., 1989), and the number of phosphate solubilizing bacteria is more important than that of fungi (Kucey, 1983). However, inoculation studies aimed to improving P nutrition in plants involved bacteria and fungi, and is commercially available in Western Canada as the phosphate inoculant Jumpstart (Philom Bios, Saskatoon, Sask.). They are sold for wheat, canola, mustard and other legumes and contain a bacterial strain of Penicillium Bilaii. (http://www.phildombios.ca/).

**PLANT GROWTH PROMOTING BACTERIA**

Although plant growth promoting bacteria occur in soil, usually their numbers are not high enough to compete with other bacteria commonly established in the rhizosphere. Therefore, for agronomic utility, inoculation of plants by target microorganisms at a much higher concentration than those normally found in soil is necessary to take advantage of their beneficial properties for plant yield enhancement. (Igual, 2001)

Figure 6

Figure 5: Soil and root interactions

(http://www.sare.org/publications/bsbc/fig3_3.jpg)
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Figure 6: Microbial activities in the soil for plant growth promotion.

In recent years, interest in soil microorganisms that can promote plant growth has been increased considerably. The use of PGPRs to control soil borne pathogens is a practice with a promising future, because the Montreal Protocol (an international treaty to protect the earth from the detrimental effects) proposes the elimination of toxic chemicals. This has forced the plant scientists to look for new alternatives to replace fertilizers. A number of different bacteria have been reported to promote plant growth, including Azotobacter sp., Azospirillum sp., Pseudomonas sp., Acetobacter sp., Burkholderia sp. and Bacillus sp. (Rodrigues and Fraga 1999)

MECHANISM OF PLANT GROWTH PROMOTION

PGPR use one or more of direct or indirect mechanisms of action to improve plant growth and health. These mechanisms can probably be active simultaneously or sequentially at different stages of plant growth. They include

1. Phosphate solubilization,
2. Biological nitrogen fixation
3. Biological control of plant pathogens
4. Improvement of other plant nutrients uptake
5. Phytohormone production like indole-3-acetic acid and indole butyric acid

The Phosphate solubilization effect seems to be the most important mechanism of plant growth promotion in moderately to fertile soils (Chabot et al., 1998). Strains from the genera Pseudomonas, Bacillus and Rhizobium are among the most powerful P solubilizers (Rodriguez and Fraga, 1999)

Rhizobia have well known beneficial symbiotic atmospheric nitrogen fixing symbiosis with legumes, have an excellent potential to be used as PGPR with non legumes (Antoun et al., 1998).

Biological control of plant pathogens and deleterious microbes, through the production of antibiotics, lytic enzymes, hydrogen cyanide, and siderophores. Induction of the systemic resistance against many pathogens, insect and nematodes is also a recent indirect mechanism of action of PGPR (Ramamoorthy et al., 2001; Zehnder et al., 2001)

They assist competition for nutrients and space which significantly improve plant health and promote growth as evidenced by increases in seedling emergence, vigor and yield (Antoun and Kloepper, 2001).

Some PGPR have the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which hydrolyses ACC, the immediate precursor of ethylene in plants (Glick et al., 1995). By lowering ethylene concentration in seedlings and thus its inhibitory effect, these PGPR stimulate seedlings root length (Glick et al., 1998).

PGPR can promote mycorrhizal functioning. Recently for example, Villegas and Fortin (2001) showed an interesting specific synergistic interaction between the Phosphate solubilizing bacterium Pseudomonas aeruginosa and the AM fungus Glomus intraradices. No synergistic effect was observed with the other two Phosphate solubilizing bacteria tested (Pseudomonas putida and Serratia plymuthica) showing the specificity of this interaction.

All these traits that can be present in PGPR, illustrate how it is complex and difficult to associate the promotion of plant growth with Phosphate solubilization, and they explain in part the reason of obtaining better responses from plant inoculated with a mixture of PGPR. (Hodge, 2000) This has also forced plant scientist to look for PGPRs having more than one above-mentioned PGPRs trait in it.

The amelioration of phosphate deficiency by the application of costly and environmentally hazardous phosphate fertilizers is not an ideal solution and has generated serious
issues about the continued viability of current agriculture practice. This has led to a search for more environmentally friendly and economically feasible strategies to improve crop production in low phosphorus soils. In an ideal manner, such strategies should enable the efficient use of phosphate solubilizing microorganisms. Several scientists have reported the ability of different bacterial species to solubilize insoluble inorganic phosphate compounds, such as tricalcium phosphate and dicalcium phosphate (Gaur and Gaind, 1999).

**ROLE OF PHOSPHATE SOLUBILIZING BACTERIA AS BIOFERTILIZER**

Biofertilizers are organisms that enrich the nutrient quality of soil. The main sources of biofertilizers are bacteria, fungi, and cyanobacteria (blue-green algae). Plants have a number of relationships with fungi, bacteria, and algae. After the introduction of chemical fertilizers during the last century, farmers were happy of getting increased yield in agriculture. But slowly chemical fertilizers started displaying their ill-effects such as leaching out, polluting water basins, destroying flora and fauna including friendly organisms, making the crop more susceptible to the attack of diseases, reducing the soil fertility and thus causing irreparable damage to the eco system (Rodrígues and Fraga 1999).

The principle behind this strategy is that microbes have various abilities which could be exploited for better farming practices. Some of them help in combat diseases while some have the ability to degrade soil complex compounds into simpler forms which are utilized by plants for their growth. They are extremely beneficial in enriching the soil by producing organic nutrients for the soil. To convert insoluble phosphates to a form accessible to the plants, like orthophosphate, is an important trait for a PGPB for increasing plant yields (Hilda et al., 2006). Microbes having the ability to dissolve appreciable amount of phosphates is not rare. Some of them are already used as commercial biofertilizers for agricultural improvements (Rodrígues and Fraga 1999). The use of microbial products has certain advantages over conventional chemicals: they are considered safer than many of the chemicals now in use; they do not accumulate in the food chain; the target organisms seldom develop resistance as is the case when chemical agents are used; and biofertilizing agents are not considered harmful to ecological processes or the environment (Rodrígues and Fraga 1999).

According to Statistics, the worldwide transaction amount of fertilizer is roughly US$40 billion. Out of this, 135 million metric tons of chemical fertilizer is applied each year, with sales volume of about US$30 billion. Although there are no clear application statistics for biofertilizer, however, its sales volume is estimated to be as much as US$3 billion. Commercial biofertilizers claiming to undergo phosphate solubilization using mixed bacterial cultures have been developed. Examples of these are: ‘Phylazonit-M’ (permission at No. 9961, 1992, by the Ministry of Agriculture of Hungary), a product containing Bacillus Megaterium; Azotobacter Chroococcum, which allows an increase in N and P supply to the plants; and the product known as ‘KYUSEI EM’ (EM Technologies, Inc.) (Rodrígues and Fraga 1999).

**PSBS AS PLANT GROWTH PROMOTERS**

The substantial number of bacterial species, mostly those associated with the plant rhizosphere, may exert a beneficial effect upon plant growth. This group of bacteria has been termed “plant growth promoting bacteria” or PGPB, and among them, some phosphatesolubilizing bacteria (PSB) are already used as commercial biofertilizers for agricultural improvements. (SubbaRao1993; Rodríguez and Fraga 1999)

Among PGPR, phosphate solubilizing bacteria (PSB) are considered as promising biofertilizers since they can supply plants with phosphorus (P) from sources otherwise poorly available. Beneficial effects of the inoculation with PSB to many crop plants have been described by numerous authors (Antoun et al., 1998; Chabot et al., 1998; Pal, 1998; Peix et
Moreover, synergistic interactions on plant growth have been observed by co-inoculation of PSB with other bacteria, such as Azospirillum (Alagawadi and Gaur, 1992; Belimov et al., 1995) and Azotobacter (Kundu and Gaur, 1984), or with vesicular arbuscular mycorrhizae (Toro et al., 1997, 1998). In addition, many Pseudomonas strains promote plant growth by mechanisms such as the production of plant growth regulators and vitamins, enhancement of plant nutrient uptake and suppression of pathogenic or deleterious organisms (Davison, 1998; Glick, 1995; O’Sullivan and O’Gara, 1992).

PHOSPHATE SUPPLY AND DEMAND

The expected annual growth rate in world demand for phosphate fertilizers is about 2.8 percent until 2012 (Figure 2), for an increase of 5 million tonnes P₂O₅ compared with 2006.

About 58 percent of this growth will take place in Asia; consumption growth in South Asia is projected to surpass growth in East Asia at almost 5 percent/year. Rapid growth will also occur in East Europe and Central Asia (from a low base) and in Latin America. Phosphate fertilizer consumption will continue to decline marginally in West Europe and Central Europe.

Figure 9

World phosphoric acid capacity is expected to increase by 18 percent (8.1 million tonnes) by 2012. The largest increase is expected towards the end of the projection period in West Asia, when significant additional supply capacity should become operational in Saudi Arabia. Other significant expansions are scheduled in Africa (Morocco), China, and Latin America. In all other regions, phosphoric acid capacity is expected to remain almost constant or increase marginally during the period (www.cfo).

PRODUCTION OF GROWTH STIMULATING PHYTOHORMONES

Plant hormones also known as plant growth substances are naturally occurring chemicals that control plant growth and development. They regulate the rate at which individual part of a plant growth, integrate growth of those parts to form the whole organism and control reproduction. Since 1937, gibberlin, ethylene, cytokinin and abscisic acid have joined auxin as phytohormones and regarded as “classical five” (Ranjan 2003).

Figure 11

The primary auxin in plants is indole-3-acetic acid. Although other compounds with auxin activity such as indole butyric acid, phenyl acetic acid are also present in plants but little is known about their physiological role (Normanly et al., 1995).

At the molecular level, auxins influence cell division, cell elongation and cell differentiation (Davies, 1995). At the macroscopic level, auxins direct vascular development, promotes apical dominance and lateral root formation, and mediate gravitropism and phototropism (Davies, 1995). Indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA) are two endogenous auxins that can be interconverted (Ludwig-Miuller, 2000; Baartel et al., 1997).
**GENETIC BASIS OF PSBS (PHOSPHATE SOLUBILIZING BACTERIA)**

The genetic basis of phosphate solubilization is not well understood. The production of organic acids is considered to be the principal mechanism for mineral phosphate solubilization. It could be assumed that any gene involved in organic acid synthesis might have an effect on this character.

Several acid phosphatase genes from Gram negative bacteria have been isolated and characterized (Rossolini et al., 1998). 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. 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. (Idris et al., 2002).

For bacteria whose genes express in E. coli, it is possible to use plasmid cloning systems to ‘trap’ PQQGDH genes from highly efficacious MPS bacteria via functional complementation. E. coli K12 and derivatives such as DH5α constitutively synthesize apoGDH but do not synthesize PQQ. Therefore, screening genomic libraries of mineral phosphate solubilization (MPS) bacteria for gluconic acid production traps PQQ biosynthesis genes (Liu et al., 1992). Conversely, screening in E. coli AG121, a Tn5 knockout of gcd in HB101 with exogenous PQQ can trap apoGDH genes. In addition, he identified several novel DNA fragments that induce gluconic acid production in DH5α or HB101, but not...
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in AG121 (Babu khan et al., 1995; krishnaraj, 2001). These clones have no sequence homology to known PQQ genes but at least two have some homology to trans-membrane or periplasmic proteins that could mediate an environmental signal. Goldstein develops a strategy to open the funnel, thereby increasing the rate of catalysis with a concomitant increase in gluconic acid production and CaP solubilization. (Goldstein, 2003)

GENETIC BASIS OF INORGANIC PHOSPHATE SOLUBILIZATION

The genetic basis of mineral phosphate solubilization i.e. the MPS phenotype is not well understood (Goldstein and Liu, 1987). Because the production of organic acids is considered to be the principal mechanism for mineral phosphate solubilization, it could be assumed that any gene involved in organic acid synthesis might have an effect on this character.

Very little is known regarding the genetic regulation governing the mineral phosphate solubilization trait. In fact, the information about the genetic or biochemical mechanisms involved in the synthesis of the GDH-PQQ halo enzyme is scant, and variations between constitutive and inducible phenotypes are observed among several bacterial species (Goldstein, 1994). Glucose, gluconate, manitol, and glycerol are among the possible inducers of the halo enzyme activity (Vanschie, 1987)

Goldstein and Liu (1987) cloned a gene from Erwinia herbicola that is involved in mineral phosphate solubilization by screening the antibiotic resistant recombinants from a genomic library in a medium containing hydroxyapatite as the source of P. The expression of this gene allowed production of gluconic acid and mineral phosphate solubilization activity in E.coli HB101. Sequence analysis of this gene (Liu, 1992) suggested its probable involvement in the synthesis of the enzyme pyrroloquinoline quinone (PQQ) synthase, which directs the synthesis of PQQ, a co-factor necessary for the formation of the holoenzyme glucose dehydrogenase (GDH)-PQQ. This enzyme catalyzes the formation of gluconic acid from glucose by the direct oxidation pathway. (Rodrigues and Fraga, 1999; Goldstein1995; Goldstein 2003)

PYRROLOQUINOLINE QUINONE (PQQ)

PQQ (4, 5-dihydro-4, 5-dioxo-1H-pyrrolo-[2, 3-] quinoline-2, 7, 9-tricarboxylic acid) PQQ is an aromatic, tricyclic ortho-quinone that serves as the redox cofactor for several bacterial dehydrogenases. (Fig. 9) Among the best-known examples are methanol dehydrogenase and glucose dehydrogenase. PQQ belongs to the family of quinone cofactors that has been recognized as the third class of redox cofactors following pyridine nucleotide- and flavin-dependent cofactors.

PQQ is a prosthetic group required by several bacterial dehydrogenases, including methanol dehydrogenase (MDH) of Gram negative methylotrophs and some glucose dehydrogenases. PQQ is derived from two amino acids, tyrosine and glutamic acid (Houck, 1991, Van Kleef, 1988), but the pathway for its biosynthesis is unknown.

Figure 13

Figure 10: Alignment of pqq gene cluster. Equivalent genes have the same pattern

(Source: http://www.chris-anthony.co.uk/reseach%20pics/pqq.jpg)

All carbon and nitrogen atoms of PQQ are derived from conserved tyrosine and glutamate residues of the PQQA peptide. R1 and R3 represent the N- and C-terminal portions of PQQA, respectively. R2 represent a three-amino-acid linker between Glu and Tyr.

PQQ is an important cofactor of bacterial dehydrogenases, linking the oxidation of many different compounds to the respiratory chain. PQQ was the first of the class of quinone cofactors that have been discovered in the last 18 years and make up the prosthetic group of quinoproteins (Duine, 1991; Klinman &Mu, 1994, Klinman, 1996). Although plants and animals do not produce PQQ themselves, PQQ has invoked considerable interest because of its presence in human milk and its remarkable antioxidant properties. Recently, the first potential eukaryotic PQQ dependent enzyme [(aminoadipic 6-semialdehyde-dehydrogenase (AASDH; U26)] has been
identified, indicating that PQQ may function as a vitamin in mammals as well (Duine 1999).

PRESENT STATUS, DISTRIBUTION AND SIGNIFICANCE OF PQQ

PQQ was discovered in 1979 from a bacterium, and afterward it was reported to be in common foods. Because PQQ-deprived mice showed several abnormalities, such as poor development and breakable skin, PQQ has been considered as a candidate for vitamin. It was a mystery, that until 2003 it was not identified as vitamin. Since the first vitamin (now called vitamin B1) was discovered in 1910 by Dr. U. Suzuki, thirteen substances have been recognized as vitamins; the latest one was vitamin B12 found in 1948. So it takes 55 years to discover “PQQ” a previously identified substance as new vitamin (Choi 2008; Kashara and Kato 2003).

After it had been established that PQQ occurs in several bacterial enzymes, a logical next question was whether it also occurs in higher organisms. Perhaps stimulated by the reports (Paz, 1988) that PQQ occurs at high levels in certain body fluids and tissues of mammalian milk, and in citrus fruits, several reports followed in which beneficial effects were ascribed to its administration, e.g. that a diet supplemented with PQQ improved the “health” of mice substantially or prevented the outbreak of certain diseases (Duine, 1999).

The quinoprotein glucose dehydrogenase (GCD) has been demonstrated in a number of microorganisms including the enteric bacteria Klebsiella pneumoniae, E. coli and Salmonella typhimurium, and in Acinetobacter, Pseudomonas, Agrobacterium and Gluconobacter species. In most cases, GCD is a membrane bound enzyme which oxidizes glucose to gluconate in the periplasmic space (Duine, 1991). The formed gluconate is subsequently taken up in a number of organisms via a specific transport system and further metabolized. The reducing equivalents from glucose are donated via PQQ to the respiratory chain. Surprisingly, in some of the organisms mentioned above only apo-GCD is found. Thus, K. pneumoniae synthesizes a holo-GCD (Neijssel et al., 1983) but the closely related E. coli and S. typhimurium can synthesize only apo-GCD but not PQQ (Hommes et al., 1984, 1986). However, apo-GCDs in these and other organisms can be converted into active holo-GCDs by the addition of extracellular PQQ (Ameyama et al., 1986; Hommes et al., 1986, 1984; Van Schie et al., 1987).

It has been concluded that organisms like E. coli and S. typhimurium lack the genes to synthesize PQQ and it remains unclear what the role of such apo-GCDs could be. It has been suggested that organisms that are unable to synthesize PQQ could scavenge it from their surroundings and activate their apo-quinoproteins (Matsushita & Adachi, 1993).

BIOSYNTHETIC ROUTE OF PQQ

The biosynthetic route of PQQ has not been elucidated yet, but it has been proposed that glutamate and tyrosine are precursors of PQQ (Houck et al., 1988, 1991; Van Kleef & Duine, 1988). In all cases studied, the pqq operon contains a small gene; in the case of K. pneumoniae, this is pqqA which can encode a small peptide of 23-29 a containing a glutamate and tyrosine residue at conserved positions. Synthesis of the small peptide has been demonstrated (Velterop, 1995) and it is thought to be the precursor of PQQ (Goosen et al., 1989, 1992; Meulenberg et al., 1992). The K. pneumoniae pqqF gene product shows similarities to proteases like E. coli protease III (or piritilysin) and insulinases and it has been proposed that the PqqF enzyme is involved in cleavage of the PqqA precursor (Meulenberg et al., 1992).

Expression of the six K. pneumoniae pqq genes in E. coli results in PQQ synthesis and an active GCD (Meulenberg et al., 1990, 1992). Most likely PQQ biosynthesis takes place in the cytoplasm and PQQ can be released into the medium. Since the PQQ binding site of GCD faces the periplasm (Yamada et al., 1993), it is clear that both complementation of E. coli with the K. pneumoniae pqq genes as well as addition of PQQ can result in an active GCD (Matsushita, 1997). From growth studies with E. coli ptsHI mutants complemented with the complete set of K. pneumoniae pqq genes or with plasmids lacking one of the six pqq genes and from measurement of PQQ biosynthesis in the growth medium, it was concluded that each of the six genes is required for PQQ biosynthesis (Velterop et al., 1995).

In Methylobacterium extorquens AM1, the genes for PQQ synthesis are found in two clusters, pqqAB (C/D) E and pqqFG (Toyama et al., 1997). These gene designations standardize the nomenclature with that of K. pneumoniae. These genes in Methylobacterium strains were formerly called pqqDGCBA (Morris et al., 1994) and pqqEF (Springer et al., 1996), respectively. In M. extorquens AM1, pqqC and pqqD are not separate genes. Instead, they are fused into a single gene, pqqCD (Toyama et al., 1997).
Transcriptional analysis of pqqA in M. extorquens AM1 showed that a short mRNA containing only pqqA was produced at a much higher level than the mRNA for pqqAB (Ramamoorthi & Mary, 1995). Similar results were obtained in the case of K. pneumoniae by using pqqTn5::lacZ operon fusions on the chromosome (Velterop et al., 1995). These results are also consistent with the hypothesis that pqqA is a PQQ precursor, because stoichiometric amounts rather than catalytic amounts are required if the peptide is the substrate for PQQ synthesis (Velterop et al., 1995).

**GENETICS OF PQQ**

Genes involved in PQQ synthesis have been cloned from Acinetobacter calcoaceticus (Goosen et al., 1989), K. pneumoniae (Meulenberg et al., 1992), Pseudomonas fluorescens CHA0 (Schnider et al., 1995), Methylobacterium organophilum DSM 760 (Biville et al., 1989) and M. extorquens AM1 (Morris et al., 1994). In A. calcoaceticus, five pqq genes were identified and sequenced, designated IV, V, I, II and III (Goosen et al., 1989). In K. pneumoniae, genes analogous to those were identified and designated pqqABCDE, and in addition, a sixth gene was found immediately downstream of pqqE, designated pqqF (Meulenberg et al., 1992). In Methylobacterium strains, a five gene cluster (designated pqqDGCBA) was identified by complementation analysis (Biville et al., 1989; Morris et al., 1994), and sequence data from M. extorquens AM1 showed that the first three of these genes (pqqDGC) were analogous to pqqABC of K. pneumoniae (Morris et al., 1994). In P. fluorescens, genes analogous to pqqFAB of K. pneumoniae have also been sequenced (Schnider et al., 1995).

**Figure 14**

Table 3: Percentage similarities between pqqBCDE genes of different bacteria

(Source: Felder et al., 2000)

In all four sequences, a small gene is present that encodes a peptide of 22-29 amino acids, which contains conserved tyrosine and glutamate residues. Since tyrosine and glutamate are the probable precursors for PQQ synthesis (Van Kleeft and Duine, 1988; Houck et al., 1991), it has been proposed that this peptide is the precursor from which PQQ is synthesized (Goosen et al., 1992). However, the biochemical steps of PQQ synthesis are still unknown. Velterop et al. (1995) examined PQQ synthesis in vitro.

A series of experiments was carried out in which cell extracts of Escherichia coli containing all but one of the Pqq proteins were combined with those containing the missing Pqq protein. PQQ was produced in only one of these sets, that involving PqqC. E. coli cells containing a clone encoding all but the PqqC protein apparently produced an intermediate of PQQ, found both in the culture medium and in the cells. However, the amount of the intermediate was low and it was unstable (Velterop1995).

(Source: Felder et al., 2000)

Genes involved in PQQ biosynthesis have been cloned from several organisms. Five A. calcoaceticus pqq genes, pqqIV, V, I, II, and III (Goosen et al., 1989, Goosen et al., 1987), and six K. pneumoniae pqq genes, pqqA, B, C, D, E, and F (Mellenberg 1990, Mellenberg et al., 1992), were cloned and sequenced. Comparison of the deduced amino acid sequences showed that the proteins encoded by the first five genes of the K. pneumoniae pqq operon (pqqABCDE) show similarity to the proteins encoded by the corresponding A. calcoaceticus genes (49 to 64% identical amino acid residues). The K. pneumoniae pqqF gene encodes a protein that shows similarity to E. coli protease III and other proteases (Mellenberg et al., 1992), but its equivalent has not yet been found in A. calcoaceticus. Recently, three M. extorquens AM1 pqq genes, pqqD, G, and C, have been cloned and sequenced (Morris et al., 1994); pqqC was only partly sequenced. The encoded proteins showed similarity to the K. pneumoniae PqqA, B, and C proteins and the A. calcoaceticus PqqIV, V, and I proteins, respectively. Four additional pqq genes have been detected in M. extorquens by isolation of mutants and complementation studies. From similar studies, six (possibly seven) pqq genes have been postulated in M. organophilum DSM760 (Biville, 1989). Finally, a DNA fragment cloned from Erwinia herbicola contained a gene encoding a protein similar to K. pneumoniae PqqE and A. calcoaceticus PqqIII (Liu, et al.,
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1992). Except for the K. pneumoniae PqqF protein, none of the Pqq proteins shows similarity to other proteins in the database. One of the pqg genes is small and may encode a polypeptide of 24 amino acids (PqgIV, A. calcoaceticus), 23 amino acids (PqgA, K. pneumoniae), or 29 amino acids (PqgD, M. extorquens AM1). Interestingly, these putative polypeptides contain conserved glutamate and tyrosine residues (positions 15 and 19, respectively, in K. pneumoniae and the equivalents in A. calcoaceticus and M. extorquens). Those residues have been suggested previously as precursors in PQQ biosynthesis. Replacement of Glu-16 by Asp and Tyr-20 by Phe in A. calcoaceticus PqqIV abolished PQQ biosynthesis. A frame shift in K. pneumoniae pqqA had the same result (Melulenberg et al., 1992). It was suggested that the PqqA/PqqIV polypeptide might act as a precursor in PQQ biosynthesis (Goosen 1989; Goosen 1992; Melulenberg et al., 1992).

In what way could a 24-amino-acid polypeptide be involved in PQQ synthesis? Its small size makes a direct enzymatic function in the conversion of glutamate and tyrosine to PQQ unlikely. A regulatory role of the gene IV product in the expression of the other pqq genes is also improbable, since previous experiments already showed that gene IV is also essential for PQQ synthesis in an E. coli strain. In these experiments, the expression of the pqq genes from A. calcoaceticus was under control of the E. coli lac promoter, which excludes a transcriptional control of these genes by the gene IV peptide. Therefore, it is likely that the small polypeptide has a more direct role in synthesis of the coenzyme (Velterop1995).

Seven genes, called pqq genes, are required for PQQ biosynthesis in M. extorquens AM1, but their functions are unknown (Moris et al., 1994; Nunn and Lidstrom 1986a, Nunn and Lidstrom 1986b). The pqqD gene encodes a small polypeptide of 29 amino acids containing conserved tyrosine and glutamate residues separated by three amino acids (Moris et al., 1994). Tyrosine and glutamate have been shown to be the precursors of PQQ biosynthesis, and it has been proposed that the peptide might serve as the substrate for PQQ biosynthesis (Goosen, 1992; Houck, 1991; Meulenberg1992).

References


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r-7.


r-10.


r-14.


r-28.
r-43.
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