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GENETIC MANIPULATION OF PLANT GROWTH-PROMOTING BACTERIA TO ENHANCE BIOCONTROL OF PHYTOPATHOGENS

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ABSTRACT

Plant growth-promoting bacteria (PGPB) control the damage to plants from phytopathogens by a number of different mechanisms including: outcompeting the phytopathogen, physical displacement of the phytopathogen, secretion of siderophores to prevent pathogens in the immediate vicinity from proliferating, synthesis of antibiotics, synthesis of a variety of small molecules that can inhibit phytopathogen growth, production of enzymes that inhibit the phytopathogen and stimulation of the systemic resistance of the plant. Biocontrol PGPB may be improved by genetically engineering them to overexpress one or more of hese traits so that strains with several different anti-phytopathogen traits which can act synergistically are created. In engineering these strains it is essential to ensure that the normal functioning of the bacterium is not impaired, i.e., that there is no problem with metabolic load.
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KEY WORDS

Biocontrol, plant growth promoting bacteria, PGPR, soil bacteria, phytopathogens

INTRODUCTION

Free-living soil and rhizosphere bacteria that are beneficial to plants are often referred to as plant growth-promoting rhizobacteria or PGPR (Kloepper et al.,1989; Glick, 1995a). A number of different bacteria may be considered to be PGPR including *Azotobacter, Azospirillum,* pseudomonads, *Acetobacter, Burkholderia, Enterobacter* and bacilli (Brown, 1974; Elmerich,1984; Kloepper et al., 1988 ; Kloepper et al.,1989; Bashan and Levanony,1990; Tang, 1994; Glick, 1995a).

PGPR can affect plant growth either directly or indirectly. 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. The direct effects of PGPR include providing the host plant with i) fixed nitrogen, ii) phosphorus and iron solubilized from the soil and iii) phytohormones such as auxins, cytokinins and gibberelins that are synthesized by the bacterium. The indirect promotion of plant growth occurs when PGPR lessen or prevent the deleterious effects of one or more phytopathogenic organisms. It was recently suggested that soil bacteria that indirectly stimulate plant growth be referred to as biocontrol-PGPB or biocontrol plant growth-promoting bacteria while bacteria that directly stimulate plant growth be referred to as PGPB (Bashan and Holguin, 1996).

Phytopathogens can reduce crop yields from 25-100%, which is an enormous potential loss of productivity. This loss is currently dealt with by the use of chemical agents, although soil fumigation, steam-treatment and solarization of soils have also been employed (Gamliel and Katan, 1992; Sivan and Chet, 1992; Chet and Inbar, 1994). Unfortunately, for most bacterial diseases, plants can be symptomless for prolonged periods. Slight changes in the environment which favor the proliferation of the bacteria may cause a rapid outbreak of the disease, creating severe epidemics that can destroy the crop. The available means of controlling field epidemics are few, decades-old, rarely effective and too expensive for low value crops. To prevent the development of epidemics it is necessary to contain the pathogen when its level is low (Bashan,1996).

Many of the chemicals that are used to control fungal and bacterial diseases of plants are hazardous to animals and humans and persist and accumulate in natural

ecosystems. It is therefore desirable to replace these chemicals agents with biological approaches that are more "friendly" to the environment. The biological approaches that are currently being developed for the control of a variety of phytopathogenic agents include the development of transgenic plants that are resistant to one or more pathogenic agent (Broglie et al., 1991; Greenberg and Glick, 1993; Zhu et al., 1994; Shah et al., 1995), and the use of biocontrol PGPB that can suppress or prevent the phytopathogen damage (O'Sullivan and O'Gara, 1992; Sivan and Chet, 1992; Sutton and Peng, 1993; Cook, 1993; Chet and Inbar, 1994; Dowling and O'Gara, 1994; Pankhurst and Lynch, 1995; McLaughlin et al.,1995).

BIOCONTROL OF PATHOGENS BY PGPB

A variety of substances produced by biocontrol PGPB have been implicated in the mechanism(s) used by these organisms to limit the damage to plants by phytopathogens. These include siderophores, antibiotics, other small molecules, and a variety of enzymes. It has been suggested (Dowling and O'Gara, 1994) that the following criteria may be used to determine if a particular compound is directly involved in biological control of fungal pathogens: "1) Mutants defective in metabolite(s) are unable to show inhibition of the pathogen in the laboratory. 2) The biocontrol ability of the mutants is reduced in the field. 3) Complementation of the mutant with wild-type DNA sequences restores biocontrol ability. 4) The purified metabolite shows fungicidal or antimicrobial properties. 5) The metabolite may be detected in situ (i.e. in the rhizosphere) when producing strains are present."

One can envision a number of different ways in which biocontrol PGPB 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.

It should be emphasized here that for the most part, the studies described here have been carried out under controlled laboratory, growth chamber or greenhouse conditions. While such studies clearly facilitate the understanding of some of the underlying mechanisms, the ultimate utility of a strategy based on a particular mechanism can only be assessed under field conditions.

Siderophores

Although iron is one of the most abundant minerals on Earth, in the soil it is unavailable for direct assimilation by microorganisms because 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 soluble iron in the soil would be much too low to support microbial growth, soil microorganisms secrete low molecular mass (~400-1000 daltons) iron-binding molecules known as siderophores which bind Fe^{+3} with a very high affinity (Kd = 10^{-20} to 10^{-5O}) (Castignetti and Smarrelli,1986) and transport it back to the microbial cell where it is taken up by means of a cellular receptor, and then make it available for microbial growth (Neilands and Leong, 1986; Briat,1992).

One way that biocontrol PGPB can prevent the proliferation of phytopathogens, and thereby facilitate plant growth, is through the production and secretion of siderophores that bind most of the Fe^{+3} that is available in the rhizosphere, and as a result effectively prevent any fungal pathogens in this immediate vicinity from proliferating because of a lack of iron (O'Sullivan and O'Gara, 1992). The bacteria that originally synthesized the siderophores take up the iron-siderophore complex using a receptor which is specific for the complex and is located in the outer cell membrane of the bacterium (O'Sullivan and O'Gara, 1992). Although fungal phytopathogens also synthesize siderophores, the fungal siderophores generally have a lower affinity for iron than do the siderophores produced by biocontrol PGPR (Schippers et al., 1987) so that biocontrol PGPR in effect out-compete fungal phytopathogens for available iron.

Unlike microbial phytopathogens, plants are not generally harmed by the localized depletion of iron in the soil caused by PGPR. Most plants can grow at much lower (about 1000-fold) iron concentrations than microorganisms (O'Sullivan and O'Gara, 1992). In addition, 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 bacterial siderophore so that it can be used by the plant (Bar-Ness et al.,1991; Bar-Ness et al.,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 biocontrol PGPB is an effective disease-suppressive agent in the laboratory under controlled conditions, its behaviour in the field is 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.

Consistent with the involvement of siderophores in fungal pathogen-caused disease suppression it was shown that: i) A mutant strain of *Pseudomonas putida* that overproduced siderophores was more effective than the wild-type bacterium in controlling a strain of *Fusarium oxysporum* that is pathogenic to tomatoes (Vandenburgh and Gonzalez, 1984). ii) A mutant strain of *Pseudomonas aeruginosa* that was selected for its lack of siderophore production no longer had the ability, shown by the wild-type, to protect tomato plants against *Pythium* damping-off (Buysens et al.,1994). iii) Increasing the amount of iron present in the soil to 40 μmol Fe^{+3}/L caused a parallel decrease in both the amount of fluorescent siderophores produced and the inhibitory effect against *Gaeumannomyces graminis* var. *tritici,* a pathogen of wheat, in a collection of 70 separate isolates of fluorescent pseudomonads (Elsherif and Grossmann, 1994). iv). A single Tn5 insertion into the genome of *Alcaligenes* sp. strain MFA1 resulted in the simultaneous loss of the ability of the bacterium to grow in the absence of iron, and to inhibit microconidial germination and germination-tube elongation of the pathogen *Fusarium oxysporum* (Martinetti and Loper, 1992). v) 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 ELISA assay to quantify the amount of siderophore from a fluorescent pseudomonad that was present in a barley rhizosphere sample (Buyer et al.,1993).

In one study, mutants of the biocontrol PGPB strain *Pseudomonas fluorescens* 2-79 deficient in either siderophore or antibiotic production or both were selected (Hamdan et al., 1991). This bacterium normally suppresses take-all, a major root disease of wheat caused by the fungal pathogen *Gaeumannomyces graminis* var. *tritici.* In all cases mutant strains that were deficient in the production of the siderophore pyoverdin controlled take-all as efficiently as did the parental strains that produced this siderophore. This result was interpreted as indicating that in this case the siderophore pyoverdin is probably not required for the suppression of take-all in wheat. Furthermore, these workers suggest that under iron limited conditions fluorescent pseudomonads can synthesize a variety of other compounds that are responsible for iron uptake and have anti-fungal activity. If fluorescent pseudomonads can produce a number of different compounds that can facilitate iron uptake, then these results are not necessarily at odds with the other reported studies, and siderophore production is still likely to be an important component of

the mechanism used by biocontrol PGPB to limit the proliferation of fungal phytopathogens.

Since each siderophore is encoded by a number of different genes, genetically engineering bacteria to produce modified siderophores is not a simple matter. On the other hand, it is possible to improve biocontrol PGPB by extending the range of iron-siderophore complexes that a particular strain can utilize so that a genetically altered biocontrol PGPB strain could take up and use siderophores synthesized by other soil microorganisms thereby giving it a competitive advantage. This was done by cloning the genes for iron-siderophore receptors from one biocontrol PGPB and introducing them into other strains (Marugg et al.,1989).

Antibiotics

One of the most effective mechanisms which a biocontrol PGPB can employ to prevent phytopathogen proliferation is the synthesis of antibiotics. The antibiotics synthesized by biocontrol pseudomonads include, but are not limited to, agrocin 84, agrocin 434, 2,4-diacetylphloroglucinol, herbicolin, oomycin, phenazines, pyoluteorin and pyrrolnitrin. The biocontrol activity of a number of strains has been shown to be directly related to the ability of the bacterium to produce one of these antibiotics. However, an antibiotic that is effective in the laboratory against one strain of a pathogenic agent may not prevent damage to the plant that occurs as a consequence of other strains of the same pathogen, and may not be as effective under more variable field conditions.

Evidence for the direct involvement of antibiotic production in biocontrol PGPB-mediated disease-suppression comes from several different types of experiments: i) In a number of instances, the antibiotics that were isolated and purified from biocontrol PGPB were shown to inhibit the same spectrum of fungal pathogens as the biocontrol strain itself (Carmi et al., 1994). ii) Non-antibiotic-producing mutants of several different disease-suppressive bacterial strains either were no longer able to prevent phytopathogen (e.g., *Gaeumannomyces graminis* var. *tritici, Pythium ultimum* and *Rhizoctonia solani)* caused damage to plants or protected the plant to a much lesser extent than the wild-type bacterium (Thomashow and Weller, 1988; Haas et al.,1991; Howie and Suslow,1991; Hamdan et al.,1991; Keel et al., 1992; Hill et al., 1994; Pierson et al., 1994). iii) In one study, it was reported that mutants of the biocontrol PGPB *Pseudomonas fluorescens* BL915 that no longer produced the antibiotic pyrrolnitrin lost the ability to prevent *Rhizoctonia solani*-induced damping-off of cotton plants (Hill et al., 1994). Moreover, when a DNA

fragment, isolated from the wild-type bacterium, that restored pyrrolnitrin synthesis to these mutants was transferred to two strains of *P. fluorescens* that did not normally synthesize this antibiotic, the transformed strains gained both the ability to synthesize pyrrolnitrin and inhibit *Rhizoctonia solani*-induced damping-off of cotton plants. iv) 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* to a greater extent than did the wild-type strain (Maurhofer et al.,1992; Schnider et al.,1994).

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

Another way in which the activity of a biocontrol PGPB strain can be enhanced is by genetic manipulation to increase the amount of antibiotic that the bacterium synthesizes. While some increase in the amount of antibiotic produced by a particular bacterium might be obtained by conventional mutagenesis and selection, more extensive manipulation of antibiotic production will in all likelihood only be obtained through the use of recombinant DNA technology.

Since a number of anti-fungal metabolites produced by pseudomonads appear to be regulated by a global regulator (Laville et al., 1992), it should be possible to enhance the antibiotic production of a biocontrol PGPB by modifying this global regulation. In fact, it was recently reported that amplification of the gene from *Pseudomonas fluorescens* CHAO encoding the housekeeping sigma factor σ^{70} both enhanced antibiotic production and improved protection against *Pythium ultimum*-induced damping-off of cucumber (Maurhofer et al., 1995; Schnider et al., 1995a). In another study, inactivation of the *pqq* genes which are involved in the biosynthesis of pyrroloquinoline quinone, a cofactor of different hydrogenases, in *Pseudomonas fluorescens* CHAO stimulated the production of the antibiotic pyoluteorin (Schnider et al., 1995b). While the precise mechanism of this stimulation is unclear, it is possible that this mutant might enhance the flux of metabolites from other metabolic pathways into the pyoluteorin biosynthesis pathway.

At the present time there is only one commercially available genetically engineered biocontrol bacterium. A modified strain of *Agrobacterium radiobacter* strain K84 has been marketed in Australia since 1989 as a means of controlling *Agrobacterium tumefaciens*-caused crown gall disease which affects stone fruit trees and almond. The antibiotic agrocin 84 that is produced by *A. radiobacter is* normally toxic to agrobacteria carrying a nopaline/agrocinopine A type Ti plasmid (Kerr, 1989; McClure et al. 1994). However, agrocin 84 resistant strains of the pathogen *A. tumefaciens* can develop if the plasmid carrying the genes for the biosynthesis of agrocin 84 is accidentally transferred from *A. radiobacter. To* avoid this possibility, the region of DNA responsible for plasmid transfer was removed from the agrocin 84 plasmid. Thus, a mutant of the biocontrol *A. radiobacter* strain was constructed which no longer can transfer the modified agrocin plasmid to pathogenic agrobacteria, thereby retaining the capacity to act as a biocontrol agent (Jones et al. 1988).

Antifungal metabolites

Biocontrol PGPB produce a wide range of low molecular weight metabolites with anti-fungal activity (Dowling and O'Gara, 1994). For example, some pseudomonads can synthesize hydrogen cyanide - to which these pseudomonads are themselves resistant - a metabolite that has been linked to the ability of those strains to inhibit some pathogenic fungi, e.g. *Thielabiopsis basicola,* the causative agent. of black root rot of tobacco (Voisard et al.,1989).

One group of researchers reported 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 the damage that is caused by various species of the fungus *Fusarium.*

Enzymes

Many plants respond to pathogen attack by synthesizing pathogenesis related (PR) proteins that can hydrolyze the cell walls of some fungal pathogens (Mauch et al., 1988). Similarly, some biocontrol PGPB strains have been found to produce enzymes including chitinase, b-1,3-glucanase, protease and lipase that can lyse

fungal cells (Chet and Inbar,1994). For example, Lim et al. (1991) isolated a strain of *Pseudomonas stutzeri* that produced extracellular chitinase and laminarinase, and found that these enzymes could digest and lyse *Fusarium solani* mycelia thereby preventing the fungus from causing crop loss due to root rot. Similarly, 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* which was able to damage fungal mycelia. Furthermore, it was recently shown (Chernin et al., 1995) that three different strains of the biocontrol PGPB *Enterobacter agglomerans* that are antagonistic to fungal pathogens including *Rhizoctonia solani,* possess a complex of four separate enzymes that is responsible for the chitinolytic activity of the bacteria. These bacteria significantly decreased the damage to cotton plants following infection with *Rhizoctonia solani.* Moreover, Tn5 mutants of one of these biocontrol strains that were deficient in chitinase activity were unable to protect the plant against damage caused by the fungal pathogen.

Since many of the enzymes from biocontrol PGPB that have been found to lyse fungal cells, including chitinases and b-glucanases, are encoded by a single gene, it should be a relatively straightforward matter to isolate some of these genes and then transfer them to other biocontrol PGPB, thereby constructing biocontrol PGPB that, for example, produce both antibiotics and fungus-degrading enzymes. In one series of experiments, a chitinase gene was isolated from the bacterium *Serratia marcescens* and then transferred into *Trichoderma harzianum* and *Rhizobium meliloti* cells (Chet and Inbar, 1994). In both cases, the transformed microorganisms expressed the chitinase and subsequently displayed increased anti-fungal activity. When the *S. marcescens* chitinase gene was introduced into a strain of *P. fluorescens* that acts as a biocontrol PGPB, the transformant stably expressed and secreted active chitinase and was an effective biocontrol strain against the pathogen *Rhizoctonia solani* (Koby et al.,1994).

Competition

In addition to the more common antibiosis mechanisms which include the disease-suppressive effects of siderophores and antibiotics, there are a number of other ways in which PGPB can inhibit phytopathogens. For example, 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 some biocontrol PGPB 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). In another study, saprophytic *P. syringae* completely protected pears against gray mold and blue mold caused by *Botrytis cinerea* and *Penicillium expansum,* respectively (Janisiewicz and Marchi, 1992). Since this effect appears to depend on applying an extremely high inoculum of the biocontrol agent compared to the pathogen, possibly making this approach impractical, it is necessary to select or develop strains that can be applied in somewhat lower numbers but multiply rapidly following environmental dissemination.

The leaf surface has a limited number of sites where a phytopathogenic bacteria can invade the tissue (Henis and Bashan, 1986). Phylosphere saprophytic bacteria that compete successfully with pathogens for these sites can often reduce disease incidence. In this regard, a good candidate for a biocontrol agent would be a non-pathogenic variant of a pathogenic organism that can compete with the pathogen for the same niche, and is also copper resistant; copper is the most common antibacterial compound used and is registered for use against almost all bacterial diseases of plants (Cooksey,1990b).

When a nonpathogenic, copper resistant Tn5 mutant of *Pseudomonas syringae* pv. *tomato,* the causal agent of bacterial speck of tomato, was co-inoculated with a pathogenic strain, the disease incidence was significantly reduced in greenhouse experiments. In addition, when infected plants were also treated with copper, the disease incidence was reduced even further (Cooksey, 1988, 1990a). Clearly, when developing biocontrol strains for use in the field it will be necessary to develop more stable strains in which the pathogenicity genes are specifically deleted rather than Tn5 inactivated and the copper resistance is chromosomally rather than plasmid encoded. Unfortunately, copper resistant strains have recently been detected in alarming rates so that copper spraying may not have much of a future in controlling foliar bacterial diseases (Bashan,1996).

Preventing ice-nucleation is a classic example of displacement of a bacterial pathogen by a biocontrol agent. Pathogenic *P. syringae* increased frost susceptibility of tomato and soybean when sprayed on leaves prior to low temperature stress in

addition to being a pathogen of these plants (Anderson et al., 1982). However, an antagonistic ice-nucleation deficient bacterium can effectively out compete a pathogenic ice-nucleating bacterium of the same species (Wilson and Lindow,1994) regardless of whether the "ice-minus" mutant is naturally occurring or genetically engineered.

An important facet of the competitiveness of a biocontrol PGPB is the ability of the bacterium to persist and proliferate. However, it is often difficult to predict the behaviour in the environment of a particular PGPB since the soil persistence of a bacterium may be influenced by a number of different factors including soil composition (Heijnen and van Elsas, 1994; Bashan et al., 1995), temperature (Sun et al.,1995; Chiarini et al.,1994) and the, presence of recombinant plasmids (Tang et al., 1994; Tang et al., 1995; Glick, 1995b). Since in cold and temperate climates many fungal phytopathogens are most destructive when the soil temperature is low, it is reasonable to expect that those biocontrol PGPB that are cold tolerant and can also function at low temperatures will be much more effective in the field than mesophilic biocontrol strains. With this in mind McBeath (1995) reported the isolation of several strains of *Trichoderma* sp. that acted as biocontrol agents at low temperatures (i.e., 4-10°C) against a range of different pathogenic fungi.

One strategy that plants sometimes use to limit root colonization by phytopathogens is through the production of active oxygen species such as the hydroxyl radical, the superoxide anion and hydrogen peroxide that can inhibit a variety of pathogen cell processes (Doke, 1983; Klotz et al., 1989; Sutherland, 1991). Plant roots may also respond to colonization by PGPB by producing active oxygen species (Katsuwon and Anderson, 1989; Katsuwon and Anderson, 1990). Phytopathogens that contain higher levels of enzymes such as superoxide dismutase, catalase and peroxidase that can reduce the amount of active oxygen species have been shown to be more effective pathogens presumably reflecting the ability of the fungus to survive this plant defense (Klotz and Hutcheson, 1992). It should therefore be possible, by genetic manipulation of biocontrol PGPB, to increase the levels of one or more of the enzymes that reduce the amount of active oxygen species (e.g., Gruber et al.,1990) so that PGPB strains with an increased root colonizing ability and hence increased effectiveness against fungal pathogens might be created.

The soil contains a large number of different microorganisms, and those strains that are able to utilize an unusual carbon or nitrogen source such as an opine, 1-aminocyclopropane carboxylate (ACC) or a xenobiotic compound (such as a herbicide or pesticide) should be able to proliferate and then persis t longer than

other microorganisms in those soils that contain such unusual compounds. For example, the ability of some PGPB to hydrolyze ACC, the immediate precursor of ethylene in plants and a compound naturally found in root exudates, may provide these strains with a competitive advantage over other microorganisms in the rhizosphere because they can use ACC as a source of nitrogen (Jacobson et al., 1994; Glick et al., 1994a, 1994b, 1995a).

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. Similarly, the presence of a herbicide, pesticide or other organic pollutant in soil may facilitate the proliferation of bacteria engineered to degrade these compounds (Brazil et al.,1995); at the same time, these chemicals may suppress the proliferation of the other microorganisms in the same soil and possibly provide a "biodegradative PGPB" with a significant competitive advantage. This strategy has the advantage that in addition to increasing the competitiveness of a biocontrol PGPB strain, it may also be a useful strategy for the biodegradation of some recalcitrant organic molecules in the soil.

A PGPB 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 countries such as Canada, this means being able to survive long cold winters and then grow at cool (soil) temperatures in the spring (~5-10°C). It was recently reported that the PGPB *P. putida* GR12-2 secretes antifreeze protein(s) into the surrounding medium when the bacterium is grown at low temperatures (Sun et al.,1995). This protein(s) may regulate the formation of ice crystals outside of the bacterium, thereby protecting it from damage that might otherwise occur at freezing temperatures. The addition, by genetic engineering, of antifreeze protein synthesizing capability to biocontrol PGPB that are otherwise unable to persist and proliferate at cold temperatures may make a bacterium more effective by permitting it to thrive under these seemingly adverse conditions.

Regardless of the sorts of genetic manipulations that are used in an effort to improve a bacterium, generally, non-transformed wild-type organisms are likely to be more persistent in the environment than transformed bacteria. Moreover, an organism that could both out-compete and out-persist non-transformed wild-type organisms might be problematic for an inoculum industry that, in order to stay in business, needs to provide PGPB to farmers on an ongoing annual basis.

Plant ethylene levels

Plants respond to a variety of different stresses including fungal phytopathogen infection by synthesizing "stress" ethylene (Abeles et al., 1992; Hyodo, 1991). In turn, the ethylene can trigger a stress/senescence response in the plant which may lead to the death of those cells that are at or near the site of the fungal infection. Stress ethylene is thought to act as a secondary messenger and can (in different tissues in different plants) stimulate senescence, leaf or fruit abscission, disease development, inhibition of growth, and/or antibiotic enzyme (e.g. chitinase) synthesis.

Many of the symptoms of a diseased plant may arise as a direct result of the stress imposed by the infection (Van Loon, 1984). That is, a significant portion of the damage to plants infected with fungal phytopathogens occurs as a result of the response of the plant to the increased levels of stress ethylene. And, not only does exogenous ethylene often increase the severity of a fungal infection but, as well, inhibitors of ethylene synthesis can significantly decrease the severity of a fungal infection. For example: i) In a study of the reaction of over 60 different cultivars and breeding lines of wheat to the fungal phytopathogen *Septoria nodorum,* increased ethylene production (as a consequence of fungal infection) was correlated with increased plant disease susceptibility (Abeles et al., 1992). ii) Cotton plants treated with chemical inhibitors of ethylene synthesis (such as L-α-(aminoethoxyvinyl)-glycine, i.e. AVG) were damaged to a much lesser extent than untreated plants by the fungal phytopathogen *Alternaria* (Bashan, 1994). iii) Melon plants that were treated with ethylene inhibitors showed decreased levels of ethylene and decreased disease severity following infection by the fungal phytopathogen *Fusarium oxysporum* (Cohen et al., 1986). iv) Pretreating cucumber plants with ethylene increased disease development with the fungus *Colletotrichum lagenarium* (Biles et al., 1990). v) Exogenous ethylene has been found to increase disease severity in *Verticillium* wilt of tomato (Cronshaw and Pegg, 1974). vi) Treatment of roses, carnations, tomato, pepper, French-bean and cucumber with ethylene inhibitors decreased disease severity when these plants were infected with the fungus *Botrytis cinerea* (Elad, 1988, 1990).

The enzyme ACC deaminase, when present in PGPB such as *P. putida* GR12-2, can act to modulate the level of ethylene in a plant (Glick et al., 1994a & b; Jacobson et al., 1994; Glick et al., 1995; Hall et al., 1996). This enzyme, which has no known function in bacteria, is probably part of a, hitherto undescribed, mechanism that

P. putida GR12-2 (and other PGPB) uses to stimulate plant growth. This could occur by ACC deaminase lowering the level of ethylene in germinating seeds or in roots thereby stimulating root development in those plants that are sensitive to ethylene (Hall et al., 1996). In support of this hypothesis it was observed that mutants of *P. putida* GR12-2 that lack ACC deaminase activity no longer stimulate the growth of canola roots. Moreover, while the growth of the more ethylene sensitive dicots is stimulated by *P. putida* GR12-2, less ethylene sensitive monocots are unaffected by the presence of *P. putida* GR12-2 (Hall et al.,1996).

P. putida GR12-2 not only directly stimulates the growth of plants under gnotobiotic conditions, but it can also limit the damage to cucumber plant due to the fungal phytopathogen *Pythium ultimum* (P. Newell and B. Glick, unpublished observations). Since *P. putida* GR12-2 does not produce any antibiotics and these experiments were performed under conditions that included a sufficient level of iron for plant growth, it is likely that this antiphytopathogenic effect is the result of some bacterial trait other than antibiotic and/or siderophore production. If ethylene mediates some of the manifestations of pathogen-induced damage in plants, ACC deaminase from *P. putida* GR12-2 may act to modulate the level of ethylene in a plant and thereby prevent or decrease the damage normally caused by phytopathogens such as *Pythium ultimum.* Should this assumption turn out to be correct then it should be possible to isolate bacterial genes for ACC deaminase and transfer them to biocontrol PGPB in order to develop biocontrol strains that employ several different anti-fungal phytopathogen mechanisms.

Systemic acquired resistance

In many plants long-lasting and broad-spectrum systemic acquired resistance to disease-causing agents including fungal pathogens can be induced by treating the plant or seed with either environmental factors, a microorganism, or by both (van Peer et al., 1991; Tuzun and Kloepper, 1994). Systemic acquired resistance can be induced by pathogens, non-pathogens, seed treatments with PGPB and by microbial metabolites. Most studies of systemic acquired resistance have been carried out against fungal pathogens; however, a few studies of pathovars of *P. syringae* suggested that this approach may have potential in the future control of foliar diseases.

In one study, inoculation with two PGPB strains (*P. putida* and *Serratia marcescens)* protected cucumber plants against *P. syringae* pv. *lachrymans,* the causal agent of bacterial angular leaf spot. Treatment of seeds or cotyledons resulted in a

significant decrease in both lesion number and size, and also a significantly decrease in the epiphytic population of the pathogen (Liu et al. 1995). In another report, inoculation of seeds with a strain of *P. fluorescens* induced protection against *P. syringae* pv. *phaseolicola,* with the greatest protection coming at the highest inoculum level tested, $>10^8$ cells/mL (Alstrom, 1991) suggesting that, in some instances, seed inoculation might offer the possibility of immunizing whole plants against bacterial foliar diseases. Of course, systemic acquired resistance induced by PGPB provides effective protection against fungal as well as bacterial pathogens (Van Peer et al., 1991).

Since PGPB appear to "turn on" the synthesis of some anti-pathogen metabolite(s) including β-1,3-glucanases, chitinases, thaumatin-related proteins and some pathogenesis-related proteins within the plant in a mechanism that does not involve any direct interaction between the PGPR and the pathogen (Kessmann et al., 1994), a better understanding of the metabolic signals that activate the synthesis of these proteins may allow for the construction of PGPB strains that act more rapidly and more efficiently in eliciting systemic acquired resistance.

Direct stimulation of plant growth and development

Numerous free-living bacteria have been shown to directly stimulate the growth of plants (Glick, 1995a). The mechanisms utilized by these bacteria are numerous and varied, and may differ from one type of organism to another e.g. from azospirilla to pseudomonads. Some of the mechanisms that different bacteria may use to promote plant growth include: nitrogen fixation, although this generally contributes only a small extent to the promotion of plant growth by the bacterium (Christiansen-Weniger and van Veen, 1991); improving mineral and water uptake; changes in the hormonal balances of plants (Fulchieri et al., 1993; Glick et al. 1994b; Xie et al., 1996); general improvement of the entire root system (Sarig et al. 1992; Bashan and Dubrovsky, 1996); and reduction of the membrane potential of the roots (e.g. Bashan, 1989; Bashan, 1990; Bashan, 1991; Bashan and Levanony, 1991). In many instances, there may not be a single major mechanism of plant growth stimulation involved. Rather, more than one mechanism may be operative, with the effects on the treated plants being additive (Bashan and Levanony, 1990; Bashan and Dubrovsky, 1996).

A healthier better developed plant is less likely to become infected with pathogens, and a less healthy or stressed plant is more susceptible to pathogens. For example, *P. syringae* pv. tomato, the causal agent of bacterial speck of tomato,

requires wounded or stressed plants for establishment and symptom production (Bashan et al., 1978). *Xanthomonas campestris* pv. *vesicatoria,* the causal agent of bacteria scab of pepper and tomato also required stressed plants as well as sufficient relative humidity for successful infection (Diab et al., 1982a, 1982b). Similarly, *Alternaria* leaf spot of cotton developed in plants already suffering severe potassium deficiency (Hillocks and Chinodya, 1989). Although many phytopathogens attack a healthy plant as well, any sort of genetic manipulation that improves the ability of a PGPB to stimulate plant growth should at the same time improve the ability of the bacterium to act as a de facto biocontrol agent.

CONCLUSION

In the past few years a considerable amount of attention has been directed toward the possibility of genetically manipulating plants so that they are able to withstand a number of different "environmental challenges" including insects, viruses, fungi, bacteria and weather (Greenberg and Glick, 1993). However, for this strategy to be truly effective each cultivar or variety of each and every crop species must be protected against a wide range of possible environmental challenges. Given the complexity of this problem, there is probably no simple and straightforward solution that includes the genetic manipulation of plants that would at this time appear to have any chance of success. On the other hand, it should be possible, using a combination of traditional mutagenesis and selection together with genetic engineering, to develop PGPB that are effective biocontrol agents against a range of different phytopathogens and can also be used with a number of different crop plants. Thus, although the genetic manipulation of PGPB may appear to be less glamorous than the genetic manipulation of plants, it may ultimately turn out to be more useful.

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