Plant-Growth-Promoting Rhizobacteria

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antibiosis, antifungal metabolite, biocontrol, competition for nutrients and niches, induced systemic resistance

Abstract

Several microbes promote plant growth, and many microbial products that stimulate plant growth have been marketed. In this review we restrict ourselves to bacteria that are derived from and exert this effect on the root. Such bacteria are generally designated as PGPR (plant-growth-promoting rhizobacteria). The beneficial effects of these rhizobacteria on plant growth can be direct or indirect. This review begins with describing the conditions under which bacteria live in the rhizosphere. To exert their beneficial effects, bacteria usually must colonize the root surface efficiently. Therefore, bacterial traits required for root colonization are subsequently described. Finally, several mechanisms by which microbes can act beneficially on plant growth are described. Examples of direct plant growth promotion that are discussed include (*a*) biofertilization, (*b*) stimulation of root growth, (*c*) rhizoremediation, and (*d*) plant stress control. Mechanisms of biological control by which rhizobacteria can promote plant growth indirectly, i.e., by reducing the level of disease, include antibiosis, induction of systemic resistance, and competition for nutrients and niches.

Contents

Rhizosphere: the (soil) layer influenced by the root

Rhizosphere effect: the fact that the bacterial density in the rhizosphere is much higher than that in the surrounding soil

Root exudate: nutrient-rich components secreted by the root

Antibiosis: mechanism of biocontrol based on secretion of antibiotics along the root

Competition for nutrients and niches (CNN): a mechanism of biocontrol based on CNN between the pathogen and the biocontrol agent

INTRODUCTION

This review focuses on rhizosphere microbes that are beneficial for the plant. Some beneficials do so by promoting plant growth directly, i.e., in the absence of pathogens. Others do this indirectly by protecting the plant against soilborne diseases, most of which are caused by fungi.

Hiltner (37) discovered that the rhizosphere, i.e., the layer of soil influenced by the root, is much richer in bacteria than the surrounding bulk soil is. These rhizosphere microbes benefit because plant roots secrete metabolites that can be utilized as nutrients. This rhizosphere effect is caused by the fact that a substantial amount of the carbon fixed by the plant, 5–21% (61), is secreted, mainly as root exudate. Although the concentration of bacteria in the rhizosphere is 10 to 1000 times higher than that in bulk soil, it is still 100-fold lower than that in the average laboratory medium. Therefore, the lifestyle of rhizobacteria is best characterized as starvation. To exert their beneficial effects in the root environment, bacteria have to be rhizosphere competent, i.e., able to compete well with other rhizosphere microbes for nutrients secreted by the root and for sites that can be occupied on the root.

Which food sources are available in the rhizosphere and how microbes use them to feed themselves are poorly understood (87). An exception is the composition of the root exudate of tomato, in which organic acids, followed by sugars, are the major components (58). The predicted role of organic acids in root colonization was confirmed (21) by the finding that mutants affected in organic acid utilization are poorer competitive root colonizers than the parental strain, whereas mutants defective in sugar utilization are indistinguishable from the parent with respect to root colonization (59).

Only a small part of the root surface is covered by bacteria (74). The most popular sites for bacterial growth are junctions between epidermal cells and areas where side roots appear. Poor rhizoplane colonization was long ago considered a factor that can limit biocontrol efficacy (77, 98). In recent years it has been proven that root colonization indeed is required for some biocontrol mechanisms, such as antibiosis (11) and CNN (competition for nutrients and niches) (44, 88).

In this review we begin with a description of how bacteria live on the root, which nutrients are available, and how the bacteria colonize the root. Competitive rhizosphere colonization is crucial for many mechanisms of action of plant-beneficial bacteria. Which bacterial traits are important for root colonization when bacteria compete with each other and with other organisms such as fungi, nematodes, and protozoa? Finally, we describe various mechanisms used by specialized beneficial rhizobacteria to positively influence plant growth. We begin with bacteria that directly, i.e., in the absence of pathogens, promote plant growth. Then we discuss so-called biocontrol bacteria, which promote plant growth because they can reduce harm caused by pathogens and therefore act as biopesticides.

NUTRIENTS FOR MICROBES IN THE RHIZOSPHERE

Organic compounds released by plant roots include amino acids, fatty acids, nucleotides, organic acids, phenolics, plant growth regulators, putrescine, sterols, sugars, and vitamins. (For an extensive review on exudate composition see Reference 87.) Data on exudate composition should be interpreted with care. (*a*) One can only find components that one is searching for unless one is lucky. For example, putrescine was not searched for until the characterization of a competitive colonization mutant suggested its presence (50). (*b*) Only exudates collected from sterile plants growing under artificial conditions, such as on sterile filter paper or in sterile plant nutrient solution, are sufficiently concentrated to be analyzed successfully. It is known that the physiological status of the plant, the presence of microbes (41, 42, 63), and the presence of products from rhizobacteria such as phenazines, 2,4-diacetylphloroglucinol, and zearalenone (70), as well as the growth substrate (40, 41, 54), influence exudate composition. (*c*) The plant not only secretes components but also takes up exudate components (41, 70).

The best-known exudate composition is probably that of tomato. Its major soluble carbon source consists of organic acids (40, 41, 56), followed by sugars (40, 57, 59) and amino acids (82). Important nitrogen sources are amino acids and putrescine (50). Root exudate can also influence the behavior of (pathogenic) fungi. Tomato root exudate as well as two of its major components, citrate and glucose, allows spores of the tomato root pathogen *Forl* (*Fusarium oxysporum* f. sp. *radicis-lycopersici*) to germinate. The additional presence of the biocontrol strain *Pseudomonas fluorescens* WCS365 delays this process (42).

RHIZOPLANE COLONIZATION

Introduction

Bacteria on the root epidermis are often covered by a mucigel layer. The introduction of a modified scanning electron microscopy procedure, which makes this layer semitransparent, enabled scientists to see bacteria on the rhizoplane (13). The use of color variants of the GFP (green fluorescent protein) (28, 52) made it possible to visualize all cells of individual microbial strains. By combining different fluorescent colors, it is possible to visualize simultaneously two bacteria (5) or one bacterium and one fungus (6) on the red autofluorescent tomato root surface.

Forl: *Fusarium oxysporum* f. sp. *radicis-lycopersici*

Studies on the mechanism of root colonization starting from bacterized seeds were facilitated by the development of a monoaxenic system (83). We used the best root colonizer known at that moment, *P. fluorescens* WCS365, as the model strain. A seed or seedling coated with one or two bacteria is planted in this monoaxenic system, which contains sterile sand and sterile plant nutrient solution without added carbon source. After growth for up to 7 days, with root exudate functioning as the only carbon source, various parts of the root were analyzed for the presence of bacteria. It appeared that the distribution of the *P. fluorescens* WCS365 bacteria varied after 7 days from 10^6 CFUs per centimeter of root near the root base to 10^2 to 10^3 CFUs per centimeter of root near the root tip. A time course showed that *P. fluorescens* WCS365 bacteria first multiplied on the seed coat. In contrast to the stem, which was not colonized, the root was gradually colonized, first by single cells that later grew out to microcolonies (13). These microcolonies, now called biofilms (4), usually consist of multiple layers of bacteria and are covered with a mucous layer.

Colonization Genes and Traits

The monoaxenic system described above (83) has been applied to identify genes and traits involved in competitive root tip colonization. After mutants with a growth defect in laboratory medium were first deleted, random Tn*5* mutants were allowed pair-wise to compete with their parental strain, *P. fluorescens* WCS365, for root tip colonization. Mutants impaired in colonization in the sand system also appeared to behave as mutants in potting soil. The mutants were analyzed genetically

Plant-growth-promoting rhizobacteria: bacteria that cause indirect plant growth promotion or biological control

and physiologically. The major competitive tomato root tip colonization traits appeared to be motility; adhesion to the root; a high growth rate in root exudate; synthesis of amino acids, uracil, and vitamin B1; the presence of the O-antigenic side chain of lipopolysaccharide; the two-component ColR/ColS sensory system; fine-tuning of the putrescine uptake system (the mutant had an impaired *pot* operon); the site-specific recombinase Sss or XerC; the *nuo* operon (the mutant had a defective NADH:ubiquinone oxidoreductase); the *secB* gene involved in a protein secretion pathway; and the type three secretion system (TTSS). Most results have been reviewed in References 57 and 58. Therefore, we only discuss newer results that further our understanding of the colonization process.

Motility, later refined to chemotaxis toward root exudate, appeared to be an important colonization characteristic. The major identified chemoattractants in tomato root exudate for *P. fluorescens* WCS365 are amino acids (L-leucine was the best) and dicarboxylic acids. Sugars are inactive. Considering their levels in root exudate, we suggested that malic acid and, to a lesser extent, citric acid are the major chemoattractants for this strain in the tomato rhizosphere (23). In root exudate of *Arabidopsis thaliana* another organic acid, L-malate, appeared to be the major chemoattractant for the biocontrol rhizobacterium *Bacillus subtilis* FB17 (75).

The growth rate in root exudate for competitive tomato root colonization mutants in the ColR/ColS two-component system is impaired when they are incubated with wild-type *P. fluorescens* WCS365. Moreover, they are supersensitive to the lipopolysaccharide (LPS)-binding antibiotic polymyxin B. They are more resistant than the wild type toward the other tested antibiotics. The genes *colR/colS* regulate the methyltransferase/*wapQ* operon located downstream. Competitive colonization is impaired in mutants in the individual methyltransferase and phosphatase genes. *wapQ* encodes a putative heptose phosphatase. It was hypothesized (20) that both gene products modify the LPS, which interacts with outer membrane porins (60). Absence of these chemical groups from the LPS results in a narrowing of the pore diameter. The modified LPS of the mutant explains the lower growth rate in exudate and the impaired competitive colonization ability, as well as a more intensive interaction with the antibiotic polymyxin B (20).

Tested mutants in both *hrcD* and *hrcR* genes of the TTSS of *P. fluorescens* SBW25 are impaired in competitive tomato root tip colonization but not when tested alone. Because attachment to seed and root in competition with the parent strain was not impaired, we suggested that the TTSS of *P. fluorescens* SBW25 pushes its needle into the cytoplasm of the plant epithelial cells to feed on these plant juices. In fact, injection of a hollow needle may have been the first function of the TTSS. We hypothesize that this system later evolved into an injection system for proteins and, after incorporating a functional motor, into rotating flagella (21). Our conclusion that the TTSS plays a role in rhizosphere competence is consistent with the finding that the biocontrol ability of *P. putida* KD against *Fusarium* in tomato and against *Pythium* in cucumber is lost when the *hrcC* gene is mutated (72).

Considering that many genes appear to be involved in competitive root colonization, the search for competitive colonization genes and traits will not easily be completed. A genomic approach is required to obtain a more complete understanding of this process.

DIRECT PLANT GROWTH PROMOTION

Direct plant-growth-promoting rhizobacteria enhance plant growth in the absence of pathogens. An excellent review on this topic has been published (91). Depending on the mechanism used, several classes of bacteria that promote plant growth can be distinguished.

Biofertilizers

Some rhizobacteria promote plant growth in the absence of pathogen pressure. Bacterial

fertilizers supply the plant with nutrients. N_2-fixing bacteria such as *Rhizobium* and *Bradyrhizobium* can form nodules on roots of leguminous plants such as soybean, pea, peanut, and alfalfa, in which they convert N_2 into ammonia, which in contrast to N_2 can be used by the plant as a nitrogen source (84, 93). *Azospirillum* is a free-living N_2-fixer that can fertilize wheat, sorghum, and maize. Despite *Azospirillum*'s N_2 fixing capacity, the yield increase caused by inoculation by *Azospirillum* is attributed mainly to increased root development and thus to increased rates of water and mineral uptake (66).

Low levels of soluble phosphate can limit the growth of plants. Some plant-growth-promoting bacteria solubilize phosphate from either organic or inorganic bound phosphates, thereby facilitating plant growth (54, 96). Several enzymes, such as nonspecific phosphatases, phytases, phosphonatases, and C-P lyases, release soluble phosphorus from organic compounds in soil. C-P lyases cleave C-P links in organophosphonates. Release of phosphorus from mineral phosphate is related to the production of organic acids, such as gluconic acid (73).

Rhizoremediators

A problem in the degradation of soil pollutants by bacteria is that such bacteria, although effective in the laboratory, poorly adapt to the conditions in bulk soil, where their primary metabolism is dependent on degradation of the pollutant. In fact they starve soon after application and then become inefficient in pollutant degradation (7). A promising strategy to solve this problem is to uncouple the energy needed for primary metabolism from the energy required for pollutant degradation. To this end, Kuiper et al. (49) developed a system called rhizoremediation (51). Their strategy was to select pollutant-degrading rhizobacteria that live on, or are close to, the root so that they can use root exudate as their major nutrient source. These authors developed a system to efficiently enrich such bacteria by starting from a crude mixture of bacteria from grass roots and subsequently alternating between selecting for growth on the pollutant naphthalene and selecting for efficient colonization of grass roots (49). One of the resulting strains, *P. putida* PCL1444, effectively utilizes root exudate, degrades naphthalene around the root, protects seeds from being killed by naphthalene, and allows the plant to grow normally. Mutants unable to degrade naphthalene do not protect the plant (49).

PQQ: pyrrolquinoline quinone

IAA: indole-3-acetic acid or auxin

Phytostimulators

Some bacteria produce substances that stimulate the growth of plants in the absence of pathogens. The best understood example is the hormone auxin. In addition, other hormones as well as certain volatiles and the cofactor pyrrolquinoline quinone (PQQ) stimulate plant growth.

The root-growth-promoting hormone auxin, as present in root exudate, is usually synthesized from the exudate amino acid tryptophan. The tryptophan concentration in exudate differs strongly among plants (41, 48). Inoculation of seeds with the auxin-generating *P. fluorescens* WCS365 did not result in an increase in the root or shoot weight of cucumber, sweet pepper, or tomato, but led to a significant increase in the root weight of radish. Radish produces at least nine times more tryptophan in its exudate per seedling than cucumber, sweet pepper, or tomato (41). An example of bacterial stimulation of radish growth is shown in **Figure 1**.

The N_2-fixing bacterium *Azotobacter paspali*, isolated from a subtropical grass species, improves growth of a variety of dicotyledonous and monocotyledonous plants. Experiments with added inorganic nitrogen suggested that plant growth promotion is caused by the production of plant growth factors such as IAA, gibberellins, and cytokinins, rather than nitrogen fixation (66).

Some rhizobacteria, such as strains from *B. subtilis*, *B. amyloliquefaciens*, and *Enterobacter cloacae*, promote plant growth by releasing volatiles (76). The highest level of growth promotion was observed with 2,3-butanediol and

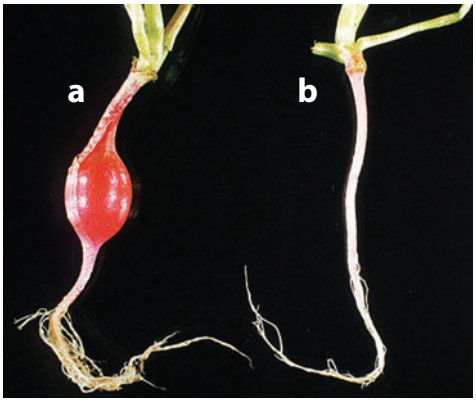

Figure 1

Plant growth promotion by a bacterium. Roots of 17-day-old radish plants from which the seeds were inoculated with (*a*) auxin-producing *Pseudomonas corrugata* SPB2184 suspended in 1% methylcellulose and with (*b*) 1% methylcellulose suspension without added microbes. Photographs courtesy of Dr. Natalya Makarova.

acetoin. Mutants of *B. amyloliquefaciens* IN937a and *B. subtilis* GB03, blocked in the biosynthesis of these compounds, were inactive in plant growth promotion. More recently, Zhang et al. (99) found that *B. subtilis* GB03 increases the photosynthetic efficiency and chlorophyll content of *A. thaliana* through the modulation of endogenous signaling of glucose and abscisic acid sensing. They concluded that the bacterium plays a regulatory role in the acquisition of energy by the plant.

The cofactor PQQ was described as a plant growth promoter (15). Synthetic PQQ promotes growth of tomato and cucumber plants. The results suggest that PQQ acts as an antioxidant in plants. However, it cannot be excluded that the effect is indirect because PQQ is a cofactor of several enzymes, e.g., involved in antifungal activity and induction of systemic resistance.

Stress Controllers

Plant-growth-promoting bacteria that contain the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase facilitate plant growth and development by decreasing plant ethylene levels. Such bacteria take up the ethylene precursor ACC and convert it into 2-oxobutanoate and NH_3. Several forms of stress are relieved by ACC deaminase producers, such as effects of phytopathogenic bacteria, and resistance to stress from polyaromatic hydrocarbons, from heavy metals such as Ca^{2+} and Ni^{2+}, and from salt and draught (33).

1-Aminocyclopropane-1-carboxylate (ACC): a precursor of ethylene

Biological control: control of diseases by living organisms

BIOLOGICAL CONTROL OF SOILBORNE PLANT DISEASES

Introduction

Plant diseases are responsible for annual crop losses at a total value of more than €200 billion (1). Resistant plants and chemicals are often used to control plant disease. Resistance does not exist against all diseases and the breeding of resistant plants takes many years. Moreover, acceptance of genetically engineered resistance is still a sensitive issue in the European Union. The use of agrochemicals is negatively perceived by consumers and supermarket chains. It is increasingly banned by governmental policies.

The use of microbes to control diseases, which is a form of biological control, is an environment-friendly approach. The microbe is a natural enemy of the pathogen, and if it produces secondary metabolites, it does so only locally, on or near the plant surface, i.e., the site where it should act. In contrast, the majority of molecules of agrochemicals do not reach the plant at all. Moreover, the molecules of biological origin are biodegradable compared with many agrochemicals that are designed to resist degradation by microbes. The term biocontrol is used not only to control diseases in living plants but also to control diseases occurring during the storage of fruits (also called postharvest control). Studies on the control of pathogens by rhizobacteria usually focus on pathogenic microorganisms. It should be noted that some rhizobacteria are also active against weeds (30) and insects (68, 81).

Soils in which a pathogen causes disease symptoms are called conducive soils.

Spontaneous control of plant diseases by bacteria in some fields was discovered at several places around the world. Some soils, called suppressive soils, contain bacteria that protect plants against fungal diseases despite the presence of disease-causing pathogens in soil. Mixing small amounts of suppressive soil with a large amount of conducive soil makes the latter soil suppressive. For more information the reader is referred to References 34 and 78.

Microbial control of plant diseases is a complex process involving not only the biocontrol microbe, the pathogen, and the plant, but also the indigenous microflora, macrobiota such as nematodes and protozoa, and the plant growth substrate such as soil, stonewool, or vermiculite. Many reviews on biocontrol have been written (10, 16, 34, 86). To act efficiently, the microbial control microbe should remain active under a large range of conditions, such as varying pH, temperature, and concentrations of different ions. These requirements are not easy to fulfill. Therefore, it is not surprising that the efficacy of several first-generation commercial biocontrol products (17) is not always sufficient. However, as our understanding of mechanisms of biocontrol and selection procedures for active strains increases, biocontrol products will improve and therefore biocontrol has a good future.

Mechanisms of Biocontrol

In our laboratory we use the tomato disease tomato foot and root rot (TFRR), caused by the fungal pathogen *Forl*, as a model system for studying mechanisms utilized by various biocontrol strains. Other groups use other model systems (26, 31, 34, 86). The following mechanisms can be distinguished.

Antagonism. Bacteria that produce antibiotics, which kill pathogens, act via antagonism if their mutants defective in structural genes in the synthesis of this antibiotic are biocontrol negative. For a bacterium to be suitable for biocontrol, it must not only synthesize and release the antibiotic, but also compete successfully with other organisms for nutrients from the root and for niches on the root to deliver the antibiotic along the whole root system (11) (**Figure 2*a***). Also, the bacterium should escape in sufficient numbers from predators feeding on rhizosphere bacteria, so-called protozoan grazers (39). Furthermore, the bacterium should produce the antibiotic in the right microniche on the root surface (71).

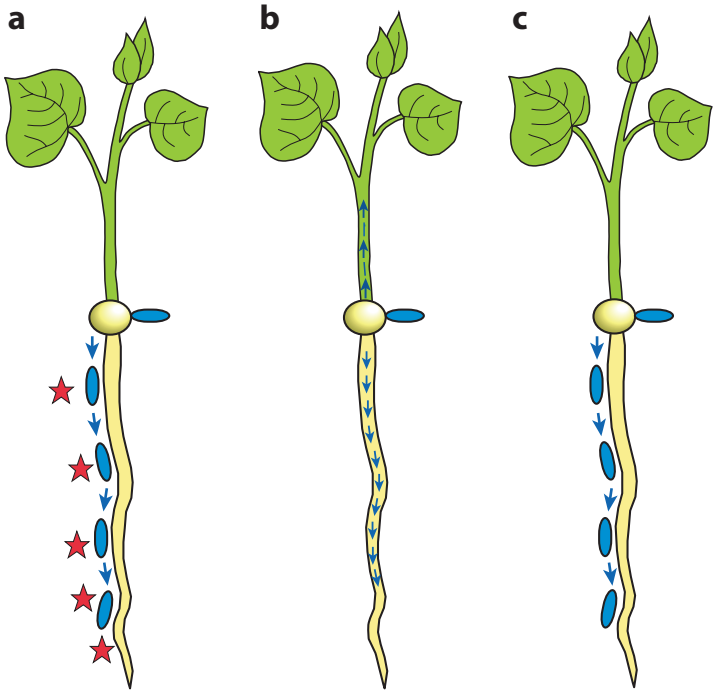

Figure 2

Illustration of the most important mechanisms of biological control of plant diseases by bacteria. In all cases illustrated here, biocontrol begins by coating seeds with the biocontrol bacterium. (*a*) Antibiosis. The bacterium colonizes the growing root system and delivers antibiotic molecules around the root, thereby harming pathogens that approach the root (*indicated by stars*). (*b*) Induced systemic resistance (ISR). Local root colonization is sufficient to induce ISR. Many bacterial products induce systemic signaling, which can result in protection of the whole plant against diseases caused by different organisms. The latter aspect of ISR resembles innate immunity in humans and animals. (*c*) Competition for nutrients and niches. Biocontrol bacteria acting through this mechanism excel in fast chemotactic movement along the growing root in their efficient hunt for root exudate components, thereby outcompeting the pathogen in scavenging nutrients and in occupying niches on the root.

Antibiotics identified in antagonistic gram-negative biocontrol bacteria include the classical compounds HCN (35); phenazines (10, 12, 62), of which the major ones are

Tomato foot and root rot (TFRR): an important disease of tomato

Phl: 2,4-diacetyl phloroglucinol

AHL: *N*-acyl homoserine lactone

ISR: induced systemic resistance

AFM: antifungal metabolite

phenazine-1-carboxylic acid and phenazine-1-carboxamide; 2,4-diacetyl phloroglucinol (Phl) (26, 86); pyoluteorin (65); and pyrrolnitrin (46). Zwittermycin A (29) and kanosamine (64) can be produced by *Bacillus cereus*. Antibiotics more recently discovered in biocontrol strains are D-gluconic acid (45) and 2-hexyl-5-propyl resorcinol (9). Volatiles other than hydrogen cyanide, such as 2,3-butanediol, or blends of volatiles produced by *Bacillus* spp. (76) or by fungi (85) can be involved in plant protection. Finally, lipopeptide biosurfactants produced by *B. subtilis* (67) and by pseudomonads (18) have been implied in biocontrol. Rhamnolipid and phenazine act synergistically against soilborne diseases caused by *Pythium* spp. (69).

Signal interference. Many bacteria only express pathogenicity/virulence factors at a high bacterial cell density, sensed when the level of quorum-sensing molecules such as homoserine lactones (AHLs) accumulate in the medium (2). AHLs are required, for example, for the synthesis of cell-wall-degrading enzymes of the pathogen *Erwinia carotovora*. Signal interference is a biocontrol mechanism based on the degradation of the AHL (53), for example, by AHL lactonases of *B. thuringiensis* strains that hydrolyze the lactone ring or by AHL acylases that break the amide link. Recently, it was shown that AHL acylases play a role in the formation of biofilms (79). Lack of biofilm formation probably makes biocontrol easier.

Predation and parasitism. Predation and parasitism, the major biocontrol mechanism used by some (fungal) *Trichoderma* species, is based on enzymatic destruction of the fungal cell wall (36). To our knowledge this mechanism has not thoroughly been identified in bacteria. Even the fungus eater *Collimonas fungivorans* likely uses CNN rather than predation and parasitism as its mechanism to control TFRR (43).

Induced systemic resistance. Interaction of some bacteria with the plant roots can result in plants resistant to some pathogenic bacteria, fungi, and viruses. This phenomenon is called induced systemic resistance (ISR) (**Figure 2*b***). ISR shares many properties with innate immunity in humans (55). (For recent reviews on ISR, the reader is referred to References 47 and 91, which focus on *Pseudomonas* and *Bacillus*, respectively.) ISR differs from SAR (systemic acquired resistance). ISR was discovered by the findings that resistance can be induced by the rhizobacterium *Pseudomonas* sp. strain WCS417r against *Fusarium* wilt of carnation (92) and by selected rhizobacteria against the fungus *Colletotrichum orbiculare* in cucumber (97). ISR is dependent on jasmonic acid and ethylene signaling in the plant (91).

Many individual bacterial components induce ISR, such as LPS, flagella, salicylic acid, and siderophores (91). More recently, cyclic lipopeptides (67), the antifungal factor Phl (38), the signal molecule AHLs (80), and volatile blends produced by *B. subtilis* GB03 and, to a lesser extent, the individual volatiles acetoin and 2,3-butanediol (76) have been added to the list. In contrast to many biocontrol mechanisms, extensive colonization of the root system is not required for ISR, as shown by the ISR (44) of *P. fluorescens* WCS365 (19) using root colonization mutants.

It is unlikely that a poor colonizer acts through antibiosis, since colonization is the delivery system for antifungal components along the root system (11). Therefore, the finding that certain strains of *B. cereus*, which are poor colonizers, are good biocontrol agents was surprising (32). However, the observation that certain antifungal metabolites (AFMs) can induce ISR explains this phenomenon. We therefore speculate that many of the *Bacillus* strains that can act as biocontrol agents, act through ISR rather than antibiosis.

Rudrappa et al. (75) reported that infection of leaves of *A. thaliana* seedlings with the foliar pathogen *P. syringae* pv. *tomato* Pst DC3000 results in enhanced secretion of L-malic acid by the roots, and that the enhanced level of L-malic acid selectively signals and recruits the beneficial rhizobacterium *B. subtilis* FB17, which is a biocontrol bacterium that protects the plant through ISR. Previously, De Weert et al. (23)

reported that another biocontrol bacterium, *P. fluorescens* WCS365, which also acts through ISR (44), shows strong chemotaxis toward the major tomato root exudate component, citric acid. In contrast to what is suggested by Rudrappa et al. (75), we do not believe that the results indicate that enhanced L-malic acid secretion selectively attracts beneficial bacteria. It is unlikely that chemotaxis to L-malic acid is a trait exclusive of beneficial bacteria.

Competition for ferric iron ions. When antibiosis is carried out on a test plate containing a medium with a low ferric iron concentration, and when the test strain inhibits fungal growth in the absence but not in the presence of added Fe^{3+} ions, the bacterial strain likely produces a siderophore, i.e., a Fe^{3+} ion-chelating molecule. Upon binding the ion, the formed siderophore-Fe^{3+} complex is subsequently bound by iron-limitation-dependent receptors at the bacterial cell surface and the Fe^{3+} ion is subsequently released and active in the cytoplasm as Fe^{2+}. Bacteria producing high concentrations of high-affinity siderophores in the rhizosphere can inhibit the growth of fungal pathogens when the Fe^{3+} concentration is low, e.g., in acid soils (77).

Competition for nutrients and niches. Competition of biocontrol bacteria with the pathogen for nutrients and niches in the rhizosphere (CNN) (**Figure 2*c***) has been suggested for decades as a possible mechanism of biocontrol, but experimental proof was lacking. Kamilova et al. (44) argued that if this mechanism exists such biocontrol strains can be selected. To this end, they applied a mixture of rhizosphere strains onto surface-sterilized seeds, which were subsequently allowed to germinate in a gnotobiotic system (83). After 1 week, the root tip, which contained the best competitive root colonizers, was removed from the seedling and the bacterial content was briefly allowed to multiply and subsequently applied onto fresh seeds for a new enrichment cycle. After three of such cycles, the isolated bacteria were as good as, or even better, in competitive root tip colonization than our model colonizer, *P. fluorescens* WCS365. They also grow efficiently on root exudate. Most of the isolates, including *Pseudomonas* strains PCL1751 and PCL1760, controlled TFRR. Mutant studies confirmed the proposed mechanism (44, 88, chapter 4).

Kamilova et al. (44) observed that one of the best competitive root-tip-colonizing strains did not control TFRR. It was concluded that efficient overall colonization of the root is not sufficient for biocontrol (44). An explanation for this phenomenon came from the work of Pliego et al. (71), who isolated two similar enhanced root colonizers, of which only one showed control of white root rot in avocado. It appeared that the strains colonized different sites on the root. Apparently, an exact minimiche on the root has to be colonized to protect the plant against the pathogen. A study on biocontrol of TFRR in stonewool showed that after 3 weeks more cells of the CNN strain *P. putida* PCL1760, which was selected for biocontrol in stonewool substrate (88, chapter 4), are present on the root compared with all other culturable bacteria combined. This illustrates the enormous protective capacity of this CNN strain.

Interference with activity, survival, germination, and sporulation of the pathogen. Fusaric acid secreted by *Forl* hyphae acts as a chemoattractant for cells of *P. fluorescens* WCS365 (22). During biocontrol of TFRR by this bacterium, the bacteria colonize the hyphae of the pathogen *Forl* extensively and form microcolonies on them (6). This colonization is likely to make the fungus less virulent. Scanning electron microscopy has shown that *P. fluorescens* WCS365 also colonizes *Forl* hyphae when incubated in root exudate. Testing different media showed that the poorer the growth medium, the more extensive the hyphae are colonized (42). This observation supports an earlier suggestion (43) that bacteria colonize hyphae to use them as a food source.

When incubated in root exudate, microconidia of *Forl* germinate. The presence of *P. fluorescens* WCS365 inhibits spore germination,

presumably because of nutrient deprivation. After growth of *Forl* hyphae in exudate, the hyphae develop microconidia, spores that can spread the pathogen through the environment. The presence of *P. fluorescens* WCS365 also leads to a reduction of this spore formation process and therefore reduces pathogen spread. In conclusion, *P. fluorescens* WCS365 inhibits activity, survival, and germination of the pathogen, colonizes its hyphae, and inhibits formation of new spores. Although these processes may not be specific for biocontrol strains, it is clear that when plants are grown in new stonewool, which is practically sterile, these effects significantly contribute to the reduction of TFRR after introduction of *P. fluorescens* WCS365 into the plant nutrient solution (42, 89).

Other Aspects of Biocontrol

To increase the efficacy of disease control, it was logical to inoculate seeds with two strains that use different mechanisms of biocontrol. In our experience such so-called cocktails never resulted in better disease control. An explanation may be that the cell numbers of each of the two bacteria on the root are reduced below the threshold level required to cause control.

Bacteria indigenous to soil compete with biocontrol strains for root colonization and produce various factors that can decrease the beneficial effect of a biocontrol strain. Because new stonewool is practically free from living microbes, it has the disadvantage that incoming pathogens destroy many plants in a greenhouse but the advantage that such a system can easily be buffered with biocontrol bacteria. For example, *P. putida* PCL1760 remains the dominant microbe on the root for at least 3 weeks and has a high affinity for stonewool (88, chapter 4). A similar effect was found in salinated desert soil in Uzbekistan, which is poor in organic matter and therefore in indigenous microflora. The indigenous microflora is rich in plant pathogens as well as potential human pathogens (3, 27). Under these circumstances, seed inoculated with biocontrol bacteria adapted to these stress conditions strongly decreases the level of plant diseases and may help to protect field-workers from exposure to pathogens (27).

Lessons from the Past to Create a Shining Future

Many bacterial strains exert their beneficial effects in laboratory culture, a lower number are successful in a laboratory greenhouse, and a much lower number are functioning under practical conditions, i.e., in a commercial greenhouse or in the field. Understanding the reasons for the failures in greenhouses and in the field may lead to the isolation of improved strains. Strains using antibiosis are easy to isolate, and therefore they have been used most often. These strains are often effective but sometimes they are not. They could fail for a number of reasons.

First, many biocontrol traits, such as root colonization, motility, and the production of AFMs, biosurfactants, chitinases, lipases, and proteases are subject to phase variation. Phase variation is the process of reversible, high-frequency phenotypic switching that is mediated by mutation, reorganization, or modification of DNA (90). Second, regulation of the syntheses of secondary metabolites is complicated and far from understood (24, 35). For example, growth temperature, salinity, and concentrations of ferric, phosphate, sulfate, and ammonia ions have a strong influence on the level of phenazine-1-carboxamide production (95). Third, antibiotics can be degraded. For example, the Phl-producing strain *P. fluorescens* CHAO produces an enzyme that removes an acetate group from Phl, resulting in a less active derivative of Phl (7). Fourth, AHLs, signal molecules required for the synthesis of several AFMs and exo-enzymes, can be degraded by enzymes from competing bacteria. Furthermore, Van Rij et al. (94) have shown that the *Fusarium* metabolite fusaric acid inhibits phenazine synthesis of *P. chlororaphis* PCL1391 and therefore its biocontrol activity. In fact fusaric acid interferes before or at the level of AHL synthesis, which is required for phenazine synthesis (94).

Last, not all fungi are simple victims of antagonistic biocontrol bacteria. Some pathogens defend themselves and become resistant. In principle, they can utilize a range of possible mechanisms, such as enzymatic inactivation of the antifungal toxin by chemical modification, repression of biosynthetic toxin genes, modification of the target of the antifungal toxin, and secretion of the antifungal toxin (25).

The above mentioned results, as well as the fact that registration of antibiotic-producing products is discouraged because of possible cross-resistance with antibiotics applied for human and animal use, suggest that biocontrol strains based on mechanisms other than antibiosis might have a better future for surviving the registration procedure and therefore becoming a product. It is not yet possible to select for strains that utilize ISR. In contrast, bacteria based on CNN can easily be selected for under the environmental conditions one wants to apply them.

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