REVIEW/SYNTHÈSE

The enhancement of plant growth by free-living bacteria

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Abstract: The ways in which plant growth promoting rhizobacteria facilitate the growth of plants are considered and discussed. Both indirect and direct mechanisms of plant growth promotion are dealt with. The possibility of improving plant growth promoting rhizobacteria by specific genetic manipulation is critically examined.

Key words: plant growth promoting rhizobacteria, PGPR, bacterial fertilizer, soil bacteria.

Résumé : Nous présentons et discutons les façons selon lesquelles les rhizobactéries promotrices de la croissance des plantes arrivent effectivement à favoriser la croissance des végétaux. Nous traitons des mécanismes directs et indirects de facilitation de la croissance des végétaux. Nous examinons de façon critique les possibilités d'améliorer ces rhizobactéries par des manipulations génétiques spécifiques.

Mots clés : rhizobactéries promotrices de la croissance des plantes, PGPR, engrais bactérien, bactéries telluriques.
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Introduction

A large number of different microorganisms are commonly found in the soil including bacteria, fungi, actinomycetes, protozoa, and algae (Paul and Clark 1989). Of these, bacteria are by far the most common type of soil microorganism, possibly because they can grow rapidly and have the ability to utilize a wide range of substances as either carbon or nitrogen sources. While many of the bacteria found in soil are bound to the surface of soil particles and are found in soil aggregates, a number of soil bacteria interact specifically with the roots of plants. In fact, the concentration of bacteria (per gram of soil) that is found around the roots of plants (i.e., in the rhizosphere) is generally much greater than the bacterial density, or concentration, that is found in the rest of the soil (Lynch 1990). This presumably reflects the presence of high levels of nutrients that are found in the zone around the roots and can be used to support bacterial growth and metabolism.

The interaction between bacteria and the roots of plants may be beneficial, harmful, or neutral for the plant, and sometimes the effect of a particular bacterium may vary as a consequence of soil conditions (Lynch 1990). Thus, for example, a particular organism that facilitates plant growth by fixing nitrogen, which is usually present in the soil in limited amounts, is unlikely to provide benefit to a plant in a setting where exogenous fixed nitrogen is added to the soil.

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While there are a number of soil bacteria that are mildly pathogenic to plants, many of the more agronomically important plant disease causing soil microorganisms are fungi, including members of such genera as *Fusarium*, *Pythium*, and *Rhizoctonia*. Despite the fact that most of the research in the area of microbial phytopathogenesis is directed toward reducing the negative impact that these organisms have on plants, knowledge of the mechanism(s) of microbial phytopathogenesis may indirectly contribute to a better understanding of some of the mechanisms used by plant growth promoting bacteria.

The bacteria that provide some benefit to plants are of two general types, those that form a symbiotic relationship with the plant and those that are free-living in the soil, but are often found near, on, or even within the roots of plants (Kloepper et al. 1988; van Peer and Schippers 1989; Frommel et al. 1991). The symbiotic bacteria, especially rhizobia, have been studied extensively, and although the world market for these microorganisms is relatively small at the present time, they have been developed as a biological means of increasing crop yields in certain circumstances (Vance 1983; Bohlool 1990; Paau 1991; Sharma et al. 1993). Beneficial free-living soil bacteria are usually referred to as plant growth promoting rhizobacteria or PGPR (Kloepper et al. 1989), or, by one group of workers in China, as yield increasing bacteria or YIB (Piao et al. 1992; Tang 1994). A number of different bacteria may be considered to be PGPR, including *Azotobacter* species, *Azospirillum* species, pseudomonads, *Acetobacter* species, *Burkholderia* species, and bacilli (Brown 1974; Elmerich 1984; Kloepper et al. 1988, 1989; Bashan and Levanony 1990; Tang 1994; Okon and Labandera-González 1994).

PGPR can affect plant growth in two different ways, indirectly or directly. The indirect promotion of plant growth occurs when PGPR lessen or prevent the deleterious effects of one or more phytopathogenic organisms. The direct promotion of plant growth by PGPR for the most part entails either providing the plant with a compound that is synthesized by the bacterium or facilitating the uptake of certain nutrients from the environment.

Collectively phytopathogens can reduce crop yields by 25–75%, which is an enormous potential loss of productivity. At present this loss is dealt with by the use of chemical agents (pesticides), although fumigation, steam treatment, and solarization of soils have also been employed (Gamliel and Katan 1992). Until recently it was believed that the advantages of using pesticides could be realized without any deleterious environmental effects. However, with the realization that many of these chemicals may be hazardous to animals and humans, and persist and accumulate in natural ecosystems, came the desire to replace these chemical agents with biological approaches that were more "friendly" to the environment. The biological approaches that are currently being developed to control a variety of phytopathogenic agents include the development of transgenic plants that are resistant to one or more pathogenic agents (Greenberg and Glick 1993) and the use of PGPR that can suppress or prevent the phytopathogenic damage (O'Sullivan and O'Gara 1992; Sivan and Chet 1992; Sutton and Peng 1993; Cook 1993).

Bacteria that act by directly stimulating plant growth have to date received relatively little attention. However, in the past few years it has become clear that the use of these organisms represents an enormous opportunity for agriculture.

Mechanisms of antibiosis

Although iron is one of the most abundant minerals on Earth, in the soil it is surprisingly unavailable for direct assimilation by microorganisms. The reason for this is that the ferric ion, or Fe^{3+}, which is the predominant form of iron in nature, is only sparingly soluble, i.e., about 10^{-18} M at pH 7.4 (Neilands et al. 1987). Since the amount of iron that is available in the soil would be much too low to support microbial growth, soil microorganisms secrete low molecular mass (~400–1000 Da) iron-binding molecules (siderophores) that bind Fe^{3+}, transport it back to the microbial cell, and then make it available for microbial growth (Neilands and Leong 1986; Briat 1992).

One way that PGPR can prevent the proliferation of phytopathogens, and thereby facilitate plant growth, is through the production and secretion of siderophores with a very high affinity ($K_d = 10^{-20}$ to 10^{-50}) for iron (Castignetti and Smarrelli 1986). The secreted siderophore molecules bind most of the Fe^{3+} that is available in the rhizosphere, and as a result effectively prevent any pathogens in this immediate vicinity from proliferating because of a lack of iron (O'Sullivan and O'Gara 1992). The bacterium that originally synthesized the siderophore takes up the iron–siderophore complex by using a receptor that is specific for the complex and is located in the outer cell membrane of the bacterium (O'Sullivan and O'Gara 1992). Evidence for this mechanism comes from a number of different studies including a report that a mutant strain of *Pseudomonas putida* overproduced siderophores and was more effective than the wild type in controlling a strain of *Fusarium oxysporum* that is pathogenic to tomatoes (Vandenburgh and Gonzalez 1984). In another report, a mutant strain of *Pseudomonas aeruginosa* that was deficient in siderophore production no longer protected tomato plants against *Pythium* damping-off (Buysens et al. 1994). Direct confirmation that PGPR in the rhizosphere actually synthesize siderophores in response to iron-limiting conditions comes from a study in which monoclonal antibodies were used to develop an enzyme-linked immunosorbent assay (ELISA) to quantify the amount of siderophore from a fluorescent pseudomonad that was present in a barley rhizosphere sample (Buyer et al. 1993).

Unlike microbial phytopathogens, plants are not generally harmed by the localized depletion of iron caused by PGPR. Most plants can grow at much lower (about 1000-fold) iron concentrations than microorganisms (O'Sullivan and O'Gara 1992) and a number of plants have mechanisms for binding the bacterial iron–siderophore complex, transporting it through the plant, and then reductively releasing the iron from the siderophore so that it can be used by the plant (Crowley et al. 1988; Bar-Ness et al. 1991, 1992; Wang et al. 1993). The ability of siderophores to act as effective disease-suppressive agents is affected by the particular crop plant, the specific phytopathogen being suppressed, the soil composition, the bacterium that synthesizes the siderophore, and the affinity of the specific siderophore for iron. Thus, even though a particular PGPR is an effective disease-suppressive agent in the laboratory under controlled conditions, its behaviour in the field is generally extremely difficult to predict. This caveat notwithstanding, there is every reason to believe that the ability of bacterial siderophores to suppress phytopathogenic organisms is an important trait that could have a significant agronomic impact.

One of the most effective mechanisms that a PGPR can employ to prevent proliferation of phytopathogens is the synthesis of antibiotics. Evidence for the direct involvement of antibiotic production in PGPR-mediated disease suppression has come from two different types of experiments. (*i*) Non-antibiotic-producing mutants of several different disease-suppressive bacterial strains were no longer able to prevent phytopathogen-caused damage to plants (Gutterson et al. 1986; Thomashow and Weller 1988; Haas et al. 1991; Howie and Suslow 1991; Keel et al. 1992). (*ii*) When an antibiotic-producing (wild type) strain of *Pseudomonas fluorescens* was genetically manipulated to overproduce the antibiotics pyoluteorin and 2,4-diacetylphloroglucinol, the resultant strain protected cucumber plants against disease caused by *Pythium ultimum* more than did the wild-type strain (Maurhofer et al. 1992; Schnider et al. 1994).

In addition to more widespread antibiosis mechanisms that include the disease-suppressive effects of siderophores and antibiotics, there are a number of different ways in which PGPR can inhibit phytopathogens. (*i*) It has been suggested that the ability of some pseudomonads to synthesize hydrogen cyanide (to which these pseudomonads are themselves resistant) may be linked to the ability of those strains to inhibit some pathogenic fungi, although the role of hydrogen cyanide in disease suppression is not considered to be firmly established (Voisard et al. 1989). (*ii*) In at least one instance it has been demonstrated that several different microorganisms including strains of *Cladosporium werneckii*, *Pseudomonas cepacia* (= *Burkholderia cepacia*), and *Pseudomonas solanacearum*

are able to hydrolyze the compound fusaric acid (Toyoda and Utsumi 1991). Fusaric acid is the causative agent of the damage to plants that occurs upon *Fusarium* infection. As a consequence of the ability to hydrolyze fusaric acid, these bacterial strains can prevent plant diseases that are caused by various species of the fungus *Fusarium*. (*iii*) Many plants respond to pathogenic attack by synthesizing enzymes that can hydrolyze the cell walls of some fungal pathogens (Mauch et al. 1988). Similarly, some PGPR strains have been found to produce enzymes that can lyse fungal cells. For example, Lim et al. (1991) isolated a strain of *Pseudomonas stutzeri* that produced extracellular chitinase and laminarinase. These enzymes could digest and lyse *Fusarium solani* mycelia thereby preventing the fungus from causing crop loss owing to root rot. In addition, Fridlender et al. (1993) were able to reduce the incidence of plant disease caused by the phytopathogenic fungi *Rhizoctonia solani*, *Sclerotium rolfsii*, and *Pythium ultimum* by using a β-1,3-glucanase-producing strain of *Pseudomonas cepacia* that was able to damage fungal mycelia. (*iv*) Competition for nutrients and suitable niches on the root surface (Kloepper et al. 1988; O'Sullivan and O'Gara 1992) is a somewhat overlooked mechanism by which PGPR may protect plants from phytopathogens. In one study Stephens et al. (1993) concluded that the "major factor influencing the ability of a pseudomonad isolate to act as a biocontrol agent against *Pythium ultimum* on sugarbeets in soil, is their ability to metabolize the constituents of seed exudate in order to produce compounds inhibitory to *P. ultimum*." They also observed that there was not necessarily any relationship between the ability of a bacterium to inhibit a fungal pathogen when the bacterium was grown in the laboratory on media that favored the production of either antibiotics or siderophores, and the biocontrol activity of the bacterium in vivo (Stephens et al. 1993). (*v*) In many plants long-lasting and broad-spectrum systemic resistance to disease-causing agents including fungal pathogens can be induced by treating the plant or seed with a PGPR (van Peer et al. 1991; Tuzun and Kloepper 1994). In this case the PGPR appears to turn on the synthesis of some antipathogenic metabolite(s) within the plant in a mechanism that does not involve any direct interaction between the PGPR and the pathogen. (*vi*) Another facet of the competitiveness of a PGPR is the ability of the bacterium to persist both free in the soil and in the rhizosphere. However, it is often difficult to predict the behaviour of a particular PGPR, since the soil persistence of a bacterium may be influenced by a number of different factors including soil composition (Heijnen and van Elsas 1994), temperature (Sun et al. 1994; Chiarini et al. 1994), and the presence of recombinant plasmids (Tang 1992; Tang et al. 1994). Clearly, for any disease-suppressive mechanism to be effective, it is important that the PGPR first be able to efficiently establish itself in the rhizosphere. Thus, for the rational design of better biocontrol strains it is important to understand precisely what traits are significant for successful root colonization under field conditions and subsequent soil persistence of the PGPR.

Mechanisms of direct growth promotion

There are several ways in which different PGPR have been reported to directly facilitate the proliferation of their plant hosts. PGPR can fix atmospheric nitrogen and supply it to plants; they synthesize siderophores that can solubilize and sequester iron from the soil and provide it to plant cells; they synthesize several different phytohormones that can act to enhance various stages of plant growth; they may have mechanisms for the solubilization of minerals such as phosphorus that then become more readily available for plant growth; and they may synthesize some less well characterized low molecular mass compounds or enzymes that can modulate plant growth and development (Brown 1974; Kloepper et al. 1986, 1989; Davison 1988; Lambert and Joos 1989; Glick et al. 1994*a*, 1994*b*). A particular PGPR may affect plant growth and development by using any one, or more, of these mechanisms.

The extensive biochemical and molecular biological studies of symbiotic diazotrophs, such as rhizobia, have served as a conceptual starting point for understanding the mechanisms of growth promotion by PGPR. Since one of the major benefits that rhizobia provide to plants is fixed nitrogen in exchange for fixed carbon (photosynthate), it was initially thought that diazotrophic PGPR might also function in this way. However, not all PGPR are diazotrophic, and many of those that are diazotrophic fix only limited amounts of nitrogen and not nearly enough to provide for their own as well as the host plant's nitrogen requirements (Hong et al. 1991*a*). Some diazotrophic PGPR provide their plant hosts with a portion of the fixed nitrogen that they require; however, even for these PGPR, nitrogen fixation is only a minor component of the benefit to the plant (Chanway and Holl 1991).

As mentioned earlier, a number of plants are able to use bacterial iron–siderophore complexes as a means of obtaining iron from the soil (Crowley et al. 1988; Bar-Ness et al. 1991, 1992; Wang et al. 1993). Without this mechanism for obtaining iron, the growth of most plants in most soils would be severely limited. However, if the effect of a PGPR on plant growth were limited to providing the plant with sufficient iron, one might expect treated plants to vary in their response to the PGPR according to differences in the amount of available iron in the soil. This is not the case, so that while bacterial siderophores undoubtedly contribute to the nutrition, and hence to the growth, of plants, in most instances this effect is probably small.

Several reports have suggested that PGPR stimulate plant growth by facilitating the uptake of minerals into the plant, phosphate in particular (Kloepper et al. 1988). However, there is some controversy regarding the mechanism(s) that PGPR employ(s) in the uptake of minerals. On the one hand, it is argued that the increased mineral uptake in plants treated with PGPR is an indirect by-product of that interaction and actually reflects a better developed root system and an overall healthier plant. On the other hand, experiments with *Azospirillum* species have suggested that this organism specifically enhances mineral uptake (Murty and Ladha 1988; Bashan et al. 1990).

It is generally assumed that PGPR stimulation of plant growth requires the binding of the bacterium to the plant root. While this is undoubtedly true for most PGPR effects, it is not always the case (Reddy and Rahe 1989; Hong et al. 1991*b*). For example, in one study significant growth promotion of onion in the field was observed even though only very low levels of root colonization by PGPR were detected. In addition, electron microscopy studies of the root elongation of germinating canola seedlings in the presence of the PGPR

Pseudomonas putida GR12-2 in growth pouches under gnotobiotic conditions indicated that bacterial adherence to the seed coat alone was sufficient to enhance root elongation during this initial phase of seedling development (Hong et al. 1991*b*). Root colonization may play another, and later, role in the plant growth promoting activity of this PGPR.

The mechanism(s) most commonly invoked to explain the various effects of PGPR on plants is (are) the production of phytohormones, and most of the attention has focused on the role of the phytohormone auxin (Brown 1974; Tien et al. 1979). Auxins are a class of plant hormones; the most common and well characterized is indole-3-acetic acid (IAA), which is known to stimulate both rapid (e.g., increases in cell elongation) and long term (e.g., cell division and differentiation) responses in plants (Cleland 1990; Hagen 990). Since plants as well as many PGPR can synthesize auxin, it is important when assessing the consequences of treating a plant with a PGPR to distinguish between the auxin synthesized by the plant in response to PGPR stimulation and the auxin synthesized by the PGPR itself (Gaudin et al. 1994). A relatively straightforward way to directly monitor the effects of bacterially synthesized auxin is to compare plants treated with either wild-type PGPR strains or mutant strains that either do not produce, or else overproduce, auxin. Thus, for example, mutant strains of the PGPR *Azospirillum brasilense* that synthesized only very low levels of IAA, when compared with the wild-type strain, no longer promoted the formation of lateral roots of wheat seedlings (Barbieri et al. 1986; Barbieri and Galli 1993). On the other hand, a mutant strain of the PGPR *Pseudomonas fluorescens* BSP53a that overproduced IAA stimulated the root development of blackcurrant softwood cuttings and inhibited that of cherry (Dubeikovsky et al. 1993). These results indicate that the growth of plants treated with an IAA-secreting PGPR is affected by the amount of IAA that the bacterium produces and that the response observed may vary from one species of plant to another. Thus, PGPR facilitate plant growth by altering the hormonal balance within the affected plant.

A hitherto unsuspected mechanism of plant growth promotion involves the plant hormone ethylene. It was recently demonstrated that the PGPR *Pseudomonas putida* GR12-2 contains the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Jacobson 1993; Jacobson et al. 1994); this enzyme hydrolyzes ACC, the immediate biosynthetic precursor of ethylene in plants. When *Pseudomonas putida* GR12-2 was chemically mutagenized, three independent mutants that lacked ACC deaminase activity were selected. Unlike the wild type, none of these selected *acd* mutants were able to promote the growth of canola seedling roots under gnotobiotic conditions, implicating the enzyme ACC deaminase in the mechanism that this bacterium uses to stimulate canola root elongation (Glick et al. 1994*a*, 1994*b*). One model that can be used to explain this observation is that *Pseudomonas putida* GR12-2 binds to seed coats (Hong et al. 1991*b*), and during seed imbibition the bacterium sequesters and then hydrolyzes ACC, thereby lowering the level of ethylene in the developing plant. For a number of different plants, ethylene stimulates germination and breaks the dormancy of the seeds (Esashi 1991), but if the level of ethylene following germination is too high, root elongation is inhibited (Jackson 1991). In addition, since many PGPR such as *Pseudomonas putida* GR12-2 synthesize indoleacetic acid, the ACC deaminase activity may prevent indoleacetic acid, which normally stimulates the enzyme ACC synthase in the plant, from increasing ethylene synthesis. Thus, when PGPR, such as *Pseudomonas putida* GR12-2, that contain the enzyme ACC deaminase are bound to the seed coat of a developing seedling, they may act as a mechanism for insuring that the ethylene level does not become elevated to the point where root growth is impaired. This bacterially facilitated stimulation of root elongation could enhance the survival of some seedlings, especially during the first few days after the seeds are planted. This model predicts that any bacterium that contains the enzyme ACC deaminase and can bind to plant seeds or roots in the soil should also be able to promote root elongation. In other words, any rhizosphere bacterium with ACC deaminase activity should also be a PGPR. In fact, when *Pseudomonas* spp. that were able to use ACC as a sole nitrogen source were isolated from several different soil samples and then tested for the ability to promote the proliferation of canola roots in sterile growth pouches, in seven out of seven cases examined a *Pseudomonas* sp. that was able to utilize ACC as a sole source of nitrogen also displayed PGPR activity (Glick et al. 1994*c*).

Several studies have suggested that a number of factors seemingly unrelated to PGPR activity may have an impact on the ability of a bacterium to promote the growth of plants, as the following examples show. (*i*) It was recently reported that a lysate of degraded sodium alginate can stimulate the elongation of barley roots (Tomoda et al. 1994). These authors suggested that some of the products of the alginate degradation may act as a signal that turns on a stress response in the plant, and that in the soil this is normally achieved by alginate-degrading bacteria (Tomoda et al. 1994). (*ii*) In another instance, Lifshitz et al. (1988) used transposon mutagenesis to select a mutant of the PGPR *Pseudomonas putida* GR12-2R3 (a spontaneous rifampicin-resistant mutant of *Pseudomonas putida* GR12-2 that behaves in a manner identical to *Pseudomonas putida* GR12-2) that was no longer able to stimulate root elongation under gnotobiotic conditions. Unfortunately, despite cloning a DNA fragment that complemented this mutant, Lifshitz et al. (1988) were unable to discern the nature of the affected gene. Subsequent studies indicated that the mutant was not defective in siderophore production, IAA synthesis, or ACC deaminase activity (B.R. Glick, C.B. Jacobson, and H. Xie, unpublished observations). (*iii*) Bayliss et al. (1993) reported the isolation of a series of transposon mutants of the PGPR *Pseudomonas putida* GR12-2R3 that had a decreased ability to promote canola root elongation. A number of the selected mutants were found to be defective in their ability to utilize a specific carbon or nitrogen compound for bacterial growth. As these workers pointed out, "plant growth promotion by PGPR in the field is likely to require the coordination of multiple phenomena" so that the selected mutants may affect the growth-promoting activity of this bacterium by a variety of different mechanisms; however, at this stage the nature of the mutagenized genes still remains to be elucidated. (*iv*) It has also been suggested that the secretion of succinic and lactic acids per se by a PGPR strain of *Pseudomonas putida* may act to directly stimulate root growth in asparagus seedlings (Yoshikawa et al. 1993). However, the precise mechanism by which these organic acids promote growth stimulation is at present unknown. (*v*) Plasmid transformation of both *Azotobacter vinelandii* and a PGPR

strain of *Pseudomonas putida* has been found to severely impair a number of biological functions, including the ability of the bacterium to stimulate plant growth (Glick et al. 1986, 1989; Renaud et al. 1989; Hong et al. 1991*a*). This indirect effect, which was termed a metabolic load, probably resulted from the synthesis of plasmid-encoded proteins siphoning off energy from the bacterium and limiting other energy-dependent processes such as nitrogen fixation, siderophore production, and growth.

Strategies to increase PGPR effectiveness

The interest in PGPR is based on both the desire to unravel a complex and fascinating biological system and the potential to utilize these organisms to increase the yields of crop plants. In the first instance, strains shown to possess specific desirable traits have been selected, tested, and then used as a component of agricultural practice, although so far to a limited extent. As more and better strains are developed and shown to be effective, the use of PGPR in agriculture will increase. Some improved PGPR strains will undoubtedly be developed by conventional mutagenesis and selection; however, this approach is intrinsically limited by the genetic material contained within the target PGPR. Thus, it is only through the use of genetic engineering that truly novel "superior" PGPR strains can be developed.

One can envision a number of different ways in which PGPR might be improved by genetic engineering. Some of these strategies are relatively straightforward, requiring the addition of only a single functional gene, while others are more complex and will require PGPR to be transformed with several genes at once.

One strategy that plants use to limit root colonization by phytopathogens is production of active oxygen species such as the hydroxyl radical, the superoxide anion, and hydrogen peroxide (Doke 1983; Klotz et al. 1989; Sutherland 1991). Plant roots may also respond to colonization by PGPR by producing active oxygen species (Katsuwon and Anderson 1989, 1990). Phytopathogens that contain higher levels of the enzymes that reduce the amount of active oxygen species, i.e., superoxide dismutase, catalase, and peroxidase, have been shown to be more effective pathogens (Klotz and Hutcheson 1992). It is therefore reasonable to expect that increasing the levels of one or more of these enzymes by the genetic manipulation of a PGPR might increase the colonizing ability and hence the effectiveness of that PGPR strain.

The soil contains a large number of different microorganisms, and those microbial strains that are able to utilize an unusual carbon or nitrogen source such as an opine, ACC, or a xenobiotic compound (such as a herbicide or pesticide) should be able to proliferate and then persist longer than other microorganisms in those soils that already contain such unusual compounds. For example, the ability of some PGPR to hydrolyze ACC may provide these strains with a competitive advantage over other microorganisms in the rhizosphere, although not in the rest of the soil, because they can use ACC as a source of nitrogen (Jacobson 1993; Jacobson et al. 1994; Glick et al. 1994*a*, 1994*b*). In addition, in an effort to engineer a more soil-persistent biocontrol bacterium, another group of researchers transferred the NAH7 plasmid, which carries the genes encoding the enzymes of the naphthalene and salicylate biodegradative pathway, into an established biocontrol strain (Colbert et al. 1993). The introduced plasmid was stably maintained and conferred increased persistence upon the host bacterium when salicylate was present in the soil (Colbert et al. 1993). Similarly, the presence of a herbicide or pesticide in soil may facilitate the proliferation of bacteria engineered to degrade these compounds; at the same time, these chemicals may suppress the proliferation of the other microorganisms in the same soil and possibly provide a biodegradative PGPR with a significant competitive advantage.

A PGPR that can stimulate plant growth in the laboratory will not necessarily have any significant impact on plants in the field unless it is able to persist and grow in the natural environment. In Canada, this means being able to survive long cold winters and then grow at cool temperatures in the spring (~5–10°C). It was recently reported that the PGPR *Pseudomonas putida* GR12-2 secretes antifreeze protein(s) into the surrounding medium when the bacterium is grown at low temperatures (Sun et al. 1994). This (these) protein(s) may regulate the formation of ice crystals outside of the bacterium, thereby protecting it (them) from damage that might otherwise occur at freezing temperatures. The addition, by genetic engineering, of antifreeze protein(s) to a PGPR that is otherwise unable to persist and proliferate at cold temperatures may make a bacterium a more effective PGPR by permitting it to thrive under these adverse conditions.

Since one of the major ways in which PGPR act as biocontrol agents is through the synthesis of antibiotics, the activity, and hence the utility, of a PGPR may be improved by providing it with genes that encode the biosynthesis of antibiotics normally encoded by other bacteria (Gill and Warren 1988). In this way it should be possible to extend the range of phytopathogens that a PGPR is able to suppress. In addition, by limiting the proliferation of other soil microorganisms, antibiotic-secreting PGPR may indirectly facilitate their own proliferation because they will have fewer competitors for limited nutritional resources.

Antibiotic biosynthesis in the rhizosphere, and throughout the rest of the soil, is not restricted to PGPR. In some instances it may be difficult for a PGPR to survive in the presence of another antibiotic-synthesizing microorganism unless the PGPR has antibiotic resistance genes that, when expressed, allow the PGPR to prevent the antibiotic from inhibiting its growth. Obviously, however, it is not possible to protect a PGPR against all possible antibiotics. Since PGPR with genetically engineered antibiotic resistance may transfer that resistance to other bacteria in the environment, it may be better to avoid the use of antibiotic resistance genes altogether.

A novel way to improve PGPR would be to use genetic engineering to extend the range of iron–siderophore complexes that a particular strain could utilize. This was done by cloning the genes for iron–siderophore receptors from one PGPR and introducing them into other strains (Marugg et al. 1989). In this way a genetically altered PGPR strain could utilize siderophores synthesized by other soil microorganisms, thereby giving it a competitive advantage.

In some instances it may be possible to utilize the ability of PGPR to colonize the roots of crop plants to deliver various biological insecticides to the plant. The roots of many plants

are attacked by insects that, because of their underground location, would normally make insecticides based on *Bacillus thuringiensis* useless as biocontrol agents. However, in one case the insecticidal toxin gene from *B. thuringiensis* subsp. *kurstaki* was introduced into a PGPR strain (a *Pseudomonas fluorescens* that was originally isolated from corn roots) so that after reintroduction into the soil, the engineered bacterium synthesized and released the insecticidal toxin into the rhizosphere and conferred protection against root-attacking insects (Obukowicz et al. 1986*a*, 1986*b*). With this construct, as long as the engineered bacteria are able to persist in the soil, they should continue to synthesize the insecticidal toxin, obviating the need for repeated sprayings of either biological or chemical insecticides.

Although it may seem that diazotrophs have a competitive advantage over nondiazotrophic strains in nitrogen-poor soils, the very large amount of energy, in the form of ATP, that is required for nitrogen fixation may actually put the diazotrophs at a disadvantage. Therefore, converting a nondiazotrophic PGPR by genetic engineering to a diazotrophic one, while technically possible, will not necessarily enhance the competitiveness of the bacterium and could even debilitate the engineered bacterium, rendering it unable to stimulate plant growth (Glick et al. 1986, 1989; Hong et al. 1991*a*).

Conclusions

Research efforts worldwide over the past 10–15 years have provided a better understanding of PGPR and the mechanisms that these bacteria use to promote plant growth, and have also renewed commercial interest in PGPR. Although only a few PGPR are commercially available at the present time (Okon and Labandera-González 1994; Tang 1994; Mohammadi 1994), within the next few years it is expected that there will be many more. The next phase in the development of this field will likely see the selection and testing of improved mutants of existing PGPR strains. Ultimately, however, significantly better strains will have to be constructed by genetic engineering. Despite the fact that these efforts are still at a very early stage, this approach should have the effect of accelerating progress in this field so that it may not be unreasonable to expect to see improved genetically engineered PGPR strains being used for crop improvement within the next 10 years.

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