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Phosphate solubilizing bacteria and their role in plant growth promotion

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Abstract

The use of phosphate solubilizing bacteria as inoculants simultaneously increases P uptake by the plant and crop yield. Strains from the genera *Pseudomonas, Bacillus* and *Rhizobium* are among the most powerful phosphate solubilizers. The principal mechanism for mineral phosphate solubilization is the production of organic acids, and acid phosphatases play a major role in the mineralization of organic phosphorous in soil. Several phosphatase-encoding genes have been cloned and characterized and a few genes involved in mineral phosphate solubilization have been isolated. Therefore, genetic manipulation of phosphate-solubilizing bacteria to improve their ability to improve plant growth may include cloning genes involved in both mineral and organic phosphate solubilization, followed by their expression in selected rhizobacterial strains. Chromosomal insertion of these genes under appropriate promoters is an interesting approach.

Keywords: Phosphate solubilization; Soil bacteria; Plant-growth-promoting bacteria; Rhizobacteria; Phosphatases; Biofertilization

1. Introduction

It is well known that a considerable number of bacterial species, mostly those associated with the plant rhizosphere, are able to exert a beneficial effect upon plant growth. Therefore, their use as biofertilizers or control agents for agriculture improvement has been a focus of numerous researchers for a number of years [1–5]. This group of bacteria has been termed 'plant growth promoting rhizobacteria' (PGPR) [6], and among them are strains from genera such as *Pseudomonas, Azospirillum, Burkholderia, Bacillus, Enterobacter, Rhizobium, Erwinia, Serratia, Alcaligenes, Arthrobacter, Acinetobacter* and *Flavobacterium.*

Stimulation of different crops by PGPR has been demonstrated in both laboratory and field trials. Strains of *Pseudomonas putida* and *Pseudomonas fluorescens* have increased

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root and shoot elongation in canola, lettuce, and tomato [7,8] as well as crop yields in potato, radishes, rice, sugar beet, tomato, lettuce, apple, citrus, beans, ornamental plants, and wheat [1,3,4,9]. Wheat yield increased up to 30% with *Azotobacter* inoculation and up to 43% with *Bacillus* inoculants, [10] and a 10–20% yield increase in the same crop was reported in field trials using a combination of *Bacillus megaterium* and *Azotobacter chroococcum* [11]. *Azospirillum* spp. have increased yield in maize, sorghum, and wheat [12–14], and *Bacillus* spp. has increased yield in peanut, potato, sorghum, and wheat [15–17].

Bacterial inoculants have been used to increase plant yields in several countries, and commercial products are currently available. For example, in Cuba, several biofertilizers are commercially produced and employed with different crops, mostly using strains of *Azotobacter*, *Rhizobium*, *Azospirillum* and *Burkholderia.*

The mechanisms by which PGPR can exert a positive effect on plant growth can be of two types: direct and indirect [5]. Indirect growth promotion is the decrease or prevention of deleterious effect of pathogenic microorganisms, mostly due to the synthesis of antibiotics [18] or siderophores [19] by the bacteria. Direct growth promotion can be through the synthesis of phytohormones [20], N_2 fixation [21], reduction of membrane potential of the roots [22], synthesis of some enzymes (such as ACC deaminase) that modulate the level of plant hormones [23], as well as the solubilization of inorganic phosphate and mineralization of organic phosphate, which makes phosphorous available to the plants [24–26]. The occurrence of this last mechanism in several PGPRs and its possible role in the overall effects on plant growth promotion will be discussed in this review.

2. Phosphate availability in soil

Phosphorus (P) is one of the major essential macronutrients for biological growth and development [27]. It is present at levels of 400–1200 $mg \cdot kg^{-1}$ of soil [28]. Its cycle in the biosphere can be described as ‘open’ or ‘sedimentary’, because there is no interchange with the atmosphere [29]. Microorganisms play a central role in the natural phosphorus cycle. This cycle occurs by means of the cyclic oxidation and reduction of phosphorus compounds, where electron transfer reactions between oxidation stages range from phosphine (-3) to phosphate ($+5$). The genetic and biochemical mechanisms of these transformations are not yet completely understood [30].

The concentration of soluble P in soil is usually very low, normally at levels of 1 ppm or less (10 M $H_2PO_4^-$) [31]. The cell might take up several P forms but the greatest part is absorbed in the forms of HPO_4^{2-} or $H_2PO_4^-$ [32].

The biggest reserves of phosphorus are rocks and other deposits, such as primary apatites and other primary minerals formed during the geological age [28,33]. For example, it is estimated that there are almost 40 million tons of phosphatic rock deposits in India [34], and this material should provide a cheap source of phosphate fertilizer for crop production [35]. 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 ferralitic soils, in which hydration and accumulation of hydrated oxides and hydroxides of Fe takes place, producing an increase of phosphorus fixation capacity [28].

Most agricultural soils contain large reserves of phosphorus, a considerable part of which has accumulated as a consequence of regular applications of P fertilizers [36]. However, a large portion of soluble inorganic phosphate applied to soil as chemical fertilizer is rapidly immobilized soon after application and becomes unavailable to plants [37]. The phenomena of fixation and precipitation of P in soil is generally highly dependent on pH and soil type. Thus, in acid soils, phosphorus is fixed by free oxides and hydroxides of aluminum and iron, while in alkaline soils it is fixed by calcium, causing a low efficiency of soluble P fertilizers, such as super calcium [31,38,39]. According to Lindsay [40], superphosphate contains a sufficient amount of calcium to precipitate half of its own P, in the form of dicalcium phosphate or dicalcium phosphate dihydrated.

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% [41]. 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 [42–44]. Other organic P compounds in soil are in the form of phosphomonoesters, phosphodiesters including phospholipids and nucleic acids, and phosphotriesters.

Among identifiable components in hydrolysates of soil extracts are cytosine, adenine, guanine, uracil, hypoxanthine, and xanthine (decomposition products of guanine and adenine). Of the total organic phosphorus in soil, only approximately 1% can be identified as nucleic acids or their derivatives [41]. Among the phospholipids, choline has been identified as one of the products of the hydrolysis of lecithin. Various studies have shown that only approximately 1–5 ppm of phospholipids phosphorus occur in soil, although values as high as 34 ppm have been detected [41].

Many of these P compounds are high molecular-weight material which must first be bioconverted to either soluble ionic phosphate (Pi, HPO_4^{2-}, $H_2PO_4^-$), or low molecular-weight organic phosphate, to be assimilated by the cell [31]. Besides this, 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 recently several reports have documented microbial P release from these sources [30,45,46].

3. Phosphate solubilizing bacteria

3.1. Mineral phosphate solubilization

Several reports have examined the ability of different bacterial species to solubilize insoluble inorganic phosphate compounds, such as tricalcium phosphate, dicalcium phosphate, hydroxyapatite, and rock phosphate [38]. Among the bacterial genera with this capacity are

Pseudomonas, Bacillus, Rhizobium, Burkholderia, Achromobacter, Agrobacterium, Micrococcus, Aereobacter, Flavobacterium and *Erwinia.*

There are considerable populations of phosphate-solubilizing bacteria in soil and in plant rhizospheres [47–50]. These include both aerobic and anaerobic strains, with a prevalence of aerobic strains in submerged soils [49]. A considerably higher concentration of phosphate-solubilizing bacteria is commonly found in the rhizosphere in comparison with nonrhizosphere soil [48,49].

Visual detection and even semiquantitative estimation of the phosphate solubilization ability of microorganisms have been possible using plate screening methods, which show clearing zones around the microbial colonies in media containing insoluble mineral phosphates (mostly tricalcium phosphate or hydroxyapatite) as the single P source. In some cases, there have been contradictory results between plate halo detection and P solubilization in liquid cultures [51–53]. However, the method can be regarded as generally reliable for isolation and preliminary characterization of phosphate-solubilizing microorganisms [48,54–57]. Gupta et al. [58] developed an improved procedure using a medium containing bromophenol blue. In this medium, yellow colored halos are formed around the colonies in response to the pH drop produced by the release of organic acids, which are responsible for phosphate solubilization. With this method, the authors reported more reproducible and correlated results than with the simple halo method.

In vitro studies of the dynamics of phosphate solubilization by bacterial strains have been carried out based on the measurement of P release into culture broth, from cultures developed using an insoluble compound as the only P source. The rate of P solubilization is often estimated by subtracting the final P concentration (minus that of an inoculated control) from the initial theoretical P supplied by the P substrate. This estimation has the disadvantage of not taking into account the P utilized by the cells during growth.

Babenko et al. [59] have isolated and grouped phosphate-solubilizing bacteria into four different types, according to kinetics and rate of P accumulation. These groups range from a linear increase of P concentration along with the growth of the culture, to oscillating behavior with variations in the soluble P levels giving rise to several peaks and troughs of P concentration. This last type of kinetic behavior has also been observed [56,60,61]. These changes in P concentration could be a consequence of P precipitation of organic metabolites [59,60] and/or the formation of organo-P compounds with secreted organic acids, which are subsequently used as an energy or nutrient source, this event being repeated several times in the culture [56]. An alternative explanation could be the difference in the rate of P release and uptake. When the rate of uptake is higher than that of solubilization, a decrease of P concentration in the medium could be observed. When the uptake rate decreases (for instance as a consequence of decreasing growth or entry into stationary phase), the P level in the medium increases again. More probably, a combination of two or more phenomena could be involved in this behavior. Thus, the P concentration in the culture broth as an indication of phosphate solubilization capacity should be viewed with caution, and a kinetic study of this parameter would offer a more reliable picture of cellular behavior toward P.

The physiology of phosphate solubilization has not been studied thoroughly. Some studies indicate that certain mineral elements play a role in this process. A critical K concentration is necessary for optimum solubilization rates [32,56], while Mg and Na seem to be important in

some fungi [32] but not in *Pseudomonas* strains [56]. The role of N and P uptake remains controversial [56,62].

Instability of the phosphate-solubilizing character of some strains after several cycles of inoculation has been reported [35,56,63]. However, the trait seems to remain stable in most isolates [64].

Table 1 summarizes the solubilization ability of different insoluble P substrates by several bacterial species. Although no accurate quantitative comparison can be made from experiments from different sources, the data suggest that *Rhizobium*, *Pseudomonas* and *Bacillus* species are among the most powerful solubilizers, while tricalcium phosphate and hydroxyapatite seem to be more degradable substrates than rock phosphate.

3.2. Organic phosphate solubilization

As discussed previously, soil contains a wide range of organic substrates, which can be a source of P for plant growth. To make this form of P available for plant nutrition, it must be hydrolyzed to inorganic P. Mineralization of most organic phosphorous compounds is carried out by means of phosphatase enzymes. The presence of a significant amount of phosphatase activity in soil has been reported [65–70]. Important levels of microbial phosphatase activity have been detected in different types of soils [71,72]. In fact, the major source of phosphatase activity in soil is considered to be of microbial origin [73,74]. In particular, phosphatase activity is substantially increased in the rhizosphere [75].

The presence of organic phosphate-mineralizing bacteria in soil has been surveyed by Greaves and Webley [76] for the rhizosphere of pasture grasses, by Raghu and MacRae [49] for rice plants, as well as by Bishop et al. [67] and Abd-Alla [77], and others.

The pH of most soils ranges from acidic to neutral values. Thus, acid phosphatases should play the major role in this process. Significant acid phosphatase activity was observed in the

Table 1
Total P accumulation in cultures of different bacterial species grown on insoluble mineral phosphate substrates (mg l^{-1})

Bacterial strain	Substrate			Reference
	$Ca_3(PO_4)_2$	Hydroxyapatite	Rock phosphate	
Pseudomonas sp.	52	nd	nd	[56]
Pseudomonas striata	156	143	22	[64]
Burkholderia cepacia	35	nd	nd	[61]
Rhizobium sp.	nd	300	nd	[115]
Rhizobium meliloti	nd	165	nd	[115]
Rhizobium leguminosarum	nd	356	nd	[115]
Rhizobium loti	nd	27	nd	[115]
Bacillus amyloliquefaciens	395	nd	nd	[121]
Bacillus polymyxa	116	87	17	[64]
Bacillus megaterium	82	31	16	[64]
Bacillus pulvifaciens	54	65	13	[64]
Bacillus circulans	11	17	6	[64]
Citrobacter freundi	16	7	5	[64]

nd indicates not determined.

rhizosphere of slash pine in two forested Spodosoils [78]. Burns [79] studied the activity of various phosphatases in the rhizosphere of maize, barley, and wheat, showing that phosphatase activity was considerable in the inner rhizosphere at acidic and neutral soil pH. Soil bacteria expressing a significant level of acid phosphatases include strains from the genus *Rhizobium* [77], *Enterobacter*, *Serratia*, *Citrobacter*, *Proteus* and *Klebsiella* [80], as well as *Pseudomonas* [81] and *Bacillus* [82].

According to Greaves and Webley [76], approximately 30–48% of culturable soil and rhizosphere microorganisms utilize phytate. On the other hand, Richardson and Hadobas [83] reported that 63% of culturable soil bacteria were able to grow on this substrate as carbon and P source on agar medium. However, of these, only 39–44% could utilize phytate as a P source in liquid medium, while a very low proportion could use it as a C source in this condition.

All of these studies provide evidence that support the role of bacteria in rendering organic P available to plants [84]. Some examples of soil bacteria capable of P release from different organic sources are shown in Table 2.

3.3. Phosphate-solubilizing bacteria as plant growth promoters

Although several phosphate solubilizing bacteria occur in soil, usually their numbers are not high enough to compete with other bacteria commonly established in the rhizosphere. Thus, the amount of P liberated by them is generally not sufficient for a substantial increase in in situ plant growth. Therefore, inoculation of plants by a target microorganism at a much higher concentration than that normally found in soil is necessary to take advantage of the property of phosphate solubilization for plant yield enhancement.

There have been a number of reports on plant growth promotion by bacteria that have the ability to solubilize inorganic and/or organic P from soil after their inoculation in soil or plant seeds [9,25,26,85–88]. The production by these strains of other metabolites beneficial to the plant, such as phytohormones, antibiotics, or siderophores, among others, has created confusion about the specific role of phosphate solubilization in plant growth and yield stimulation

Table 2
Phosphate mineralization from P-substrates by some soil bacterial species

Bacterial strain	Substrate	Enzyme type	Reference
Pseudomonas fluorescens	Non-specific	Acid phosphatase	[81]
Pseudomonas sp.	Non-specific	Acid phosphatase	[81]
Burkholderia cepacia	Non-specific	Acid phosphatase	[61]
Enterobacter aerogenes	Non-specific	Acid phosphatase	[80]
Enterobacter cloacae	Non-specific	Acid phosphatase	[80]
Citrobacter freundi	Non-specific	Acid phosphatase	[80]
Proteus mirabalis	Non-specific	Acid phosphatase	[80]
Serratia marcenscens	Non-specific	Acid phosphatase	[80]
Bacillus subtilis	Inositol phosphate	Phytase	[83]
Pseudomonas putida	Inositol phosphate	Phytase	[83]
Pseudomonas mendocina	Inositol phosphate	Phytase	[83]
Pseudomonas fluorescens	Phosphonoacetate	Phosphonoacetate hydrolase	[45]
Bacillus licheniformis	D-α-glycerophosphate	D-α-glycerophosphatase	[82]
Klebsiella aerogenes	Phosphonates	C-P Lyase	[30]

[1,10]. However, at present, there is evidence supporting the role of this mechanism in plant growth enhancement. For example, several soil microorganisms, including bacteria, improve the supply of P to plants as a consequence of their capability for inorganic or organic P solubilization [24,36,89]. Considering that P availability is a limiting step in plant nutrition [38], this evidence suggests a fundamental contribution of phosphate-solubilizing bacteria to plant nutrition and, therefore, to the improvement of plant growth performance.

Chabot et al. [90] demonstrated growth stimulation of maize and lettuce by several microorganisms capable of mineral phosphate solubilization. A strain of *Burkholderia cepacia* showing no indoleacetic acid production, but displaying significant mineral phosphate solubilization and moderate phosphatase activity [61] has improved the yield of tomato, onion, potato, banana, citrics, and coffee, among other cultivars, in field tests, and is currently being used as a commercial biofertilizer in Cuba (Martínez A. et al., personal communication).

Furthermore, several examples of simultaneous growth promotion and increase in P uptake by plants as the result of phosphate-solubilizing bacteria inoculations have been reported. Inoculation with two strains of *Rhizobium leguminosarum* selected for their P-solubilization ability has been shown to improve root colonization and growth promotion and to increase significantly the P concentration in lettuce and maize [91,92]. Chabot et al. concluded that the phosphate-solubilization effect of *Rhizobia* and other mineral phosphate-solubilizing microorganisms seems to be the most important mechanism of plant growth promotion in moderately fertile and very fertile soils. On the other hand, a strain of *Pseudomonas putida* also stimulated the growth of roots and shoots and increased ^{32}P-labeled phosphate uptake in canola [89]. Inoculation of rice seeds with *Azospirillum lipoferum* strain 34H increased the phosphate ion content and resulted in significant improvement of root length and fresh and dry shoot weights [93]. Simultaneous increases in P uptake and crop yields have also been observed after inoculation with *Bacillus firmus* [87], *Bacillus polymyxa* [25] and *Bacillus cereus* [94], and others.

An alternative approach for the use of phosphate-solubilizing bacteria as microbial inoculants is the use of mixed cultures or co-inoculation with other microorganisms. Several studies demonstrate the beneficial influence of combined inoculation of phosphate-solubilizing bacteria and *Azotobacter* on yield, as well as on nitrogen (N) and P accumulation in different crops [95,96]. Co-inoculation of *Pseudomonas striata* and *Bacillus polymyxa* strains showing phosphate-solubilizing ability, with a strain of *Azospirillum brasilense*, resulted in a significant improvement of grain and dry matter yields, with a concomitant increase in N and P uptake, compared with separate inoculations with each strain [97]. Also, phosphate-solubilizing *Agrobacterium radiobacter* combined with nitrogen fixer *Azospirillum lipoferum* produced improved grain yield of barley compared with single inoculations in pot and field experiments [98]. These authors concluded that mixed inoculants provided more balanced nutrition for the plants, and that the improvement in N and P uptake was the major mechanism involved. This evidence points to the advantage of the mixed inoculations of PGPR strains comprising phosphate-solubilizing bacteria.

On the other hand, it has been postulated that some phosphate-solubilizing bacteria behave as mycorrhizal helper bacteria [99,100]. In this regard, several studies have shown that phosphate-solubilizing bacteria interact with vesicular arbuscular mycorrhizae (VAM) by releasing phosphate ions in the soil, which causes a synergistic interaction that allows for better

exploitation of poorly soluble P sources [101–103]. It is likely that the phosphate solubilized by the bacteria could be more efficiently taken up by the plant through a mycorrhizae-mediated bridge between roots and surrounding soil that allows nutrient translocation from soil to plants [104]. In fact, Toro et al. [105], using radioactive ^{32}P labeling, demonstrated that phosphate-solubilizing bacteria associated with VAM improved mineral (N and P) accumulation in plant tissues. These authors suggested that the inoculated rhizobacteria could have released phosphate ions from insoluble rock phosphate and/or other P sources, which were then taken up by the external VAM mycelium.

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.), a mixed inoculum including lactic acid bacteria, the lactic acid being the agent for mineral phosphate solubilization.

Considerable evidence supports the specific role of phosphate solubilization in the enhancement of plant growth by phosphate-solubilizing bacteria. However, not all laboratory or field trials have offered positive results. For example, an inoculant using *Bacillus megaterium* var. *phosphoricum*, was applied successfully in the former Soviet Union and India, but it did not show the same efficiency in soils in the United States [106]. Undoubtedly, the efficiency of the inoculation varies with the soil type, specific cultivar, and other parameters. The P content of the soil is probably one of the crucial factors in determining the effectiveness of the product.

4. Mechanisms of phosphate solubilization

4.1. Solubilization of mineral phosphates

It is generally accepted that the major mechanism of mineral phosphate solubilization is the action of organic acids synthesized by soil microorganisms [35,107–112]. Production of organic acids results in acidification of the microbial cell and its surroundings. Consequently, Pi may be released from a mineral phosphate by proton substitution for Ca^{2+} [31]. The production of organic acids by phosphate solubilizing bacteria has been well documented. Among them, gluconic acid seems to be the most frequent agent of mineral phosphate solubilization. It is reported as the principal organic acid produced by phosphate solubilizing bacteria such as *Pseudomonas* sp. [56], *Erwinia herbicola* [113], *Pseudomonas cepacia* [114] and *Burkholderia cepacia* (Rodriguez et al., unpublished results). Another organic acid identified in strains with phosphate-solubilizing ability is 2-ketogluconic acid, which is present in *Rhizobium leguminosarum* [35], *Rhizobium meliloti* [115], *Bacillus firmus* [109], and other unidentified soil bacteria [107]. Strains of *Bacillus liqueniformis* and *Bacillus amyloliquefaciens* were found to produce mixtures of lactic, isovaleric, isobutyric, and acetic acids. Other organic acids, such as glycolic, oxalic, malonic, and succinic acid, have also been identified among phosphate solubilizers [56,109].

There is also experimental evidence that supports the role of organic acids in mineral phosphate solubilization. Halder et al. [35] showed that the organic acids isolated from a culture of *Rhizobium leguminosarum* solubilized an amount of P nearly equivalent to the

amount that was solubilized by the whole culture. Besides this, treatment of the culture filtrates from several *Rhizobium* strains with pepsin or removal of proteins by acetone precipitation did not affect phosphate release capacity, showing that this was not an enzymatic process. However, neutralization with NaOH destroyed the solubilization activity [115]. Based on these findings, following the cloning of mineral phosphate solubilization genes, Goldstein [31,116] has proposed that the direct periplasmic oxidation of glucose to gluconic acid, and often 2-ketogluconic acid, forms the metabolic basis of the mineral phosphate solubilization phenotype in some Gram negative bacteria.

Alternative possibilities other than organic acids for mineral phosphate solubilization have been proposed based on the lack of a linear correlation between pH and the amount of solubilized P [27,117,118]. In addition, no significant amounts of organic acid production could be detected from a phosphate solubilizer fungus, *Penicillium* sp. [56]. Studies have shown that the release of H^+ to the outer surface in exchange for cation uptake or with the help of H^+ translocation ATPase could constitute alternative ways for solubilization of mineral phosphates.

Other mechanisms have been considered, such as the production of chelating substances by microorganisms [47,107] as well as the production of inorganic acids, such as sulphidric [47,119], nitric, and carbonic acid [120]. However, the effectiveness of these processes has been questioned and their contribution to P release in soil appears to be negligible [119,121].

4.2. Mineralization of organic phosphorus

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 [45]. The microbial mineralization of organic phosphorus is strongly influenced by environmental parameters; in fact, moderate alkalinity favors the mineralization of organic phosphorus [41].

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 [30,45,46].

The mineralization of these compounds is carried out by means of the action of several phosphatases (also called phosphohydrolases). These dephosphorylating reactions involve the hydrolysis of phosphoester or phosphoanhydride bonds. The phosphohydrolases are clustered in acid or alkaline. The acid phosphohydrolases, unlike alkaline phosphatases, show optimal catalytic activity at acidic to neutral pH values. Moreover, they can be further classified as specific or nonspecific acid phosphatases, in relation to their substrate specificity. Rossolini et al. [122] recently published a comprehensive review of bacterial nonspecific acid phosphohydrolases. The specific phosphohydrolases with different activities include: 3′-nucleotidases and 5′-nucleotidases [123]; hexose phosphatases [124]; and phytases [125]. A specific group of P releasing enzymes are those able to cleave C-P bonds from organophosphonates [30,45,46,126].

Some phosphohydrolases are secreted outside the plasma membrane, where they are either released in a soluble form or retained as membrane-bound proteins. This localization allows them to act as scavenging enzymes on organic phosphoesters that are components of high molecular weight material (i.e. RNA and DNA) and cannot cross the cytoplasmic membrane. This material can be first converted to low molecular weight components, and this process may occur sequentially i.e. the transformation of RNA and DNA to nucleoside monophosphate via RNase and DNase respectively, followed by the release of P and organic by-products via phosphohydrolases, providing the cell with essential nutrients [31].

5. Genetics of phosphate solubilizing bacteria

5.1. Genetics of mineral phosphate solubilization

The genetic basis of mineral phosphate solubilization (i.e. the Mps^+ phenotype) [57] is not well understood. 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.

Goldstein and Liu [57] 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 [113] 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.

Following a similar strategy, a mineral phosphate solubilization gene from *Pseudomonas cepacia* was isolated [127]. This gene (*gabY*), whose expression also allowed the induction of the mineral phosphate solubilization phenotype via gluconic acid production in *Escherichia coli* JM109, showed no apparent homology with the previous cloned PQQ synthetase gene [113,128], but it did with a permease system membrane protein. The *gabY* gene could play an alternative role in the expression and/or regulation of the direct oxidation pathway in *Pseudomonas cepacia*, thus acting as a functional mineral phosphate solubilization gene in vivo.

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 holoenzyme is scant, and variations between constitutive and inducible phenotypes are observed among several bacterial species [31]. Glucose, gluconate, manitol, and glycerol are among the possible inducers of the holoenzyme activity [129].

Concerning the possible effect of soluble P on the expression of the phosphate-solubilizing activity, Goldstein and Liu [57] found that the mineral phosphate solubilization trait in *E. herbicola* is induced by P starvation and repressed by elevated exogenous P levels (complete repression achieved at P concentrations >20 mM). Coincidentally, a *Burkholderia cepacia* strain showed reduced expression of tricalcium phosphate solubilization at increasing phos-

phate concentration >2 mM, and finally failed to express any solubilization ability at P levels between 30 and 40 mM (Rodríguez, unpublished results). However, Halder et al. [35] found no effect of soluble P up to 6 mM on rock phosphate solubilization in cultures of *Rhizobium leguminosarum.* Mikanova et al. [130] isolated a number of phosphate solubilizing bacteria, some of them exhibiting repression of this trait under the presence of soluble P and others showing no repression effect at concentrations up to 50 mM. These data thus suggest that P availability could regulate mineral phosphate solubilization in some species and have no effect in others. This aspect needs to be investigated in more detail, in particular for soil bacterial isolates.

6. Genetics of organic phosphate mineralization

Different patterns of phosphatase activity are widespread in bacteria, particularly in those belonging to the family *Enterobacteriaceae.* The production of these enzymes is often controlled by complex regulatory mechanisms, so that the enzyme activity is detectable only under specific environmental conditions. In fact, a comprehensive understanding of the properties, regulation, and role of these enzymes is still lacking; even in *Escherichia coli* and *Salmonella typhimurium*, which are the most thoroughly investigated in this regard [131], only some genes have been cloned, sequenced, and studied for their effects on regulation.

The principal mechanism for the regulation of phosphatases production is the regulation by inorganic phosphate (Pi) concentration (i.e. phosphate-repressible phosphatases). This mechanism has been best studied in the alkaline phosphatase (gene *phoA*) of *E. coli*, which is suddenly and fully induced when the Pi concentration decreases from 100 mM to 0.16 mM [132]. The mechanism involves a Pi transport operon as a regulatory element, in addition to the sensor-activator operon. The genes controlled by Pi and activated by PhoB constitute the PHO regulon [133].

Another Pi repressible bacterial phosphatase is the alkaline phosphatase of *Morganella morganii*, produced under conditions of low-Pi availability, which, according to its regulation and the molecular mass of its polypeptide components, is probably similar to that of *E. coli* [134]. *Pseudomonas fluorescens* MF3, *Providencia stuartii*, and *Providencia rettgeri* also produce alkaline phosphatase activity, which is repressed by phosphate [80,81]. Some authors suggest that the regulation of the expression of phosphatase genes in other genus belonging to the family *Enterobacteriaceae* may be similar to the *pho* genes from *E. coli*, based on the high degree of conservation of the promoter structure between these genes. For example, the sequence in the –35 region of *phoC* (encoding for an acid phosphatase of *Zymomonas mobilis*) was remarkably similar to that of the 'pho box' in *E. coli*. In the best alignment, 12 of 18 bases were conserved in *Zymomonas mobilis phoC*, and five conserved bases in the –10 region were identical [135].

The cleavage of the C-P bond from organophosphonates by phosphonoacetaldehyde hydrolase and C-P lyases is also inducible only under conditions of phosphate limitation [136,137].

According to Kier et al. [138], the production of the PhoN enzyme (class A acid phosphatase) of *Salmonella enterica* serovar *typhimurium* is moderately induced by Pi starvation. However, evidence has also shown that this gene is under the control of the *phoP-phoQ* two-

component regulatory system [139,140], which promotes transcription of *phoN* and other PhoP activated genes under low environmental Mg^{2+} concentrations [141].

Some *Enterobacteriaceae* species, such as *Morganella morganii* and *Providencia stuartii*, show a peculiar pattern of phosphatase activity consisting of the high-level phosphate-irrepressible production of acid phosphatase activity (HPAP phenotype) [142,143]. *Morganella morganii* produces a major phosphate-irrepressible class A acid phosphatase (named PhoC), which is associated with the HPAP phenotype and a minor phosphate-irrepressible class B acid phosphatase (named NapA). The regulation of the class B enzyme is apparently similar to that of class B phosphatase of *E. coli* [134]. Another example of a Pi irrepressible phosphatase-encoding gene is the *phoD* gene of *Zymomonas mobilis*, which is expressed constitutively [144,145].

These findings indicate that most of alkaline phosphatases found in the family *Enterobacteriaceae* are Pi-repressible, while many of the acid phosphatases are Pi-irrepressible. Other regulatory systems have been proposed for some bacterial phosphatases. In *Pseudomonas fluorescens* MF3, it was determined that the expression of the *apo* gene, which encodes an acidic phosphatase enzyme, was regulated by the growth temperature. The finding of a co-regulation mechanism at the transcriptional level suggests the existence of a new regulatory mechanism for these genes (whose expression is maximal at 17.5°C) as a response to the growth temperature [146]. Furthermore, the *apy* gene of *Shigella flexneri*, encoding an ATP diphosphohydrolase or apyrase, and other related alleles present in virulent *Shigella* spp. and enteroinvasive *E. coli* strains, is expressed in a thermoregulated manner [147].

According to the results of Rossolini et al. [148], in *E. coli* MG1655 the production of the p27 enzyme (acid phosphatase, class B, probably corresponding to the product of the *napA* gene found in this species, [149] appears to be switched off when cells were grown on glucose, and turned on when growth was supported by alternative carbon sources. This behavior suggests that expression of the p27 enzyme is regulated in a complex fashion.

Finally, positive regulation by cyclic adenosine monophosphate (cAMP) and the cAMP receptor protein (CRP) was proposed by Kier et al. [138] for two enzymes produced by *Salmonella typhimurium*, an acid hexose phosphatase and a cyclic phosphodiesterase. This mechanism was proposed by Pradel and Boquet [124,150] for the expression in vivo of the *E. coli agp* gene, which encodes a periplasmic acid glucose-1-phosphate phosphatase. In addition, a negative control by cAMP has been found for the pH 2.5 acid phosphatase gene (appA) from *E. coli* [151].

All of the available evidence indicates that the regulation of phosphatase enzymes is a complex matter that requires considerable additional research. In any event, the existing knowledge about *Enterobacteriaceae* phosphatases constitutes a basis for better understanding and for further exploration of the rules governing phosphatase expression in soil bacteria.

6.1. Isolation and characterization of acid phosphatase encoding genes

The isolation of bacterial phosphatase-encoding genes has been carried out by means of expression cloning systems based on histochemical screening of genomic libraries. These procedures allow quick recognition of clones harboring and expressing the enzymatic activity.

A system based on an indicator medium (named TPMG) containing the phosphatase substrate phenolphthalein diphosphate (PDP) and the stain methyl green (MG) was developed

by Riccio et al. [152]. This medium allows identification of the phosphatase positive phenotype (pho^+) as green-stained colonies, while the phosphatase negative (pho^-) clones grow as unstained colonies. This system has been used for the isolation of several bacterial phosphatase-encoding genes from different species, such as *Providencia sturatii, Providencia rettgeri* and *Morganella morganii* [134,149,152,153].

Another system for expression cloning of bacterial phosphatase-encoding genes 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. This system was used by Pond et al. [135] for cloning an acid phosphatase-encoding gene (*phoC*) from *Zymomonas mobilis* [154]. Groisman et al. [154] cloned the structural gene for the pH 2.5 acid phosphatase (*appA*) of *E. coli* by a method consisting of an in vivo shotgun cloning technique to amplify directly the genes responsible for high level para-nitrophenyl-phosphate (pNPP) hydrolysis (phosphatase activity). Colonies that stained yellow were considered to be acid phosphatase-positive clones. This technique was also used by Pradel and Boquet [124] to clone the *agp* gene, encoding a periplasmic acid glucose phosphatase of *E. coli.*

By using different expression cloning systems, 14 nonspecific acid phosphatase encoding genes from different bacterial species have been isolated [122]. Sequence analysis of the cloned phosphatase genes and other characteristics has allowed the classification of nonspecific phosphohydrolases into three different families: class A, class B, and class C phosphatases [134,149,153].

High homology at the sequence level has been detected in class A phosphatase genes from *M. morganii* and *P. stuartii*, suggesting that these genes are vertically derived from a common ancestor [122]. The existence of various conserved domains and a signature sequence motif for each family (A, B, and C) of bacterial phosphatases has been confirmed [122].

In addition, several other phosphatase genes have been isolated from *Escherichia coli.* These include: *ushA*, which encodes a 5′-nucleotidase [123]; *agp*, which encodes an acid glucose-1-phosphatase [124,150]; and *cpdB*, encoding the 2′–3′ cyclic phosphodiesterase [155]. A gene from *Providencia stuartii* and *Providencia rettgeri* that encodes the 43-kDa acid-hexose phosphatase [152], as well as a gene cluster involved in the synthesis of specific P-releasing enzyme from organophosphonate substrates (C-P lyase) [30] have also been cloned. These genes may be an interesting source for the further genetic manipulation of soil phosphate-solubilizing bacteria.

7. Future prospects

Phosphate-solubilizing bacteria play an important role in plant nutrition through the increase in P uptake by the plant, and their use as PGPR is an important contribution to biofertilization of agricultural crops. Accordingly, further investigation is needed to improve the performance and use of phosphate-solubilizing bacteria as bacterial inoculants.

Greater attention should be paid to studies and application of new combinations of phosphate-solubilizing bacteria and other PGPR for improved results. The mechanisms explaining the synergistic interaction should be a matter of further research to elucidate the biochemical basis of these interactions. On the other hand, genetic manipulation of phosphate-solubilizing

bacteria to improve their phosphate-solubilizing capabilities and/or the introduction of this trait in strains with other plant growth promoting effects is not only important, but also seems to be practically feasible.

In addition, the selection by classical genetic methods of mutants with increased production of organic acids and/or phosphatase activity, could constitute an effective approach that can not be underestimated. Genetic manipulation by recombinant DNA technology seems to offer a feasible approach for obtaining improved strains. Cloning of genes involved in mineral phosphate solubilization, such as those influencing the synthesis of organic acids, as well as phosphatase encoding genes, would be the first step in such a genetic manipulation program. Subcloning of these genes in appropriate vectors and their transfer and expression (or overexpression) in target host strains could be a successful procedure for improving the phosphate solubilization capabilities of selected strains. Recipient strains should be selected either for the expression of a certain phosphate-solubilizing activity, which is to be improved, or for the presence of some other important trait involved in plant growth promotion that would favorably complement the potential to release P from insoluble substrates.

Future research should also investigate the stability and performance of the phosphate solubilization trait once the bacteria have been inoculated in soil, in both natural and genetically modified strains. The survival and establishment of the introduced strain can be affected by low competitiveness, thus limiting the effectiveness of application [156]. On the other hand, the putative risk involved in the release of genetically engineered microorganisms in soil is a matter of controversy, in particular with regard to the possibility of horizontal transfer of the inserted DNA to other soil microorganisms [157]. For these reasons, the use of genetic reporter systems, such as bioluminescence genes [158,159], or green fluorescent protein genes [160] is crucial in studying the fate and survival of the strain in soil.

Genetic engineering of the phosphate solubilizing character must eventually be directed to the chromosomal integration of the gene for higher stability of the character and to avoid horizontal transfer of the inserted gene in soil. This strategy would also prevent the risk of metabolic load caused by the presence of the plasmid in the bacterial cell [161]. On the other hand, chromosomal integration may have the disadvantage of a low expression of the activity, due to the low copy number of the gene, in comparison with plasmid-harbored genes. An alternative to this situation might be the integration of multicopies of the target gene. Additionally, the use of powerful and species-specific promoters, which could be activated under the specific environmental conditions of soil is another interesting approach to successful gene expression in the engineered strain.

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