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BIOLOGICAL CONTROL OF SOILBORNE PLANT PATHOGENS IN THE RHIZOSPHERE WITH BACTERIA

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INTRODUCTION

Biological control of soilborne pathogens by introduced microorganisms has been studied for over 65 years (9, 49), but during most of that time it has not been considered commercially feasible. Since about 1965, however, interest and research in this area have increased steadily (9), as reflected by the number of books (10, 47, 49, 152) and reviews about it (11, 26, 30, 106, 143, 153, 173, 174, 183) that have appeared. Concurrently, there has been a shift to the opinion that biological control can have an important role in agriculture in the future, and it is encouraging that several companies now have programs to develop biocontrol agents as commercial products. This renewed interest in biocontrol is in part a response to public concern about hazards associated with chemical pesticides.

Microorganisms that can grow in the rhizosphere are ideal for use as biocontrol agents, since the rhizosphere provides the front-line defense for roots against attack by pathogens. Pathogens encounter antagonism from rhizosphere microorganisms before and during primary infection and also during secondary spread on the root. In some soils described as microbiologically suppressive to pathogens (172), microbial antagonism of the pathogen is especially great, leading to substantial disease control. Although pathogen-suppressive soils are rare, those identified are excellent examples of the full potential of biological control of soilborne pathogens.

In the last 15 years, several examples of bacteria capable of providing substantial disease control in the field have been reported, and at times control approaches that in suppressive soils. These more recent successes in biological control, which are in contrast to less successful attempts early in this century (49), result in part from a greater understanding of the rhizosphere and the selection of strains more adapted to growing there. Bacterial biocontrol agents improve plant growth by suppressing either major or minor pathogens. Major pathogens produce the well-known root or vascular diseases with obvious symptoms (163). Minor pathogens are parasites or saprophytes that damage mainly juvenile tissue such as root hairs and tips and cortical cells (163), and the disease symptoms are not obvious. Within the category of minor pathogens, Schippers et al (170) distinguished the parasitizing minor pathogens from the nonparasitizing deleterious rhizosphere microorganisms (DRMO). DRMO include deleterious rhizobacteria (DRB) (184) and deleterious fungi. Other discussions of this topic are available (30, 173, 174, 183). This review examines the current successes and problems of biological control of soilborne pathogens with bacteria in the rhizosphere. This chapter also discusses possible reasons for inconsistent performance of biocontrol agents in the field and approaches to help realize the full potential of bacteria in plant-disease control. It focuses on the mechanisms by which introduced bacteria suppress pathogens and traits that may contribute to their ability to colonize roots.

BACTERIAL BIOCONTROL AGENTS

Bacteria shown or thought to have potential for biocontrol occur in many genera, including *Actinoplanes* (72, 186), *Agrobacterium* (111, 112, 143, 144, 194), *Alcaligenes* (69, 227, 229), *Amorphosporangium* (72), *Arthrobacter* (125, 142, 177), *Azotobacter* (140), *Bacillus*, (4, 24, 25, 31, 38, 40, 64, 71, 122, 126, 138, 139, 189, 197, 198, 200, 203, 204), *Cellulomonas* (210a), *Enterobacter* (88, 126, 146, 149, 179, 199, 201, 202), *Erwinia* (179), *Flavobacterium* (46, 126), *Hafnia* (177, 178, 179), *Micromonospora* (72), *Pseudomonas* (31, 32a, 45, 48, 67, 68, 70, 76, 79, 80, 93, 94, 97, 108, 109, 114, 115, 116, 118, 121, 141, 146, 150, 182, 216, 218, 220, 223, 226, 228, 229), *Pasteuria* (28, 106, 164, 165, 181), *Rhizobium* and *Bradyrhizobium* (39, 195, 196), *Serratia* (177, 178, 179), *Streptomyces* (25, 43, 138, 139, 161, 210a), and *Xanthomonas* (46, 126). Obviously, biocontrol agents are not limited to a specific bacterial group; however, given the diversity of the rhizosphere microflora, it is probable that the full spectrum of potentially effective strains has barely been explored. Many of these biocontrol agents are effective under field conditions.

The premier example of a bacterial biocontrol agent is *Agrobacterium*

radiobacter strain 84, which controls crown gall caused by *A. tumefaciens* (111). Strain 84 is the first bacterium used commercially for biocontrol, and it has been successful worldwide. This biocontrol system has been reviewed extensively (112, 143, 144, 194), and therefore is only mentioned in this review.

Bacillus spp. have been tested on a wide variety of plant species for ability to control diseases. They are appealing candidates for biocontrol because they produce endospores that are tolerant to heat and desiccation. Of greatest interest is *B. subtilis* A13, which was isolated by Broadbent et al (25) from lysed mycelium of *Sclerotium rolfsii* (24). Strain A13 is inhibitory in vitro to several plant pathogens and has improved the growth of many plant species in steamed and natural soils (24, 25, 229). As a seed treatment, it increased the yield of carrots by 48%, oats by 33% (139) and peanuts up to 37% (198). *B. subtilis* A13 appears to improve plant growth by suppressing major and minor pathogens and possibly also by directly stimulating plant growth (24, 197, 198). Since 1983, *B. subtilis* A13 has been sold as a treatment for peanut under the name QUANTUM-4000 (Gustafson, Dallas, Texas; B. L. Kirkpatrick, personal communication) (197).

Currently, *Pseudomonas* spp. are receiving much attention as biocontrol agents. The worldwide interest in this group of bacteria was sparked by studies initiated at the University of California, Berkeley, during the 1970s. In 1978, Burr et al (31) reported that strains of *P. fluorescens* and *putida*, applied to seed pieces, improved the growth of potatoes. These findings were confirmed (121), and extended to sugarbeets (185) and radish (118). In summarizing results from field tests, Schroth & Hancock (174) reported that the fluorescent pseudomonads increased the yield of potato 5–33%, of sugarbeet 4–8 tons per hectare, and root weight of radish 60–144%. These strains and similar strains were given the name plant growth-promoting rhizobacteria (PGPR). The term *rhizobacteria* was coined for bacteria with the ability to colonize roots aggressively (173, 174).

PGPR are thought to improve plant growth by colonizing the root system and preempting the establishment of or suppressing DRMO on the roots (173, 174, 184). Studies in the Netherlands suggest that PGPR promote potato growth primarily by suppressing cyanide-producing DRMO (14, 170). Dutch workers have also demonstrated that the frequency of potato production in a field affects the ability of PGPR to improve plant growth. When potatoes were grown in the same field every third year (short potato rotation), yields were 10–15% less than when grown every sixth year (long potato rotation), and 30% less when cropped continuously (170, 171). The PGPR improved potato growth and yield in short- but not long-rotation soils (15, 78, 80, 170, 171). DRMO are thought to achieve populations required for disease in short- but not long-rotation soils, thus accounting for the rotational effect.

Fluorescent *Pseudomonas* strains also suppress major pathogens of plants (48, 76, 93, 94, 114, 159, 182, 210, 216, 218, 220, 223, 226, 229). One example is biological control of take-all, a root disease of wheat caused by *Gaeumannomyces graminis* var. *tritici*. Take-all is probably the most important root disease of wheat. Owing to the lack of host-plant resistance and of economical chemical controls, biological control with bacteria is being studied intensively (36, 38, 50, 216, 220, 223). Weller & Cook (216) isolated fluorescent pseudomonads from wheat roots grown in soil from a field where take-all decline had occurred. Take-all decline (a natural form of biocontrol) is expressed as a spontaneous diminution in the severity of take-all and concomitant increase in yield with monoculture of wheat (50). Fluorescent pseudomonads were tested for control of take-all because these bacteria may have a role in take-all decline (50). *P. fluorescens* 2-79 (NRRL B-15132) alone or in combination with 13-79 (NRRL B-15134) suppressed take-all on both spring and winter wheat when applied as a seed treatment (216, 220). Yields were increased an average of 17% in experimental plots (216) and 11% in commercial-scale tests (50; R. J. Cook, D. M. Weller, unpublished findings). The combination of strains was superior to either strain alone in about 50% of the tests (216). The addition of a third strain, *P. fluorescens* R4a-80 (NRRL B-15133), enhanced the effectiveness of the treatment even more (R. J. Cook & D. M. Weller, unpublished findings). The combination of strains may better simulate the natural microflora responsible for take-all decline. Like the PGPR strains, 2-79 is an aggressive root colonist and can be isolated from treated wheat throughout the growing season. When applied as a seed treatment, strain 2-79 comprised 50% of the total population of fluorescent pseudomonads on seminal roots for a period of two months after planting (212).

Pseudomonads that are PGPR and those that suppress major pathogens should not be considered as functionally separate groups of bacteria. Many strains suppress both major and minor pathogens.

Selection of Candidate Bacteria

Because of the time and expense required for field testing, better methods are needed to select potential field-effective strains. Rhizosphere bacteria with the ability to provide biological control appear to comprise less than 10% of the total population of bacteria in the rhizosphere (173, 174, 185, 218). The chance of selecting effective strains may be improved initially by first isolating bacteria from the same environment in which they will be used, for example, selecting from a pea rhizosphere if the target pathogen causes a root disease of pea. Isolating bacteria from pathogen-suppressive soils (172) may increase the chances of finding effective strains even more (49). There is evidence that pseudomonads have a role in the suppressiveness of certain soils

to fusarium wilt of flax, radish, and cucumber (167), take-all of wheat (50), and black root rot of tobacco (182). The percentage of fluorescent pseudomonads suppressive to take-all in a greenhouse bioassay (219, 220) was greater when the bacteria were isolated from roots of wheat grown in suppressive soils (from fields that had undergone take-all decline) than in nonsuppressive soils.

Since no general relationship exists between the ability of a bacterium to inhibit a pathogen in vitro and suppress disease caused by that pathogen in vivo (11, 173, 223), strains producing the largest zones of inhibition on agar media do not always make the best biocontrol agents. Thus, when attempting to develop a biocontrol system, prescreening strains on agar media may not be useful.

Some in vitro assays have been modified to more closely simulate natural conditions. Rhodes et al (158) developed a tuber-slice assay to prescreen biocontrol agents of potato pathogens. In this assay, antagonist and pathogen are co-inoculated on a potato slice and the amount of decay is compared to that caused by the pathogen alone. Randhawa & Schaad (157) developed a seedling-bioassay chamber that permits studies of antagonists and fungal and bacterial pathogens on roots grown in a petri dish.

Selection of potential field-effective strains can be further facilitated by use of greenhouse assays. Greenhouse methods have been developed to screen antagonists of *G. graminis* var. *tritici* (220) and *Pythium* spp. (218) on wheat, *Erwinia carotovora* on potato (225), and *Phytophthora megasperma* f. sp. *glycinea* on soybean (129). Important parameters of the assays are usually inoculum potential of the pathogen (220), environmental conditions (i.e. temperature and moisture content of the soil), and dose of the candidate bacterium (225).

Another approach is to screen strains for ability to colonize roots without concern for their biocontrol activity (107). The assumption is that biocontrol agents of root pathogens should be good root colonists. Scher et al (169) devised a closed-tube soil assay to assess the ability of bacteria to colonize maize roots. Kloepper et al (117) reported a method to select superior spermosphere colonists, which involved monitoring the populations of introduced bacteria in the spermosphere of soybean after the bacteria were introduced on the seed or into the soil.

Formulation of Bacterial Biocontrol Agents

Biological control depends upon the establishment and maintenance of a threshold population of bacteria on planting material or in soil, and a drop in viability below that level may eliminate the possibility of biological control (29, 183, 225). Many soil edaphic factors, including temperature (131, 216), soil moisture (31, 34, 65, 221), pH (221), and clay content (33, 35, 135,

160), influence the survival and establishment of the bacteria and their interaction with the pathogen. The way in which the bacteria are cultured and then processed will affect their viability and tolerance to adverse conditions once applied. Concerns about inoculum viability are less with *Bacillus* spp. than with gram-negative bacteria since *Bacillus* produces endospores, making it more easily formulated (110). Formulation problems with gram-negative biocontrol agents will be similar to those that have been faced in developing rhizobia, which are sensitive to drying and heat (41, 57, 136, 151). Peat and other carriers (41, 193) developed for rhizobia may be useful. A granular peat formulation of a *P. fluorescens* (Dagger™G, Ecogen Inc., Langhorne, Pennsylvania) shows promise for control of seedling pathogens of cotton (J. L. McIntyre, personal communication). Entrapment of bacteria in polymer gels (17, 75, 145) such as xanthan gum or alginate also has potential. Kloepper & Schroth (119) developed a dried formulation of PGPR strains for potatoes by mixing bacteria with xanthan gum and then adding talc.

Possible Reasons for Inconsistent Performance

It is encouraging that there are now so many examples of biological control with bacteria in the field. Unfortunately, one characteristic that is common to most biocontrol systems with introduced bacteria is the inconsistency of disease control, illustrated in Table 1. Using yield as a common measure, it is apparent that plant growth is not always improved, and that the level of improvement varies greatly from test to test. A multitude of factors could account for inconsistent results, given the complex interactions among host, pathogen, antagonist, and the environment. Three possibilities are discussed below.

LOSS OF ECOLOGICAL COMPETENCE Ecological competence is the ability of a bacterium to compete and survive in nature (175). Many bacterial traits (most of them unknown) contribute to ecological competence in the rhizosphere and loss of any one can reduce the ability of the bacteria to become established or function on or near the root. Important traits can be lost when a bacterium is grown in vitro (175). For example, bacteria in nature, and when first isolated, are surrounded by a capsular exopolysaccharide (EPS) (52), but EPS-deficient mutants arise spontaneously in vitro and eventually predominate in a culture because they multiply faster. Such mutants might be less able to survive when used as biocontrol agents. Repeated culturing of fluorescent pseudomonads in vitro can result in a loss of field efficacy, possibly related to changes in cell and colony morphology, loss of cell surface structures, or reduction in antibiotic and siderophore production (32a, 175; D. M. Weller, unpublished findings).

Table 1 Effectiveness of bacterial biocontrol agents

Bacteria[a]	Crop	Pathogen	Number of significant trials/ total[b]	Average yield increase (%)	Range of increase (%)	References
Bacillus pumilus	wheat	*Gaeumannomyces graminis*	1/4	35	0 to 114	38
Pseudomonas sp. TL-3	potato	DRMO	6/11	10	–7 to 24	31, 121
Pseudomonas sp. SH-5	sugarbeet	DRMO	5/9	12	–3 to 32	183, 185
Pseudomonas putida W4P63	potato	*Erwinia carotovora*	1/3	7	0 to 12	226
Pseudomonas fluorescens E6	zinnia	DRMO	19/23	36	–3 to 136	228
Pseudomonas fluorescens 2–79	wheat	*Gaeumannomyces graminis*	2/3[c]	17	6 to 27	216
P. fluorescens 13–79			6/10[d]	11	–1 to 25	R. J. Cook, D. M. Weller, unpublished

[a]All studies were conducted in the field except that with *P. fluorescens* E6 which was conducted in the greenhouse.
[b]Level of significance, $P = 0.1$.
[c]Small scale experimental plots.
[d]Commercial trials. In one of the 10 trials, *P. fluorescens* R4a-80 was included in the mixture.

TARGET PATHOGEN ABSENT OR NONTARGET PATHOGEN INTERFERENCE Because bacterial biocontrol agents improve plant growth by reducing damage from pathogens, a positive response to their introduction does not occur when the target pathogen(s) is absent, or when environmental conditions are unsuitable for disease development. This is clearly illustrated by the studies of PGPR on potatoes in the Netherlands that were described earlier; PGPR strains *P. fluorescens* WCS374 and *P. putida* WCS358 and other strains improved potato growth and yield in short- but not long-rotation soils (15, 78, 80, 170, 171). The performance of the PGPR strains would appear very inconsistent if cropping history were not considered. Similarly, the average yield increase of peanut treated with *B. subtilis* A13 was 12% for 11 fields with a poor rotation (legumes in one of two previous years), but only 3.4% for 5 fields with a good rotation (no legumes grown in two previous years) (197, 198).

The effect of pathogens other than the target pathogen is another concern. If a bacterium suppresses only one pathogen, but another becomes predominant, the treatment will appear ineffective. R. J. Cook & D. M. Weller (unpublished findings) found that the failure to obtain a significant growth response in wheat with *P. fluorescens* Q72a-80, applied to control pythium root rot, occurred because *Rhizoctonia solani* AG-8 was also present, and the pseudomonad is not effective against rhizoctonia root rot. Thus, an understanding of the pathogens in the agroecosystem and the conditions that favor each is essential.

VARIABLE ROOT COLONIZATION BY BACTERIA It is generally assumed that root colonization by introduced bacteria is essential for biocontrol of root pathogens and that increasing the population of an introduced bacterium on the root should enhance disease control (183). Unfortunately, only a few studies (29, 32a, 114, 120, 126a, 184, 226) have tried to assess the effect of bacterial population size on pathogen population and of disease severity on roots. Xu & Gross (226) applied *P. putida* W4P63 to potato seedpieces and monitored its population and that of *Erwinia carotovora* on roots in the field. Populations of W4P63 ranged between 10^4 and 10^5 cfu(colony forming units)/g root, while the population of *E. carotovora* on these same roots was only 10% of that on roots with no W4P63. Bull (29) treated wheat seeds with increasing dosages of *P. fluorescens* 2-79 (approximately 0, 10^2, 10^4, 10^6, 10^8 cfu/seed) and planted these seeds in natural soil infested with the take-all pathogen. There was a direct linear relation between dose of 2-79 on the seed and the population of 2-79 that developed on the root. Further, there was an inverse relationship between the population of 2-79 on the root and the number of take-all lesions. The study by Bull (29) demonstrates conclusively that the extent of take-all control is directly related to root colonization.

Spatial-temporal colonization patterns of introduced bacteria on individual roots offer the best analysis of a strain's colonization capability, population stability on the root, and the extent of colonization. Such patterns are rarely determined because the studies are extremely labor intensive (8). Instead, bacterial populations on roots usually are determined from pooled samples of roots (79, 97, 107, 114, 121, 185, 212, 226). This type of sampling, however, results in an overestimation of the mean population of introduced bacteria, since the bacteria populations are lognormally rather than normally distributed among root systems of different plants (8,132) and among individual roots of a single plant (8, 29). Populations of introduced PGPR strains A1 or SH5 on root systems of individual potato or sugarbeet seedlings varied by a factor of 10-100 (132). Populations of *P. fluorescens* 2-79 on individual roots of wheat seedlings (29) also varied up to 1000-fold, and 20–40% of the roots were not colonized four weeks after planting (29). Even on a single root, populations of introduced bacteria may vary several log units along the axis (length) of a root, with the greatest numbers usually occurring near the inoculum source and decreasing toward the root tip (8, 131, 213). When samples are pooled, the root system or root with the largest population provides a disproportionate number of bacteria to the mean and gives a perception that root colonization is better than what has actually occurred.

Variable root colonization by introduced bacteria, including colonization from plant to plant, and root to root on a given plant, is probably a main reason for inconsistent control by biocontrol agents.

ROOT COLONIZATION

For an introduced bacterium to be a root colonizer, how much of the root must it colonize, and for how long? What population size must it reach? Rigid guidelines for a root colonizer do not exist and establishing ones that would fit all biocontrol systems would be difficult. Scher et al (169) defined colonizers of corn roots as bacteria that attain cfu $> 5 \times 10^3$/g root. I suggest that so far as introduced bacteria are concerned, in general, a root colonizer is a bacterium that when introduced becomes distributed along the root in natural soil, propagates, and survives for several weeks in the presence of competition from the indigenous rhizosphere microflora. This definition eliminates bacteria that are transient in the rhizosphere, or that can establish themselves on roots only in the absence of competition. In this review, the term root colonization includes colonization of the root (internal or surface) as well as the rhizosphere soil by the introduced bacteria. The bacteria are probably not restricted to either location (8).

Ahmad & Baker (2) used the term rhizosphere competence to describe the ability of biocontrol agents to grow and function in the rhizosphere. Rhizo-

sphere competence might also be thought of as the relative root-colonizing ability of a strain. Thus, rhizosphere competence varies among bacteria, with strains unable to colonize roots being rhizosphere incompetent. The rhizosphere competence of a strain can be quantified by measuring the population it attains on a root and/or by determining the length or number of roots colonized. Thus, strains can be compared on this basis.

The Process of Root Colonization

Howie et al (96) hypothesized that colonization of wheat roots by *P. fluorescens* occurs in two phases. In phase I, the bacteria attach to, and are then transported on the elongating root tip, and in phase II the bacteria spread locally and proliferate to the limits of the niche in competition with indigenous organisms and survive. This process may apply to other biocontrol systems.

Phase I begins as introduced bacteria on seeds or seedpieces come into contact with the emerging roots. Some kind of attachment of the cells to the root surface may be essential for initiation of phase I and would also assure priority access to root exudates (29, 105). As the root elongates, some of the bacteria are carried along with the root tip, while others are left behind as a source of inoculum on older portions of the root (213). Bacterial multiplication at the tip ideally would permit transport of bacteria as long as the roots grows, but without multiplication, transport would occur only until the initial inoculum at the root tip is diluted out. Evidence for phase I includes the observation by Bull (29) that bacteria from diverse ecological niches, such as *P. fluorescens* 2-79 and Q72a-80 (biocontrol agents isolated from roots of wheat), *Escherichia coli, P. syringae, Xanthomonas campestris, Erwinia carotovora,* and *E. herbicola* (which are not biocontrol agents), attached equally well to wheat roots. Further, after they had been applied to wheat seed, and after the seed had subsequently been planted in natural soil, these bacteria were detected along roots in the absence of percolating water in nearly equal numbers four days after planting. Finally populations of all strains along individual roots declined linearly from seed to tip (29).

Transport of bacteria by the root tip is sometimes inefficient and tips do not always become or remain colonized (8, 29). Bull (29) sampled seminal roots emerging from wheat seed treated with *P. fluorescens* 2-79 soon after planting and could not detect the bacterium on 20–40% of individual seminal roots. Loss of introduced bacteria from the tip may occur from physical removal, as the tip displaces or realigns soil particles, or because of adsorption to the soil particles, or owing to competition from indigenous bacteria (8). The primary constraint may be the inability of bacteria at times to multiply rapidly enough to keep pace with the root tip, which extends rapidly through the soil (2–9

cm/day) by expansion of cells in the zone of elongation (10–20 times their original length) (73).

The ultimate fate of an introduced bacterium is governed to a large extent by its ability to compete with the indigenous microflora during phase II. Rhizosphere-competent bacteria will multiply and survive on the root, whereas incompetent bacteria are rapidly displaced. Using the same bacteria described above to treat wheat seed, Bull (29) showed that after 14 days populations of the biocontrol agents (*P. fluorescens* strains) were much greater than those of bacteria that are not biocontrol agents on roots in natural soil, but the populations of all the strains were nearly the same on roots in pasteurized soil. Dupler & Baker (65) reported that *P. putida* N-1R colonized the radish rhizosphere less efficiently when the bacteria were added to a biologically active soil than when the same soil was air dried prior to the test to reduce microbiological activity; competition would have been greater in the former soil.

Nutrients rather than space are thought to be the limiting factor in competition among bacteria during rhizosphere colonization. Direct observations of roots from soil show that most of the root surface is open space and remains uncolonized (22). Bacteria tend to congregate in grooves between cells where nutrients may be most abundant (22). Since the carrying capacity of the rhizosphere is limited, an introduced strain must preempt the establishment of indigenous bacteria if it is to become established. Thus, in response to an introduced strain, the total population of rhizosphere microorganisms may not change, but rather the composition of the population is altered (212).

Factors Affecting Root Colonization

The distribution of introduced bacteria along the root during phase I, and their propagation and survival during phase II, are profoundly affected by abiotic and biotic factors. Howie et al (96) studied the effect of rhizosphere matric potential on phases I and II of wheat-root colonization by seed-applied *P. fluorescens* 2-79. The greatest populations developed on roots at −0.3 bars in one soil and at −0.7 bars in two other soils. They suggested that −0.3 to −0.7 bars was the range in which oxygen availability and turgor potential of the cells and/or nutrient availability were optimal for bacterial-cell growth. Interestingly, strain 2-79 spread from seeds onto roots even in soil at −4.0 bar matric potential, and extrapolations from the data predicted that downward spread could occur in soil down to −7.0 bars. Populations detected at −4.0 bars and lower were thought to reflect primarily phase I, since it is unlikely that cells would be unable to maintain turgor potential for growth at such matric potentials (90, 96). From the practical standpoint, it is encouraging

that introduced bacteria can spread from planting material to roots over such a wide range of matric potentials.

Transport of bacteria along with the elongating root (phase I) does not require percolating water (96); nevertheless, such water enhances bacterial movement (8, 42, 126, 155). In an elegant field experiment, Bahme & Schroth (8) monitored the spatial distribution of seedpiece-applied *P. fluorescens* A1-B on potato roots before and after irrigation. The water moved cells of A1-B from the seedpiece into the soil and redistributed the populations of A1-B that had become established on roots prior to irrigation, which resulted in increased populations near the root tips. These researchers (8) speculated that percolating water could serve to renew populations of introduced bacteria at the root tip.

The movement of bacteria through soil by means of water is affected by bacterial characteristics such as cell shape, size, buoyancy, motility (21, 222), and electrostatic charge (135), as well as by soil type, pore-size distribution, and water content and pH of the soil (8, 21, 222). Channels left by old roots or worms (133) and possible gaps at the root-soil interface created by the diurnal shrinking and swelling of roots might allow more rapid downward movement of bacteria in water than occurs along roots in a more uniform soil matrix (155). Thus the effect of water movement on introduced bacteria might best be studied in the field or with intact soil cores from the field.

The optimal temperature for growth of *Pseudomonas fluorescens* and *P. putida* in vitro is 25–30°C, but root colonization by these bacteria is generally greatest below 20°C (29, 132). Microbial activity in the soil increases as soil temperatures increase; thus better colonization at lower temperatures probably reflects less competition from indigenous microflora. With regard to pH, the growth of these bacteria shows a similar pattern. While they tend to grow best in vitro at neutral pH or above, colonization of wheat roots by *P. fluorescens* 2-79 was greater at rhizosphere pH 6–6.5 than at 7.0 or above (95), possibly because of less competition from indigenous rhizosphere bacteria at the more acid pH.

Plant genotype influences the quantity and composition of the rhizosphere microflora (6, 7, 147), possibly through differences in root exudates (55), and manipulating host genotype may offer some opportunity to improve the efficiency or consistency of root colonization by introduced bacteria. For example, wheat lines S-615 and Rescue, both susceptible to common root rot, harbored larger numbers of rhizosphere bacteria than did the resistant Apex. Substitution of a chromosome pair 5B from Apex for its homologue in S-615 resulted in the chromosome-substitution line S-A5B that is as resistant as Apex to root rot. Further, the indigenous rhizosphere bacterial population on S-A5B was also similar to that of Apex; the resistant lines had a higher

percentage of bacteria that were antagonists of *Cochliobolus sativus* than the susceptible lines (6, 147). Root colonization by introduced bacteria is also affected by host genotype. Weller (215) has demonstrated considerable differences among wheat cultivars in their ability to support root colonization by 2-79, and he found that populations were 100-fold greater on the most supportive cultivar, Wampum, as compared to the least supportive cultivar, Brevor.

Indigenous microorganisms may also enhance root colonization by introduced bacteria. The populations of indigenous gram-negative bacteria (27, 209, 212), of *Pseudomonas* spp. (212), and of seed-applied *P. fluorescens* (29, 212) were larger on roots infected by *G. graminis* var. *tritici* than on healthy roots. Electron microscopy showed that the bacteria proliferate in the lesions, probably owing to the greater availability of nutrients in these microsites (162). Introduced *P. fluorescens* are stimulated to an even greater extent than the indigenous bacteria (212). Even a single take-all lesion can enhance the population of *P. fluorescens* 2-79 10-fold per cm of root (29). This is of practical significance, since the infected tissues are where the inhibitory bacteria are needed the most. Colonization of lesions provides considerable protection against secondary spread of the take-all fungus on the roots.

Rhizosphere Competence Traits

Besides a suitable rhizosphere environment, successful long-term root colonization requires that introduced bacteria possess rhizosphere competence traits involved in attachment, distribution, growth, and survival. Bacterial traits that contribute to rhizosphere competence are mostly unknown, but some that may be important are surface polysaccarides, fimbriae, flagella, chemotaxis, osmotolerance, and ability to utilize complex carbohydrates.

CELL SURFACE POLYSACCHARIDES Polysaccharides present at the bacterial-cell surface are required for the establishment of some bacteria-plant associations. Several different exopolysaccharides are important in the attachment of *A. tumefaciens* to plant cells (62, 63, 137, 192) (an initial step in pathogenesis) and in the nodulation of legumes by *Rhizobium* (37, 58, 127, 176). Cellulose fibrils anchor *Agrobacterium tumefaciens* to the plant-cell surface (137, 188) and may mediate attachment of *Rhizobium leguminosarum* to pea root hairs (176). Other bacteria, including *Pseudomonas* spp., also produce cellulose fibrils (59). *A. tumefaciens* strains carrying mutations in either of two chromosomal virulence loci, designated *chvA* and *chvB*, were impaired in attachment and were avirulent (62, 63). The *chvB* locus is required for the synthesis of the extracellular polysaccharide, cyclic 1,2-β-*D*-glucan (156). Interestingly, homology exists between the *chv* genes of *A. tumefaciens* and DNA from *Azospirillum brasilense* and *A. lipoferum* (205,

211), free-living, nitrogen-fixing bacteria that also attach to the root surfaces (19, 61, 102) A cosmid library of *A. brasilense* also complemented *R. meliloti* mutants deficient in the production of the EPS succinoglycan (J. Vanderleyden, personal communication), an exopolysaccharide required for nodulation in *Rhizobium*. These studies suggest that the early phases in the interaction between *Azospirillum* and plant roots may have some similarities to those that occur with *Agrobacterium* and *Rhizobium*. These similarities possibly extend to bacteria that are able to provide biocontrol.

Bacteria in various ecological niches, including the rhizosphere, are normally surrounded by EPS (52, 53, 74) that binds cells together and thus mediates the formation of microcolonies (53). The EPS protects cells against desiccation, antibacterial agents, and predators, and aids the cells by concentrating nutrients and ions (52, 53, 187). Such a structure presumably could help an introduced bacterium avoid displacement by indigenous microorganisms.

FIMBRIAE (PILI) These proteinaceous, filamentous appendages function in bacterial binding to animal cells and inert surfaces (101). Fimbriae also mediate adhesion of N_2-fixing strains of *Klebsiella* and *Enterobacter* to roots of grasses and cereals, thus helping to establish these bacteria-plant associations (86, 87, 123, 124). Vesper & Bauer (207) observed fimbriae on *Bradyrhizobium japonicum* and *Rhizobium trifolii*, and they also demonstrated a correlation between the number of fimbriated cells in populations and the number of cells that attached to soybean roots. A mutant of *B. japonicum*, with an increased percentage of fimbriated cells in its population, showed greater attachment to roots and improved root colonization (208). The take-all suppressive strain *P. fluorescens* 2-79 produces fimbriae (206, L. S. Thomashow, D. M. Weller, unpublished findings) that mediate attachment to corn roots (206) and may also be involved in phase I transport on wheat roots.

FLAGELLA The importance of flagella in the movement of bacteria in the soil and along roots has long been debated and studied (89, 180, 224). Regardless of their function, it is unlikely that movement mediated by flagella can occur in soil drier than −0.5 bars because water films become too thin and water-filled pores too small and discontinuous (81, 224). Howie et al (96) found that nonflagellated mutants of *P. fluorescens* strains R7z-80R, R1a-80R, and R4a-80R colonized wheat roots in two different soils to the same extent as the respective wild types at both −0.2 bars (favorable for motility) and −2.0 bars (unfavorable for motility). This finding indicates clearly that flagella are not essential for bacterial movement along wheat roots. Likewise, *P. putida* RW3 and a nonflagellated Tn*5* mutant applied as seed treatments developed similar populations on soybean roots (F. M. Scher, personal

communication). In contrast, De Weger et al (60) reported that each of four nonmotile Tn*5* mutants of *P. fluorescens* WCS374 applied to 1-cm-long roots of potato-stem cuttings developed significantly lower populations than the parental strain on roots at a depth of 8 cm. They concluded that motility is required to colonize growing potato roots.

These conflicting findings on the role of flagella could be due to differences in bacterial strains, plant species, or physical conditions of soil, particularly moisture. Howie et al (96) conducted their investigation at precisely controlled matric potentials. In contrast, de Weger et al (60) did not control matric potential and allowed wetting of the soil from the bottom; if the soil had a substantially greater matric potential than that in the study by Howie et al, bacterial movement could have been greater. It is obvious from these two reports that when bacterial traits potentially important in root colonization are being studied, soil conditions should be controlled as rigorously as possible to avoid the introduction of multiple experimental variables.

CHEMOTAXIS At a soil-matric potential suitable for motility, chemotaxis toward seed or root exudates may contribute to the ability of bacteria to colonize roots. Chemotaxis may be especially important when the bacteria are added to soil or in the seed furrow, and thus initially are not in contact with the plant. Scher et al (168) demonstrated chemotaxis of fluorescent pseudomonads to soybean seed exudates in water-saturated soil; they found that *P. putida* RW1 moved 1 cm toward a soybean seed in 12 hr. *Azospirillum lipoferum* showed evidence of chemotaxis to wheat-root exudates and sucrose in vitro (91). In sterile soil at near field capacity, *A. brasilense* Cd and *P. fluorescens* 82011 each migrated several centimeters toward wheat roots but moved less in the soil when wheat roots were absent (18). Rhizobia are attracted to substances in plant-root exudates (56, 77, 100), and chemotaxis may help guide them to infection sites (82). Solby & Bergman (180) demonstrated that a motile but nonchemotactic mutant of *R. meliloti* in sterile soil spread only slightly better than a nonmotile mutant and much more poorly than the parental strain. *P. fluorescens* and *P. putida* were attracted to substances from conidia of *Cochliobolus victoriae* and sclerotia of *Macrophomina phaseolina* (5).

OSMOTOLERANCE Tolerance to dry soil and low osmotic potential may aid the survival of some bacteria in the rhizosphere. With an actively transpiring plant, the matric potential at the root-soil interface fluctuates and may become very low during periods of high evapotranspiration (154). In a study of "drought-resistant" bacteria (i.e. ones which survived dry conditions for over 15 days) and "drought-sensitive" bacteria (i.e. ones which died in 1–4 days under dry conditions), the resistant strains showed generally greater osmo-

tolerance than did the susceptible strains (44). Loper et al (131) demonstrated a relationship between osmotolerance and population size for eight *Pseudomonas* strains on potato roots. In contrast, W. J. Howie & T. V. Suslow (personal communication) found that *P. putida* MK280 and an osmosensitive mutant colonized roots of cotton grown in soil at −1.8 bars equally well. If osmotolerance does aid survival of some introduced bacteria on roots, then it may be possible to enhance survival by developing proline-overproducing strains. Proline is an osmoprotectant in *Salmonella typhimurium* and other organisms. Some proline-overproducing mutants of *S. typhimurium, E. coli,* and *K. pneumoniae* acquired increased osmotolerance (54, 103).

COMPLEX CARBOHYDRATE UTILIZATION Although most root exudates are readily metabolized by introduced and indigenous bacteria, fewer organisms can degrade the root-tip mucilages that consist, in part, of complex carbohydrates such as cellulose, hemicellulose, and pectin (74). The ability to use these carbohydrates might provide a competitive advantage to introduced bacteria, since it might permit more efficient colonization of the root tip during phase I. Mutants of *Trichoderma harzianum* with increased cellulase production had a greater competitive saprophytic ability and rhizosphere competence as compared to the wild type (2, 3). Such mutants may be more competitive because of enhanced utilization of cellulose on the root.

MECHANISMS OF PATHOGEN SUPPRESSION

Substrate Competition and Niche Exclusion

Competition for nutrients supplied by root and seed exudates probably occurs in most interactions between bacteria and pathogens on the root and is responsible at least to some small degree for the observed biocontrol by introduced bacteria (68, 69, 183). Large populations of bacteria established on planting material and roots become a partial sink for nutrients in the rhizosphere, thus reducing the amount of carbon and nitrogen available to stimulate spores of fungal pathogens or for subsequent colonization of the root (68, 69). Fluorescent pseudomonads are especially suited to "mopping up" nutrients, since they are nutritionally versatile and grow rapidly in the rhizosphere (214).

Suslow (183) suggested that niche exclusion is potentially an important mechanism of antagonism of DRB by PGPR (184). Certain areas on the root, such as cell junctions and points of emergence of lateral roots, appear to be favored for colonization by many kinds of bacteria, including DRB, because root exudates are abundant there. Inoculating planting material with PGPR presumably prevents or reduces the establishment by DRB at these sites (183). Although *Agrobacterium* strain 84 suppresses *A. tumefaciens* mainly

by production of agrocin 84 (112), physical blockage of infection sites also may contribute to biocontrol (51, 66).

Siderophores

Siderophores are low molecular weight, high affinity iron (III) chelators that transport iron into bacterial cells (128, 148). When grown under low-iron conditions, fluorescent pseudomonads produce yellow-green, fluorescent siderophores (of the pyoverdine type) and membrane-receptor proteins that specifically recognize and take up the siderophore-iron complex (32, 92, 134).

Kloepper et al (116) were the first to demonstrate the importance of siderophore production as a mechanism of biological control. Subsequently, siderophores have been shown to be involved in the suppression of formae speciales of *Fusarium oxysporum* (12, 13, 67, 68, 115, 166, 167, 179), *G. graminis* var. *tritici* (50, 115, 217, 219, 223), *Pythium* spp. (20, 130), and DRMO (15, 116, 120, 170). Because siderophores sequester the limited supply of iron (III) in the rhizosphere, they limit its availability to pathogens and ultimately suppress their growth (173, 174).

The availability of iron (III) in the soil declines logarithmically with increasing soil pH. Thus siderophore-mediated suppression should be greater in neutral and alkaline soils than in acid soils (12, 13). Pathogens are thought to be sensitive to suppression by siderophores for several reasons: (*a*) they produce no siderophores of their own; (*b*) they are unable to use siderophores produced by the antagonists or by other microorganisms in their immediate environment; (*c*) they produce too little siderophore or a siderophore with a lower affinity for iron than those of the antagonists; or (*d*) they produce a siderophore that can be used by the antagonist, but they are unable to use the antagonist's siderophore (32, 128, 134, 167, 173, 174). Several recent reviews have summarized the evidence supporting a role for siderophores in biocontrol (12, 13, 128). The most convincing evidence has been the fact that siderophore-minus mutants are less suppressive to pathogens in the rhizosphere than parental strains (15, 16, 20, 116, 120, 130).

Antibiotics

Antibiotics play a major role in disease suppression by some bacteria. Agrocin 84 is a kind of antibiotic and mediates suppression of *A. tumefaciens* by *A. radiobacter* strain 84 in wound tissue; this topic has been thoroughly reviewed (112, 194). Another example is phenazines produced by some fluorescent pseudomonads suppressive to take-all of wheat. *P. fluorescens* 2-79 inhibits *G. graminis* var. *tritici* in vitro and is suppressive to take-all in the field when applied as a seed treatment (216). The phenazine-type antibiotic produced by *P. fluorescens* 2-79 was reported to be a dimer of phenazine-l-carboxylate

(83); however, in another study (23) the structure was reported to be the monomer form. This antibiotic inhibits *G. graminis* var. *tritici* in vitro at less than 1 μg/ml; it also inhibits several other wheat-root pathogens. Tn*5* mutants of 2-79 deficient in production of this phenazine were significantly less suppressive of take-all than the parental strain (by about 60–90%, depending on the soil used in the test). Complementation of the phenazine mutants with cosmid clones from a 2-79 library fully and coordinately restored both phenazine production and suppressiveness of take-all (190, 191). A similar experimental approach was used to demonstrate the importance of phenazine-l-carboxylate and 2-hydroxy phenazine-l-carboxylate to the suppressiveness of take-all by *P. aureofaciens* 30-84 (L. S. Pierson & L. S. Thomashow, unpublished findings).

P. fluorescens Hv37a produces an antifungal compound (Afu) inhibitory to *Pythium ultimum*. Synthesis of the compound is regulated by glucose (104) and depends on expression of at least five genes (84, 85). An isogenic mutant of Hv37a deficient in Afu production was significantly less effective than the parental strain in protecting cotton against *Pythium ultimum* (98; W. J. Howie & T. V. Suslow, personal communication). A β-galactosidase gene fusion into one of the genes responsible for antibiotic biosynthesis showed that the gene was expressed in the cotton spermosphere. The level of expression was greatest on seeds in soil at pH 6–8 and at 20°C (99; W. J. Howie & T. V. Suslow, personal communication).

Antibiotic-deficient mutants have also been used to demonstrate the importance of antibiotic production in biological control by other bacteria. *P. putida* M17, which produces an unidentified antibiotic inhibitory to *Erwinia* spp., controlled potato-tuber soft rot, but an antibiotic-negative mutant of M74 was less effective (48). *P. fluorescens* R1a-80 (NRRL B-15135) produces an unknown antibiotic that inhibited *G. graminis* var. *tritici* in vitro; and nitrosoguanidine (50, 217, 219) or Tn*5* mutants (A. R. Pobobloskv & A. H. Elingbow, personal communication) deficient in antibiotic production were less suppressive of take-all in vivo than the parental strain.

Howell & Stipanovic (93, 94) demonstrated that the purified antibiotics pyoluteorin and pyrrolnitrin, obtained from *P. fluorescens* Pf-5, provided the same protection of cotton against damping-off by *Pythium ultimum* or *Rhizoctonia solani* as did the bacterium. *Streptomyces hygroscopicus* var. *geldanus* suppressed rhizoctonia root rot of pea and produced the antibiotic geldanamycin, which inhibited *Rhizoctonia solani* in vitro. Rothrock & Gottlieb (161) presented direct evidence that the antibiotic was produced in soil by *Streptomyces hygroscopicus*. The topic of antibiotics and biological control is reviewed by Fravel (74a) in this volume.

Interestingly, in studies where mutants have been used to determine the role of antibiotics or siderophores in disease suppression, loss of either compound

has had little or no effect on the ability of the bacteria to colonize roots (15, 16, 120, 130, 219). Thus at least on a short-term basis, these compounds do not appear to be important as factors in root colonization. Further work is needed to assess the role of these compounds in longterm colonization.

Induced Resistance

P. fluorescens CHAo produces both antibiotics and siderophores (1), but suppression by this bacterium of black root rot of tobacco (caused by *Thielaviopsis basicola)* (182) appears to be mediated mainly by the production of hydrogen cyanide (G. Defago, personal communication). Mutants of CHAo deficient in HCN production were less suppressive than the parental strain; hydrogen cyanide on and in the root is thought to induce resistance in tobacco to *Theilaviopsis*. Kempe & Sequeira (109) suggested that resistance to a virulent strain of *P. solanacearum* was induced in potato by treatment of seed pieces with avirulent or incompatible strains of *P. solanacearum* or *P. fluorescens* (109).

It is important to remember that in a given biological agent more than one mechanism may operate to suppress a pathogen, and the relative importance of a particular mechanism may vary with the physical or chemical conditions in the rhizosphere (219).

CONCLUSION AND PROSPECTS FOR FUTURE RESEARCH

In order to develop bacterial biocontrol agents for commercial use, the consistency of their performance must be improved. Accomplishing this will require research in many diverse areas, because biological control is the culmination of complex interactions among the host, pathogen(s), antagonist, and environment.

Research to identify bacterial traits that function in plant colonization and pathogen antagonism is critically important, and molecular genetics offers the best approach to such studies. For example, transposon mutagenesis can be used to generate mutants deficient in single traits that are of interest. The mutants can then be evaluated to establish the importance of those traits to the biocontrol ability of that strain. Identifying important traits allows more efficient selection of new strains. Further, such traits can be altered to make a strain more effective. For example, the demonstration that phenazine antibiotics play a major role in suppression of take-all by *P. fluorescens* 2-79 and *P. aureofaciens* 30-84 has led to a search for superior antagonists among strains producing multiple phenazines and for mutants of 2-79 and 30-84 that produce altered or novel phenazines (L. S. Thomashow & D. M. Weller). Ultimately, the possibility exists of genetically engineering superior biocon-

trol agents by moving genes from one bacterium to another. However, this approach should be viewed with cautious optimism since bacterial determinants that are of interest, such as antibiotics and siderophores, are not simple one-gene products (113). Additional research is also needed on soil physical and chemical factors that influence both root colonization and the expression of traits important to antagonism in the rhizosphere. By identifying these factors, it may be possible to manipulate them in the field so as to enhance root colonization. Finally, more research on formulation and delivery of the bacteria is needed. The challenge is to develop inexpensive, easily applied preparations that remain viable under less than optimal conditions. It must be kept in mind that growers cannot be expected to buy new equipment or to modify equipment or farming practices substantially to accommodate a biological treatment.

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