Antibiosis and Antagonism of *Sclerotinia homoeocarpa* and *Drechslera poae* by *Pseudomonas fluorescens* Pf-5 In Vitro and In Planta

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ABSTRACT

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Pseudomonas fluorescens strain Pf-5, which produces several antifungal metabolites, including the antibiotics pyoluteorin, pyrrolnitrin, and 2,4-diacetylphloroglucinol, was tested for its ability to inhibit *Sclerotinia homoeocarpa* (causal agent of dollar spot) and *Drechslera poae* (causal agent of 'melting-out') in vitro and in turfgrass; Tn5 mutants with altered antibiotic production also were tested. Inhibition in vitro differed with the medium used, but both fungi generally were inhibited by Pf-5. In most cases, a mutant deficient in pyoluteorin but not pyrrolnitrin or 2,4-diacetylphloroglucinol was as inhibitory as Pf-5, whereas a pyrrolnitrin-deficient mutant was less inhibitory than Pf-5 in most fungus/medium combinations. High-performance liquid chromatography analysis of cul-

ture extracts showed that bacterial genotype and nutrition have an interactive effect on antibiotic production, such that conditions causing an increase in one antibiotic may increase or decrease another. The purported deficiencies for the pyrrolnitrin- and pyoluteorin-deficient mutants were confirmed. In *S. homoeocarpa*-infested grass clippings incubated in a moist chamber, Pf-5 reduced mycelial growth, whereas the pyrrolnitrin-deficient mutant did not and the pyoluteorin-deficient mutant was intermediate. In greenhouse experiments, Pf-5 reduced dollar spot disease incidence in bentgrass and bluegrass when sprayed over inoculated turf. In grass clippings infested with *D. poae* and incubated in a moist chamber under favorable conditions for spore production, Pf-5 did not reduce significantly the number of spores produced compared with the non-treated control. However, Pf-5 reduced melting-out disease incidence and severity in bluegrass inoculated with spores of *D. poae* under greenhouse conditions.

Dollar spot disease caused by *Sclerotinia homoeocarpa* F.T. Bennett is a destructive and widespread disease of turfgrasses, including warm- and cool-season species (2). The taxonomic position of the causal agent has been in question since the disease was first described; however, no name change was recommended until further studies have been conducted on the teleomorph of this species (2). Dollar spot occurs on putting greens with *Agrostis* sp. and may damage other turfgrasses in golf fairways, home lawns, and amenity turf areas (27). On golf courses, control of dollar spot is reportedly more expensive than that of any other disease (30). Biological control of dollar spot by strains of *Enterobacter cloacae* (20), *Gliocladium virens* (6), and several other bacterial and fungal antagonists has been investigated (3). Strains of *Pseudomonas fluorescens* and *P. lindbergii*, tested for control of *S. homoeocarpa* and *Bipolaris sorokiniana* on the phylloplane of *Poa pratensis* (Kentucky bluegrass), prevented infection by *S. homoeocarpa*, only the *P. lindbergii* strain was antagonistic to *B. sorokiniana* (8).

Drechslera poae (Baudys) Shoemaker causes a spring and fall disease of Kentucky bluegrass in temperate regions (2). The disease has a leaf-spot stage in cool, wet weather (2,30). Leaf spot incidence is usually higher on the sheath than on leaf blades and, in warmer weather, is often so extensive that the leaf is girdled and drops from the plant ('melting-out' phase) (2). Grass clippings constitute the main source of inoculum for epidemics (4). Biological control for this disease has not been reported.

Pseudomonas species are known to have a significant role in suppression of some diseases (11,13), apparently via production of

antifungal metabolites such as antibiotics (7,17,28), pyoverdine siderophores (5), cyanide (31), and ammonia (9). The biocontrol agent *P. fluorescens* Pf-5, originally isolated from a cotton rhizosphere (10), produces cyanide, pyoverdine siderophores, and the antifungal antibiotics pyoluteorin, pyrrolnitrin, and 2,4-diacetylphloroglucinol (15,21,22); it may produce other antibiotics as well. Antibiosis by *Pseudomonas* species in vitro has been demonstrated in studies with pure cultures and pure-culture filtrates (11,22). In addition, modern tools of microbial genetics and chemical analysis have provided evidence for antibiosis as a mechanism for biological control. For example, Tn5 mutants deficient in antibiotic production have displayed less pathogen inhibition than the wild-type (1,22,26), and antibiotic production has been verified in some cases by high-performance liquid chromatography (HPLC) (1,11,26). In addition, antibiotics have been isolated from soil and plant roots (16,29) and quantified with thin-layer chromatography and HPLC. Nowak-Thompson et al. (21) isolated the antibiotics pyrrolnitrin, pyoluteorin, and 2,4-diacetylphloroglucinol from culture extracts of *P. fluorescens* Pf-5; they noted that 2,4-diacetylphloroglucinol production was enhanced in a medium containing glucose and reduced in a medium containing glycerol.

The objectives of this study were to (i) assess the role of antibiotics in antagonism of *S. homoeocarpa* and *D. poae* in pure culture by *P. fluorescens* Pf-5, using Tn5 mutants; (ii) detect and quantify antibiotics produced by Pf-5 and its mutants in liquid culture; (iii) evaluate the bacteria's ability in vitro to colonize infested grass clippings and control mycelial growth (in the case of *S. homoeocarpa*) or conidia production (in the case of *D. poae*); and (iv) assess the effectiveness of antagonistic bacteria to control or reduce dollar spot (in bentgrass and bluegrass) and leaf spot (in bluegrass) on turfgrass under greenhouse conditions.

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MATERIALS AND METHODS

Organisms and media. *S. homoeocarpa* was isolated from infected bentgrass and maintained on $1/4$ -strength potato dextrose agar ($1/4$ -PDA) at 4°C. *D. poae* from the American Type Culture Collection (ATCC60573), Rockville, MD, was maintained on dried silica gel at 4°C. *Pyrenophora tritici-repentis* (Died.) Drechs., isolated from wheat in Kansas, was used as a positive control for in vitro experiments only. *P. fluorescens* strain Pf-5 (originally isolated by Howell and Stipanovic [10]) was obtained from J. E. Loper, as were the following Tn5 mutants of Pf-5 (15): JL3985 (pyrrolnitrin deficient) (22), JL4274 (pyoluteorin deficient), and JL4135 (antibiotic nonproducer strain). All bacterial strains were stored at -80°C. The media used for bacterial growth included King's medium B (KMB) agar or broth (14), dilute cornmeal agar (DCMA, 1.7 g of Difco [Detroit] cornmeal agar and 13.5 g of agar per liter [22]) or broth (DCMB), 523 agar or broth (12), and nutrient agar or broth amended with 2% (wt/vol) glucose (NAGluc or NBgluc) or 1% (wt/vol) glycerol (NAGlyc or NBglyc) (15). Media for fungal growth were potato dextrose broth (PDB; Difco), $1/4$ -PDA, and V8-juice agar (18). When necessary, antibiotics were added to KMB in the following concentrations: ampicillin (Ap) at 150 mg/liter; streptomycin (Sm) at 100 mg/liter; kanamycin (Km) at 150 mg/liter; and cycloheximide (Ch) at 50 mg/liter. Pf-5 is naturally resistant to Ap, Sm, chloramphenicol, and tetracycline (22); the Tn5 mutants are additionally resistant to Km.

Antagonism in vitro. Bacteria were grown in KMB broth overnight at 28°C with shaking, and 0.1 ml of this broth culture was transferred into 5 ml of fresh broth and grown under the same conditions for three more hours. Cells were washed by centrifugation and resuspended in 0.1 M MgSO₄. One 10- μ l drop of the suspension (10^8 cells per ml) was applied to the surface of each of several plates of DCMA, 523, and NAGluc. Plates were incubated at 28°C for 2 days, individually exposed to vapor of 0.25 ml of chloroform for 30 min to kill bacterial cells, and vented for 60 min in a flow hood.

To prepare mycelial suspensions, separate cultures of *S. homoeocarpa*, *Pyrenophora tritici-repentis*, and *D. poae* were grown for 2, 4, and 10 days at 28, 20, and 18°C, respectively, in 8 ml of PDB, and each was ground in 30 ml of PDB in a sterile blender. The mycelial suspension (2 ml) of each fungus was mixed with 6 ml of molten overlay DCMA containing 0.1 M FeCl₃ (to eliminate inhibition by bacterial siderophores [22]), and the mixture was poured over the surface of each agar plate containing killed bacterial cells. The radial distance between the edge of the bacterial colony and the edge of the fungal growth (*S. homoeocarpa*, *Pyrenophora tritici-repentis*, or *D. poae*) was measured after 2, 3, or 4 days, respectively. One bacterial strain was inoculated per agar plate, and four replicates of each treatment (fungus/bacterial strain combination) were evaluated in each experiment. Each experiment was done twice, with similar results that were combined for statistical analysis. Experiments were treated as blocks.

Antibiotic production in culture. *P. fluorescens* Pf-5 or its mutants were grown at 28°C with shaking in 5 ml of NBgluc, NBglyc, 523 broth, or DCMB. After 96 h of growth, cells and supernatants were separated by centrifugation. Cells were extracted once in 1 ml of acetone. The acetone extracts were reduced to dryness in vacuo, redissolved in 300 μ l of methanol, and analyzed by HPLC. Supernatants were acidified to pH 2 with 1 M HCl and extracted three times with 1 ml of ethyl acetate; in the first extraction, the broth was saturated with NaCl (80 mg/ml) to make the water phase more polar. Organic phases were dried with anhydrous MgSO₄, reduced to dryness in vacuo, and redissolved in 300 μ l of methanol for analysis by HPLC.

The HPLC system (Waters Corp., Milford, MA) included a photodiode array detector (Waters model 996) and a Nova-Pak C₁₈ 3.9 $\times$ 150-mm column. Samples were run in an isocratic mobile phase (45% H₂O, 30% acetonitrile, and 25% methanol [21]) at a flow rate of

1.0 ml/min. Chromatograms at or near absorption maxima were obtained at 225 nm (pyrrolnitrin), 272 nm (2,4-diacetylphloroglucinol), and 325 nm (pyoluteorin), and spectra were compared with those of antibiotic standards provided by Nowak-Thompson et al. (21) to verify identity. Detection limits for 2,4-diacetylphloroglucinol, pyoluteorin, and pyrrolnitrin, expressed as micrograms per milliliter of original culture broth, were 0.05, 0.3, and 0.8, respectively.

Plant material. Four-month-old Kentucky bluegrass (seeding rate 240 kg/ha) and Penncross bentgrass (*Agrostis* sp.) (seeding rate 160 kg/ha) were used for in planta experiments. Grasses were grown in a 50:50 sand/soil mixture in tall cylindrical pots (8 cm diameter) in the greenhouse. Pots were placed in a mist chamber for 10 (bentgrass) or 15 days (bluegrass) for seed germination and then were moved to a greenhouse bench. The grass was cut every week, and grass clippings were collected for use in laboratory studies or as substrate for fungal inoculum. Plants were fertilized with Peters fertilizer (Fogelsville, PA) (20-20-20, N-P-K) (7.5 g/m²) every 2 weeks.

Antagonism in infested grass clippings. The objectives of this experiment were to measure bacterial growth in infested and non-infested grass clippings during 15 days and to measure the survival and activity of *S. homoeocarpa* (by mycelial growth) and *D. poae* (by spore production) in grass treated with the antagonist. To produce infested grass clippings, 2-cm bluegrass pieces sterilized with propylene oxide were placed in contact with *S. homoeocarpa*, *D. poae*, or sterile agar blocks (control treatments) in a moist chamber. *S. homoeocarpa*-infested clippings were incubated at 28°C for 4 days, and *D. poae*-infested clippings were incubated at 18°C for 10 days. After inoculated grass pieces were colonized thoroughly by the fungus mycelium, infested or noninfested grass pieces were dipped individually in a suspension of washed bacteria (*P. fluorescens* Pf-5 at 10^8 CFU/ml) or in water and then incubated in moist chambers. Grass in the *S. homoeocarpa* experiment was incubated in the dark at 28°C for 15 days, whereas that in the *D. poae* experiment was incubated for 3 days under fluorescent light and then in the dark for 12 more days (to stimulate conidia production) at 18°C. In *S. homoeocarpa* experiments, the three mutants of Pf-5 also were evaluated. Four replicate chambers per treatment in each experiment were placed in an incubator in a completely randomized design.

To periodically evaluate growth of the bacteria on grass clippings, two replicate grass pieces from each treatment were placed together in 1.5 ml of 0.1 M MgSO₄, sonicated, and diluted at each sampling time. Aliquots were spread on plates of KMB-Ap-Sm-Ch or KMB-Km-Ch when mutants were evaluated, and bacteria colonies were counted after 2 days. The experiment was done twice, with similar results that were combined for statistical analysis.

D. poae conidia production in infested grass clippings was evaluated every 3 days beginning on day 6 after the treatments were applied. Conidia were counted in the same suspensions produced by sonicating grass pieces for bacterial evaluation. Each observation had four replications, and the experiment was done twice, with similar results that were combined for statistical analysis.

S. homoeocarpa mycelial growth was measured after 15 days of exposure to Pf-5 or its mutants as follows: a grass piece from each treatment replication was taken from the moist chamber and placed in contact with three previously sterilized grass pieces that were dipped in bacterial suspension (Pf-5 or the mutants at 10^8 CFU/ml) or water. Grass pieces were arranged end-to-end on a plastic screen suspended in a moist chamber in the dark at 28°C. Mycelial growth from one end of the infested grass piece through the length of the other three grass pieces was observed daily under a dissecting microscope for a period of 20 days. Two-factor repeated measures analysis was used, with bacterial strains (Pf-5, its mutants, and nontreated control) as whole plots and time as the subplot. Each observation had six replications, and the experiment was done twice, with similar results that were combined for statistical analysis with experiments treated as blocks.

In planta antagonism of *S. homoeocarpa* on bentgrass and bluegrass. To evaluate the ability of Pf-5 to reduce turf infection by mycelium of *S. homoeocarpa* originating from infested grass clippings, experiments were done separately with bentgrass and bluegrass. Grass clippings were autoclaved and then infested with *S. homoeocarpa* by placing them in contact with colonized agar blocks in a moist chamber at $23 \pm 2^\circ\text{C}$ for 10 to 15 days. This infested grass was air-dried overnight under sterile air and cut into 5×5 -mm pieces. These inoculum pieces were dipped in a suspension of washed Pf-5 cells (10^8 CFU/ml) or in water before placing them in pots of turf (three inoculum pieces per pot). Preliminary experiments showed that dipping inoculum in antagonist was not sufficient to control dollar spot; therefore, this treatment was combined with one or two additional applications of Pf-5 as a spray to the turf. After treatment, pots were placed on a greenhouse bench and misted with water from 6:00 a.m. to 8:00 p.m. each day, for 10 (bentgrass) or 25 days (bluegrass). Mist was applied for 20 s every 10 min in alternating cycles of wetting (2 h) and drying (2 h).

Disease incidence in bentgrass was evaluated on day 10 by estimating the percent blighted turf area in the pot with the aid of a transparent grid with 20 squares. In bluegrass, disease incidence was evaluated on day 25 by counting the number of lesions on all leaves in a pot. At 3-day intervals, bacterial populations on living leaves were estimated by washing five leaves from each treatment replication in 1.5 ml of 0.1 M MgSO_4 , sonicating, and diluting as described previously. Five replicate pots per treatment were arranged in a completely randomized design in a mist chamber in the greenhouse. Each experiment was done twice, and the results were combined for statistical analysis, with the two trials treated as blocks.

In planta antagonism of *D. poae* on bluegrass. *D. poae* was grown on V8-juice agar in the dark at 18°C . After 15 days, the mycelium was flattened with a sterile glass rod, and the plates were placed under fluorescent light for 3 days, then in the dark for 3 days, after which conidia were collected in sterile water. To evaluate the ability of *P. fluorescens* Pf-5 to protect grass leaves from infection by spores of *D. poae*, turf was sprayed with the *D. poae* conidial suspension, followed immediately by a spray of the antagonist. Preliminary experiments (23) indicated that one application of Pf-5 on day 6 or three applications on days 1, 3, and 6 significantly reduced leaf spot incidence. Therefore, the following treatments were applied: one spray of the antagonist (10^8 or 10^{10} CFU/ml) on day 6 or three sprays (10^8 CFU/ml) on days 1, 3, and 6. Three control treatments were included: inoculated turf sprayed with water and noninoculated turf sprayed with bacteria or water. Pots of turf were covered individually with sterile plastic bags and placed in a growth chamber at 18°C , with 12 h of dark per 12 h of light each day. Disease incidence and severity were evaluated on day 16 by counting the lesions on all leaves in each pot. To assess bacterial populations, dilution plating from each treatment replication was done (as previously described) every 3 days. Disease incidence and severity data were analyzed, using a completely randomized design. Each observation had five replications, and the experiment was done twice, with similar results that were combined for statistical analysis.

RESULTS

Antagonism in vitro. Inhibition zones in *Pyrenophora tritici-repentis* caused by Pf-5 and the mutants were consistent with previously reported results (22); Pf-5 was inhibitory to this fungus on

TABLE 1. Inhibition zones in *Pyrenophora tritici-repentis*, *Drechslera poae*, and *Sclerotinia homoeocarpa* caused by *Pseudomonas fluorescens* Pf-5 and its mutants

Strain	Radius of inhibition zones (mm) in mycelial growth ^y								
	<i>Pyrenophora tritici-repentis</i>			<i>D. poae</i>			<i>S. homoeocarpa</i>		
	DCMA ^z	523 ^z	NAgluc ^z	DCMA	523	NAgluc	DCMA	523	NAgluc
Pf-5	26 a	32 a	21 a	17 a	28 a	0 b	21 a	27 a	10 a
JL3985	0 b	26 a	1 b	0 b	19 b	1 b	7 b	26 a	5 b
JL4274	20 a	32 a	16 a	20 a	24 ab	6 a	19 a	24 a	4 b
JL4135	0 b	0 b	0 b	0 b	0 c	0 b	0 c	0 b	0 c

^y Values within a column followed by the same letter do not differ ($P = 0.05$) by Duncan's new multiple range test.

^z DCMA = dilute cornmeal agar; 523 = medium 523; and NAgluc = nutrient agar + 2% (wt/vol) glucose.

TABLE 2. Antibiotic production in culture by *Pseudomonas fluorescens* Pf-5 and its mutants^w

Bacterial strain	Antibiotic ^x	DCMB medium ^y		523 medium ^y		NBgluc medium ^y		NBglyc medium ^y	
		Supernatant	Cells	Supernatant	Cells	Supernatant	Cells	Supernatant	Cells
Pf-5	plt	5.1, 68 ^z	0	0	0	8.2, 10	0	6.6, 13	0
	phl	0.1, 2	0	305, 366	0	326, 392	4.2, 5	0.5, 1	0
	pn	2.0, 26	0	1.4, 1	0.7, 1	0	4.1, 5	1.8, 4	1.2, 2
JL3985	plt	6.8, 205	0	81, 108	10, 14	42, 126	6.0, 18	6.6, 20	9.1, 27
	phl	17, 518	0	313, 418	54, 72	382, 1147	8.3, 25	36, 107	0.2, 0.5
	pn	0	0	0	0	0	0	0	0
JL4274	plt	0	0	0	0	0	0	0	0
	phl	0.1, 2	0	438, 548	9.3, 12	546, 769	8.1, 11	67, 153	1.5, 4
	pn	1.4, 28	0	1.6, 2	0.7, 1	0	2.5, 4	0	0
JL4135	plt	0	0	0	0	0	0	0	0
	phl	0	0	0	0	0	0	0	0
	pn	0	0	0	0	0	0	0	0

^w Pf-5 and its mutants were incubated with shaking at 28°C for 96 h in 5 ml of broth and supernatant and cells were separated for extraction and high-performance liquid chromatography (HPLC) analyses.

^x HPLC retention times were 2.5 min for pyoluteorin (plt); 4.6 min for 2,4-diacetylphloroglucinol (phl); and 9.8 min for pyrrolnitrin (pn).

^y DCMB = dilute cornmeal broth; 523 = 523 broth; NBgluc = nutrient broth supplemented with 2% glucose (wt/vol); and NBglyc = nutrient broth supplemented with 1% (wt/vol) glycerol.

^z Data are expressed as micrograms per milliliter of culture volume (from supernatant or cells produced in that volume) and, in italics, as nanograms per 10^6 CFU (supernatant or cells). Bacterial density (CFU per milliliter) varied with strain and medium.

all three media (Table 1), and nonantibiotic producer strain JL4135 was not inhibitory. Pyrrolnitrin-deficient strain JL3985 was not inhibitory on DCMA and only slightly so on NAgLuc, and pyoluteorin-deficient strain JL4274 was as inhibitory as the Pf-5 wild-type on all three media.

The pattern of inhibition zones in *D. poae* (Table 1) was similar in most respects to that in *Pyrenophora tritici-repentis*. On DCMA, strains that produce pyrrolnitrin were inhibitory, whereas JL3985 and JL4135 were not. Each antibiotic-producing strain was inhibitory on 523 medium, although JL3985 was less so than the wild-type. On NAgLuc, nonpyrrolnitrin producers (JL3985 and JL4135) were noninhibitory, as observed for *Pyrenophora tritici-repentis*, but unlike the latter fungus, *D. poae* was not affected by Pf-5 and only slightly affected by JL4274 on this medium.

S. homoeocarpa was inhibited by Pf-5 on all three media (Table 1) and was unaffected by nonantibiotic producer JL4135. On DCMA, JL3985 (nonpyrrolnitrin producer) was somewhat inhibitory to this fungus, and JL4274 (nonpyoluteorin producer) was as inhibitory as the wild-type strain. All three antibiotic producers were equally inhibitory on medium 523 (as observed for *Pyrenophora tritici-repentis*). On NAgLuc, inhibition of *S. homoeocarpa* by JL3985 was similar to that by JL4274, and both were less inhibitory than Pf-5.

Extraction from liquid culture. HPLC analysis verified that, in the media and growth conditions we used (liquid shake-culture at 28°C), strain JL3985 produced no detectable pyrrolnitrin, strain JL4274 produced no detectable pyoluteorin, and strain JL4135 produced no pyrrolnitrin, pyoluteorin, or 2,4-diacetylphloroglucinol (Table 2).

For the antibiotics that a given strain could produce, growth medium had a pronounced effect on production. Pf-5 produced moderate amounts (2 to 5 µg/ml) of pyrrolnitrin and pyoluteorin in DCMB and a small amount of 2,4-diacetylphloroglucinol (0.1 µg/ml). In 523, Pf-5 produced a large amount of 2,4-diacetylphloroglucinol (300 µg/ml), moderate amount of pyrrolnitrin (1.4 µg/ml), but no detectable pyoluteorin. In NBgluc, production of 2,4-diacetylphloroglucinol was high (>300 µg/ml), that of pyoluteorin was moderate (8 µg/ml), and pyrrolnitrin was produced but remained in the cells rather than being exported to the medium. Antibiotic production by Pf-5 in NBglyc was generally similar to that in DCMB (Table 2).

Fig. 1. Mycelial growth of *Sclerotinia homoeocarpa* in grass clippings treated with *Pseudomonas fluorescens* Pf-5, its mutants (JL4135, JL3985, or JL4274), or water (control). Vertical bars are ±1 standard error of the mean.

Strain JL3985 produced a moderate amount of pyoluteorin in DCMB, but 2,4-diacetylphloroglucinol production by this strain was higher than that of the wild-type (Table 2). On 523 and NBgluc, JL3985 produced high levels of 2,4-diacetylphloroglucinol and large amounts of pyoluteorin. Antibiotic production in NBglyc was similar to that in DCMB.

Strain JL4274 was similar to Pf-5 in antibiotic production in DCMB, with the exception that no pyoluteorin was produced. As observed for Pf-5, growth in 523 or NBgluc stimulated increased 2,4-diacetylphloroglucinol production. Production of pyrrolnitrin by JL4274 in these two media was similar to that in DCMB; again, pyrrolnitrin produced in NBgluc remained in the cells. In NBglyc, 2,4-diacetylphloroglucinol production was moderately high, but no pyrrolnitrin was detected.

The different strains shown in Table 2 did not grow to the same population sizes in all media (data not shown); populations (CFU per milliliter) of JL3985 were only 35 to 65% of those for Pf-5 in the various media, except 523 (in which all strains grew to similar population sizes), and populations of all strains were ~10-fold lower in DCMB than in the other media. When antibiotic levels are calculated per bacterial population unit (Table 2, italicized data), pyrrolnitrin production in DCMB (by Pf-5 and JL4274) was 5- to 10-fold higher than in other media, 2,4-diacetylphloroglucinol production by JL3985 in NBgluc was greater than in any other strain/medium combination, and the highest levels of pyoluteorin per CFU were produced by JL3985 grown in DCMB, 523, or NBgluc.

Antagonism in infested grass clippings. The analysis of variance (ANOVA; data not shown) of *S. homoeocarpa* mycelial growth in grass clippings treated with Pf-5 and its mutants (Fig. 1) showed significant interaction in treatment by day. Average mycelial growth in grass clippings treated with Pf-5 was less than in the other treatments, except for strain JL4274 at 4 and 8 days. Mycelial growth in grass clippings treated with strain JL4274 was less than in grass clippings treated with strain JL3985; however, the difference was not statistically significant, except on day 12.

Population levels of Pf-5 and its mutants on *S. homoeocarpa*-infested and noninfested grass clippings are shown in Figure 2. The ANOVA for bacterial population size showed significant ($P =$

Fig. 2. Population size of *Pseudomonas fluorescens* Pf-5 and its mutants in grass clippings infested (solid lines) and noninfested (dashed lines) with *Sclerotinia homoeocarpa* mycelium. Statistical analysis of regression line slopes and intercepts for bacterial growth curves, within noninfested and infested grass clippings, showed no significant differences ($P = 0.05$) among bacterial strains. Vertical bars are ±1 standard error of the mean.

0.05) interaction for fungus (i.e., infested and noninfested grass clippings) by day and no significant interaction for fungus by bacterial strain, bacterial strain by day, or fungus by bacterial strain by day. The main effects for fungus treatment and days were significant, but the main effect for bacterial strain was not significant. The bacterial population sizes in infested grass clippings were significantly lower than those in noninfested grass clippings. In infested and noninfested grass clippings, the average population size of strain JL3985 was 0.7 to 1 log unit lower than those of Pf-5, JL4274, or JL4135, but statistical analysis of slopes and intercepts of the regression lines within noninfested or infested grass clippings showed no significant differences among bacterial strains.

In the *D. poae* experiment, average conidia production in Pf-5-treated clippings was lower (7×10^5 conidia per g of grass) than in nontreated clippings (10×10^5 conidia per g of grass) (23), but the difference was not statistically significant (*t* test, $P = 0.05$).

Antagonism of *S. homoeocarpa* on bentgrass and bluegrass. Dollar spot incidence in bentgrass (Table 3) was significantly lower in the treatment in which fungal inoculum was dipped in the antagonist and the turf was sprayed with Pf-5 two times (days 3 and 6) than in the treatment in which fungal inoculum was dipped in the antagonist and turf was sprayed with Pf-5 once (day 3) or in the treatment in which fungus inoculum was dipped in water and the turf was sprayed with Pf-5 once (day 1). Disease incidences in the latter two treatments were not significantly different from each other but were significantly lower than that of the control treatment (no bacteria). Nine days after inoculation, the population size of Pf-5 in turf sprayed two times (days 3 and 6) was 7.8 log units, ~1 log unit higher than that of turf sprayed with Pf-5 once (day 3) and ~2 log units higher than that of turf sprayed with Pf-5 once (day 1) (Fig. 3A).

In bluegrass, dollar spot disease incidence was reduced by treatment with Pf-5 (Table 4). Although the reduction was not significant at $P = 0.05$, we describe the reduction as significant ($P = 0.10$) given the consistent effect in two experiments and the high variability in this experimental material. The reduction was similar in the treatment in which fungus inoculum was dipped in antagonist and the turf was sprayed with Pf-5 twice and in the treatment in which inoculum was not dipped in the antagonist but the turf was sprayed with Pf-5 once (day 1). Nine days after inoculation, the population size of Pf-5 in bluegrass (Fig. 3B) sprayed twice (days 3 and 6) was 6.4 log units, ~1 log unit higher than that of turf sprayed with Pf-5 once (day 3) and ~2 log units higher than that of turf sprayed once (day 1). In 9 days, bacterial populations applied only once in infested bentgrass (Fig. 3A) and bluegrass (Fig. 3B) dropped from 8.5 to 5.4 log units and from 7.7 to 4.6 log units, respectively. By day 9, bacterial populations applied only

once (day 3) dropped ~1.6 log units in bentgrass and 2.3 log units in bluegrass.

Antagonism of *D. poae* in bluegrass. Disease incidence and severity of *D. poae* leaf spot on bluegrass (Table 5) was significantly lower in turf sprayed with Pf-5 than in the nontreated control. There were no significant differences among Pf-5 treatments, whether turf was sprayed once (day 6) or three times (days 1, 3, and 6) or whether the bacteria concentration was 10^8 or 10^{10} CFU/

Fig. 3. Population size of *Pseudomonas fluorescens* Pf-5 in **A**, bentgrass and **B**, bluegrass in a greenhouse. Solid lines indicate turf inoculated with *Sclerotinia homoeocarpa*; dashed lines indicate noninoculated turf (ck). Inoculum consisted of grass clippings colonized by the fungus. Inoculum pieces were dipped in a suspension of washed Pf-5 cells (10^8 CFU/ml) (I) or dipped in water (N) before placing them in pots of turf. Pf-5-treated turf received one or two additional antagonist applications (0.5 ml of bacterial suspension at 10^8 CFU per ml per pot of turf) as a spray. Vertical bars are ± 1 standard error of the mean.

TABLE 3. Dollar spot incidence in bentgrass treated with *Pseudomonas fluorescens* Pf-5 under greenhouse conditions

Fungal inoculum ^w	Antagonist application to inoculum ^x	Time of antagonist application to growing turf ^y	Disease incidence on day 10 (%) ^z
0	—	0	0.0
0	—	Day 1	0.0
+	ck	0	59.6 a
+	ck	Day 1	20.3 b
+	+	Day 3	22.2 b
+	+	Days 3 and 6	8.7 c

^w Turf was inoculated (+) with three pieces (5×5 mm) of *Sclerotinia homoeocarpa* inoculum or was noninoculated (0).

^x Fungal inoculum was dipped in water (ck) or in bacterial suspension (Pf-5 at 10^8 cells per ml) (+). — = no inoculum.

^y Day(s) on which Pf-5 suspension (10^8 cells per ml) or water (0) was sprayed over turf. Fungal inoculum was applied on day 1.

^z Mean of two experiments. Values followed by a common letter do not differ ($P = 0.05$) by Duncan's new multiple range test.

ml. Fifteen days after fungal inoculation in this experiment, Pf-5 population size (Fig. 4) in the treatment sprayed once (day 6; 10^{10} CFU/ml) was about 0.3 log units higher than in turf sprayed three times (days 1, 3, and 6; 10^8 CFU/ml) or in turf sprayed once (day 6; 10^8 CFU/ml). Population size on noninoculated turf sprayed once (day 1) with 10^8 CFU/ml was ~2 log units lower than the latter population sizes.

DISCUSSION

The responses of *S. homoeocarpa*, *D. poae*, and *Pyrenophora tritici-repentis* to *P. fluorescens* strain Pf-5 and its Tn5 mutants (Table 1) suggest that mycelial growth of these fungi is inhibited by pyrrolnitrin, although not exclusively by this antibiotic. The complexity of nutritional effects on antibiotic production (Table 2), the possibility that a given fungus may be sensitive to more than one antibiotic, as well as the possibility that additional unknown antibiotics may be produced by these bacteria makes it difficult to assign antagonism effects to specific antibiotics. Furthermore, we lacked sufficient purified antibiotics to test them directly against fungi on the various media. Inhibition of *Pyrenophora tritici-repentis* in our tests is consistent with its known inhibition (22) by pyrrolnitrin. However, this fungus also was inhibited by JL3985 on medium 523 in the absence of detectable pyrrolnitrin. This inhibition might have been due to the very high level of pyoluteorin (Table 2), perhaps as a threshold effect in combination with a high level of 2,4-diacetylphloroglucinol; 2,4-diacetylphloroglucinol alone did not seem to be very toxic to this fungus (com-

parison of Tables 1 and 2 for JL3985 on NBgluc). Alternatively, Pf-5 and its mutants may have produced an unknown (thus unmeasured) antibiotic on medium 523 (inhibition zones were prominent for all antibiotic-producing strains on 523). Inhibition of *D. poae* also appeared to be coincident with pyrrolnitrin production, except there was little or no inhibition where pyrrolnitrin was retained within the cells; perhaps the leakage of pyrrolnitrin from chloroform-treated cells in the antagonism assay was sufficient to inhibit the more sensitive *Pyrenophora tritici-repentis* but not *D. poae*. Inhibition of the latter fungus in the absence of pyrrolnitrin, in the strain/medium combinations JL4274/Naglug, JL3985/523, and JL3985/Naglug, might have been due to threshold effects of particularly high concentrations of 2,4-diacetylphloroglucinol, alone or in combination with high levels of pyoluteorin. Inhibition of *S. homoeocarpa* was similarly consistent with sensitivity to pyrrolnitrin in relatively low amounts (comparison of Tables 1 and 2 for JL4274 on DCMA). However, the observed inhibition on all media by the three antibiotic-producing strains indicates that this fungus is additionally sensitive to other antibiotics. The modest inhibition zones around JL3985 on DCMA and Naglug might be explained by the fungus' sensitivity to pyoluteorin or 2,4-diacetylphloroglucinol or to another unknown antibiotic. The larger zone observed for JL3985/523 compared with JL3985/Naglug suggests a nondetected antibiotic produced on 523.

Although our results are not definitive with respect to activity spectra and interactions of the antibiotics, the results indicate clearly that antibiosis is involved in antagonism of the fungi by Pf-5 in vitro and that the inhibition is complex in mechanism and expression under different nutrition environments. Further, antibiotic production appears to be affected by temperature (J. E. Loper, personal communication), as suggested by the differences between antibiotic production observed by us and other researchers who used cooler incubation temperatures (21,22). Interpretation is complicated further by the possibility (not demonstrated) that nutrition conditions may affect the ability of the fungi to detoxify antibiotics. Therefore, although it is very problematical to extrapolate

TABLE 4. Dollar spot incidence in bluegrass treated with *Pseudomonas fluorescens* Pf-5 under greenhouse conditions

Fungal inoculum ^w	Antagonist application to inoculum ^x	Time of antagonist application to growing turf ^y	Mean disease incidence on day 25 (%) ^z
0	—	0	0.0
0	—	Day 1	0.0
+	ck	0	74.3 a
+	ck	Day 1	44.0 b
+	+	Day 3	53.7 ab
+	+	Days 3 and 6	43.9 b

^w Turf was inoculated (+) with three pieces (5 × 5 mm) of *Sclerotinia homoeocarpa* inoculum or was noninoculated (0).

^x Fungal inoculum was dipped in water (ck) or in bacterial suspension (Pf-5 at 10^8 cells per ml) (+). — = no fungal inoculum.

^y Day(s) on which Pf-5 suspension (10^8 cells per ml) or water (0) was sprayed over turf. Fungal inoculum was applied on day 1.

^z Mean of two experiments. Values followed by a common letter do not differ ($P = 0.10$) by Duncan's new multiple range test.

TABLE 5. Disease incidence and severity in bluegrass inoculated with *Drechslera poae* conidial suspension and treated with *Pseudomonas fluorescens* Pf-5 under growth-chamber conditions

<i>D. poae</i> ^v	Antagonist application ^w		Mean disease incidence (%)	Mean disease severity ^y
	CFU/ml	Day(s) applied ^x		
0	...	...	0.0	0.00
0	10^8	1	0.0	0.00
+	...	...	20.1 a ^z	0.23 a
+	10^8	6	3.1 b	0.13 b
+	10^{10}	6	2.8 b	0.09 b
+	10^8	1, 3, and 6	2.8 b	0.11 b

^v Turf in each pot was sprayed with 0.5 ml of *D. poae* conidial suspension (3×10^3 spores per ml) (+) or was sprayed with water (0).

^w Pf-5 was sprayed over turf (0.5 ml of bacterial suspension per pot).

^x Day(s) on which the antagonist was applied to the turf. *D. poae* conidia were sprayed on the turf on day 1.

^y Total number of lesions in a pot of turf divided by the total number of leaves in a pot.

^z Values within a column followed by the same letter do not differ ($P = 0.05$) by Duncan's new multiple range test.

Fig. 4. Population size of *Pseudomonas fluorescens* Pf-5 on bluegrass in a growth chamber. Solid lines indicate turf inoculated with a *Drechslera poae* conidial suspension; dashed line indicates noninoculated turf. Turf in each treatment received 0.5 ml of bacterial suspension (10^8 CFU/ml) in each spray, except the first treatment, which received a higher bacterial concentration (10^{10} CFU/ml). Vertical bars are ± 1 standard error of the mean.

antibiotic production from liquid culture to growth on a natural substrate such as grass, it appears, nonetheless, that antibiotics are likely to be important in antagonism on such substrates. For example, our observations of reduced antagonism by the pyrrolnitrin-deficient mutant (Fig. 1) suggests that this antibiotic is effective and may be produced by the wild-type on grass leaves. Attempts to recover and quantify antibiotics from inoculated grass clippings were unsatisfactory (F. Rodriguez and W. F. Pfender, unpublished data), because of incomplete extraction and chromatography interference due to organic components in the leaves (23).

In an additional experiment that was replicated but not repeated (23) (data not shown), the Tn5 mutants of Pf-5 were evaluated by spraying them on pots of turf inoculated with *D. poae* spores. Disease incidence and severity in turf treated with strain Pf-5 or pyoluteorin-deficient strain JL4274 were significantly lower than in turf treated with pyrrolnitrin-deficient strain JL3985, nonantibiotic-producing strain JL4135, or in the control treatment. These observations also support a role for antibiosis, specifically by pyrrolnitrin, in antagonism.

Alternatively, the lack of inhibition by strain JL3985 could be due to low population levels; in experiments with *S. homoeocarpa*, the average population size of strain JL3985 was ~1 log unit lower (not statistically significant) than that of Pf-5, JL4274, and JL4135. However, in agar plates, inhibition zones caused by strain JL3985 were significantly smaller on DCMA and NAGluc than the ones produced by Pf-5 and strain JL4274. In similar experiments with *Pyrenophora tritici-repentis*, Pfender et al. (22) found that populations of Pf-5 and JL3985 on agar test plates were similar. We, therefore, suggest that the greater inhibition by Pf-5 than by JL3985 in grass clippings was due to pyrrolnitrin production and that research to optimize in situ production of this antibiotic would be beneficial.

Whereas Pf-5 reduced mycelial growth of *S. homoeocarpa* in grass clippings, it did not reduce significantly the number of conidia of *D. poae* produced on this substrate. The grass clippings were colonized thoroughly by *D. poae* mycelium before the treatments were applied, and it is unknown whether the bacterial antibiotics can influence sporulation from vigorous mycelium. Also, the temperature at which the experiment was carried out (18°C) was more favorable for pathogen spore production than for bacterial growth.

Despite the lack of *D. poae* sporulation inhibition by Pf-5 and the inability of the bacteria to completely prevent growth of *S. homoeocarpa*, disease suppression in the greenhouse was obtained by spraying the bacteria on living turf. For *S. homoeocarpa*, our data suggest that bacteria applied to infested grass clippings are not adequate for disease suppression, which required at least one spray of Pf-5 onto foliage. For *D. poae*, application of the antagonist on day 6 after inoculation seemed to be important in reducing disease incidence, reflecting perhaps sensitivity of *D. poae* at a critical stage in germination and infection. Zhou and Paulitz (32) reported an effect of *Pseudomonas* spp. on spore germination in *Pythium aphanidermatum*.

The smaller percentage of dollar spot disease reduction in bluegrass compared with bentgrass might be due to the lower survival rate of Pf-5 in the bluegrass turf microenvironment. In 6 days, the population of bacteria sprayed once over turf dropped ~1.6 log units in bentgrass but 2.3 log units in bluegrass. Reduced survival could be related to the fact that the bluegrass canopy is less dense than in bentgrass, with consequent differences in temperature and humidity. In addition, bacterial cells could have been washed off by the mist more easily in bluegrass than in the denser bentgrass. Alternatively, because the disease took more time to develop in bluegrass than in bentgrass, it is possible that one more spray of Pf-5 on day 9 or the same number of sprays at a later time would have resulted in more efficient control of the disease.

Further research to measure population sizes and antagonistic activity of Pf-5 and its mutants under field conditions is necessary

to assess the utility of Pf-5 and pyrrolnitrin in suppression of dollar spot in bentgrass and 'melting out' in bluegrass. Two important factors for antagonist establishment and growth are the environmental conditions (temperature, moisture, nutrients, and pH) and the organism's ability to compete with existing microbial communities. In addition, the antagonist must be compatible with other management inputs (pesticides and fertilizers), and its activities must not be inhibited by cultural practices used in turf maintenance (19). Populations of Pf-5 in bentgrass and bluegrass fell quickly with time (Fig. 3), and the application of high populations was ineffective in preliminary studies (23). Therefore, the possibility of transferring the genes for antibiotic production into other bacterial strains better suited than Pf-5 for survival and activity in the turf microenvironment could be a promising alternative.

If biological control through antibiosis is to be effective under field conditions, antibiotics must be produced in concentrations adequate for fungal inhibition and be active against the target pathogens during periods favorable for plant infection. Thus, parameters affecting antibiotic production need to be more fully understood. Sarniguet et al. (24,25) have demonstrated that in Pf-5 a regulatory gene homologous to the *rpoS* gene in *Escherichia coli* is involved in the regulation of pyrrolnitrin production (24). Tn5 mutations in this regulatory region show pleiotropic effects that include sensitivities of stationary phase cells to stress caused by hydrogen peroxide, osmotic shock, or aging (25); thus, production of pyrrolnitrin is regulated coordinately with several traits induced in response to environmental stress (24,25). In addition to environmental effects on antibiotic production, possible destruction of antibiotics by factors such as sunlight also should be considered. Therefore, it is necessary to study the influence of nutritional, chemical, and physical factors in the turf microenvironment and their interaction on the production and persistence of antibiotics by biocontrol bacteria such as Pf-5. The understanding of these interactions may provide insights into better methods of manipulating the microbial environment to enhance the antibiotic-producing capability of biocontrol bacteria and improve control strategies.

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