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Molecular mechanisms of defense by rhizobacteria against root disease

R. JAMES COOK*, LINDA S. THOMASHOW, DAVID M. WELLER, DEBBIE FUJIMOTO, MARK MAZZOLA, GITA BANGERA, AND DAL-SOO KIM

United States Department of Agriculture, Agricultural Research Service, Root Disease and Biological Control Research, 367 Johnson Hall, Washington State University, Pullman, WA 99164-6430; and Washington State University, Department of Plant Pathology, Pullman, WA 99164-6430

ABSTRACT **Genetic resistance in plants to root diseases is rare, and agriculture depends instead on practices such as crop rotation and soil fumigation to control these diseases. "Induced suppression" is a natural phenomenon whereby a soil due to microbiological changes converts from conducive to suppressive to a soilborne pathogen during prolonged monoculture of the susceptible host. Our studies have focused on the wheat root disease "take-all," caused by the fungus *Gaeumannomyces graminis* var. *tritici*, and the role of bacteria in the wheat rhizosphere (rhizobacteria) in a well-documented induced suppression (take-all decline) that occurs in response to the disease and continued monoculture of wheat. The results summarized herein show that antibiotic production plays a significant role in both plant defense by and ecological competence of rhizobacteria. Production of phenazine and phloroglucinol antibiotics, as examples, account for most of the natural defense provided by fluorescent *Pseudomonas* strains isolated from among the diversity of rhizobacteria associated with take-all decline. There appear to be at least three levels of regulation of genes for antibiotic biosynthesis: environmental sensing, global regulation that ties antibiotic production to cellular metabolism, and regulatory loci linked to genes for pathway enzymes. Plant defense by rhizobacteria producing antibiotics on roots and as cohabitants with pathogens in infected tissues is analogous to defense by the plant's production of phytoalexins, even to the extent that an enzyme of the same chalcone/stilbene synthase family used to produce phytoalexins is used to produce 2,4-diacetylphloroglucinol. The defense strategy favored by selection pressure imposed on plants by soilborne pathogens may well be the ability of plants to support and respond to rhizosphere microorganisms antagonistic to these pathogens.**

Growth and reproduction of the same plant species at the same sites year after year is the norm in natural plant communities. Crop rotation (alternate, pure-stand plantings of taxonomically very different plant species) is a novelty of agriculture used to manage soilborne pathogens and has no ecological equivalent in natural plant ecosystems. The continual presence of the same and related plant hosts in natural plant communities should favor pathogens below as well as above ground, yet curiously natural selection has produced abundant examples of useful genetic resistance to above-ground but not to below-ground pathogens. Selection pressure imposed by soilborne pathogens may favor a different defense strategy—namely, plants with the ability, during monoculture, to support and respond to populations of rhizosphere microorganisms antagonistic to their pathogens. The natural plant defense provided by microorganisms in the rhizosphere deserves much more scientific attention, both because of its importance in plant ecology and because of its potential as a practical means to improve the health and productivity of crop plants.

We have focused on wheat rhizosphere bacteria (rhizobacteria) active against the pathogenic, soilborne fungus *Gaeumannomyces graminis* var. *tritici*, which is the cause of the root disease of wheat known as "take-all." These beneficial bacteria are associated with a spontaneous take-all decline that occurs after two or three successive outbreaks of the disease with continuous cropping to wheat (1). Take-all decline is the most studied of several documented cases of "induced suppression" to a plant pathogen that develops in soil with monoculture of the susceptible host (2). The suppressiveness to take-all holds even when virulent inoculum of the pathogen is added to soil diluted by as much as 1:100 with disease-conducive soil (3). Studies of this suppression have pointed to a role of rhizobacteria of fluorescent *Pseudomonas* spp. inhibitory to the take-all pathogen (3–5). A population shift toward both higher numbers and a higher percentage of wheat rhizosphere-inhabiting fluorescent *Pseudomonas* spp. inhibitory *in vitro* to *G. graminis* var. *tritici* occurs as the soil undergoes conversion from conducive to suppressive to take-all (6). Yield increases of up to 33% have been obtained in performance trials using a seed-treatment method of application of fluorescent *Pseudomonas* spp. in fields infested with the take-all pathogen (refs. 7 and 8; R.J.C. and D.M.W., unpublished results).

Some of the earliest studies on mechanisms of plant defense by rhizobacteria focused on the ability of the antagonist to produce siderophores with high affinity for iron, such as pyroverdin, thought to starve the pathogen for iron during its prepenetration and penetration activities in the rhizosphere (9–11). Competition for iron by rhizobacteria of fluorescent *Pseudomonas* spp. can potentiate competition for carbon by nonpathogenic fusaria interacting with the pathogen *Fusarium oxysporum* in the rhizosphere of plants in fusarium wilt-suppressive soils (12). In most cases, however, success in plant defense by these beneficial fluorescent pseudomonads also requires the ability to produce one or more antibiotics (13).

Role of Antibiotics in Take-All Suppression

Production of phenazine-1-carboxylate (PCA) accounts for 50–90% of take-all suppression by *Pseudomonas fluorescens* 2-79 (14), a strain obtained originally from the rhizosphere of

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Abbreviations: PCA, phenazine-1-carboxylate; Phz$^-$, phenazine-deficient; Phl, 2,4-diacetylphloroglucinol; ORF, open reading frame; CHS/STS, chalcone synthase/stilbene synthase.

*To whom reprint requests should be addressed.

wheat grown in a take-all suppressive soil (15). Phenazine-deficient (Phz^-) mutants produced by Tn*5* mutagenesis failed to inhibit *G. graminis* var. *tritici in vitro* and were coordinately reduced in their ability to suppress take-all on wheat plants *in situ*. However, when these mutants were restored for the ability to produce PCA by complementation with homologous DNA from a genomic library of strain 2-79, the complemented strains were also fully restored for the ability both to inhibit *G. graminis* var. *tritici in vitro* and to suppress take-all *in situ*. Similar results have been obtained using these same protocols with *Pseudomonas chlororaphis* strain 30-84, which produces three phenazines (16), and *P. fluorescens* strain Q2-87, which suppresses take-all by production of 2,4-diacetylphloroglucinol (Phl) (17, 18). Mazzola *et al.* (19) found by the converse experimental approach that, with certain interesting exceptions, isolates of *G. graminis* var. *tritici* more and less sensitive to PCA *in vitro* were, respectively, more and less suppressed by strain 2-79 *in situ*. Similarly those isolates of the pathogen more and less sensitive to Phl *in vitro* were, respectively, more and less suppressed by strain Q2-87 *in situ*.

Direct evidence for *in situ* production of antibiotics in the rhizosphere was provided using high-pressure liquid chromatography; phenazines were detected in extracts from roots and associated soil when seeds used to produce the plants were treated with Phz^+ strains of 2-79 and 30-84 but not when treated with Phz^- mutants of these strains (20).

Mazzola *et al.* (21) used the Phz^- mutants and Phz^+ wild-type and complemented strains of 2-79 and 30-84 to show the importance of antibiotic-producing ability for ecological competence of these rhizobacteria. They started with a one-time introduction of each strain genetically marked (to facilitate monitoring) into samples of natural or pasteurized soil with and without *G. graminis* var. *tritici* and then planted the soils five times to wheat on a 20-day cycle at 15°C. Phz^- strains declined significantly faster than Phz^+ strains and were near or below the level of detection by the fifth cycle of wheat in natural soil in the absence of added inoculum of *G. graminis* var. *tritici*. In contrast, Phz^- and Phz^+ strains survived equally well in pasteurized soil, pointing to the importance of competition with indigenous, rhizosphere microorganisms in the more rapid decline of Phz^- strains in the natural soil. Antibiotic-producing ability was also less critical to maintenance of populations of the introduced strains in the natural soil in the presence than in the absence of *G. graminis* var. *tritici*. This could mean that nutrients in the form of root-lesion exudates were so much more plentiful than from healthy roots as to override the importance of competition from indigenous rhizosphere microorganisms, at least during the five 20-day cycles of the experiments. Evidence has been accumulating for decades to suggest that defense by a natural microbiota accounts for certain examples of susceptible plants and animals remaining surprisingly free of infections in spite of ample exposure to virulent inoculum of pathogens. However, these results unambiguously show that antibiotic production in natural habitats plays a significant role in both plant defense by and ecological competence of soil microorganisms providing this defense.

Antibiotic Genes and Their Regulation

A few model systems have begun to emerge on the genetics of antibiotic production by rhizobacteria, and from these, certain key elements and similarities are becoming apparent. Genes tend to be clustered and at least some regulatory genes are linked. In addition, there appear to be three levels of regulation: a primary level that involves environmental sensing, a secondary (or intermediate) level that ties regulation of antibiotic biosynthesis into other metabolic processes through global regulation and cellular homeostasis, and a highly specific tertiary level involving regulatory loci that are linked and divergently transcribed from the structural genes for antibiotic biosynthesis genes. These principles can be illustrated with the oomycin A model (22).

P. fluorescens Hv37a provides protection of germinating cotton seeds against damping-off caused by *Pythium ultimum*. At least 50% of the activity of Hv37a is due to its production of the antibiotic oomycin A (23), the biosynthesis of which requires glucose and involves the expression of at least eight genes. At the primary level of regulation, glucose sensing depends on the membrane-bound enzyme glucose dehydrogenase, the activity of which is mediated by the products of the *afuAB* operon. When glucose is present, the product of *afuP* at the second regulatory level activates expression of the tertiary regulator *afuR*, enabling transcription of the divergently transcribed biosynthetic operon *afuEFG*. In the uninduced cell, the product(s) of the secondary regulator *cin* prevents transcription of *afuR* and *afuEFG*, apparently by limiting the activity of the *afuP* gene product. The tertiary level of regulation also includes a positive feedback loop thought to depend on the product of *afuR*, with oomycin A as a coinducer, making biosynthesis of the antibiotic cell-density dependent.

P. chloroaphis strain 30-84 produces PCA, 2-hydroxyphenazine-1-carboxylic acid, and 2-hydroxyphenazine (24). At least four clustered genes are present in the region necessary for antibiotic biosynthesis: *phzB*, encoding a 55-kDa protein involved in the biosynthesis of PCA; *phzC*, encoding a 19-kDa protein involved in the biosynthesis of 2-hydroxyphenazine-1-carboxylic acid (24); and two regulatory genes, *phzR* and *phzI*, situated upstream of *phzB* and *phzC* and with sequence similarity to *luxR* and *luxI*, respectively (25, 26). *phzR* as the tertiary-level regulator encodes a 27-kDa protein similar to the transcriptional activator *lasR* of *Pseudomonas aeruginosa*. *phzR* and *phzI* appear to be functionally analogous to *luxR* and *luxI* in that *phzR* activates transcription of phenazine genes in trans and in response to the product of the *phzI* gene. The *luxR* family of positive transcriptional activators is thought to control the expression of target genes in a cell density-dependent manner by sensing the accumulation of a diffusible homoserine lactone autoinducer encoded by *luxI* (27). This provides a mechanism by which expression of phenazine biosynthetic genes is cell-density dependent. The production of phenazine antibiotics is known to be influenced by a variety of environmental conditions, but the specific signals that function at the primary level of antibiotic synthesis in this strain are not understood at the molecular level.

At least two loci are required for production of PCA by *P. fluorescens* strain 2-79 (26). The first, which encompasses the major biosynthetic region, consists of two adjacent, divergently transcribed units of approximately 5 kb and 0.75 kb that are strongly and weakly expressed, respectively, under conditions favorable for production of PCA. These regions are thought to be structurally and functionally analogous to *phzB* and *phzR*, respectively. Preliminary DNA sequence analysis within the *phzB* region has shown similarity to genes encoding *p*-aminobenzoate and anthranilate synthases (V. Kseuzenko, D. Mavrodi, and L.S.T., unpublished results), which is consistent with the active participation in the proposed PCA biosynthetic pathway.

A second locus contains an intermediate-level regulatory gene designated *phzP* that is unlinked to the major biosynthetic genes and broadly distributed among pseudomonads, including those that do not produce phenazines. Mutants in *phzP* exhibit multiple phenotypic changes, including loss of production of PCA and altered colony morphology. In its pleiotropic effects, its size of ≈2.7 kb, and its constitutive expression, *phzP* resembles *lemA* (28), the product of which is thought to function in conjunction with the product of the *gacA* gene as a two-component signal transduction system controlling diverse metabolic processes in *Pseudomonas* (28, 29). The *gacA* locus was originally described in *P. fluorescens* strain CHA0, a

biocontrol agent that inhibits fungal pathogens through production of hydrogen cyanide and the antibiotics pyrrolnitrin, pyoluteorin, and Phl; mutants in *gacA* were deficient in production of all of these inhibitory substances (28). Subsequent studies have shown that *gacA* also controls extracellular protease and phospholipase C in strain CHA0 (30); and it controls colony morphology and production of pyrrolnitrin, cyanide, chitinase, and gelatinase in *P. fluorescens* BL915, a biocontrol agent active against *Rhizoctonia solani* (31). Similar pleiotropic antifungal metabolite-defective mutants have been described in *P. fluorescens* Pf-5, a biocontrol agent effective against *Pythium ultimum* and *R. solani*; these mutants failed to produce pyoluteorin, pyrrolnitrin, Phl, and hydrogen cyanide (32), and they contained transposon insertions in a locus designated *apd*, which is closely related by DNA sequence analysis to *lemA* (33). A second global regulator from strain Pf-5 has high nucleotide similarity with the *rpoS* gene from *Escherichia coli*, which encodes a σ factor with a central role in gene regulation during stationary growth phase; mutants defective in the Pf-5 *rpoS* homologue failed to produce pyrrolnitrin, overproduced pyoluteorin and Phl, and were affected in ecological fitness and biological control activity (34).

P. fluorescens Q2-87 suppresses take-all through production of the broad-spectrum phenolic antibiotic, Phl (17, 18). Six open reading frames (ORFs) have been identified within a 6.5-kb segment of DNA from strain Q2-87 that is sufficient to transfer Phl biosynthetic capability to recipient strains of *Pseudomonas* spp. that did not previously produce the antibiotic (ref. 35; G.B. and L.S.T., unpublished results). ORFs 1 and 2 predict proteins PS1 and PS2 of 389 and 349 amino acids, respectively. Comparison of the derived protein sequences with the nonredundant data base at the National Center for Biotechnology Information (National Library of Medicine) revealed highly significant similarity of PS1 to thiolases that reversibly catalyze the removal or addition of C_2 units to fatty acyl thioester chains. PS2 had strong similarity with members of the chalcone synthase/stilbene synthase (CHS/STS) family of plant enzymes (35) that catalyze the condensation of acyl subunits to substrates such as coumaroyl-CoA in the biosynthesis of flavonoids and phytoalexins by plants. The active site of the CHS/STS enzymes resides in the N-terminal portion of the protein, and a signature sequence of unknown function is conserved in the C-terminal part of all members of the family; PS2 contains both of these conserved domains. These sequence similarities are consistent with PS1 and PS2 functioning in acyl condensation reactions of the sort expected in the synthesis of Phl, a probable polyketide product.

We recognized early in our work that plant defense by antibiotic-producing plant-associated microorganisms in infection courts or as secondary colonists of lesions is analogous to plant defense by production of phytoalexins. The findings of Bangera *et al.* (35) suggest even more than functional and biochemical parallels; they suggest the possibility of gene exchange by plants and their bacterial symbionts or, more likely, a common evolutionary beginning between these two mechanisms of defense. PS2 may be the first protein to be identified in prokaryotes that shows such extensive similarity to the plant CHS/STS protein family. It also is significant that it has little similarity to β-ketoacyl synthases that catalyze similar condensing reactions in prokaryotes.

The PS3 protein showed similarity to members of a large superfamily of transmembrane solute facilitators specific for simple sugars, oligosaccharides, organic acids, organophosphate esters, and drugs. Among the most well-studied representatives of the drug efflux subfamily of permeases are the highly related tetracycline-H^+ antiporters driven by protonmotive force. PS3 exhibits conserved structural features of these integral membrane permeases, including the presence of a central hydrophilic loop surrounded on either side by strongly hydrophobic α-helices. These conserved features suggest that PS3 may function in the export of Phl.

Genes involved in drug efflux typically are negatively regulated; the transport and repressor genes are divergently transcribed from tandem or overlapping promoters. In the Phl locus, ORF 4 is divergently transcribed from ORF 3 and encodes PS4, which has sequence similarity with the Tn*10* family of tetracycline-resistance repressors. This suggests that the Phl locus may share certain organizational and functional features of the tetracycline-resistance locus; the significant difference is that the putative biosynthetic genes encoding PS1 and PS2, as well as the still-uncharacterized genes for PS5 and PS6, have been inserted between the transporter and repressor regions in the Phl locus. This placement of Phl biosynthetic genes downstream of the promoter region, between the permease and repressor genes, presumably would provide both a regulated promoter and a mechanism for export of the antibiotic.

Conservation of Antibiotic-Producing Ability

There is almost unlimited biodiversity by way of genetically different microorganisms that can be isolated from habitats such as the rhizosphere and shown to have activity as members of the community of plant-associated microorganisms suppressive to plant pathogens. In contrast, genetically and geographically very different strains of rhizobacteria commonly produce the same antibiotics.

For example, a role of the antibiotic Phl in plant defense has been shown or suggested for at least five different plant pathogens, including in the Ukraine for *Pseudomonas aurantiaca* against *F. oxysporum* (36, 37), in Switzerland for *P. fluorescens* strain CHA0 against *Thielaviospsis basicola* and *G. graminis* var. *tritici* (38, 39), in Ireland for *Pseudomonas* sp. F113 against *Pythium ultimum* (40, 41), in Texas for *P. fluorescens* Pf-5 against *Pythium ultimum* and *R. solani* (32), and in Washington for *P. fluorescens* Q2-87 against *G. graminis* var. *tritici* (17, 18). Three of six other strains of fluorescent pseudomonads isolated from the same take-all suppressive soil in Washington State as Q2-87 and selected for biological activity against *G. graminis* var. *tritici* also produce Phl (42).

Stabb *et al.* (43) found that 11% of *Bacillus cereus* representing isolates collected worldwide produce the antibiotic zwittermicin. Of the two phenazine-producing strains used in our studies—namely, *P. fluorescens* strain 2-79 and *P. chlororaphis* strain 30-84—both were isolated from the rhizosphere of wheat grown in take-all suppressive soil, but 2-79 is from Washington State and 30-84 is from Kansas (W. Bockus, personal communication). Phenazine antibiotics are well-known secondary metabolites of fluorescent *Pseudomonas* spp. The ability to produce the antibiotic pyrrolnitrin is also conserved among pseudomonads (31, 38, 44, 45).

The ability to produce antibiotics provides the means by which microorganisms defend their habitats, whereas the existence of such genetic diversity of strains with the ability to produce these antibiotics provides the means by which populations of these plant-associated microorganisms have adapted to such a diversity of soil habitats worldwide. This can explain why an antibiotic-producing ability has been conserved in such a diversity of genetic backgrounds.

Induced Resistance

Certain rhizosphere-inhabiting strains of bacteria induced a resistance response in the leaves to the pathogen *Colletotrichum orbiculare* when applied to cucumber seeds (46) and the production of β-1,3-glucanases and chitinases in the leaves of tobacco when applied to tobacco seeds (47). van Peer *et al.* (48) reported that *Pseudomonas* sp. WCS417r induced resistance in carnation to the wilt pathogen *F. oxysporum* f. sp. *dianthi*,

including increased accumulation of phytoalexins in the stems. *P. fluorescens* strain CHA0 produces salicylate (52), which has been implicated in other research (49) as an important signal in the classical systemic resistance response. The possibility of induced resistance points to a role of the host plant as an active participant, rather than simply a passive support system, in this method of defense.

Prospects for Genetic Improvement

Like cultivar development for crop plants, knowing what specific traits to breed and screen for can lead more quickly to better strains. There are also pitfalls to this approach, however, such as to overlook strains with totally unexpected or unpredicted activity. We found such a strain in *P. fluorescens* Q69c-80, which is not inhibitory to *G. graminis* var. *tritici in vitro* yet provides significant defense against take-all *in situ* and has performed more consistently and over a wider geographic area in Washington State than any other strain field-tested to date (D.M.W. and R.J.C., unpublished results). Significantly, when biosynthetic genes for production of either PCA or Phl were transferred on plasmids into this strain, the recombinants exhibited a slight but statistically significant improvement in biological control compared to the parental strain (50). Strain Q69c-80 could be particularly useful as an experimental system as well as a vehicle for strain improvement.

One problem for these symbionts is the same problem encountered for *Rhizobium* and *Bradyrhizobium* spp. with improved nitrogen-fixation efficiency: introduced strains are unable to compete in the rhizosphere with indigenous species of bacteria. Real advances in overcoming this ecological problem must await more knowledge of the genetic control of processes such as plant–symbiont communications and the induction of antibiotic production.

Antibiotic biosynthetic genes also can be used as transgenes expressed in the crop plants themselves. Traits such as antibiotic production for defense against root disease ideally should be placed under control of a root-specific promoter to avoid the potential problems of a toxicant produced in the harvested product used as food or feed. Any attempt to use this method of defense in agriculture must also take into account the importance of multiple mechanisms of suppression by these rhizobacteria. There is considerable variation in sensitivity to PCA and Phl within the populations of *G. graminis* var. *tritici* (19). A mechanism of defense that is not effective against all pathogens or all genotypes of the same pathogen will provide selection pressure in favor of resistant types, and the method of control will fail.

Recently Kim *et al.* (51) discovered a *Bacillus* sp. reported as L324-92 with antibiotic activity against every species, anastomosis group, and strain of the three major root pathogens responsible for take-all, rhizoctonia root rot, and pythium root rot targeted in our program for biological control on wheat. This strain has also shown activity against all three root diseases in growth chamber tests and has produced significant increases in yield of wheat in field tests. Equally significantly, this strain can grow at temperatures down to 10°C or slightly lower, which makes it unique among *Bacillus* spp., which tend not to grow at these temperatures. The three wheat root diseases targeted by our program typically occur when soils are cool as well as moist. The discovery of this strain and its broad-spectrum effectiveness against wheat root diseases gives but a glimpse of the enormous untapped potential for discovery of strains or traits and their deployment in defense against soilborne plant pathogens.

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