A Genomic Region from *Pseudomonas fluorescens* Pf-5 Required for Pyrrolnitrin Production and Inhibition of *Pyrenophora tritici-repentis* in Wheat Straw

William F. Pfender, Jennifer Kraus, and Joyce E. Loper

First author: Department of Plant Pathology, Kansas State University, Manhattan 66506; second and third authors: USDA, ARS, Horticultural Crops Research Laboratory, 3420 NW Orchard Ave., and Department of Botany and Plant Pathology, Oregon State University, Corvallis 97330.

Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the USDA and does not imply its approval to the exclusion of other products that may also be suitable.

Contribution 93-227-J from the Kansas Agricultural Experiment Station, Kansas State University, Manhattan.
Accepted for publication 11 June 1993.

ABSTRACT

Pfender, W. F., Kraus, J., and Loper, J. E. 1993. A genomic region from *Pseudomonas fluorescens* Pf-5 required for pyrrolnitrin production and inhibition of *Pyrenophora tritici-repentis* in wheat straw. Phytopathology 83:1223-1228.

Pseudomonas fluorescens strain Pf-5, which produces many antifungal metabolites including pyrrolnitrin, inhibited mycelial growth of *Pyrenophora tritici-repentis* in agar culture and suppressed ascocarp formation by the fungus on axenically infested wheat straw kept moist during incubation. Purified pyrrolnitrin was similarly inhibitory. A Tn*5* mutant of Pf-5 that did not produce detectable levels of pyrrolnitrin did not inhibit *P. tritici-repentis* on a culture medium or on wheat straw. A genomic region of Pf-5 was cloned that restored the pyrrolnitrin-deficient Tn*5* mutant to pyrrolnitrin production and antagonism to *P. tritici-repentis* in culture. Pf-5 did not persist or suppress *P. tritici-repentis* on naturally infested wheat straw incubated under simulated field conditions of fluctuating moist and dry periods.

Pyrenophora tritici-repentis (Died.) Drechs., the causal agent of tan spot disease of wheat (*Triticum aestivum* L.), overwinters saprophytically in wheat-straw residue resting upon the soil surface. Ascospores in residueborne ascocarps serve as the primary inoculum during the following season, and tan spot epidemic severity is affected by the amount of primary inoculum present in the field (1). Biological control agents capable of reducing the number of ascocarps of *P. tritici-repentis* in straw could therefore reduce disease severity. In previous studies (26,28), several fungi inhibited inoculum production by *P. tritici-repentis* in straw. Bacterial biocontrol agents, however, including those that produce antifungal antibiotics, have not been examined for possible antibiosis of the fungus in straw.

Wheat straw was among the first natural substrates known to support the production of antibiotics by soil microorganisms. In 1956, Wright demonstrated that gliotoxin was produced by *Trichoderma viride* Pers.:Fr. that inhabited wheat-straw residue in soil (37). More recently, the in situ production of antibiotics by soil microbes and a role for antibiotics produced by bacterial or fungal antagonists in biological control of several soilborne fungal phytopathogens have been demonstrated (7,18,35). Nevertheless, the use of bacterial antagonists to inhibit a phytopathogen in its residueborne saprophytic phase and the possibility that straw-colonizing antagonists produce antibiotics in concentrations adequate to suppress such pathogens have not been explored.

In a preliminary screen of known antibiotic-producing bacterial biocontrol strains, *Pseudomonas fluorescens* (Trevisan) Migula strain Pf-5 (13) inhibited *P. tritici-repentis* in culture and on wheat straw maintained under conditions of high humidity (Pfender, *unpublished*). Pf-5 produces several antifungal antibiotics, including pyrrolnitrin and pyoluteorin (13), cyanide, a pyoverdine siderophore, and, when cultured on nutrient agar amended with glucose, an uncharacterized compound designated antibiotic 3

© 1993 The American Phytopathological Society

(20). The activity spectra of these antibiotics differ; for example, pyrrolnitrin inhibits *Rhizoctonia solani* Kühn (13) and *Botrytis* spp. (15), pyoluteorin inhibits *Pythium ultimum* (14) and *Pyricularia oryzae* (34), and antibiotic 3 inhibits *R. solani* and *P. ultimum* (20). Pyoverdine siderophores inhibit mycelial growth of many phytopathogenic fungi in iron-limited environments and have differential effects on spore germination (23).

In this study, we investigated the antagonism of *P. tritici-repentis* by *P. fluorescens* Pf-5. Our goals were to determine whether antagonism of this fungus by Pf-5 is associated with production of a particular antibiotic, to assess the effectiveness of the antagonism in infested wheat straw, and to localize component(s) of the genetic information for antibiotic production by Pf-5. To this end, we identified mutants deficient in pyrrolnitrin production, assessed their inhibition of *P. tritici-repentis* in culture and in straw, and identified a genomic region required for pyrrolnitrin production by strain Pf-5. Portions of this work were reported in an abstract (24).

MATERIALS AND METHODS

Organisms and media. *P. tritici-repentis* isolate 6R180 was isolated from infected wheat near Manhattan, Kansas. The fungus was maintained on one-fourth strength potato-dextrose agar (1/4 PDA; Difco Laboratories, Detroit, MI) at 4 C. Isolate J1 of *R. solani* was obtained from C. Howell (USDA, Cotton Research Laboratory, College Station, TX), maintained as sclerotia in dry soil, and cultured on PDA. *P. fluorescens* strain Pf-5, which was originally isolated from a cotton rhizosphere, also was obtained from C. Howell (13). Pf-5 is naturally resistant to 100 mg/L of chloramphenicol, 50 mg/L of streptomycin, 100 mg/L of ampicillin, and 100 mg/L of tetracycline. *Pseudomonas putida* strain G1-rif, which produces no known antibiotics, was obtained from L. Thomashow (USDA, Root Disease and Biological Control Research Unit, Pullman, WA). Strains of *Pseudomonas* spp. and *Escherichia coli* were stored in nutrient broth containing 15% glycerol at −80 C.

Media used for bacterial growth were Luria-Bertani (LB) broth and LB agar (31), King's medium B (KMB) broth and KMB agar (19), and medium 523 agar (17). Media for fungal growth included dilute cornmeal agar (DCMA, 1.7 g Difco corn meal agar and 13.5 g Difco Bacto agar per liter), potato-dextrose broth (PDB), and PDA (Difco). Nutrient agar (Difco) was amended with 1% (w/v) glycerol (NaGly). When appropriate, antibiotics were incorporated into the media at the following concentrations: chloramphenicol (Cm), 100 mg/L; streptomycin (Sm), 50 mg/L; kanamycin (Km), 50 mg/L; tetracycline (Tc), 20 mg/L; and ampicillin (Ap), 100 mg/L.

Transposon mutagenesis and screening. Tn*5* mutagenesis of Pf-5 with pLG221 (4) was carried out as described previously (20). Mating mixtures containing putative Tn*5* insertion mutants were spread either on nutrient agar containing Km and Sm or on minimal medium 925 (21) containing Km and 1% (w/v) *myo*-inositol as a sole carbon source. In some cases, medium 925-inositol was amended with 5 mg/L of 5-fluoro-DL-tryptophan (Sigma Chemical Co., St. Louis, MO), a toxic analogue of tryptophan, to select for 5-fluoro-DL-tryptophan–resistant mutants. Because tryptophan is a precursor of pyrrolnitrin, we reasoned that 5-fluoro-DL-tryptophan–resistant mutants may produce concentrations of pyrrolnitrin that are less than or that exceed concentrations produced by Pf-5. Inhibition of *R. solani* J-1, which is sensitive to pyrrolnitrin, by strain Pf-5 and Tn*5*-insertion mutants was evaluated on medium 523 by an agar overlay method described previously (20).

Recombinant DNA techniques. An 8.5-kb *Eco*RI fragment that contained the Tn*5* insert and flanking sequences of the Tn*5*-insertion mutant JL3985 was cloned for use as a probe in identifying the corresponding wild-type genomic region of Pf-5. pJEL1995 was constructed by ligating *Eco*RI-digested genomic DNA of JL3985 to *Eco*RI-digested pBR322, transforming competent *E. coli* strain DH5α (31) with ligated DNA, and selecting recombinants on LB medium containing Km. A genomic library, constructed by cloning Pf-5 DNA that had been partially digested with *Sau*3AI into the *Bam*HI site of the cosmid vector pLAFR3 (33), was screened by colony hybridization to identify clones that hybridized to plasmid pJEL1995. Filters for colony hybridization were prepared with Whatman 541 paper (Whatman International Ltd., Maidstone, England) and hybridized with a ^{32}P-labeled probe as described by Gergen et al (8). The cosmids were mobilized from *E. coli* HB102 (11) donors into *P. fluorescens* or *P. putida* in triparental matings with pRK2013 (6) as a helper plasmid. Transconjugants of *P. fluorescens* containing pLAFR3 derivatives were selected on KMB containing 100 mg/L of Cm and 200 mg/L of Tc.

Transformation of *E. coli*, restriction digests, ligations, electrophoresis in Tris-phosphate-EDTA, and plasmid DNA isolation by alkaline-lysis were carried out by standard methods (31). Total genomic DNA was isolated from saturated KMB broth cultures of strain Pf-5 and derivatives by the CTAB method (2). Enzymes were from Bethesda Research Laboratories (BRL, Gaithersberg, MD). Nylon membranes (Nytran, Schleicher & Schuell, Keene, NH) were used to prepare Southern blots of plasmid and genomic DNA according to the supplier's directions. Probes were prepared from cloned fragments with biotinylated dTTP and a nick translation kit (BRL) or with ^{32}P-dCTP and a random primer labeling kit (BRL) and purified with a D50 column (International Biotechnologies Inc., New Haven, CT). Biotinylated pJEL1605 served as a Tn*5* probe (20).

Analysis of antibiotics produced by *P. fluorescens* in culture. Antibiotics produced by Pf-5 and derivative strains were extracted from the agar medium (523 or DCMA) and bacterial cells of 2-day-old cultures, as described previously (20). The extract from one petri dish was dissolved in 20 μl of methanol, and 5 μl was spotted onto reversed-phase thin-layer chromatography (TLC) plates (KC18F, Whatman); plates were developed in acetonitrile-methanol-water (1:1:1) and sprayed with diazotized sulfanilic acid for visualization of compounds (30). Purified pyrrolnitrin (from J. Roitman, USDA, Albany, CA) and pyoluteorin (purified by preparative TLC from a culture extract by published methods [13]) served as standards. Pyrrolnitrin and pyoluteorin standards were observed as maroon and brown spots with R_f values of 0.28 and 0.75, respectively. The amount of pyrrolnitrin in a sample was estimated by comparison of the size and color intensity of the visualized pyrrolnitrin spot with size and color of spots produced by 0.1, 0.7, and 5.0 μg pyrrolnitrin on the TLC plate. The detection limit for pyrrolnitrin by this method was 0.1 μg.

Antagonism of *P. tritici-repentis* in culture. Bacterial strains to be tested for fungal inhibition were grown by the transfer of 100 μl of a KMB broth culture (grown overnight) into 5 ml of fresh broth and incubation with shaking at 28 C for 3 h. Cells were washed and resuspended in 0.1 M $MgSO_4$ at 10^8 cells per milliliter. A 10-μl drop of the suspension, filter-sterilized suspension, or pyrrolnitrin-acetone solution was applied to the surface of DCMA or 523 agar medium. After incubation for 2 days at 28 C, plates were exposed to chloroform vapors for 30 min to kill bacterial cells and then vented for 60 min. To prepare a mycelial suspension of *P. tritici-repentis*, the fungus was grown for 4 days at 22 C as a still culture in 8 ml of PDB and then comminuted in 30 ml of PDB in a sterile blender. Two milliliters of the mycelial suspension was mixed with 6 ml of molten overlay agar (DCMA containing 0.1 mM $FeCl_3$ to eliminate inhibition by bacterial siderophores), and the mixture was poured over the surface of the agar plate bearing the killed bacterial cells. After 48 h at 22 C, the radial distance between the edge of the bacterial colony and the edge of fungal growth was measured. Two bacterial strains (Pf-5 and one mutant) were inoculated per agar plate; duplicate plates were evaluated in each experiment, and each experiment was done at least twice.

In some experiments, the population size of bacteria on the agar media was assessed before bacterial cells were killed. Individual spots of bacterial growth and the underlying agar were removed daily with a cork borer and suspended in sterile water. The suspension was diluted, and aliquots of the dilutions were spread on KMB agar plates.

Antagonism of *P. tritici-repentis* on wheat straw. The effects of Pf-5, derivative strains, and pyrrolnitrin on ascocarp production by *P. tritici-repentis* were evaluated in wheat straw by two methods. In the first, straw was autoclaved and then infested with *P. tritici-repentis;* antagonism was tested under constant moisture conditions. In the second, antagonism was evaluated on straw that was naturally infested with *P. tritici-repentis* and incubated under fluctuating moisture conditions. To prepare bacterial inoculum for both methods, the strains were grown on NaGly plates for 24 h at 28 C. The cells were then washed in 0.1 M $MgSO_4$ and resuspended in water at 10^8 cells per milliliter.

Antagonism in axenically infested straw. Pieces of wheat straw grown in the greenhouse were autoclaved, infested with *P. tritici-repentis,* and incubated under moisture conditions favorable for mycelial growth but not for ascocarp production, as described previously (25). Straw pieces (2 cm) were moistened between wet paper towels for 20 min, dipped individually into a bacterial suspension for 10 s, and the excess suspension was squeezed out of the straw lumens. Untreated checks were dipped in water. Some straw pieces were dipped in a solution of purified pyrrolnitrin (75 μg/ml, approximately 50 μl per straw piece). Treated straw pieces were placed on moist, sterilized perlite in petri dishes with two straw pieces per replicate dish and three replicate dishes per treatment. The closed petri dishes were incubated under ambient laboratory lighting at 24 C for 18 days; then the *P. tritici-repentis* ascocarps larger than 250 μm on each straw were counted with the use of a dissecting microscope (25×). To estimate the final bacterial populations on the straw, the two replicate straws from each dish were placed together in 30 ml of 0.1 M $MgSO_4$, sonicated, and diluted. Aliquots were then spread on plates of KMB-Sm-Ap or KMB-Km agar. The experiment was performed twice with similar results, which were combined for analysis. The experiments were treated as blocks.

Antagonism in naturally infested straw. The straw of mature wheat (cv. TAM 105) was collected at harvest from a field near Manhattan, Kansas, in which a high incidence of tan spot had been observed on the wheat prior to harvest. The straw was stored dry at room temperature for up to 6 mo before use. The upper two internodes of the stems (sheath and culm) were cut into 2-cm-long pieces. Bacterial suspensions were applied to the straw pieces as described above. Treated straws were placed onto the surface of wet perlite in pots (4 × 4 cm), five straws per pot. Three replicate pots per treatment were placed in a completely randomized design on a greenhouse bench enclosed by plastic sheeting and were misted for 8 s every 32 min for 24 h, then permitted to dry for 48 h. Additional cycles of intermittent wetting (24 h) and drying (≥48 h) were applied; after the fifth wetting cycle, straws were treated again with freshly prepared bacterial suspensions and returned to their respective pots. After a total of 13 cycles of wetting and drying, the ascocarps were counted as described. The experiment was performed twice with similar results, which were combined for analysis. The experiments were treated as blocks.

At various times during the experiments, the population size of applied bacteria in additional pots of straw was estimated (as described above) in a destructive sampling; at the end of the experiment, populations were estimated on the straws used for ascocarp counts.

RESULTS

Identification of Tn*5*-insertion mutants of Pf-5 that varied in antibiotic production. On medium 523, strain JL3985 (a Tn*5* insertion mutant of Pf-5) produced an inhibition zone against *R. solani* that was larger than that produced by Pf-5. TLC analysis of culture extracts showed that Pf-5 produced pyrrolnitrin and pyoluteorin when grown on DCMA or medium 523. But on these media, strain JL3985 did not produce detectable concentrations of pyrrolnitrin and produced more pyoluteorin than did Pf-5, as assessed by the size and color intensity of spots on developed TLC plates (Table 1). In addition, JL3985 produced antibiotic 3 on medium 523. In contrast, Pf-5 produced this antibiotic on glucose-amended nutrient agar but not on 523 (20). Both Pf-5 and JL3985 produce a fluorescent siderophore when grown in KMB. JL3985 had a single Tn*5* insertion located in an 8.5-kb *Eco*RI fragment of genomic DNA (Table 1).

Of the Tn*5* mutants that were selected for 5-fluoro-DL-tryptophan resistance before being screened for inhibition against *R. solani,* one strain (JL4062) did not produce a zone of inhibition, and one strain (JL4064) produced a zone greater than that produced by Pf-5. JL4062 produced a pyoverdine siderophore but no detectable pyrrolnitrin, pyoluteorin, or antibiotic 3 on medium 523 (Table 1). This pleiotropic mutant also had a colony morphology (colonies larger, lighter in color, and with a brighter fluorescence under UV light than colonies of Pf-5) that is characteristic of the antibiotic production (Apd^-) mutants described previously (20). JL4064 produced less pyrrolnitrin than did Pf-5. By comparing thin-layer chromatographs of extracts from JL4064 and Pf-5 with standards of known concentration, we estimate that JL4064 and Pf-5 produce approximately 0.25 and 2.5 μg, respectively, of pyrrolnitrin per 30-ml agar-plate culture. JL4064 produced more pyoluteorin than did Pf-5, as indicated by the larger spot on TLC plates, and produced antibiotic 3 on 523 agar (a medium on which Pf-5 does not produce detectable concentrations of antibiotic 3). JL4064 had a single insertion of Tn*5* located in a 21-kb *Eco*RI fragment of genomic DNA (Table 1).

Antibiosis of *P. tritici-repentis* in agar culture. *P. tritici-repentis* was sensitive to ≥0.2 μg of purified pyrrolnitrin applied to overlay plates; a 25-mm inhibition zone was produced by 2 μg of the antibiotic. Strain Pf-5 grown on 523 agar or DCMA inhibited

TABLE 1. Antibiotic production and inhibition of *Pyrenophora tritici-repentis* by *Pseudomonas fluorescens* Pf-5 and derivative strains

	Antibiotic production[x]			Radius of inhibition zone for *P. tritici-repentis*[y] (mm)		Size of Tn*5*-containing fragment (kb)		
Strain	Pyr	Plt	A3	DCMA	523	*Eco*RI	*Bam*HI	Doubling time[z] (min)
Pf-5 (wild-type)	+	+	−	24 a	26 a	...	...	51 bc
JL4140	+	−	−	25 a	28 a	10.1	5.2, 4.6	56 ab
JL4062	−	−	−	0 c	1 c	ND	ND	64 a
JL4064	(+)	+	+	9 b	16 b	21.0	16.0, 4.6	51 bc
JL3985	−	+	+	0 c	18 b	8.5	6.6, 2.8	45 c
JL3985(pJEL1884)	+	+	ND	26 a	ND	ND	ND	ND

[x]Pyr = Pyrrolnitrin; Plt = pyoluteorin; A3 = antibiotic 3; − = antibiotic not detected in culture extracts; + = antibiotic detected in culture extracts; (+) = production estimated to be 0.1 times that of Pf-5; ND = not determined. Production of Pyr and Plt were tested on dilute cornmeal agar (DCMA) and medium 523; production of antibiotic 3 was tested on medium 523 only. Strain JL3985(pJEL1884) was tested on DCMA only. All strains produced a pyoverdine siderophore on King's medium B (KMB).

[y]Inhibition zone radii with *P. tritici-repentis* as overlay of bacterial culture grown on DCMA or medium 523. Values within a column followed by the same letter do not differ ($P = 0.05$) according to Duncan's new multiple range test.

[z]In KMB broth at 28 C. Values within a column followed by the same letter do not differ ($P = 0.05$) according to Duncan's new multiple range test.

mycelial growth of the fungus (Table 1), presumably due to production by Pf-5 of antibiotics on these media. Sterile filtrates of the Pf-5 cell suspension that were placed directly on the agar surface did not inhibit *P. tritici-repentis* (data not shown); thus, the fungal antagonism was caused by compounds produced by Pf-5 on the agar medium and not by those that may have been present in the initial inoculum. Strain JL4140, which is deficient specifically in pyoluteorin production (20), was as inhibitory to the fungus as was Pf-5. Strain JL3985, which did not produce pyrrolnitrin but overproduced pyoluteorin, did not inhibit the pathogen on DCMA. Population sizes of JL3985 and Pf-5 on agar surfaces were similar over the period of the assay. At 24, 48, and 72 h after the plates were inoculated, the populations of Pf-5 were 7.0, 7.7, and 8.0 $\log_{10}$ cfu, respectively; whereas those of JL3985 were 7.3, 7.9, and 8.0 $\log_{10}$ cfu, respectively. Antagonism of *P. tritici-repentis* by JL4064, which produced small quantities of pyrrolnitrin, was intermediate to that by Pf-5 or the pyrrolnitrin-deficient mutant JL3985 on DCMA. On medium 523, both JL3985 and JL4064 inhibited the pathogen, although to a lesser extent than did the wild-type Pf-5 (Table 1). Inhibition of *P. tritici-repentis* by JL3985 and JL4064 on medium 523 may have been due to the production of antibiotic 3 by these strains. Mutant JL4062, which produced no detectable antibiotics, did not inhibit *P. tritici-repentis* on either medium.

Antagonism of *P. tritici-repentis* in straw. Application of strain Pf-5, JL4140, or purified pyrrolnitrin to axenically colonized straw suppressed ascocarp production by *P. tritici-repentis* under conditions of constant moisture (Table 2). Strain JL4064, which produced small quantities of pyrrolnitrin in culture, also suppressed ascocarp production by the pathogen. Neither the pyrrolnitrin-deficient strain JL3985 nor the Apd$^-$ strain JL4062 suppressed ascocarp production by the fungus. Eighteen days after the straw was inoculated, the population size of JL3985 was approximately one log unit lower than that of Pf-5 on the straw pieces.

Relatively few ascocarps (an average of 1.4 per 2 cm of straw) were produced by *P. tritici-repentis* on naturally infested wheat straw exposed to cycles of alternate drying and wetting; this level of ascocarp production is typical of that produced on field-collected material under these conditions (38). Treatment of straw with *P. fluorescens* Pf-5 or its derivatives did not decrease ascocarp production by *P. tritici-repentis* under these conditions (Table 2). Population sizes of applied bacteria decreased from 6.3–7.3 $\log_{10}$ cfu per straw to 3.4–4.6 $\log_{10}$ cfu per straw during the 3 wk after application. Eight weeks after application to straw, bacterial population sizes were near the limit of detection (2.0 $\log_{10}$ cfu per straw). Indigenous microflora and desiccation stress may have reduced the population size of *P. fluorescens* on the naturally infested straw.

TABLE 2. Effects of *Pseudomonas fluorescens* Pf-5 and derivative strains on ascocarp production by *Pyrenophora tritici-repentis* in wheat straw[w]

	Axenically colonized straw		Naturally infested straw	
Treatment[x]	Ascocarps per straw[y]	Bacterial population[z] ($\log_{10}$ cfu per straw)	Ascocarps per straw[y]	Bacterial population[z] ($\log_{10}$ cfu per straw)
None (check)	21.2 a	...	1.4 a	...
Pf-5	0.0 b	6.2 bc	1.0 a	4.5 a
JL4140	0.2 b	6.3 c	1.1 a	4.6 a
JL4062	12.2 a	5.8 b	2.5 a	ND
JL4064	1.8 b	6.1 bc	1.4 a	ND
JL3985	14.7 a	5.3 a	1.7 a	3.4 b
Pyrrolnitrin	0.5 b	...	0.7 a	...

[w] Axenically colonized straw was incubated under conditions of constant moisture, and naturally infested straw was incubated under alternate drying and wetting.

[x] Each straw was treated with 3×10^6 cfu of bacteria or approximately 3.8 μg of pyrrolnitrin.

[y] Values are averages of two experiments with three replicates per experiment. There were two axenically colonized straws or five naturally infested straws per replicate. Values within a column followed by the same letter do not differ ($P = 0.05$) according to Duncan's new multiple range test.

[z] Colony-forming units per straw 18 days after axenically colonized straws were treated and 21 days after naturally infested straws were treated. ND = Not determined. Values within a column followed by the same letter do not differ ($P = 0.05$) according to Duncan's new multiple range test.

Identification of a genomic region involved in pyrrolnitrin production by Pf-5. From a genomic library of Pf-5, two cosmids (pJEL1884 and pJEL1993) were identified that hybridized to the 8.5-kb *Eco*RI fragment containing the Tn*5* insertion and flanking sequences of JL3985. The identity of cloned genomic regions as homologs of that mutagenized in JL3985 was confirmed by restriction mapping and Southern analysis (Fig. 1). The Tn*5* insertion in JL3985 mapped to a 2.8-kb *Eco*RI fragment that was present in both hybridizing clones. Because genomic DNA of strain JL3985 had two *Bam*HI fragments of 6.6 and 2.8 kb that hybridized to Tn*5*, we expected that the genomic DNA of Pf-5 would contain a single *Bam*HI fragment of 3.7 kb that would hybridize to pJEL1995. In contrast, genomic DNA of Pf-5, pJEL1884, and pJEL1993 contained an 8.5-kb *Bam*HI fragment that hybridized to pJEL1995. Thus, a *Bam*HI recognition site was associated with the Tn*5* insertion of JL3985 and was located at the site of Tn*5* insertion (Fig. 1). Although elucidation of the mechanism by which the *Bam*HI site was introduced into the genome of JL3985 was beyond the scope of this study, several possibilities exist. Cytosine, the nucleotide at the 3′ end of the recognition sequence of *Bam*HI, is also at the 5′ end of the inverted repeat of Tn*5* (3). Thus, an insertion of Tn*5* into a target sequence containing the other five bases recognized by *Bam*HI would introduce a *Bam*HI restriction site at the site of insertion. Alternatively, an error in repair replication associated with Tn*5* transposition (3) also could introduce a restriction site. Nevertheless, the presence in pJEL1884 and pJEL1993 of *Eco*RI and *Hin*dIII fragments of the expected size that hybridized to pJEL1995 provided compelling evidence that fragments cloned in these cosmids were identical to the mutagenized region of JL3985.

Conjugal transfer of pJEL1884 and pJEL1993 from *E. coli* strain DH5α into JL3985 was unsuccessful. Conjugal transfer of pJEL1884 from *E. coli* strain HB102 into JL3985, however, occurred at a low frequency (approximately 10^{-7} transconjugants per recipient). In contrast to strain JL3985, transconjugant JL3985(pJEL1884) inhibited mycelial growth of *P. tritici-repentis* on DCMA at a level similar to the inhibition by the wild-type Pf-5 (Table 1). Production of pyrrolnitrin by strain JL3985(pJEL1884) was comparable to that of strain Pf-5, as determined by TLC analysis (Fig. 2). Although JL3985 overproduced pyoluteorin on 523 and DCMA, JL3985(pJEL1884) produced pyoluteorin at a level slightly less than that of Pf-5, as assessed by the examination of thin-layer chromatographs. Transconjugants of *P. putida* strain G1-rif harboring pJEL1884, however, did not produce detectable levels of pyrrolnitrin or inhibit *P. tritici-repentis* on DCMA. Thus, the genomic region cloned in pJEL1884 was not sufficient to confer detectable pyrrolnitrin production to *P. putida*.

DISCUSSION

Mycelial growth of *P. tritici-repentis* was inhibited by pyrrolnitrin, one of the antibiotics produced by *P. fluorescens* strain

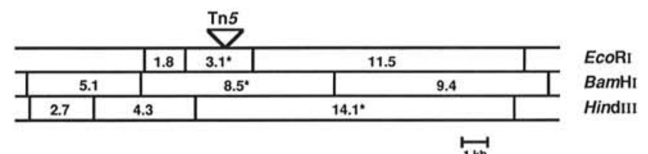

Fig. 1. Restriction-site map of cosmid clone pJEL1884 from *Pseudomonas fluorescens* Pf-5. Numbers indicate size in kilobase pairs, and asterisks indicate fragments that hybridized with the DNA flanking the site of Tn*5* insertion in mutant strain JL3985.

Pf-5. Derivative strains of Pf-5, obtained by Tn*5* mutagenesis, differed in antibiotic production and in antagonism of *P. tritici-repentis*. On DCMA medium, the fungus was not inhibited by JL3985 (which produced no pyrrolnitrin) or by JL4062 (which produced no antibiotics), was partially inhibited by JL4064 (which produced an intermediate amount of pyrrolnitrin), and was maximally inhibited by the strains that produced wild-type levels of pyrrolnitrin. Populations of Pf-5 and JL3985 on agar test plates were similar; thus, lack of inhibition by JL3985 could not be explained by lack of growth (see also growth rates in Table 1). There was no correlation of antagonism with pyoluteorin production by the mutants; thus, pyoluteorin production was not involved in inhibition of *P. tritici-repentis* by strain Pf-5. In addition to pyrrolnitrin, Pf-5 produced other compounds toxic to *P. tritici-repentis* under some nutrient conditions, as indicated by the inhibitory activity of JL3985 on medium 523 despite the inability of this strain to produce pyrrolnitrin. Pf-5 derivatives that differed in antagonism to *P. tritici-repentis* all produced a pyoverdine siderophore; furthermore, the overlay inhibition assay was conducted under conditions of iron sufficiency. Therefore, the antifungal activity of Pf-5 was not attributed to a pyoverdine-mediated iron competition.

Pyrrolnitrin, Pf-5, and pyrrolnitrin-producing derivative strains of Pf-5 suppressed ascocarp production by *P. tritici-repentis* in axenically infested straw that was incubated under conditions of constant moisture and temperature and low light intensity. Strains JL3985 and JL4062, which did not produce pyrrolnitrin, did not inhibit the fungus under these conditions. The population size of JL3985 was approximately one log unit less than that established by Pf-5 on wheat straw. Although it is possible that the lack of inhibition by JL3985 was due to its smaller population size, large population size alone did not cause the suppression by effective strains; the population size established by the ineffective strain JL4062 was similar to that of the effective strains JL4140 and Pf-5 (Table 2). The findings of this study extend those of others that have suggested that pyrrolnitrin is produced in situ by *Pseudomonas* spp. in concentrations adequate to suppress certain phytopathogenic fungi (12,15,16). Strains of *P. cepacia* suppress damping-off of radish caused by *R. solani* (12) or seedling blight of corn caused by *Drechslera maydis* (16), whereas non-pyrrolnitrin-producing mutants derived from these strains do not suppress disease.

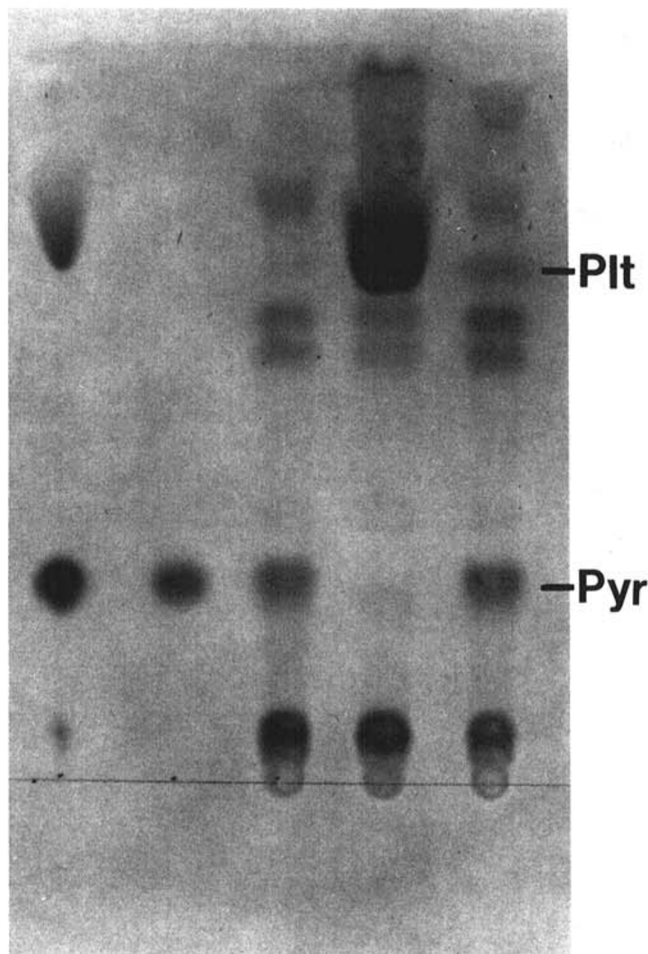

Fig. 2. Reversed-phase thin-layer chromatograph of extracts from 10-ml agar cultures of *Pseudomonas fluorescens* Pf-5 and derivative strains, oversprayed with diazotized sulfanilic acid. Lane 1, purified pyrrolnitrin plus purified pyoluteorin; lane 2, 0.7 μg pyrrolnitrin; lane 3, culture extract from the restored mutant JL3985(pJEL1884); lane 4, culture extract from pyrrolnitrin-deficient mutant JL3985; and lane 5, culture extract from wild-type Pf-5. Pyr = pyrrolnitrin; Plt = pyoluteorin.

Tryptophan is a precursor of pyrrolnitrin (39), and certain tryptophan-analogue-resistant mutants of *Pseudomonas* spp. have elevated cellular pool sizes of tryptophan and thus are altered in their accumulation of pyrrolnitrin (5) or indole acetic acid (32). Strain JL4062, which produced no known antifungal compounds, and strain JL4064, which produced trace levels of pyrrolnitrin and overproduced pyoluteorin, were selected originally for resistance to the tryptophan-analogue 5-fluoro-DL-tryptophan and were then screened for antagonism of *R. solani*. Although both strains differed from Pf-5 in the production of pyrrolnitrin, neither differed from Pf-5 in this characteristic alone. Due to the multiple antifungal compounds produced by strain Pf-5 and the complexity of their regulation, attempts to obtain pyrrolnitrin biosynthesis mutants by the traditional approach of screening for lack of antagonism of an indicator fungus have not been straightforward. The described overlay method for testing antagonism to *P. tritici-repentis* on DCMA, however, appears to provide an assay for pyrrolnitrin deficiency without interference from siderophores, pyoluteorin, or antibiotic 3.

In *Pseudomonas* spp., the transcription of genes for the biosynthesis of antifungal metabolites is controlled by one or more regulatory genes that are clustered with biosynthesis genes (9,29) or located distally in the genome (22). Among factors known to influence antibiotic production by *Pseudomonas* spp. are concentrations of glucose (10) and trace metals (36). In at least some strains, the product of a global regulatory gene is required for the production of several antifungal compounds, presumably acting as a transcriptional activator of biosynthetic genes (22; Gaffney, *personal communication*). Mutations in regulatory genes that control the transcription of one or more antibiotic biosynthesis pathways will have pleiotropic effects (22), such as those associated with the genetic lesion of the Apd$^-$ mutant JL4062.

Strain JL3985, which did not produce detectable levels of pyrrolnitrin and overproduced pyoluteorin and antibiotic 3 on certain media, was selected initially for its enhanced antagonism against *R. solani*. The pleiotropic nature of the mutation suggests that the Tn*5* insertion of JL3985 may be in a regulatory gene, such as those discussed above, that differentially regulates the production of pyrrolnitrin and other antifungal compounds. If the Tn*5* insertion of JL3985 is located in a pyrrolnitrin biosynthetic gene, however, the coincident effect of enhancing pyoluteorin and antibiotic 3 production may be explained by effects of pyrrolnitrin on expression of genes involved in production of other antibiotics. Although antifungal compounds are known to affect expression of genes required for their own biosynthesis (9), we are not aware of cases in which such compounds influence the expression of genes for the biosynthesis of a second antifungal compound. Because such effects are plausible, however, the possibility that JL3985 has a mutation in a gene encoding an enzyme in the biosynthetic pathway of pyrrolnitrin cannot be excluded.

A genomic region involved in pyrrolnitrin production of strain Pf-5 was identified in this study by hybridization to regions flanking the Tn*5* insertion of JL3985 and by restoration to wild type of the Pyr$^-$ phenotype of JL3985. Nevertheless, the cloned region did not confer pyrrolnitrin production to a strain of *P. putida*. Thus, it is clear that the biosynthetic and regulatory genes required for pyrrolnitrin production are not wholly contained on the cloned genomic region. A region involved in pyrrolnitrin

production by *P. cepacia* also has been studied (16), although, as with the genomic region of *P. fluorescens* described here, the biosynthetic or regulatory function of the region is unknown.

Pyrrolnitrin and Pf-5 effectively suppressed ascocarp formation by *P. tritici-repentis* on axenically colonized straw incubated under controlled conditions, but neither suppressed the pathogen on naturally infested straw under conditions simulating a field environment. The physical conditions of the test—heat, light, and repeated leaching from simulated rainfall—may have decreased persistence of the antibiotic in straw. Furthermore, the population size of Pf-5 declined precipitously (by three log units in the first 3 wk of incubation) on the naturally infested straw. Indeed, very small populations of fluorescent pseudomonads were associated with wheat-straw residue collected from agricultural fields in Kansas (Pfender and Wootke, *unpublished*). Nevertheless, other bacterial genera that are commonly found in microbial communities of wheat-straw residue in the field may persist on straw (27) and thus be suitable as antagonists of residueborne phytopathogenic fungi. Because the potential importance of antibiotic production in suppression of *P. tritici-repentis* was demonstrated here, future research may focus on antibiotic-producing strains of bacterial genera that are more commonly associated with wheat-straw residue in the field. Such strains may be obtained by the screening of indigenous microflora or possibly by the introduction of genes for antibiotic production into a strain that proliferates on wheat-straw residue under field conditions. Genes for pyrrolnitrin production may be useful in derivation of a suitable antagonist, and such use will depend on expression of all genes required for regulation and biosynthesis of pyrrolnitrin in a heterologous host.

LITERATURE CITED

1. Adee, E. A., and Pfender, W. F. 1989. The effect of primary inoculum level of *Pyrenophora tritici-repentis* on tan spot epidemic development in wheat. Phytopathology 79:873-877.
2. Ausbel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Smith, J. A., Seidman, J. G., and Struhl, K. 1987. Current Protocols in Molecular Biology. John Wiley & Sons, New York.
3. Berg, D. E., and Berg, C. M. 1983. The prokaryotic transposable element Tn*5*. Bio/Technology 1:417-435.
4. Boulnois, G. J., Varley, J. M., Sharpe, G. S., and Franklin, F. C. H. 1985. Transposon donor plasmids, based on ColIb-P9, for use in *Pseudomonas putida* and a variety of other Gram negative bacteria. Mol. Gen. Genet. 200:65-67.
5. Elander, R. P., Mabe, J. A., Hamill, R. L., and Gorman, M. 1971. Biosynthesis of pyrrolnitrins by analogue-resistant mutants of *Pseudomonas fluorescens*. Folia Microbiol. (Prague) 16:156-165.
6. Figurski, D. H., and Helinski, D. R. 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. Proc. Natl. Acad. Sci. U.S.A. 76:1648-1652.
7. Fravel, D. R. 1988. Role of antibiosis in the biocontrol of plant diseases. Annu. Rev. Phytopathol. 26:75-91.
8. Gergen, J. P., Stern, R. H., and Wensink, P. C. 1979. Filter replicas and permanent collections of recombinant DNA plasmids. Nucleic Acids Res. 7:2115-2136.
9. Gutterson, N. 1990. Microbial fungicides: Recent approaches to elucidating mechanisms. Crit. Rev. Biotechnol. 10:69-90.
10. Gutterson, N., Ziegle, J. S., Warren, G. J., and Layton, T. J. 1988. Genetic determinants for catabolite induction of antibiotic biosynthesis in *Pseudomonas fluorescens* HV37a. J. Bacteriol. 170:380-385.
11. Hickman, M. J., Orser, C. S., Willis, D. K., Lindow, S. E., and Panopoulos, N. J. 1987. Molecular cloning and biological characterization of *recA* gene from *Pseudomonas syringae*. J. Bacteriol. 169:2906-2910.
12. Homma, Y., and Suzui, T. 1989. Role of antibiotic production in suppression of radish damping-off by seed bacterization with *Pseudomonas cepacia*. Ann. Phytopathol. Soc. Jpn. 55:643-652.
13. Howell, C. R., and Stipanovic, R. D. 1979. Control of *Rhizoctonia solani* on cotton seedlings with *Pseudomonas fluorescens* and with an antibiotic produced by the bacterium. Phytopathology 69:480-482.
14. Howell, C. R., and Stipanovic, R. D. 1980. Suppression of *Pythium ultimum*-induced damping-off of cotton seedlings by *Pseudomonas fluorescens* and its antibiotic, pyoluteorin. Phytopathology 70:712-715.
15. Janisiewicz, W. J., and Roitman, J. 1988. Biological control of blue mold and gray mold on apple and pear with *Pseudomonas cepacia*. Phytopathology 78:1697-1700.
16. Jayaswal, R. K., Fernandez, M. A., Visintin, L., and Upadhyay, R. S. 1991. Transposon Tn*5*-259 mutagenesis of *Pseudomonas cepacia* to isolate mutants deficient in antifungal activity. Can. J. Microbiol. 38:309-312.
17. Kado, C. I., and Heskett, M. G. 1970. Selective media for isolation of *Agrobacterium, Cornyebacterium, Erwinia, Pseudomonas,* and *Xanthomonas*. Phytopathology 60:969-976.
18. Keel, C., Schnider, U., Maurhofer, M., Voisard, C., Laville, J., Burger, U., Wirthner, P., Haas, D., and Défago, G. 1992. Suppression of root diseases by *Pseudomonas fluorescens* CHA0: Importance of the bacterial secondary metabolite 2,4-diacetylphloroglucinol. Mol. Plant-Microbe Interact. 5:4-13.
19. King, E. O., Ward, M. K., and Raney, D. E. 1954. Two simple media for the demonstration of pyocanin and fluorescin. J. Lab. Clin. Med. 44:301-307.
20. Kraus, J., and Loper, J. E. 1992. Lack of evidence for a role of antifungal metabolite production by *Pseudomonas fluorescens* Pf-5 in biological control of Pythium damping-off of cucumber. Phytopathology 82:264-271.
21. Langley, R. A., and Kado, C. I. 1972. Studies on *Agrobacterium tumefaciens*. Conditions for mutagenesis by n-methyl-n′-nitro-n-nitrosoguanidine and relationships of *A. tumefaciens* mutants to crown gall induction. Mutat. Res. 14:277-286.
22. Laville, J., Voisard, C., Keel, C., Maurhofer, M., Défago, G., and Haas, D. 1992. Global control in *Pseudomonas fluorescens* mediating antibiotic synthesis and suppression of black root rot of tobacco. Proc. Natl. Acad. Sci. U.S.A 89:1562-1566.
23. Loper, J. E., and Buyer, J. S. 1991. Siderophores in microbial interactions on plant surfaces. Mol. Plant-Microbe Interact. 4:5-13.
24. Pfender, W. F., Kraus, J., and Loper, J. E. 1991. Role of pyrrolnitrin in antagonism of *Pyrenophora tritici-repentis* by *Pseudomonas fluorescens* strain Pf-5. (Abstr.) Phytopathology 81:1346.
25. Pfender, W. F., Pacey, C. A., and Zhang, W. 1988. Saprophytic growth and pseudothecia production by *Pyrenophora tritici-repentis* in plant tissue held at controlled water potentials. Phytopathology 78:1205-1210.
26. Pfender, W. F., Sharma, U., and Zhang, W. 1991. Effect of water potential on microbial antagonism to *Pyrenophora tritici-repentis* in wheat residue. Mycol. Res. 95:308-314.
27. Pfender, W. F., and Wootke, S. L. 1988. Microbial communities of *Pyrenophora*-infested wheat straw as examined by multivariate analysis. Microb. Ecol. 15:95-113.
28. Pfender, W. F., Zhang, W., and Nus, A. 1993. Biological control to reduce inoculum of the tan spot pathogen *Pyrenophora tritici-repentis* in surface-borne residues of wheat fields. Phytopathology 83:371-375.
29. Pierson, L. S., III, and Thomashow, L. S. 1992. Cloning and heterologous expression of the phenazine biosynthetic locus from *Pseudomonas aureofaciens* 30-84. Mol. Plant-Microbe Interact. 5:330-339.
30. Roitman, J. N., Mahoney, N. E., and Janisiewicz, W. J. 1990. Production and composition of phenylpyrrole metabolites produced by *Pseudomonas cepacia*. Appl. Microbiol. Biotechnol. 34:381-386.
31. Sambrook, J., Fritsch, E. F., and Maniatis, T. A. 1989. Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
32. Smidt, M., and Kosuge, T. 1978. The role of indole-3-acetic acid accumulation by alpha methyl tryptophan-resistant mutants of *Pseudomonas savastanoi* in gall formation on oleanders. Physiol. Plant Pathol. 13:203-214.
33. Staskawicz, B. J., Dahlbeck, D., Keen, N., and Napoli, C. 1987. Molecular characterization of cloned avirulence genes from Race 0 and Race 1 of *Pseudomonas syringae* pv. *glycinea*. J. Bacteriol. 169:5789-5794.
34. Takeda, R. 1958. *Pseudomonas* pigment. I. Isolation and properties of pyoluteorin. J. Ferment. Technol. Osaka 36:281-286.
35. Thomashow, L. S., and Weller, D. M. 1988. Role of phenazine antibiotic from *Pseudomonas fluorescens* in biological control of *Gaeumannomyces graminis* var. *tritici*. J. Bacteriol. 170:3499-3508.
36. Weinberg, E. D. 1990. Roles of trace metals in transcriptional control of microbial secondary metabolism. Biol. Met. 2:191-196.
37. Wright, J. M. 1956. The production of antibiotics in soil. III. Production of gliotoxin in wheat straw buried in soil. Ann. Appl. Biol. 44:461-466.
38. Zhang, W. 1992. Effect of moisture, fungal antagonism, and their interaction on ascocarp production by *Pyrenophora tritici-repentis* in wheat straw: Laboratory and conservation-tillage field studies. Ph.D. diss. Kansas State University, Manhattan.
39. Zhou, P., Mocek, U., Siesel, B., and Floss, H. G. 1992. Biosynthesis of pyrrolnitrin. Incorporation of ^{13}C,^{15}N double-labelled D- and L-tryptophan. J. Basic Microbiol. 32:209-214.