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Contribution of Phenazine Antibiotic Biosynthesis to the Ecological Competence of Fluorescent Pseudomonads in Soil Habitats

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Phenazine antibiotics produced by *Pseudomonas fluorescens* 2-79 and *Pseudomonas aureofaciens* 30-84, previously shown to be the principal factors enabling these bacteria to suppress take-all of wheat caused by *Gaeumannomyces graminis* var. *tritici*, also contribute to the ecological competence of these strains in soil and in the rhizosphere of wheat. Strains 2-79 and 30-84, their Tn*5* mutants defective in phenazine production (Phz^-), or the mutant strains genetically restored for phenazine production (Phz^+) were introduced into Thatuna silt loam (TSL) or TSL amended with *G. graminis* var. *tritici*. Soils were planted with three or five successive 20-day plant-harvest cycles of wheat. Population sizes of Phz^- derivatives declined more rapidly than did population sizes of the corresponding parental or restored Phz^+ strains. Antibiotic biosynthesis was particularly critical to survival of these strains during the fourth and fifth cycles of wheat in the presence of *G. graminis* var. *tritici* and during all five cycles of wheat in the absence of take-all. In pasteurized TSL, a Phz^- derivative of strain 30-84 colonized the rhizosphere of wheat to the same extent that the parental strain did. The results indicate that production of phenazine antibiotics by strains 2-79 and 30-84 can contribute to the ecological competence of these strains and that the reduced survival of the Phz^- strains is due to a diminished ability to compete with the resident microflora.

Successful colonization of the rhizosphere by an introduced microorganism usually requires that the microbe not only be well adapted to the rhizosphere but that it also have some selective advantage over the many indigenous microorganisms with potential to colonize that rhizosphere. Characteristics that may enhance the establishment and/or survival of introduced bacteria include a high growth rate relative to that of the indigenous microbial population (7), resistance to adverse environmental conditions (11, 25) or starvation (1, 21), cell motility (14), production of substances that aid in adherence to plant roots (2, 43, 44), and the production of antibiotics (3, 9).

A diverse group of soil microorganisms produces antibiotics when grown in culture (16). It has been suggested that antibiotics may contribute to the survival of microorganisms in soil habitats (3, 9), but support for this concept has been equivocal (16, 51, 52). The inability to recover antibiotics other than from sterilized or nutrient-amended soils traditionally has been cited as evidence that antibiotics are not likely to have a significant role in interactions among microorganisms in natural soils because such soils typically are nutrient impoverished (16). However, antibiotics produced by introduced microorganisms have been detected in natural soils in association with substrates such as seeds (53, 55) or wheat straw (9, 54). The expression of *afuE*, a biosynthesis gene correlated with production of oomycin A by *Pseudomonas fluorescens* HV37a, was detected when this bacterium was introduced into soil as a cotton seed treatment (19). Thomashow et al. (40) recovered phenazine-1-carboxylic acid (PCA) from wheat roots and associated rhizosphere soil colonized by *P. fluorescens* 2-79 or *Pseudomonas aureofaciens* 30-84 but not from roots colonized by isogenic Tn*5* mutants of strain 2-79 or 30-84 deficient in phenazine biosynthesis (Phz^-). Although most soil habitats are deficient in organic substrate, antibiotics may be ecologically important in microsites that possess sufficient substrate, especially carbon sources, to support microbial production of these compounds (4). The rhizosphere, spermosphere, and plant debris are sites that provide sufficient nutrients to support microbial growth. Therefore, these habitats are not only capable of supporting antibiotic production but also likely to be the primary sites of interaction among soil microorganisms (49).

Take-all decline is a natural form of biological control characterized by a spontaneous reduction in the incidence of take-all of wheat, caused by *Gaeumannomyces graminis* var. *tritici*, after one or two severe outbreaks of the disease during continuous wheat monoculture (34). Fluorescent pseudomonads are thought to be, at least in part, responsible for the biological control. Numerous studies have demonstrated the ability of fluorescent pseudomonads to suppress soilborne plant diseases (47). Strains 2-79 and 30-84, when applied as a wheat seed treatment, provide biological control of take-all (12, 48). *P. fluorescens* 2-79 produces the antibiotic PCA (8, 17), and *P. aureofaciens* 30-84 produces mainly PCA but also lesser amounts of 2-hydroxy-phenazine-1-carboxylic acid and 2-hydroxy-phenazine (30). Production of phenazine antibiotics in the rhizosphere is the primary means by which these strains protect wheat roots against infection by *G. graminis* var. *tritici* (30, 39).

Although antibiotic production by strains 2-79 and 30-84 is essential for the biological control of take-all, the contribution of these compounds to the survival of the bacteria in competition with indigenous soil microorganisms is un-

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Source or reference
P. fluorescens		
2-79	Phz$^+$ Rif^s	48
2-79RN$_{10}$	Phz$^+$ Rif^r	48
2-79-B46	2-79RN$_{10}$::Tn*5* Phz$^-$	39
2-79.8A-140	2-79RN$_{10}$::Tn*5* Phz$^-$	This study
2-79-B46R	2-79-B46 Phz$^+$ recombinant	39
P. aureofaciens		
30-84	Phz$^+$ Rif^s	30
30-84R	Phz$^+$ Rif^r (spontaneous)	30
30-84.44-8	30-84R::Tn*5* Phz$^-$	30
30-84.44-8R	30-84.44-8 Phz$^+$ recombinant	30
30-84Z	Phz::*lacZ* chromosomal fusion, Phz$^-$ Rif$^+$	38
E. coli HB101	F$^-$ *hsdS20* (r_B^- m_B^-) *supE44 recA13 ara-14 proA2 lacYI galK2 rpsL20 xyl-5 mtl-5* λ	Bethesda Research Laboratories
Plasmids		
pLAFR3	Tc^r	36
pPHZ6.3	pLAFR3 containing *P. fluorescens* 2-79RN$_{10}$ genomic DNA, Tc^r	This study
pRK2073	Tra$^+$ Sp^r	5

[a] Rif^r, Sp^r, and Tc^r indicate resistance to rifampin, spectinomycin, and tetracycline, respectively.

known. PCA has broad-spectrum activity against bacteria and fungi (17, 18) and therefore may be a factor in the ability of producer pseudomonads to compete with other soil or rhizosphere microorganisms. It has been suggested that antibiotic production may aid in the initial colonization of a substrate by soil microorganisms (3). However, Phz$^-$ derivatives of 2-79 and 30-84 were present in the rhizosphere of wheat at populations comparable to those of their respective Phz$^+$ parent strains during the initial 7 to 10 days of plant growth after introduction of these strains as a wheat seed treatment (30, 39). Similarly, Howie and Suslow (19) found no significant differences between populations established initially in the rhizosphere of cotton by an antibiotic-producing parental strain, *P. fluorescens* Hv37aR2, and isogenic mutants deficient in antibiotic biosynthesis. The advantage of antibiotic production may only become apparent over longer time periods; this has not been examined in the work to date. The objective of this study was to evaluate the role of phenazine biosynthesis in the ecological competence of *P. fluorescens* 2-79 and *P. aureofaciens* 30-84 in competitive soil and rhizosphere environments and over experimental periods of longer duration than those which have previously been studied.

MATERIALS AND METHODS

Organisms and plasmids. The bacterial strains and plasmids used in this study are described in Table 1. The rifampin-resistant derivatives of *P. fluorescens* 2-79 and *P. aureofaciens* 30-84 were used as phenazine-producing wild-type strains in this study. Although the phenazine-deficient strain 2-79-B46 is prototrophic with a growth rate in minimal media equal to that of the parental strain, it is unclear whether this mutant is impaired specifically in phenazine biosynthesis (39). The Tn*5* insertion in 2-79-B46 resides outside the major phenazine biosynthetic locus, in a gene of unknown function (39). Therefore, the Phz$^-$ strain 2-79.8A-140, with Tn*5* present in the phenazine structural locus, was constructed to verify the results obtained with strain 2-79-B46. A 6.3-kb *Eco*RI fragment from the major phenazine biosynthetic locus of strain 2-79 (37) was ligated into the vector pLAFR3 (37) to form pPHZ6.3. Random mutations were introduced into pPHZ6.3 with a defective λ::Tn*5* phage (13). A plasmid containing a Tn*5* insertion in the 6.3-kb *Eco*RI fragment was identified by restriction enzyme analysis. This plasmid, pPHZ6.3::Tn*5*-8A, was introduced into strain 2-79 by triparental conjugation with the helper strain *Escherichia coli* HB101(pRK2073) (5). Transconjugates were selected on nutrient yeast extract (NBY) agar (45) containing rifampin, kanamycin, and tetracycline at 75, 50, and 25 μg/ml, respectively. Homologous recombination, with Tn*5* present in the chromosomal copy of the biosynthetic locus, occurred after growing transconjugates for several generations in LB broth without antibiotic selection. The desired recombinants (kanamycin resistant and tetracycline sensitive) were replica plated onto NBY–2% glucose agar. Loss of phenazine production was confirmed by loss of colony pigmentation and absence of a UV-absorbing shadow around the bacterial colony under long-wave (365-mm) UV irradiation. Strain 2-79 was used as the phenazine-producing control. The resulting Phz$^-$ strain was designated 2-79-8A.140.

The isolate of *G. graminis* var. *tritici* was derived from a single ascospore and stored at 4°C on one-fifth-strength potato dextrose agar (4 g of glucose, extract of 40 g of fresh potatoes, and 20 g of agar). The inoculum used to infest soil was prepared by growing the fungus on sterilized oat grains as previously described (50).

Soil and soil treatment. All experiments were conducted in Thatuna silt loam (TSL; 17.6% sand, 64% silt, 18.4% clay [pH 6.15]) obtained from the Washington State University Plant Pathology Research Farm, Pullman. Soil was collected from the upper 15 cm of the soil profile and passed through a 0.5-cm-mesh screen prior to use. Oat grain inoculum of *G. graminis* var. *tritici* was ground in a Waring blender and passed through a series of screens of various mesh. Particles ranging from 0.25 to 0.5 mm in diameter were used to infest soil (0.45 g of inoculum per 100 g of soil) (50). Natural TSL amended with oat inoculum of *G. graminis* var. *tritici* is hereafter referred to as infested TSL. Pasteurization of soil was conducted by treating moist TSL with a steam-air mixture (95°C) for 90 min.

Individual soil samples were treated with a single Phz$^+$ or Phz$^-$ bacterial strain that was derived from either *P. fluorescens* 2-79 or *P. aureofaciens* 30-84. All bacterial strains were grown on NBY agar at 25°C. After 48 h, 10 ml of sterile water was added to each plate and bacteria and water were scraped into a sterile test tube. Cells, collected from a single plate, were washed twice in sterile distilled water, collected by centrifugation, and then suspended in 120 ml of sterile water. The bacterial suspension was applied to 13 kg of either noninfested TSL or infested TSL as an atomized mist produced by use of a chromatography sprayer and compressed air. Soil was mixed thoroughly during application and dispensed into conical plastic tubes (20.7 by 4 cm [top diameter]; Ray Leach Cone-tainer, Canby, Oreg.) with each tube holding approximately 100 g of soil. Bacterial populations were estimated by collecting five samples from each soil treatment 48 h after bacterial application and preparing serial dilutions of the soil in buffer (0.1 g of each sample to 10 ml of sterile 0.0125 M K_2HPO_4 buffer [pH 7.1]). Aliquots (0.1

ml) of appropriate serial dilutions were spread on King's medium B agar (KMB) (20) amended with rifampin (100 μg/ml) and cycloheximide (100 μg/ml).

Experimental design and growth conditions. Seeds of the spring wheat cultivar Fielder were immersed in a 25% solution of commercial bleach (5.25% sodium hypochlorite) for 3 min, rinsed for 30 min under running tap water, and air dried. Approximately 10 g of seed and 6 ml of sterile water were added to a sterile petri dish (9-cm diameter) lined with Whatman no. 4 filter paper. After 24 h at 20°C, seeds (radicles just emerging) were removed from the dish and one seed was sown in the soil of each tube to a depth of approximately 2 cm. Tubes supported in a hanging position in racks were placed in a controlled-environment chamber at 15°C and with a 12-h photoperiod. Soil matric potential was monitored with a tensiometer (Irrometer, Inc., Riverside, Calif.), and dilute (1:3, vol/vol) Hoaglund's solution (macroelements only) was applied to maintain the soil matric potential no drier than −0.1 MPa.

Soil treatments were arranged in a randomized complete block design with 50 tubes (plants) per soil treatment replicated twice for a total of 100 plants per soil treatment. Plants were grown for 20 days, at which time 10 of the 50 plants for each soil treatment were harvested from each replicate and sampled for population size of the introduced strains as described below. The shoots of the plants not harvested were excised at the soil surface, and the soil and the associated root system in each tube was decanted into a 0.473-liter paper cup, shaken vigorously to aerate and mix, and then returned to the same tube. This "cultivated" soil was incubated for 4 days at 15°C and then replanted with pregerminated wheat seeds. The process of plant growth and harvesting was repeated for a total of either three or five cycles that extended over 68 and 116 days, respectively. As with enrichment procedures in general, the growing of wheat as successive short-term plantings in the same soil increases populations of indigenous wheat rhizosphere microorganisms (32). Therefore, the cycling process exposed the introduced strains to progressively more intense competition from the indigenous rhizosphere colonists over the successive cycles of wheat. All experiments were repeated one time.

Sampling procedures. After each 20-day period of growth, 10 randomly selected plants were removed from the plastic tubes and shaken vigorously to remove all but the most firmly adhering soil. Shoot height from the point of seed attachment to the tip of the longest (second true) leaf was measured. The root segments 3 to 5 cm below the seed from both the radicle and either the −2A or −2B seminal root (22) were excised and placed in 10 ml of sterile phosphate buffer. The root segments and associated rhizosphere soil were sonicated in an ultrasonic cleaner for 60 s, and then serial dilutions of the root wash were plated onto KMB containing rifampin and cycloheximide. Plates were incubated at 20 to 25°C, and colonies were enumerated after 48 h. Root segments were stored in 95% ethanol and later viewed with a compound microscope for the presence of lesions caused by *G. graminis* var. *tritici*.

A portion of the bulk soil from each of the 10 tubes was collected in an 8-ml glass vial as the wheat seedlings were harvested. Soil (0.1 g [dry weight]) was suspended in 10 ml of sterile phosphate buffer, and populations of introduced strains were determined by plating aliquots of serial dilutions onto KMB supplemented with antibiotics as described above. Serial dilutions of the bulk-soil suspension were also plated onto 1/10-strength Difco tryptic soy agar (26) and NPC agar (KMB amended with novobiocin [45 μg/ml], penicillin G [45 μg/ml], and cycloheximide [100 μg/ml]) (33), and colonies were counted after 48 h of incubation at 25°C. Populations of total indigenous aerobic bacteria and total indigenous fluorescent pseudomonads were estimated by subtracting the number of colonies on plates of KMB containing rifampin and cycloheximide (introduced strain) from the number on plates of tryptic soy agar (total aerobic) and from the number of fluorescent colonies on NPC agar (total fluorescent pseudomonads), respectively.

Survival of phenazine-producing and nonproducing strains in soil in the absence of plant roots. Sieved TSL was treated with either strain 30-84 or its Phz^- derivative 30-84Z in the manner described above. Treated soils were placed in the plastic tubes and exposed to the same conditions of temperature and light as described above but without plants. Tubes were arranged in a completely randomized design, and 10 samples were collected for each soil treatment at 10-day intervals for 70 days. Populations of bacteria were determined as described above.

RESULTS

Disease development. Take-all was most severe in *G. graminis* var. *tritici*-infested soils during the first and second plant-harvest cycles. The incidence of take-all declined rapidly during subsequent plant-harvest cycles as evidenced by a reduction in the number of lesions observed on roots of plants grown in infested TSL (data not shown). The average height of plants grown in infested TSL increased from 12.5 to 20.9 cm over the five successive plant-harvest cycles of wheat as the incidence of take-all declined. In contrast, plants grown in TSL without added inoculum of *G. graminis* var. *tritici* generally were healthy after each of the five cycles. The exception was one experiment with natural TSL treated with derivatives of strain 2-79 in which a decline in average plant height from 20.1 cm at the end of cycle 1 to 16.1 cm at the end of cycle 5 was associated with root lesions caused by *Rhizoctonia solani* AG8 and *G. graminis* var. *tritici*. The TSL used in this experiment contained a low concentration of naturally occurring inoculum of both *R. solani* and *G. graminis* var. *tritici* that apparently increased with each successive cycle of wheat.

Populations of indigenous bacteria in bulk soil. Populations of indigenous aerobic bacteria did not differ significantly ($P = 0.05$) among soil samples treated with Phz^+ strains or their respective Phz^- derivatives (data not shown). Similarly, populations of indigenous fluorescent pseudomonads did not differ significantly whether the soil was amended with a Phz^+ or Phz^- derivative of the same strain. Populations of total aerobic bacteria in natural TSL prior to planting were approximately 10^8 CFU/g of soil. After five cycles of wheat, the populations of indigenous aerobic bacteria were near 10^9 CFU/g of soil for all treatments. Populations of indigenous fluorescent pseudomonads were approximately 10^6 CFU/g of soil prior to planting. The population of indigenous fluorescent pseudomonads increased 0.3 to 1 log unit after two plant-harvest cycles but thereafter declined, and after five plant-harvest cycles their population was about 10^6 CFU/g of soil for all treatments (data not shown).

Rhizosphere populations of introduced strains. At the end of cycle 1 in natural TSL, the population of strain 2-79 was significantly larger than either that of 2-79-B46 or that of 2-79-B46R, and there was no significant difference between the populations of the latter two strains (Fig. 1A). However, at the completion of cycles 2 through 5, populations of the

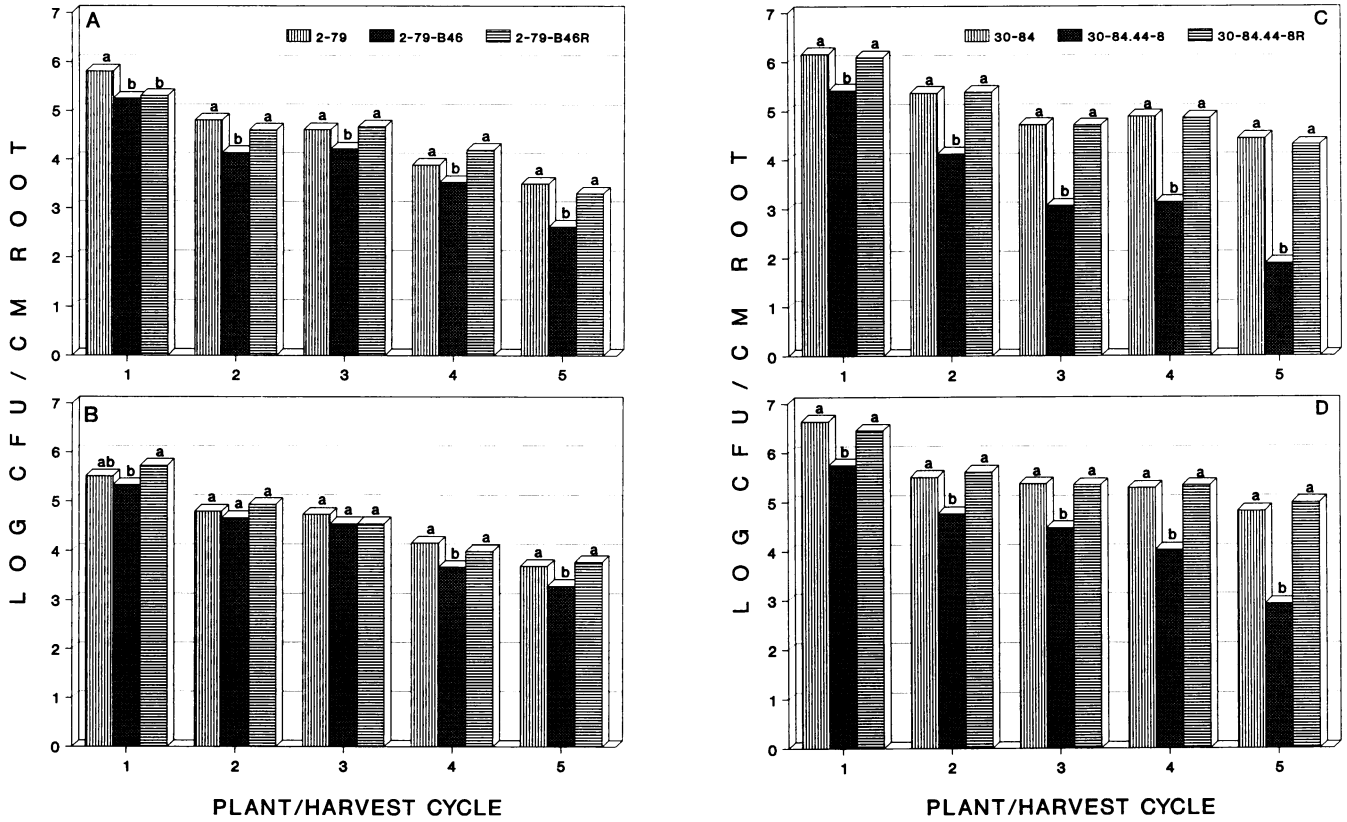

FIG. 1. Populations of strains derived from *P. fluorescens* 2-79 (A and B) and *P. aureofaciens* 30-84 (C and D) in the rhizosphere of wheat grown in TSL (A and C) or in infested TSL (B and D). Each strain was established in a separate soil sample, and rhizosphere populations of the introduced strains were determined after each of five 20-day plant-harvest cycles as described in Materials and Methods. Means for the same cycle designated with the same letter are not significantly different ($P = 0.05$) according to Fisher's protected least significant difference.

Phz^+ strains 2-79 and 2-79-B46R did not differ significantly from each other but both were significantly ($P = 0.05$) larger than that of strain 2-79-B46 (Phz^-). In infested TSL, rhizosphere populations of 2-79 (Phz^+) and 2-79-B46 (Phz^-) were similar for cycles 1 to 3, but thereafter the population of the Phz^- mutant declined more rapidly to populations significantly smaller than those of either of the two Phz^+ strains (Fig. 1B). No significant differences were observed between rhizosphere populations of the two Phz^+ strains over the five cycles of wheat grown in infested TSL.

In a separate study, rhizosphere populations of the Phz^- strain 2-79-8A.140 followed a pattern similar to that observed for 2-79-B46 in relation to that of the parent, strain 2-79. The population of 2-79-8A.140 was significantly ($P = 0.05$) smaller (approximately 0.4 log CFU/cm of root) than that of 2-79 at each of the three successive cycles of wheat (data not shown).

Populations of strains 30-84 and 30-84.44-8R (Phz^+) were significantly larger than that of 30-84.44-8 (Phz^-) at the end of all plant-harvest cycles (Fig. 1C and D). In natural TSL, the difference between rhizosphere populations of Phz^+ and Phz^- derivatives increased progressively with each successive plant-harvest cycle, and by cycle 5 the population of 30-84.44-8 was almost 3 orders of magnitude smaller than the populations of the two Phz^+ strains (Fig. 1C). In infested TSL, the difference in population size between Phz^- and Phz^+ strains increased more slowly than that in natural TSL, but after the fifth cycle, the population of 30-84.44-8 was still nearly 2 orders of magnitude smaller than that of either of the Phz^+ strains (Fig. 1D). Plants grown in infested TSL maintained significantly ($P = 0.05$) larger populations of all three strains derived from 30-84 than did plants grown in natural TSL. Populations of strains 30-84 and 30-84.44-8R were comparable over the five cycles of wheat.

Populations of introduced strains in bulk soil planted with wheat. In bulk (nonrhizosphere) soil planted with wheat, populations of the introduced strains followed trends that were similar to those observed in the rhizosphere of wheat. Initial populations (cycle 0) were approximately 10^7 and 10^8 CFU/g of soil for strains derived from 2-79 and 30-84, respectively. Populations of all introduced bacteria declined progressively over the 116-day duration of the experiment, whether in natural or infested TSL. However, populations of the Phz^- strains declined more rapidly than did those of the corresponding Phz^+ strains (Fig. 2). Differences between populations of Phz^+ and Phz^- strains were apparent at an earlier cycle of wheat for derivatives of 30-84 than for those of 2-79 and earlier in natural than infested TSL (Fig. 2).

Populations of 2-79 and 2-79-B46R were significantly larger than those of 2-79-B46 after cycle 2 in natural TSL, and this difference increased with each successive cycle of wheat (Fig. 2A). In infested TSL, no significant difference was observed between populations of 2-79 and 2-79-B46 through cycle 2 (Fig. 2B). However, the populations of both Phz^+ strains were significantly larger than that of the Phz^- strain after each of the subsequent plant-harvest cycles.

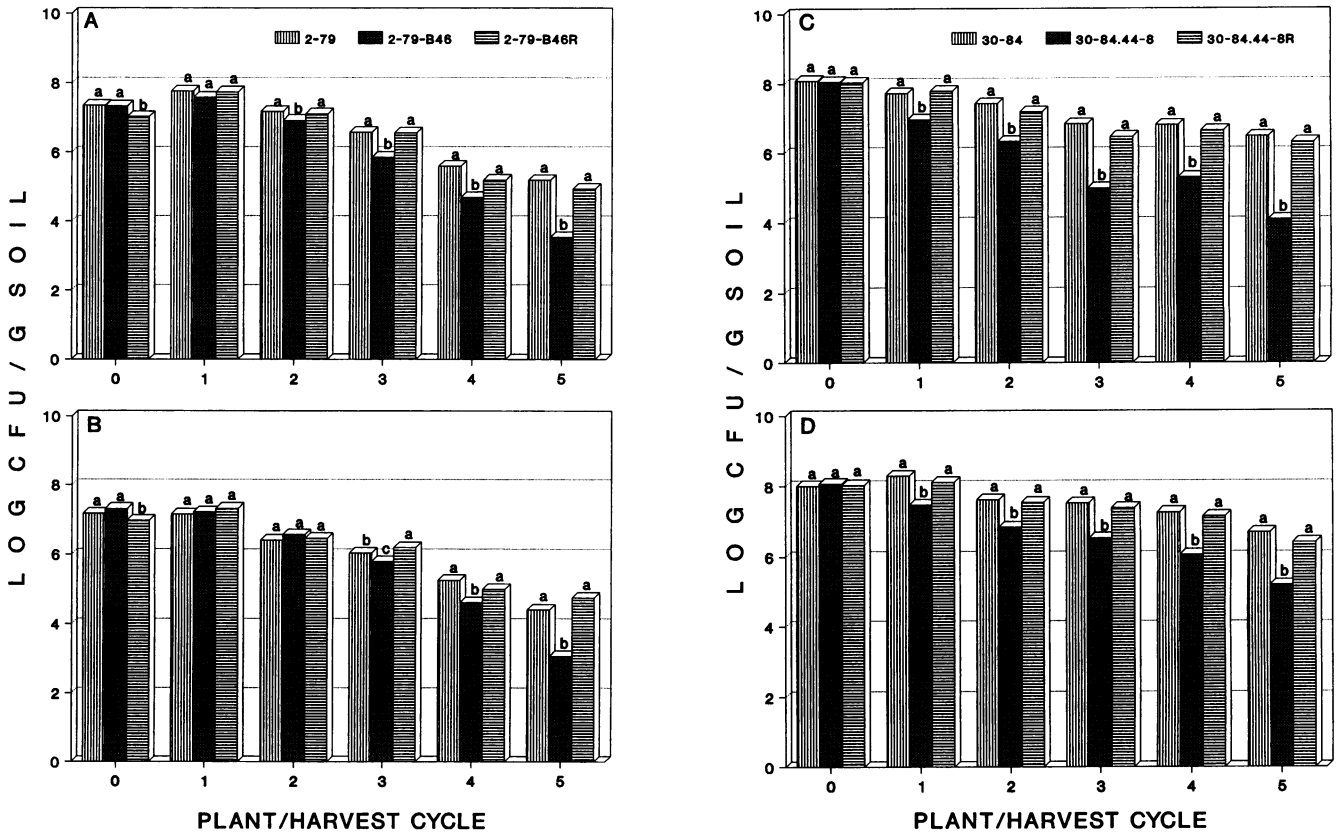

FIG. 2. Soil populations of strains derived from *P. fluorescens* 2-79 (A and B) and *P. aureofaciens* 30-84 (C and D) in TSL (A and C) or in infested TSL (B and D). Each strain was established in a separate soil sample, and populations of the introduced strains were determined after each of five 20-day plant-harvest cycles as described in Materials and Methods. Means for the same cycle designated with the same letter are not significantly different ($P = 0.05$) according to Fisher's protected least significant difference.

Strains 2-79 and 2-79-B46R generally maintained similar soil populations throughout the 116-day duration of the experiment (Fig. 2).

The experiment was repeated (data not shown) and, while populations of the introduced strains followed the same basic trends in infested TSL, a deviation from the initial results obtained with strains derived from 2-79 was observed in natural TSL during the later plant-harvest cycles. Populations of the Phz^+ strains in natural TSL were significantly larger than that of the Phz^- strain after one plant-harvest cycle. This difference increased during the second cycle and then remained unchanged through cycle 4. Surprisingly, there was no significant difference between soil populations of 2-79 and 2-79-B46 at the end of cycle 5; this development coincided with the occurrence of root lesions caused by indigenous populations of *R. solani* AG-8 and *G. graminis* var. *tritici* during cycles 4 and 5.

The population of strain 30-84.44-8 (Phz^-) was smaller than that of strains 30-84 and 30-84.44-8R after the first plant-harvest cycle in both natural and infested TSL (Fig. 2C and D). However, the population of the Phz^- strain declined more rapidly in the absence than in the presence of *G. graminis* var. *tritici* over the next four plant-harvest cycles as determined by a pairwise comparison of the slope parameter of regression. Populations of the introduced strains were significantly ($P = 0.05$) larger in infested TSL than they were in natural TSL for cycles 1 through 3, but thereafter there were no significant differences between populations of these strains in the two soils. As observed for strain 2-79, populations of strain 30-84 and its Phz^+ restored derivative were similar over the entire 116 days (Fig. 2).

Rhizosphere and soil populations in pasteurized soil. After each of three cycles of wheat, plants grown in pasteurized TSL (reduced microbial competition) supported larger rhizosphere populations of both 30-84 and the Phz^- mutant 30-84.44-8 than did plants grown in natural TSL (Fig. 3). Rhizosphere populations of these two strains in pasteurized soil did not differ significantly after each of the three plant-harvest cycles (Fig. 3A). In contrast, in natural TSL, the population of 30-84 was larger than that of 30-84.44-8 after each of the three successive plant-harvest cycles of wheat (Fig. 3B).

Bulk soil populations of strains 30-84 and 30-84.44-8 were significantly ($P = 0.05$) larger in pasteurized TSL than populations of the respective strains in natural TSL after all three plant-harvest cycles (Fig. 3C and D). In pasteurized TSL, populations of 30-84 and 30-84.44-8 remained unchanged (approximately 10^9 CFU/g of soil) through two plant-harvest cycles, and there were no significant differences between soil populations of the Phz^+ and Phz^- strains after cycles 1 and 2 (Fig. 3C). At the end of the third plant-harvest cycle, the population of both the Phz^+ and Phz^- strains in pasteurized TSL had declined 1 to 2 orders of magnitude from the initial population established in this soil, and the population of strain 30-84 was larger by 0.8 log CFU/cm of root than that of its Phz^- derivative (Fig. 3C). In

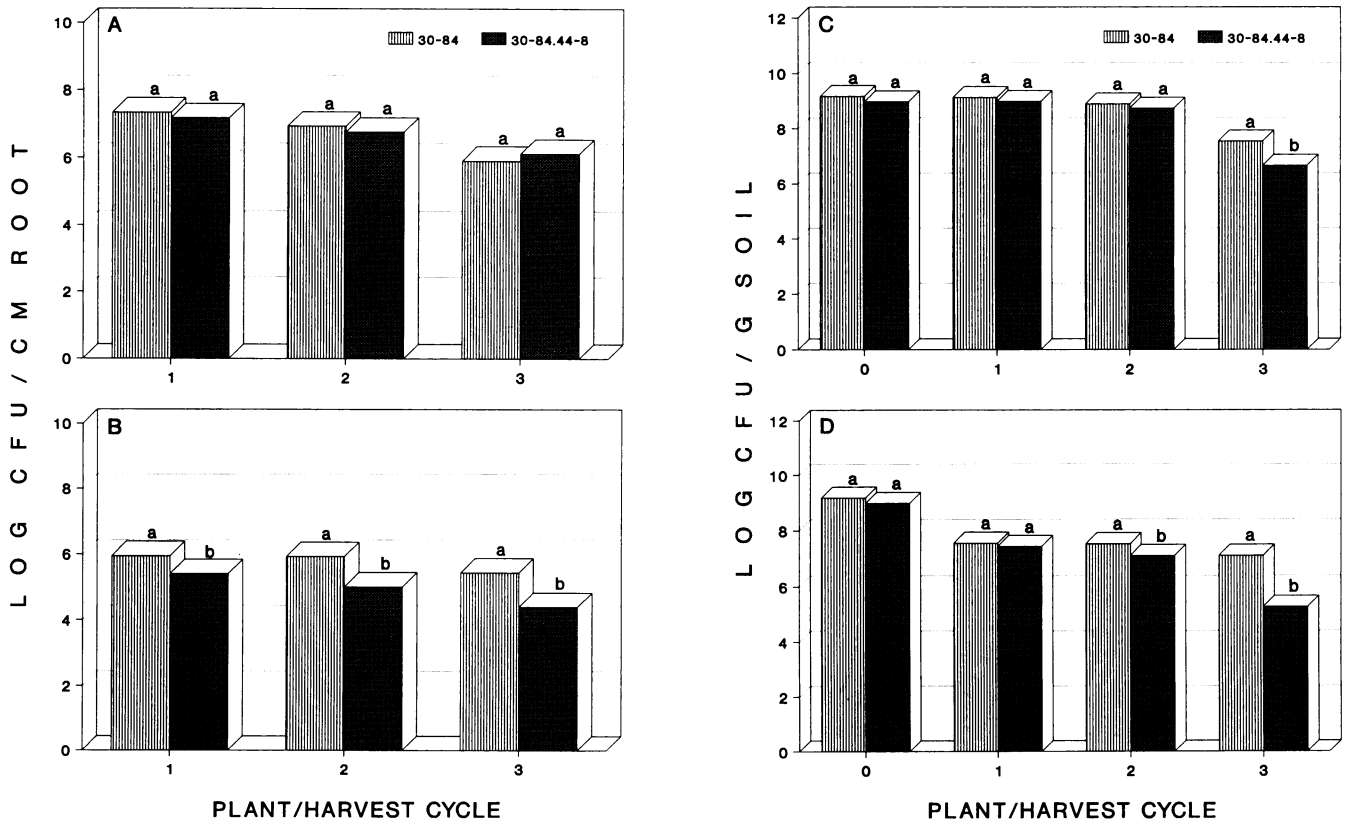

FIG. 3. Rhizosphere (A and B) or bulk-soil (C and D) populations of *P. aureofaciens* 30-84 (Phz^+) and 30-84.44-8 (Phz^-) in natural TSL (B and D) or TSL pasteurized with moist heat at 95°C for 90 min (A and C). Each strain was established at an initial population of approximately 10^9 CFU/g of soil (cycle 0), and the respective infested soils were planted with three successive cycles of wheat. Populations of the introduced strains were determined after each 20-day cycle of plant growth as described in Materials and Methods. Means for the same cycle designated with the same letter are not significantly different ($P = 0.05$) according to Fisher's protected least significant difference.

contrast, in natural TSL, populations of both 30-84 and 30-84.44-8 had already declined by almost 2 orders of magnitude after only one plant-harvest cycle (Fig. 3D). In natural TSL, the population of 30-84 remained relatively constant after the first plant-harvest cycle while that of 30-84.44-8 continued to decline. The population of 30-84 in natural TSL was significantly larger than that of 30-84.44-8 after cycles two and three (Fig. 3D).

Soil populations of introduced strains in the absence of plant roots. Soil populations of strains 30-84 and 30-84Z (Phz^-) declined slightly and similarly over 40 days (Fig. 4). The population of strain 30-84 continued this same gradual decline after 40 days, whereas the population of strain 30-84Z dropped precipitously to a size significantly smaller than that of strain 30-84 (Fig. 4). After 70 days, the population of 30-84Z was 3 orders of magnitude smaller than the population of 30-84.

DISCUSSION

Biosynthesis of phenazine antibiotics contributed to the ecological competence of both *P. fluorescens* 2-79 and *P. aureofaciens* 30-84. The loss of phenazine-producing ability in these two strains resulted in a corresponding reduction in their ability to survive in natural TSL and to colonize the rhizosphere of wheat in competition with indigenous soil and rhizosphere microorganisms. Further, the restoration of phenazine production by genetic complementation coordinately restored rhizosphere colonization and persistence in the complemented strains to a level equivalent to that of the parental strains. The recent works of Thomashow and Weller (39), Thomashow et al. (40), Bull et al. (10), and Pierson and Thomashow (30) demonstrate conclusively that phenazine antibiotics play a role in the biological control of take-all of wheat. The results of our study show that these antibiotics can also be critical to the ecological competence of the producing bacterium. Our goal is to harness antibiotic-producing bacteria for biological control in the rhizosphere, but it is evident that the bacteria evolved this trait for quite a different purpose, namely, as a means to survive under conditions of intense competition in soil and in the rhizosphere.

In contrast to our findings, previous studies have reported that the loss of antibiotic-producing ability did not alter the ability of *Pseudomonas* spp. to colonize the rhizosphere when strains were introduced on seed or seed pieces (19, 23, 30, 39). However, populations of the introduced strains were monitored for a period of no longer than 14 days and only one planting cycle. The results of our study confirm that antibiotic-negative strains may attain populations no different from those of their antibiotic-producing counterparts if studied on a short-term basis. This may be a function of the large populations of the introduced strains that have been established in either soil or on seed in both this and previous studies (19, 30, 39). It is possible that if these strains were established at populations that approximate those present in

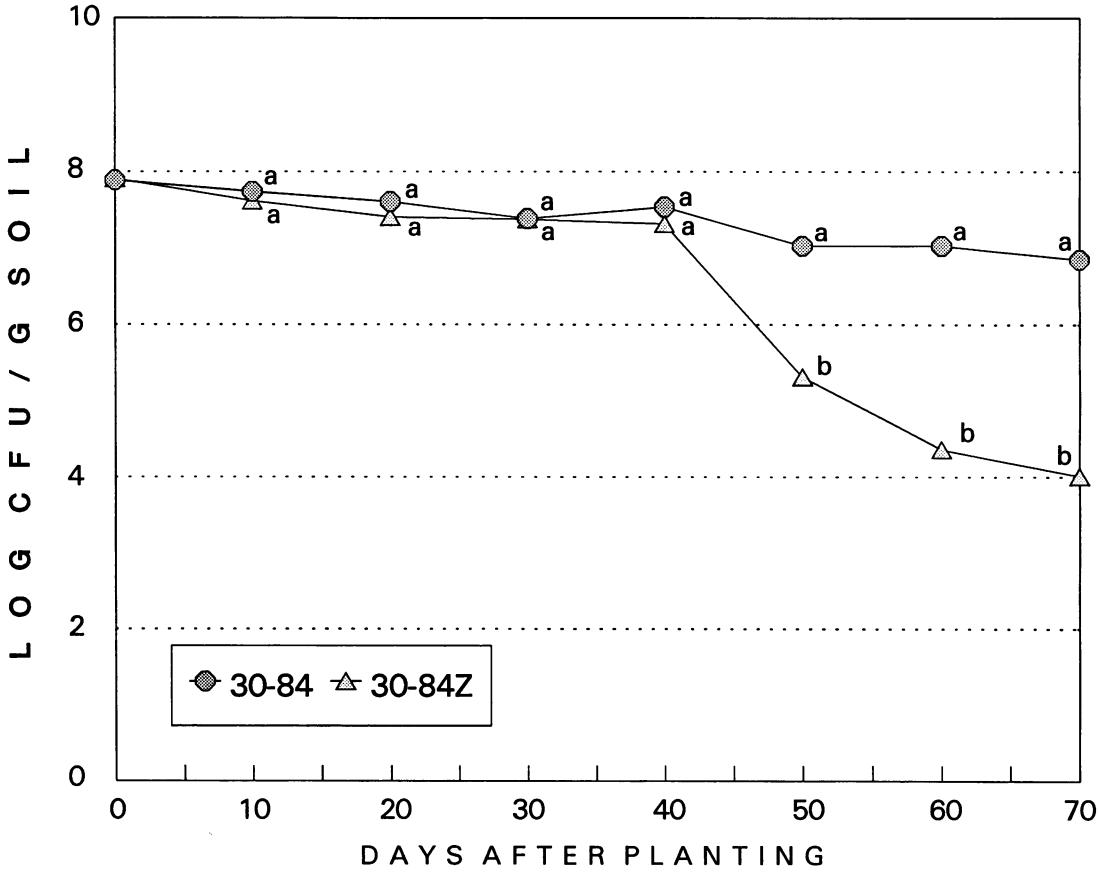

FIG. 4. Populations of *P. aureofaciens* 30-84 and 30-84Z (Phz$^-$) in natural soil in the absence of plant roots. Strains were established in soil at initial populations of approximately 10^8 CFU/g of soil. Means for the same day designated with the same letter are not significantly different ($P = 0.05$) according to Fisher's protected least significant difference.

natural soil, differences between populations of antibiotic-producing strains and the antibiotic-deficient mutants may become apparent earlier in the growing period. There has been increasing interest in determining the contribution of particular compounds, including siderophores, pigments, antibiotics, or unidentified gene products (such as those of avirulence genes), to the survival of bacteria in association with plants. Our findings indicate that time periods of several hours or even several days may not be sufficient to accurately assess the importance of a particular trait for the survival of a bacterium in natural environments.

Studies in natural TSL demonstrated that the production of phenazine antibiotics contributed positively to the persistence of strains 2-79 and 30-84 in soil habitats. However, these studies did not address the mechanism by which phenazine antibiotics enhance the survival of the producing strain. The phenazine antibiotics produced by strains 2-79 and 30-84 have excellent activity against a variety of microorganisms, including a number of fungi and bacteria that are commonly isolated from the rhizosphere of wheat (17, 18). Therefore, studies were conducted in soil pasteurized with moist heat to test the hypothesis that the enhanced survival of phenazine-producing strains was due to an improved capability to compete with indigenous microorganisms. Pasteurization reduced the level of microbial competition as evidenced by the fact that populations of the introduced strains were significantly larger in pasteurized than in natural TSL. In this environment of reduced microbial competition, the Phz$^-$ strain was able to colonize the rhizosphere of wheat to essentially the same extent as the Phz$^+$ strain. In contrast, in natural TSL possessing a high level of microbial competition relative to that present in pasteurized TSL, the ability of the Phz$^-$ strain to colonize the rhizosphere of wheat was markedly reduced in comparison to that of the Phz$^+$ strain. These findings support our hypothesis that phenazine production contributes to the survival of 2-79 and 30-84 by enhancing their ability to compete with the resident microflora.

Although production of phenazine antibiotics contributed to the persistence of both strain 2-79 and strain 30-84, there appeared to be strain-specific differences in the relative contribution of phenazines to the survival of the two strains. Differences between populations of Phz$^+$ and Phz$^-$ strains occurred at an earlier plant-harvest cycle and were of a larger magnitude for strain 30-84 than for strain 2-79. These differences may result from several factors intrinsic to these particular strains. Strain 2-79 produces a single phenazine antibiotic, PCA, while strain 30-84 produces PCA, 2-hydroxy-phenazine-1-carboxylic acid, and 2-hydroxy-phenazine (30). Toohey et al. (41) found that the hydroxyphenazines produced by *P. aureofaciens* are more toxic than PCA to bacteria and algae. *Bacillus subtilis* and *Staphylococcus aureus* were completely inhibited by 2-hydroxyphenazine-1-carboxylic acid at a concentration of 100 μg/ml, while PCA failed to significantly inhibit the growth of these bacteria in liquid culture. It is possible that production of phenazine

antibiotics may contribute more significantly to the overall competitiveness of strain 30-84 than to that of strain 2-79 because of the greater activity of the hydroxyphenazines against soil and rhizosphere microorganisms.

Differences in the relative contribution of phenazine antibiotic production to the survival of strains 2-79 and 30-84 may have resulted from differences in the regulation of phenazine biosynthesis. Growth conditions have a significant effect on the production of phenazines, and conditions favorable for phenazine biosynthesis can vary among strains (42). Production of PCA by some strains of *P. aureofaciens* is sensitive to phosphate concentration (24), but production of phenazines by *Pseudomonas phenazinium* is not regulated by phosphate (28). It is possible that the physical-chemical conditions of the soil in TSL were more favorable to the production of phenazine antibiotics by strain 30-84 than to that by strain 2-79. If so, the loss of phenazine-producing ability would appear to have a greater effect on the survival of strain 30-84 than on that of strain 2-79.

The positive contribution of phenazine antibiotics to the survival of a particular bacterium in soil habitats may raise questions concerning nontarget effects when phenazine-producing strains are introduced into soil for the biological control of plant pathogens. The introduction of phenazine-producing fluorescent *Pseudomonas* spp. was previously shown to have no impact on the ability of VA mycorrhizal fungi to colonize roots of cucumber (29). In raw soil, the population of introduced *Trichoderma harzianum* was not altered by the addition of *P. fluorescens* 2-79 at a population as large as 10^7 CFU/g of soil (6). In our study, bulk-soil populations of indigenous aerobic bacteria and fluorescent pseudomonads were the same in soil whether Phz^+ or Phz^- derivatives of the same strain had been introduced. However, the interaction between introduced strains and indigenous microorganisms is likely to occur at sites that provide sufficient substrate for microbial growth and antibiotic production such as the rhizosphere or plant debris (4, 19, 40, 54, 55). Therefore, a thorough evaluation of the effects of introduced phenazine-producing strains on populations of indigenous microbial communities will require the sampling of these specific microsites. Qualitative changes in the composition of plant-associated bacterial communities were previously observed after the introduction of the biological control agent *Bacillus cereus* UW85n (15). These changes included an increase in the number of strains isolated that possessed pectolytic activity, resistance to certain antibiotics, and the ability to produce various extracellular enzymes. The sampling methods employed in our study would not detect such variation in the composition of bacterial communities resulting from the introduction of Phz^+ strains.

Phenazine production appeared to be less critical to the survival of the introduced strains when wheat roots were infected with *G. graminis* var. *tritici* than when roots were healthy. Differences between populations of Phz^- and Phz^+ strains were observed earlier and were of a greater magnitude in natural TSL than in infested TSL. Roots infected with *G. graminis* var. *tritici* support larger populations of bacteria than do healthy wheat roots (27, 35, 46). Similarly, in our study, roots infected with *G. graminis* var. *tritici* supported larger populations of the introduced strains than did healthy wheat roots. The increased proliferation of bacteria on wheat roots infected with *G. graminis* var. *tritici* is presumed to be the result of increased leakage of nutrients from lesions induced by this fungus (31). Thus, the nutrient status of a healthy wheat rhizosphere is likely to be very distinct from that which exists in root lesions induced by *G. graminis* var. *tritici*. Differences in substrate availability and composition can influence both antibiotic production and microbial competition and, therefore, may account for the enhanced survival of Phz^- strains observed in the presence rather than in the absence of *G. graminis* var. *tritici*. Messenger and Turner (28) observed that phenazine production by *P. phenazinium* declined during periods of rapid growth in vitro and suggested that phenazines are of little benefit to the producer bacterium when resources are plentiful. Leakage of nutrients from root lesions induced by *G. graminis* var. *tritici* may have created a temporary condition of excess substrate availability. However, as take-all declined with the successive plant-harvest cycles, antibiotic-producing ability again became more critical to survival of the introduced strains as the population of microorganisms possessing the ability to compete for these nutrients increased. This may explain, in part, the relatively greater advantage of phenazine biosynthesis to the survival of strains 2-79 and 30-84 on healthy wheat roots in comparison to that observed on roots infected with *G. graminis* var. *tritici* during the initial cycles of wheat.

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