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Role of antibiotics and siderophores in biocontrol of take-all disease of wheat

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Abstract

Both antibiotics and siderophores have been implicated in the control of soilborne plant pathogens by fluorescent pseudomonads. In *Pseudomonas fluorescens* 2-79, which suppresses take-all of wheat, the importance of the antibiotic phenazine-1-carboxylic acid was established with mutants deficient or complemented for antiobiotic production and by isolation of the antibiotic from the roots of wheat colonized by the bacteria. Genetic and biochemical studies of phenazine synthesis have focused on two loci; the first is involved in production of both anthranilic acid and phenazine-1-carboxylic acid, and the second encodes genes involved directly in phenazine synthesis. Because the antibiotic does not account fully for the suppressiveness of strain 2-79, additional mutants were analyzed to evaluate the role of the fluorescent siderophore and of an antifungal factor (Aff, identified as anthranilic acid) that accumulates when iron is limiting. Whereas strains producing only the siderophore conferred little protection against take-all, Aff^+ strains were suppressive, but much less so than phenazine-producing strains. Iron-regulated nonsiderophore antibiotics may be produced by fluorescent pseudomonads more frequently than previously recognized, and could be partly responsible for beneficial effects that were attributed in the past to fluorescent siderophores.

Introduction

Certain strains of fluorescent pseudomonads, when introduced on seed or planting material, control plant diseases caused by soilborne pathogens or promote plant growth by suppressing deleterious rhizosphere microorganisms (Schippers *et al.*, 1987; Weller, 1988). These beneficial strains compete aggressively for sites on roots or in the rhizosphere where nutrients are available. Occupation of these sites allows introduced bacteria both to preempt establishment of pathogens on the root (niche exclusion; Suslow, 1982) and to reduce the amount of substrate available to pathogens (Foster, 1986; Weller, 1988). Competition for sites and nutrients is an underlying mechanism that confers a low to moderate level of suppressiveness to most introduced bacteria, but it does not account fully for their effectiveness as biocontrol agents.

Beneficial pseudomonads also antagonize pathogens by producing one or more of a variety of metabolites that include antibiotics (Fravel, 1988; Weller, 1988) siderophores (Leong, 1986; Schippers *et al.*, 1987) and other substances such as cyanide (Voisard *et al.*, 1989). The improvement and eventual commercialization of fluorescent pseudomonads as biocontrol agents depends in part on understanding and exploiting the mechanisms involved in these antagonistic interactions among bacteria, pathogens and their plant hosts.

Mechanisms in the control of take-all

Take-all, caused by *Gaeumannomyces graminis* var. *tritici*, is an important root and crown disease of wheat worldwide. Take-all decline, the spontaneous diminution in disease and increase in yield that occurs with wheat monoculture (Shipton, 1975), provides a natural model for biological control of take-all. Take-all decline results from the accumulation of a microflora antagonistic to *G. g.* var. *tritici* (Cook and Rovira, 1976; Gerlagh, 1968; Shipton, 1975), and fluorescent pseudomonads are an important component of the suppressive microflora. Roots of wheat grown in a take-all suppressive soil have larger populations of fluorescent pseudomonads antagonistic to *G. g.* var. *tritici* than roots grown in soils conducive to the disease. Further, strains from suppressive soils provide greater protection against take-all than do strains from conducive soils (Weller *et al.*, 1988). This strong association between inhibition of *G. g.* var. *tritici in vitro* and suppression of take-all, which differs markedly from the more usual lack of correlation (reviewed in Fravel, 1988) between antagonism *in vitro* and in soil, supports the idea (Weller *et al.*, 1988) that strains from roots grown in suppressive soils already have been selectively enriched by the infected root-pathogen complex for rhizosphere competence, antagonism to *G. g.* var. *tritici* and other uncharacterized traits essential for biocontrol activity. Such strains are a logical starting point for analyses of the mechanisms important in take-all suppression.

Role of phenazine antibiotics

P. fluorescens 2-79, isolated originally from a suppressive soil, inhibited *G. g.* var. *tritici* on both King's medium B and potato dextrose agar, used to detect siderophore and antibiotic production, respectively. Chemically derived mutants defective in either activity also were less suppressive of take-all, suggesting that both antibiotics and siderophores have a role in biocontrol (Weller *et al.*, 1988). A greenish-yellow antibiotic purified from cultures of strain 2-79 initially was identified as a dimer of phenazine-1-carboxylic acid (PCA) (Gurusiddaiah *et al.*, 1986) but later was shown to exist as the monomer (Brisbane *et al.*, 1987). The antibiotic was inhibitory to a variety of fungi, including the wheat pathogens *G. g.* var. *tritici*, *Rhizoctonia solani* and several *Pythium* spp. (Gurusiddaiah *et al.*, 1986).

The relationship between PCA production and take-all suppression in 2-79 was further investigated using transposon mutants defective in synthesis of the antibiotic (Phz^-). These mutants were noninhibitory to the pathogen *in vitro* and much less suppressive of take-all than 2-79 (Thomashow and Weller, 1988). Genetic analysis of six mutants revealed that each contained a single Tn*5* insertion and that the insertions in two of the mutants were closely linked. These mutants, when complemented *in trans* with homologous DNA from a 2-79 genomic library, regained the ability to produce PCA (although only at about half the wild-type level) and to inhibit the pathogen *in vitro*. Introduction of the wild-type sequences into the mutant genomes fully restored PCA production *in vitro* and suppressiveness of take-all.

Phz^- mutants were detected on roots in numbers similar to the parental strain in both steamed and natural soils, indicating that differences in suppressiveness were not due to failure of the mutants to colonize the root and the rhizosphere. Although antibiotic production apparently does not contribute to competitiveness in the short term (11–13 days), its role in long-term colonization remains uncertain. Results from this and other (Schippers *et al.*, 1987; Voisard *et al.*, 1989) studies suggest that uncharacterized and unselected rhizospere competence traits need not be lost during genetic manipulations *in vitro*. The loss of such traits, if frequent, would limit the value of the molecular approach in genetic analyses of biocontrol determinants as well as future strain improvements.

Isolation of antibiotic from the rhizosphere of wheat

Although many microorganisms from the soil produce inhibitory substances *in vitro*, there is little direct evidence that they do so in nature (Williams, 1982). Further, almost all of the evidence for the involvement of antibiotics in biocontrol is indirect or circumstantial (reviewed

in Fravel, 1988; Weller and Thomashow, 1989). Because of the fundmental uncertainty about the role of antibiotics in natural habitats, it was important to demonstrate directly that PCA production on roots of wheat was responsible for suppression of take-all.

Washings from the roots and rhizosphere soil of seedlings colonized by 2-79 were analyzed for the presence of PCA after fractionation by high pressure liquid chromatography. The antibiotic was identified by its chromatographic retention time and UV-visible spectrum, both of which were indistinguishable from those of purified PCA. In growth chamber studies, seedlings colonized by strain 2-79 in steamed soil yielded about tenfold more PCA (up to 350 ng/g root with adhering soil) than seedlings in natural soils (28–43 ng) in the presence or absence of *G. g.* var. *tritici*. The population size of 2-79 on roots from steamed soil (1.4×10^8 colony-forming units (cfu)/g root) also was larger than on roots from natural soil (approximately 1.6×10^7 and 2×10^6 cfu/g root in the presence and absence, respectively, of the pathogen). Seedlings grown in a virgin field plot without introduced *G. g.* var. *tritici* were directly comparable both in terms of PCA yield and 2-79 population with seedlings grown without introduced pathogen in the growth chamber. No antibiotic was detected from roots of seedlings colonized by a Phz$^-$ mutant or grown from nontreated seed. Finally, in both steamed and natural soils, roots from which PCA was recovered had significantly less disease than roots from which no PCA was isolated, indicting that suppression of take-all is related directly to the presence of the antibiotic in the rhizosphere.

The finding that quantities of PCA sufficient to reduce disease were present on roots in the absence of the pathogen was somewhat unexpected. Previous studies (Weller, 1983) had documented the proliferation of strain 2-79 on roots infected with *G. g.* var. *tritici*, leading to the hypothesis (Weller *et al.*, 1988) that PCA production occurs mainly within take-all lesions enriched by the leakage of nutrients, and functions to limit secondary infections by the fungus. This model is consistent with earlier observations (Cook *et al.*, 1986) that microorganisms in take-all suppressive soil limit disease progress rather than initiation, an idea originally put forth by Shipton (1975). Recently, however, an inverse relationship between the number of take-all lesions and the population size of 2-79 present on roots was demonstrated (Bull, 1987), suggesting that if large enough populations are established early enough, even lesion initiation may be limited. No such effect was observed when Phz$^-$ mutants were introduced. The finding that populations of 2-79 on uninfected roots can produce biologically relevant amounts of PCA strongly implicates the antibiotic in reducing infection incidence.

That significant take-all control occurred in the presence of as little as 25–30 ng of PCA per gram of root is consistent with efficient delivery of the antibiotic to the pathogen whether in lesions or prior to their development, but a more complex targeting mechanism probably is needed in the latter case. Bacteria grow on roots as discrete microcolonies in sites enriched in root exudates, lysates or mucilage (Foster, 1986), and the antibiotic also is likely to be more concentrated in these areas. If the passages by which fungi gain access to the root are favored sites for bacterial growth, as proposed by Foster (1986), then occupation of such sites by PCA-producing antagonists would position them strategically for antibiotic delivery.

Relative importance of antibiotics and siderophores

Disease ratings of seedlings treated with Phz$^-$ mutants of 2-79 are intermediate between those of the parental strain and the nontreated control (Thomashow and Weller, 1988), indicating that PCA is not the sole factor responsible for suppressiveness. Siderophores produced by fluorescent pseudomonads have been implicated as a control mechanism for many pathogens (reviewed in Leong, 1986; Schippers *et al.*, 1987) including *G. g.* var. *tritici* (Kloepper *et al.*, 1980; Weller *et al.*, 1988; Wong and Baker, 1984), although not all studies have supported their importance in take-all control (*e.g.* Brisbane and Rovira, 1988). To evaluate the importance of the fluorescent siderophore produced by strain 2-79, Hamdan (1988) characterized mutants deficient in production of PCA (Phz$^-$), the fluorescent

siderophore (Flu^-) or both factors (Phz^-Flu^-). These studies revealed that two Phz^- mutants, 2-79-B46 and 2-79-782 (Thomashow and Weller, 1988) also failed to produce an antifungal factor (Aff) that accumulated in iron-limited cultures of 2-79 but was distinct from the fluorescent siderophore. Mutants at this locus therefore were $Phz^-Aff^-Flu^+$ as compared to phenazine mutants at other loci which were $Phz^-Aff^+Flu^+$. Moreover, $Phz^-Aff^+Flu^-$ mutants were inhibitory to *G. g.* var. *tritici* on King's medium B whereas $Phz^-Aff^-Flu^+$ strains were not, indicating that the Aff factor, rather than the fluorescent siderophore, is responsible for inhibition by strain 2-79 *in vitro* under iron-limiting conditions.

Iron availability in soil increases as a function of decreasing pH, and siderophore production occurs only in environments low in available iron. Suppressiveness associated with siderophore activity therefore is expected to be greatest in high pH soils low in available iron (reviewed in Baker *et al.*, 1986). In contrast, strain 2-79 requires iron for phenazine production (Gurusiddaiah *et al.*, 1986; Weller *et al.*, 1988) and activity of the antibiotic *in vitro* increases with decreasing pH (Brisbane and Rovira, 1988). Because of the potential impact of pH and iron availability on factors contributing to suppressiveness, Hamdan's strains were compared for ability to control take-all in two soils, one of high pH (7.6) and low extractable iron (6.75 μg/g) and the other of low pH (5.5) and higher extractable iron (93.7 μg/g).

In both soils, seedlings treated with Phz^- mutants were significantly more diseased than those treated with strain 2-79 regardless of the Flu or Aff phenotype (Hamdan, 1988). The relationship between soil pH and suppressiveness due to PCA was examined further with strain 2-79 or a $Phz^+Aff^+Flu^-$ mutant in steamed soil adjusted to pH values from 4.9 to 8.0. Both strains suppressed take-all over the full pH range tested (B.H. Ownley and D.M. Weller, personal communication). These results provide additional evidence for the importance of PCA as a major determinant of suppressiveness, but conflict with *in vitro* data and theoretical considerations (Brisbane *et al.*, 1987; Brisbane and Rovira, 1988) suggesting that PCA might be ineffective in alkaline environments. Perhaps the simplest explanation for this apparent discrepancy is that the pH in the microsites where the bacteria and the pathogen interact may be significantly lower than that of the surrounding bulk soil, or even of the rhizosphere in general.

Comparisons of a $Flu^-Phz^+Aff^+$ mutant with 2-79, and of $Flu^-Phz^-Aff^+$ and $Flu^+Phz^-Aff^+$ mutants, revealed no significant difference in suppressiveness between the paired strains. Further, a $Flu^+Phz^-Aff^-$ mutant was no more protective than one deficient in all three factors at either pH 7.6 or 5.5 (Hamdan, 1988). Collectively, these results based on the use of genetically defined mutants suggest that the fluorescent siderophore has a minor role, if any, in control of take-all. This conclusion differs from that of a previous report (Weller *et al.*, 1988) implicating the siderophore in disease suppression. However, the earlier study used less-chacterized, chemically derived nonfluorescent mutants that also were somewhat reduced in inhibition of *G. g.* var. *tritici in vitro* in potato dextrose agar, suggesting that they may have been pleiotropically altered in antibiotic production.

Seedlings treated with $Aff^+Flu^+Phz^-$ or $Aff^+Flu^-Phz^-$ strains were less diseased than when treated with Aff^- counterparts of these strains. The differences were much smaller than those between Phz^+ and Phz^- strains and were significant only in the pH 7.6 soil, however (Hamdan, 1988), indicating that Aff contributes much less than PCA to suppressiveness, and then only under certain conditions. The ability of Aff^+ strains to provide protection in soil at pH 7.6 but not at pH 5.5 may be related to the greater availability in the latter soil of iron, which negatively controls accumulation of the Aff product *in vitro*.

Although factors negatively regulated by iron apparently are of much less importance than the phenazine antibiotic in control of take-all by 2-79, iron-sensitive mechanisms contribute substantially to pathogen suppression or plant growth promotion by fluorescent pseudomonads in other systems (Leong, 1986; Schippers *et al.*, 1987). Siderophores generally have been considered responsible for these phenomena because

beneficial effects were correlated with their presence or presumed presence, and these effects were reversed upon addition of iron. A few recent studies (Loper, 1988; Schippers *et al.*, 1987) using well-defined mutants have clearly documented the importance of siderophores in biocontrol, but the methods in many of the earlier studies would not have distinguished between effects due to siderophores and those due to antibiotics such as Aff, both of which would have been present under the same conditions. The ability to produce nonsiderophore inhibitors in response to iron limitation may be more broadly distributed among fluorescent pseudomonads than previously realized. Nonfluorescent Tn*5* mutants of *P. fluorescens* M4-80R and *P. putida* L30b-80 remained inhibitory to *G. g.* var. *tritici* in iron-limited media (Hamdan, 1988), and an antibiotic fungistatic to *Pythium ultimum* in iron-limited cultures of *Pseudomonas* sp. NZ130 recently was described (Gill and Warren, 1988). If such substances are of common occurrence, then the relative contribution of siderophores to disease control may need to be reevaluated.

Genetics and biochemistry of phenazine production

Genetic and biochemical studies of phenazine production have focused on two loci, the first of which is required for production of both PCA and the antifungal factor Aff. The ability of single Tn*5* insertions at this locus to cause multiple phenotypic changes serves as an important reminder that even 'clean' transposon mutants can be pleiotropically altered, and the effects can be both relevant and not immediately apparent.

The Aff factor from 2-79 cultures was chromatographically and spectrally indistinguishable from anthranilic acid. Further, anthranilate was inhibitory to *G. g.* var. *tritici* at concentrations comparable to those in 2-79 culture supernates. Anthranilate is the first intermediate in tryptophan biosynthesis and is derived from chorismate, which also is a phenazine precursor. Although the enzyme responsible for the initial step in phenazine synthesis shares features in common with anthranilate synthase (AS), anthranilate previously was thought not to be a phenazine precursor (reviewed in Turner and Messenger, 1986). However, recent studies of AS in *P. aeruginosa* do not support this conclusion. *P. aeruginosa* has two pairs of related but distinguishable genes for the two heterodimeric subunits of AS. One pair, now designated *phn*AB, complements AS mutants of *Escherichia coli* (Crawford *et al.*, 1986) and is required for production of the phenazine pyocyanin, whereas the other pair, *trp*E and *trp*G, mainly functions in tryptophan biosynthesis (D. Essar, pers. comm.). These results indicate that anthranilate is an early intermediate in phenazine synthesis, and suggest that the Tn*5* insertions in Phz$^-$Aff$^-$ mutants of 2-79 (which do not have a tryptophan requirement) may be in structural or regulatory genes for *phn*AB. Presumably, anthranilate synthesized by the products of these genes then is converted to PCA by a pathway that requires iron for its full expression or activity. Whether iron also has a direct positive or negative influence on the *phn*AB locus *per se* is presently uncertain.

The second locus of interest was identified originally in *P. aureofaciens* 30-84, a take-all suppressive strain that produes two 2-hydroxyphenazine derivatives as well as PCA. The phenazines also are important determinants of suppressiveness in strain 30-84 (L.S. Pierson, pers. comm.). The 30-84 cosmid pLSP259 complemented several different Phz$^-$ mutants of both 30-84 and 2-79, including a 30-84 mutant that produced only PCA and not the 2-hydroxy derivatives. This suggested that pLSP259 might contain genes for at least part of a phenazine biosynthetic pathway. Structural and functional analyses subsequently defined contiguous regions of 2.8 kb and 1.9 kb required for production of PCA and 2-hydroxy-phenazine-1-carboxylic acid, respectively, and an unlinked region necessary for production of 2-hydroxyphenazine. When a subcloned 9.2 kb *Eco*R1 fragment containing the 2.8 and 1.9 kb regions was introduced into *E. coli*, PCA and 2-hydroxyphenazine-1-carboxylic acid were produced (L.S. Pierson, pers. comm.). Further, a 6.3 kb *Eco*R1 fragment subcloned from pPHZ173, a 2-79 cosmid homologous to pLSP259, also was expressed in *E. coli*, again resulting in PCA production. These results pro-

vide strong evidence that the cloned sequences from 30-84 and 2-79 contain genes involved directly in phenazine biosynthesis, and demonstrate the feasibility of mobilizing and expressing such genes in nonproducer organisms.

Future directions

Understanding the mechanisms involved in disease suppression is an essential first step toward improved biological control with existing strains as well as the selection or development of new agents. In strains 2-79 and 30-84, efforts now are focused on identifying the biochemical and genetic mechanisms that regulate phenazine synthesis and the environmental factors that may limit antibiotic production or activity. The goal of these studies is to find ways to manipulate the bacteria, the environment, or both to increase the level or consistency of performance. New and potentially superior biocontrol strains already are being identified on the basis of phenazine production, and hybridization probes based on phenazine biosynthetic genes could increase the speed and reliability of the screening process. Finally, traditional and molecular genetic techniques are available both to modify the kinds and amounts of phenazines by existing strains and to introduce and express phenazine biosynthetic genes in nonproducer organisms that have other desirable attributes. Such engineered strains will have immediate value as research tools and may represent a key to future generations of improved biocontrol agents.

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