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An Interspecies Signaling System Mediated by Fusaric Acid Has Parallel Effects on Antifungal Metabolite Production by *Pseudomonas protegens* Strain Pf-5 and Antibiosis of *Fusarium* spp.

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Pseudomonas protegens **strain Pf-5 is a rhizosphere bacterium that suppresses soilborne plant diseases and produces at least seven different secondary metabolites with antifungal properties. We derived mutants of Pf-5 with single and multiple mutations in biosynthesis genes for seven antifungal metabolites: 2,4-diacetylphoroglucinol (DAPG), pyrrolnitrin, pyoluteorin, hydrogen cyanide, rhizoxin, orfamide A, and toxoflavin. These mutants were tested for inhibition of the pathogens *Fusarium verticillioides* and *Fusarium oxysporum* f. sp. *pisi*. Rhizoxin, pyrrolnitrin, and DAPG were found to be primarily responsible for fungal antagonism by Pf-5. Previously, other workers showed that the mycotoxin fusaric acid, which is produced by many *Fusarium* species, including *F. verticillioides*, inhibited the production of DAPG by *Pseudomonas* spp. In this study, amendment of culture media with fusaric acid decreased DAPG production, increased pyoluteorin production, and had no consistent influence on pyrrolnitrin or orfamide A production by Pf-5. Fusaric acid also altered the transcription of biosynthetic genes, indicating that the mycotoxin influenced antibiotic production by Pf-5 at the transcriptional level. Addition of fusaric acid to the culture medium reduced antibiosis of *F. verticillioides* by Pf-5 and derivative strains that produce DAPG but had no effect on antibiosis by Pf-5 derivatives that suppressed *F. verticillioides* due to pyrrolnitrin or rhizoxin production. Our results demonstrated the importance of three compounds, rhizoxin, pyrrolnitrin, and DAPG, in suppression of *Fusarium* spp. by Pf-5 and confirmed that an interspecies signaling system mediated by fusaric acid had parallel effects on antifungal metabolite production and antibiosis by the bacterial biological control organism.**

Pseudomonas is a heterogeneous genus of *Gammaproteobacteria* composed of species with diverse ecological roles; certain strains are members of the plant microbiome contributing to plant growth and health. Our studies focus on the soil bacterium *Pseudomonas protegens* Pf-5, which is known for its capacity to suppress plant diseases and produce a large spectrum of metabolites with antibiotic activity (1, 2). The antibiotics produced by Pf-5 include pyrrolnitrin (3), pyoluteorin (4), analogs of rhizoxin (5, 6), hydrogen cyanide (7), 2,4-diacetylphloroglucinol (DAPG) (8), monoacetylphloroglucinol (MAPG) (9) (an intermediate in the DAPG biosynthetic pathway [10, 11]), the lipopeptide orfamide A (12), and toxoflavin (13). The antibiotics produced by Pf-5 have distinct but overlapping activity spectra, and it is likely that multiple compounds contribute to the interactions of Pf-5 with target plant pathogens that result in disease suppression. Commonly, the role of an antibiotic in biological control is assessed by comparing the level of disease suppression provided by a wild-type strain versus an antibiotic-deficient mutant (14). For strain Pf-5, however, this approach has not been highly successful in providing evidence for the roles of antibiotics in biological control: mutations in a specific biosynthetic locus, which eliminate the capacity of the bacterium to produce a single antibiotic, can exhibit wild-type levels of biological control activity (7). We hypothesize that the large spectrum of antibiotics produced by Pf-5 provides both enhanced potency and redundancy in the suppression of plant disease. Consequently, biological control may not depend on the production of any single antibiotic but may involve the production of several antibiotics. Here, we developed and characterized a set of mutants needed to test that hypothesis; collectively, the mutants provide the tools for a systematic approach to identify the specific antibiotics contributing to Pf-5's capacity to suppress plant disease.

In *Pseudomonas* spp., the production of antibiotics and other exoproducts requires the Gac/Rsm signal transduction system, which controls the expression of target genes through a complex signal transduction pathway involving regulatory RNAs and translational repression (15). Due to the preeminent role of the response regulator GacA in this pathway, *gacA* mutants of Pf-5 do

Received 7 August 2015 Accepted 3 December 2015

Accepted manuscript posted online 11 December 2015

Citation Quecine MC, Kidarsa TA, Goebel NC, Shaffer BT, Henkels MD, Zabriskie TM, Loper JE. 2016. An interspecies signaling system mediated by fusaric acid has parallel effects on antifungal metabolite production by *Pseudomonas protegens* strain Pf-5 and antibiosis of *Fusarium* spp. Appl Environ Microbiol 82:1372–1382. doi:10.1128/AEM.02574-15.

Editor: J. L. Schottel, University of Minnesota

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We dedicate this article to the late Aline A. Pizzirani-Kleiner, without whom this study could not have been done.

Supplemental material for this article may be found at http://dx.doi.org/10.1128/AEM.02574-15.

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not produce any of the antibiotics produced by wild-type Pf-5 (6, 12, 13, 16–18). Consequently, compared to wild-type strains, *gacA* mutants can reveal the combined contributions of antibiotics and other exoproducts to a range of biological activities of *Pseudomonas* spp. In this study, we built upon the well-characterized effects of a *gacA* mutation on the phenotypes and transcriptome of Pf-5 (16, 17) to construct a mutant set that represents all seven of the known *gacA*-regulated antibiotics produced by Pf-5 (i.e., pyrrolnitrin, pyoluteorin, analogs of rhizoxin, hydrogen cyanide, DAPG and its intermediate MAPG, orfamide A, and toxoflavin). As proof of principle, we used the mutant set to identify the specific antibiotics responsible for inhibition of *Fusarium*, a genus of filamentous ascomycete fungi that includes many plant pathogens of global agricultural importance.

Diseases caused by *Fusarium* spp. include wilts, rots, cankers, and blights of numerous agronomic, horticultural, and forest plants grown in agricultural, landscape, and natural settings (19). *F. verticillioides* causes ear rot of maize and sorghum (20) and Pokkah Boeng disease of sugarcane (21). Members of the *F. oxysporum* species complex cause wilt diseases of more than one hundred plant species (19). Diseases caused by these *Fusarium* spp. are difficult to control with existing fungicides or soil fumigants, and many agricultural plants lack resistance to them (20, 22). To meet the need for alternative disease management strategies, scientists have turned to microorganisms that inhibit *Fusarium* spp. in search of biological control agents (22). *Pseudomonas* spp. are prominent members of the suppressive soil microflora, as components of *Fusarium*-suppressive soils (23–25) or as strains applied to seeds, soil, or plant growth media to achieve biological control (23, 26–29). Among the strains of *Pseudomonas* spp. effective in biological control are *P. protegens* Pf-5 and the closely related strain *P. protegens* CHA0 (30). These strains of *P. protegens* produce a nearly identical spectrum of antibiotics, with the exception of the rhizoxin analogs, which are produced by Pf-5 but not by CHA0. The production of hydrogen cyanide contributes to the capacity of strain CHA0 to suppress crown and root rot of tomato caused by *F. oxysporum* f. sp. *radicis-lycopersici* (31, 32), but the antibiotic(s) responsible for inhibition of *Fusarium* spp. by strain Pf-5 remains unknown.

Many *Fusarium* spp. produce toxic secondary metabolites, such as trichothecenes, fumonisins (33), and fusaric acid (FA) (34). These mycotoxins can be produced by the fungus in infected plant tissues, thereby contaminating agricultural products, rendering them unacceptable for food or feed. Beyond their roles as virulence factors in some plant diseases, mycotoxins are thought to enhance the fitness of the producing fungi by other mechanisms, such as protection from oxidative stress (35) and from microbial competitors in natural habitats (36). During the saprotrophic and parasitic phases of their life cycle, *Fusarium* spp. encounter many environmental stresses, including exposure to antifungal metabolites produced by their microbial coinhabitants (37). An early example of the role of mycotoxins in defense against antibiosis was provided by Duffy and Défago in 1997, who demonstrated that FA produced by isolates of *F. oxysporum* f. sp. *radicis-lycopersici* acts as a chemical signal that represses DAPG production by *P. protegens* CHA0 (28). Since that discovery was reported, others have substantiated that FA represses the production of DAPG (31, 38) and phenazines (39) and alters the production of pyoverdine siderophores (40, 41) by strains of *Pseudomonas* spp. FA acts, directly or indirectly, by influencing the expression of biosynthesis genes, including genes for the biosynthesis of DAPG (42–44) and phenazine-1-carboxylate (39).

The purpose of this study was 3-fold. Our first goal was to identify the specific antifungal compounds responsible for antibiosis of *Fusarium* spp. by Pf-5 using a set of mutants with deletions in each of the seven known antifungal compounds produced by Pf-5 under the control of GacA. Second, we set out to evaluate the influence of FA on the production of these antifungal compounds, thereby extending knowledge of the effects of this mycotoxin to a larger set of secondary metabolites produced by this bacterium. Our final objective was to determine the influence of FA on antibiosis against *F. verticillioides* by Pf-5 and derivative strains.

MATERIALS AND METHODS

Microorganisms and culture conditions. *P. protegens* strain Pf-5 was provided by Charles Howell, who isolated it from soil in College Station, TX (3). Pf-5 and derivative strains (Table 1) were stored in Difco nutrient broth (Becton, Dickinson and Company, Sparks, MD) containing 15% glycerol at −80°C. Fresh cultures were started from frozen stocks for each experiment by plating on King's medium B (KBM) (45) and incubating plates at 27°C. Other media used in this study were Difco potato dextrose agar (PDA) (Becton, Dickinson and Company, Sparks, MD), Difco potato dextrose broth (PDB) (Becton, Dickinson and Company), PCG (46), and nutrient yeast broth (8 g Difco nutrient broth and 5 g Bacto yeast extract) supplemented with 1% glycerol (NYBGly) (47).

Fusarium verticillioides T4-0, which was isolated from corn seed in Oregon, USA, was a gift from Cynthia Ocamb (Oregon State University). *Fusarium oxysporum* f. sp. *pisi* R2-238 (race 2) and R5 (race 5), which were isolated from pea near Mount Vernon, WA, USA, were provide by Lyndon Porter (Washington State University). *Fusarium* spp. were maintained at room temperature in pure culture on PDA immersed in sterilized water. Inoculum for experiments was grown by transferring a PDA plug from these maintenance cultures to fresh PDA and incubating plates at 27°C for 5 days.

Mutant construction. A set of mutants was assembled to compare to wild-type Pf-5 for the inhibition of *Fusarium* spp. (Table 1). Mutants were constructed using an overlap extension PCR method (48) followed by a mating and selection process described previously (16, 49, 50). Briefly, the DNAs flanking a target gene were amplified and combined using overlap extension PCR to generate a DNA fragment with a deletion in the target gene. The resulting DNA fragment was cloned into the suicide plasmid pEX18Tc (51) in *Escherichia coli* S17-1 (52), and the recombinant plasmid was introduced into Pf-5 by conjugation, selecting for tetracycline resistance. The fragment containing the deletion was integrated into the Pf-5 genome by homologous recombination, and resolved merodiploids were selected by tetracycline sensitivity and growth on LB containing 5% sucrose due to the presence of genes for tetracycline resistance and levan sucrase on pEX18Tc. The sequence of each mutated locus was confirmed to be as expected by performing PCR across the deletion site and sequencing the resultant product. Primers used to construct and confirm mutations were published previously (9, 16, 49, 50, 53) or are listed in Table 2. Multiple mutants were created by repetition of the mating, selection, and confirmation process. Certain mutants were constructed by a process involving a gentamicin-resistant intermediate, in which the gentamicin resistance gene was evicted using Flp recombinase, resulting in a target gene deletion flanked by 85- to 86-bp Flp recombinase target (FRT) sites (48). Mutants having these FRT sequences are noted in Table 1, and a detailed description of this method for generating Pf-5 mutants has been published (49).

Antagonism assays. Suppression of *Fusarium* spp. by Pf-5 and derivative strains was assessed from a dual-culture assay on PDA supplemented with 0.1 mM $FeCl_3$ (PDA-Fe). $FeCl_3$ was added to PDA to avoid the influence of siderophores produced by Pf-5 on growth of *Fusarium* spp. Each petri plate was inoculated with 10 μl of a bacterial suspension (op-

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TABLE 1 *Pseudomonas protegens* Pf-5 and derivative strains used in this study

Strain	Designation	Description[a]	Reference(s)
Pf-5	JL4585	Soil isolate; produces Ofa, Prn, Plt, HCN, MAPG, DAPG, Rzx	3, 68
Mutants of Pf-5			
Δ*gacA*	JL4577	Insertion of *aphI* at the site of a 626-bp (nt 1–626) deletion in *gacA*; altered in the many phenotypes regulated by GacA; Km^r	16
Δ*ofaA*	JL4807	1,143-bp deletion internal to *ofaA*; has FRT scar; Ofa^-	16
Δ*phlA*	LK023	639-bp deletion of BglII fragment internal to *phlA*; $DAPG^-$, $MAPG^-$	9
Δ*phlD*	JL4804	162-bp deletion in *phlD*; has FRT scar; $DAPG^-$, Plt^-	49, 9
Δ*pltA*	JL4805	275-bp deletion in *pltA*; has FRT scar; Plt^-	50
Δ*prnC*	JL4793	86-bp insertion of FRT site into *prnC*; Prn^-	50
Δ*rzxB*	JL4808	1,342-bp deletion in *rzxB*; has FRT scar; Rzx^-	50
Δ*hcnB*	JL4809	239-bp deletion in *hcnB*; has FRT scar; HCN^-	53
Δ*phlD* Δ*prnC*	JL4830	$DAPG^-$, $MAPG^-$, Plt^-, Prn^-; derived by adding a *phlD* deletion to JL4793	This study
Δ*phlA* Δ*prnC*	LK026	$DAPG^-$, $MAPG^-$, Prn^-	50
Δ*phlA* Δ*rzxB*	LK027	$DAPG^-$, $MAPG^-$, Rzx^-	50
Δ*phlD* Δ*rzxB*	JL4901	$DAPG^-$, $MAPG^-$, Plt^-, Rzx^-, derived by adding an *rzxB* deletion to JL4804	This study
Δ*prnC* Δ*rzxB*	JL4902	Prn^-, Rzx^-; derived by adding an *rzxB* deletion to JL4793	This study
Δ*phlD* Δ*rzxB* Δ*prnC*	JL4844	Prn^-, $DAPG^-$, $MAPG^-$, Plt^-, Rzx^-; derived by adding an *rzxB* deletion to JL4830	This study
Δ*phlA* Δ*rzxB* Δ*prnC*	LK031	Prn^-, $DAPG^-$, $MAPG^-$, Rzx^-	50
Δ*phlD* Δ*rzxB* Δ*prnC* Δ*pltA*	JL4855	Prn^-, $DAPG^-$, $MAPG^-$, Plt^-, Rzx^-; derived by adding a *pltA* deletion to JL4844	This study
Δ*phlD* Δ*rzxB* Δ*prnC* Δ*hcnB* Δ*pltA*	JL4865	Prn^-, $DAPG^-$, $MAPG^-$, Plt^-, Rzx^-, HCN^-; derived by adding an *hcnB* deletion to JL4855	This study
Δ*phlD* Δ*rzxB* Δ*prnC* Δ*hcnB* Δ*pltA* Δ*ofaA*	JL4909	Prn^-, $DAPG^-$, $MAPG^-$, Plt^-, Rzx^-, HCN^-, Ofa^-; derived by adding an *ofaA* deletion to JL4865	This study
Δ*phlD* Δ*rzxB* Δ*prnC* Δ*hcnB* Δ*pltA* Δ*ofaA* Δ*toxB*	JL4932	Prn^-, $DAPG^-$, $MAPG^-$, Plt^-, Rzx^-, HCN^-, Ofa^-, Tox^-; derived by adding a *toxB* deletion to JL4909	This study

[a] Phenotype abbreviations: DAPG, 2,4-diacetylphloroglucinol; HCN, hydrogen cyanide; MAPG, monoacetylphloroglucinol; Ofa, orfamide A; Plt, pyoluteorin; Prn, pyrrolnitrin; Rzx, rhizoxin derivatives; Tox, toxoflavin. Mutants of Pf-5 containing deletions in *ofaA*, *phlD*, *pltA*, *prnC*, *rzxB*, *hcnB*, *and toxB* have FRT scars (85- to 86-bp Flp recombinase target sites) in those genes. In-frame deletions were generated in *phlA*, and the deleted gene does not have an inserted FRT sequence. Primers used to generate and confirm each mutation are provided in the reference to the corresponding single mutant or in Table 2. nt, nucleotides.

tical density at 600 nm [OD_{600}] = 0.05, approximately 10^8 CFU ml^{-1}) from each of three strains: Pf-5 (wild type), a Δ*gacA* mutant (JL4577), and one other mutant of Pf-5. In some experiments (specified in Results), the medium was supplemented with 0.5 mM FA (Sigma-Aldrich, St. Louis, MO). A PDA plug (2 cm in diameter) from a culture of *Fusarium* spp. was placed on the agar surface at the center of the plate. Inhibition of the *Fusarium* spp. was measured after 5 days of incubation at 27°C. The *Fusarium* growth inhibition index was calculated according to the formula $[1 - (x1/x2)] \times 100$ for Pf-5 and derivative strains, as illustrated in Fig. 1. Each experiment was done at least twice, with four replicate petri dishes for each inhibition assay in all experiments.

Quantification of secondary metabolite production by *P. protegens* Pf-5 by HPLC. Cultures of *P. protegens* Pf-5 or derivative strains were grown at 20°C or 27°C with shaking (200 rpm) in culture tubes (18-mm diameter) containing 5 ml NYBGly amended with 0.35 mM $ZnSO_4$ (NYBGly-Zn), a medium conducive to the production of DAPG, MAPG, and pyoluteorin by *P. protegens* strain CHA0 (47). In our preliminary experiments, we found that the medium was also conducive to the production of pyrrolnitrin, orfamide A, and rhizoxin WF-1360F, the primary rhizoxin analog produced by Pf-5. Cultures were harvested after 48 h of growth because the six metabolites most relevant to this study could be detected consistently from cultures of Pf-5 harvested at this time. For experiments comparing mutants to wild-type Pf-5, cultures were grown at 20°C because the production of at least one secondary metabolite, pyoluteorin, is optimized at this temperature (54). Eight cultures were grown for each strain, and cultures from four tubes were pooled prior to centrifugation, resulting in two pooled replicates. For experiments evaluating the influence of FA on secondary metabolite production, cultures were grown at 27°C, which allowed comparison to the antagonism assays done at that temperature. Four replicate cultures were grown for each treatment. Culture supernatants and pellets were extracted separately, as described previously (9, 18), and extracts were combined prior to high-pressure liquid chromatography (HPLC) analysis. In brief, 4 ml of culture was centrifuged to separate the supernatant and pellet. Supernatants were extracted twice with 2 ml ethyl acetate. In one experiment noted in Results, culture supernatants were acidified to pH 2.0 with 1 M HCl prior to extraction, which can enhance extraction of some secondary metabolites such as pyoluteorin but also results in degradation of rhizoxin (6, 55). Pellets were extracted with 4 ml acetone following sonication and centrifugation. Extracts were dried down separately under vacuum. Supernatant and pellet extracts were then resuspended in methanol, combined, and dried down under vacuum. For HPLC analysis, combined extracts were resuspended in 50 μl of 75% aqueous acetonitrile, sonicated, and centrifuged. Analysis of metabolites was performed on 5 μl per sample. Analysis of Pf-5 metabolites was performed on a Shimadzu Prominence HPLC using a Synergi Fusion-RP 80A column (4.6 by 250 mm, 4 mm; Phenomenex). The mobile phase consisted of H_2O with 0.1% acetic acid (solvent A) and acetonitrile (solvent B). A linear gradient was applied over 45 min from 20% to 100% solvent B, followed by isocratic elution at 100% solvent B for 10 min. The flow rate was 1.5 ml min^{-1}, and a Shimadzu SPD-M20A photodiode array detector was used for UV detection at 210, 254, 270, and 310 nm. For quantitative analysis, standard curves for each metabolite were constructed from injections of five concentrations, ranging from 5 ng to 1 mg, of standards using a linear-fit model (Shimadzu EZStart software, version 7.3 SP1). Pure orfamide A and rhizoxin WF-1360F were provided by Harald Gross (University of Tübingen, Germany), pure pyoluteorin was provided by Brian Nowak-Thompson (Cornell College, Mt. Vernon, IA, USA), and pure MAPG and DAPG were provided by Christoph Keel (Lausanne University, Switzerland).

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TABLE 2 Primers used in this study

Function and primer	Locus tag	cDNA target	Sequence (5′→3′)
RT-qPCR			
zwfq F	PFL_4610	*zwf*	ATCTGGCGCTGCGTAAGCTG
zwfq F			TGTCGCTCTGGGTGTTGGAG
phlDqI F	PFL_5957	*phlD*	ACGTACTGATCGTGTCC
phlDqI R			CTTGATGTAGTGCTCGC
pltA2-qF	PFL_2787	*pltA*	GACCTCGGCAGATTCC
pltA2-qR			GGGTCTTTTCTCCGA
prnCq F	PFL_3606	*prnC*	GACTTCCGCCTATGGG
prnCq R			ATGAGGGCGTGAATCC
hcnAq F	PFL_2577	*hcnA*	CAACTTCGATATTCAGCCG
hcnAq R			GTGGGCTCCGTTTCAG
rzxBq F	PFL_2989	*rzxB*	CGTTTCGACTCCCAAG
rzxBq R			CGTTTCGACTCCCAAG
pvdAq F	PFL_4079	*pvdA*	CCCACGTTCACGATTTG
pvdAq R			GCTTGAGGTAGTTGACGA
pchC2 qF	PFL_3490	*pchC*	CCACAACGGTTGATCG
pchC2 qR			AGGCATAGGCTTCGTC
Derivation of *toxB* mutation			
Gm-F			CGAATTAGCTTCAAAAGCGCTCTGA
Gm-R			CGAATTGGGGATCTTGAAGTTCCT
JR Pf5 1033 UpF1			TCAGACAAGCAAGCTTCGACCAGCACACCGAGATCA
JR Pf5 1034 UpR1			TCAGAGCGCTTTTGAAGCTAATTCGGCACAACAGCCACTACCTGCAA
JR Pf5 1034 DnF1			AGGAACTTCAAGATCCCCAATTCGGATGCCGTTGTCCTTCAGGG
JR Pf5 1035 DnR1			TCAAGCAAGCAAGCTTGACCCGGCATTTCTACTTTTGC
Confirmation of *toxB* mutation			
1034 5′F			CACGGTGTCGGTTTCCACG
1034 3′R			CATCGACGACCTGGTGACC

Effect of pyrrolnitrin and rhizoxin on the germination of *Fusarium* microconidia. Microconidia of *Fusarium* spp. were produced by inoculating 50 ml of PDB contained in 250-ml flasks with an agar plug from a 5-day culture on PDA. PDB cultures were incubated for 2 weeks at 25°C with shaking (100 rpm) under continuous light. Cultures were passed through 8 layers of sterile cheesecloth. The residue was then placed on a sterile 2-μm filter and washed with sterile water, and spores were suspended in sterile water to a final density of 20,000 spores ml^{-1}. Fifty microliters of the spore suspension was placed in each well of a 96-well tissue culture plate to a final concentration of 10^3 spores per well. Pyrrolnitrin (Sigma-Aldrich, St. Louis, MO) was suspended in methanol to a concentration of 1 mg ml^{-1} and diluted with sterile distilled water (dH_2O) to yield final concentrations used in the experiments. To each well containing the microconidia, 50 μl of the pyrrolnitrin stock or diluted solutions was added to yield final concentrations of 0, 0.05, 0.1, 0.5, 1.0, 2.5, and 5.0 μg ml^{-1} pyrrolnitrin. The control was diluted methanol at a concentration equivalent to the highest level of pyrrolnitrin tested. Rhizoxin WF-1360F was suspended in dimethyl sulfoxide (DMSO) to a concentration of 10 mg ml^{-1} and diluted with sterile dH_2O to yield final concentrations used in the experiments. To each well containing the microconidia, 50 μl of rhizoxin WF-1360F stock or dilutions was added to yield a final concentration of 0, 5, or 20 μg ml^{-1}. The control was DMSO at a concentration equivalent to that present in the solution containing highest level of rhizoxin WF-1360F tested. Plates were incubated at 27°C, and microscopic observations were recorded at 20 h. There were four replicates at each concentration, and the experiment was done three times with essentially equivalent results.

Assessing the influence of FA on antibiotic and siderophore production by *P. protegens* Pf-5. A stock solution of FA was prepared by dissolving 1 g of FA in 5 ml of methanol, bringing the concentration to 10 mg ml^{-1} with sterile water, and adjusting the pH to 6.5 with 5 N NaOH. FA was added to the medium immediately prior to inoculation with *P. protegens*, and controls received a corresponding volume of a methanol-water solution. Cultures were grown for 48 h at 27°C on a rotary shaker (200 rpm) in 5 ml of liquid medium. Bacterial growth was assessed throughout the experiment by measuring the optical density at 600 nm. Secondary metabolites were extracted from four replicate cultures and quantified by HPLC as described above.

In preliminary experiments, we evaluated the influence of FA on Pf-5 in PCG medium, which was used to assess the influence of FA on the related strain *P. protegens* CHA0 (28, 31). Strain Pf-5 did not grow as well as CHA0 in PCG medium, establishing a final optical density at 600 nm of 2.3, versus 4.0 reported for CHA0. Therefore, we tested the influence of FA on secondary metabolite production by Pf-5 in NYBGly-Zn (28) and PDB supplemented with 0.1 mM $FeCl_3$ (PDB-Fe) (pH 4.3), as these media supported growth and secondary metabolite production by Pf-5. No influence of 0.5 mM FA on the growth of Pf-5 was observed in these two media.

RNA extraction and quantitative reverse transcriptase PCR (RT-qPCR). Pf-5 was grown in 20 ml of NYBGly-Zn and FA (0 and 0.5 mM) at 27°C with shaking (200 rpm). RNA was extracted from cultures at late exponential growth phase (OD_{600} = 3.0, 12 h), and stationary growth phase (OD_{600} = 7.5, 24 h) using an RNeasy kit (Qiagen) with an adapted protocol supplied by the manufacturer. These two time points were selected because in our previous studies, genes for the biosynthesis of many antibiotics by Pf-5 were expressed at late exponential phase and stationary phase, and this expression was correlated with antibiotic production (9, 16, 17, 56). RNA samples were subjected to an on-column DNase treatment (RNeasy minikit with DNase I; Qiagen), and removal of DNA was confirmed by PCR. RNA quality was verified on a BioAnalyser 2100 (Agilent, Palo Alto, CA) by the Center for Genome Research and Biocomputing (CGRB) Core Laboratories, Oregon State University, Corvallis, OR.

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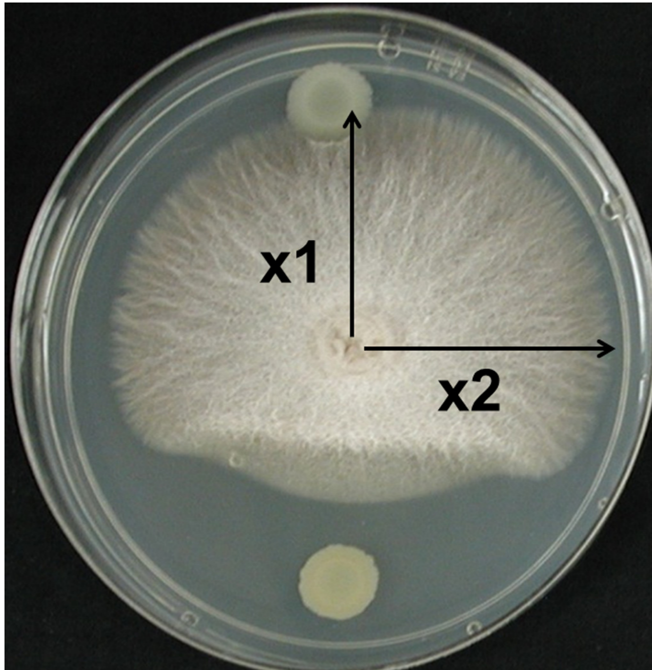

FIG 1 Inhibition of *Fusarium verticillioides* T4-0 by *P. protegens* Pf-5 (bottom) and a Δ*gacA* mutant (top) on PDA amended with 10^{-4} M $FeCl_3$. Percent inhibition was calculated from measurements of fungal radial growth toward ($x1$) versus perpendicular to ($x2$) the bacterial colony according to the formula $[1 - (x1/x2)] \times 100$.

Total RNA (1 to 10 μg) was reverse transcribed into cDNA using random hexamer primers (Invitrogen, Life Technologies, Grand Island, NY) and 200 U Superscript II RNase H− reverse transcriptase (Invitrogen) according to the procedure supplied with the enzyme.

Quantitative PCR was performed on the cDNA using SYBR green on a Roche LightCycler (Roche Diagnostics Corporation, Indianapolis, IN, USA). Primers (Table 2) were designed through LightCycler probe design software (Roche). Melting curve analysis of products was used to verify amplification of a specific product. The *zwf* gene (PFL_4610), which encodes glucose-6-phosphate 1-dehydrogenase, was selected as the reference gene because we have observed little variation in *zwf* expression in several studies of transcriptome of Pf-5 on seed and in culture or in many RT-qPCR experiments (17, 56). The fluorescence-per-cycle data for each reaction were imported into the LinRegPCR program (57, 58). LinRegPCR was used to determine the cycle threshold (C_T) value for each reaction and to determine the average amplification efficiency of each primer set. Only primers with amplification efficiencies of 1.6 or greater were used. The Pfaffl method (59) was used to determine a relative quantification of the target genes in comparison to the reference gene. Results were evaluated statistically using the relative expression software tool (60).

RESULTS AND DISCUSSION

Inhibition of *Fusarium* spp. by *P. protegens* Pf-5 and derivative strains deficient in antibiotic production. Pf-5 suppressed mycelial growth of three isolates of *Fusarium* spp. by 30% to 40% on PDA-Fe, whereas a Δ*gacA* mutant of Pf-5 suppressed mycelial growth by less than 10% on this medium (Fig. 2). These results confirm that a diffusible antifungal compound(s) produced by Pf-5 under the positive control of the Gac/Rsm signal transduction system is largely responsible for antibiosis, as established previously for Pf-5 and many other strains of *Pseudomonas* spp. (61). To identify the specific compounds contributing to suppression of *Fusarium* spp., we generated and evaluated a panel of mutants (Table 1) having single or multiple mutations in all known antibiotic biosynthetic gene clusters expressed under the positive control of GacA (16, 17).

To characterize the mutant set, we compared the antibiotic production profiles of representative mutants to that of wild-type Pf-5. Cultures were grown in NYBGly-Zn, a medium conducive to

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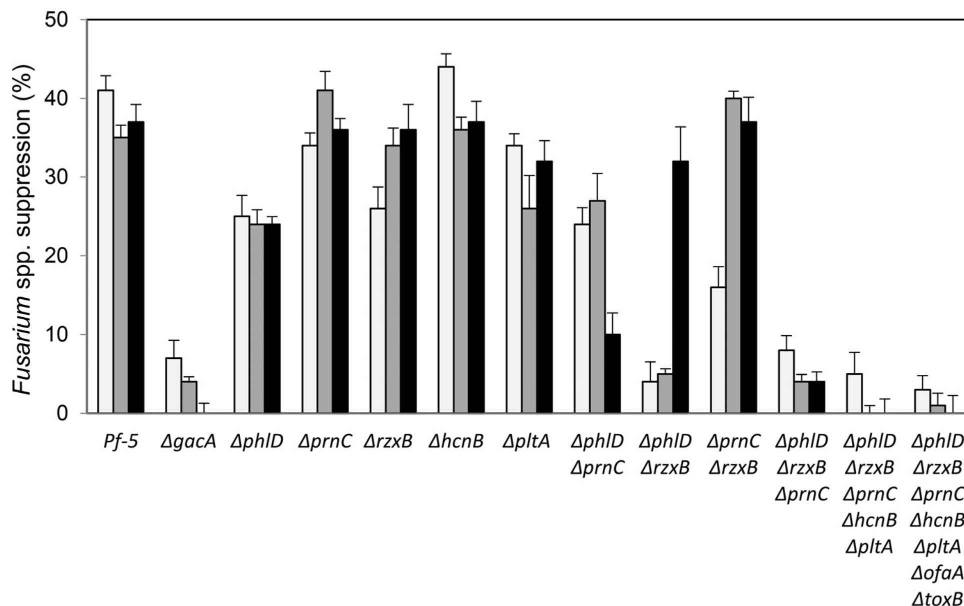

FIG 2 Inhibition of *Fusarium* spp. by *P. protegens* Pf-5 and derivative strains on PDA amended with 10^{-4} M $FeCl_3$. Pf-5 and derivatives having deletions in specified genes are shown on the *x* axis. The percent inhibition of *F. verticillioides* isolate T4-0 (white bars), *F. oxysporum* f. sp. *pisi* race 2 isolate R2-238 (gray bars), and *F. oxysporum* f. sp. *pisi* race 5 isolate R5 (black bars) was determined as shown in Fig. 1. Cultures were grown for 5 days at 27°C before radial growth of *Fusarium* spp. was measured. Means represent four replicate plates, and error bars represent the standard error of the mean.

TABLE 3 Concentrations of antibiotics produced in culture by *P. protegens* Pf-5 and derivative strains having mutations in biosynthetic genes[a]

Expt and strain/genotype	Designation	Concn, μg ml^{-1} (mean ± SEM)[b]					
		2,4-Diacetylphloroglucinol	Monoacetylphloroglucinol	Pyoluteorin	Pyrrolnitrin	Rhizoxin WF-1360F	Orfamide A
1							
Pf-5	JL4585	0.10 ± 0.04	0.13 ± 0.06	8.0 ± 2.1	1.2 ± 0.3	4.4 ± 0.4	100 ± 14
Δ*prnC*	JL4793	0.47[c]	0.73[c]	7.6 ± 0.4	BD	5.6 ± 0.7	110 ± 14
Δ*pltA*	JL4805	7.2 ± 0.4	5.6 ± 0.6	BD	1.0 ± 0.1	8.1 ± 0.4	150 ± 3
Δ*rzxB*	JL4808	0.04 ± 0.01	0.07 ± 0.02	8.0 ± 0.7	1.1 ± 0.1	BD	100 ± 7
Δ*hcnB*	JL4809	9.5 ± 0.9	12 ± 1	11 ± 7	1.6 ± 0.3	36 ± 11	270 ± 20
Δ*phlA*	LK023	BD	BD	7.2 ± 1.8	1.2 ± 0.2	3.8 ± 0.5	99 ± 11
2							
Pf-5	JL4585	0.26 ± 0.15	0.65 ± 0.36	8.0 ± 0.5	1.5 ± 0.1	4.5 ± 0.1	12 ± 1
Δ*ofaA*	JL4807	0.03 ± 0.01	0.09 ± 0.03	7.5 ± 0.6	1.6 ± 0.3	2.4 ± 0.1	0.73 ± 0.01
Δ*phlA* Δ*rzxB* Δ*prnC*	LK031	BD	BD	8.0 ± 0.1	BD	BD	110 ± 2
3							
Pf-5	JL4585	0.05 ± 0.02	0.14 ± 0.1	5.9 ± 0.4	1.3 ± 0.1	3.8 ± 0.1	92 ± 1
Δ*gacA*	JL4577	BD	BD	BD	BD	BD	BD
Δ*phlD*	JL4804	BD	BD	BD	0.63 ± 0.04	4.0 ± 0.2	130 ± 7
Δ*phlD* Δ*rzxB* Δ*prnC* Δ*hcnB* Δ*pltA* Δ*ofaA* Δ*toxB*	JL4932	BD	BD	BD	BD	BD	0.54 ± 0.11
4							
Pf-5	JL4585	1.4 ± 0.4	2.4 ± 0.6	5.5 ± 0.6	1.1 ± 0.1	5.0 ± 0.1	110 ± 7
Δ*rzxB* Δ*prnC*	JL4902	0.30 ± 0.21	0.9 ± 0.4	6.2 ± 0.6	BD	BD	90 ± 1
Δ*phlA* Δ*rzxB*	LK027	BD	BD	5.8 ± 0.1	1.2 ± 0.1	BD	88 ± 4
5							
Wild type	JL4585	0.16 ± 0.04	0.33 ± 0.07	4.0 ± 2.9	0.70 ± 0.51	2.3 ± 1.4	56 ± 32
Δ*phlD* Δ*prnC*	JL4830	BD	BD	BD	BD	7.9 ± 0.8	130 ± 14
Δ*phlD* Δ*rzxB* Δ*prnC*	JL4844	BD	BD	BD	BD	BD	130 ± 7
Δ*phlD* Δ*rzxB* Δ*prnC* Δ*hcnB* Δ*pltA*	JL4865	BD	BD	BD	BD	BD	300 ± 1
Δ*phlD* Δ*rzxB*	JL4901	BD	BD	BD	1.0 ± 0.1	BD	120 ± 1

[a] *P. protegens* Pf-5 or derivative strains were grown in 5 ml of NYBGly supplemented with 0.35 mM zinc sulfate (NYBGly-Zn). Cultures were incubated for 48 h at 20°C with shaking at 200 rpm before the concentrations of the antibiotics were quantified by HPLC.

[b] The values represent the mean and standard error of the mean for two replicates, each composed of four broth cultures. Values were rounded to two significant digits. BD, below detection, with detection limits being approximately 0.02 μg ml^{-1} for each compound.

[c] The compound was detected in only one of the two replicates. The value shown is one half the concentration determined from the replicate in which the compound was detected.

the production of six of Pf-5's known antibiotics, and extracts of culture supernatants were evaluated by HPLC (Table 3). The antibiotic production profiles of Pf-5 varied somewhat between experiments, so Pf-5 was included in each experiment as a reference for comparison to the mutants evaluated. As expected, derivatives of Pf-5 having deletions in biosynthesis genes for five metabolites (JL4865, Δ*phlD* Δ*rzxB* Δ*prnC* Δ*hcnB* Δ*pltA*) or seven metabolites (JL4932, Δ*phlD* Δ*rzxB* Δ*prnC* Δ*hcnB* Δ*pltA* Δ*ofaA* Δ*toxB*) lacked production of the corresponding five or seven compounds (Table 3). At the time this experiment was done, we had not yet established that Pf-5 produces toxoflavin and did not have a method in place to quantify toxoflavin, so toxoflavin production is not reported in Table 3. We have since demonstrated that a Δ*toxB* mutant of Pf-5 is deficient in the production of toxoflavin, a compound with broad antimicrobial activity and phytotoxicity (13). Derivatives of Pf-5 with individual deletions lacked production of the expected metabolite, and all but the Δ*phlD* mutant continued to produce all of the other compounds (Table 3). As described previously, the type III polyketide synthase PhlD is required for the production of both pyoluteorin and DAPG by Pf-5 (9). PhlD synthesizes phloroglucinol, an intermediate in the biosynthesis of DAPG. Phloroglucinol is not an intermediate in pyoluteorin biosynthesis but instead is involved in regulation of the pyoluteorin biosynthesis gene cluster in Pf-5 (56). Our HPLC analysis of the Δ*phlD* mutant confirmed that PhlD is essential for MAPG, DAPG, and pyoluteorin production (Table 3) and also demonstrated that the Δ*phlD* mutant produces orfamide A and rhizoxin at levels similar to those produced by wild-type Pf-5. We also observed quantitative differences in the production of several antibiotics by certain mutants with single deletions. For example, the Δ*ofaA* mutant produced less DAPG, MAPG, and rhizoxin WF-1360F than wild-type Pf-5. In contrast, the Δ*hcnB* mutant produced higher levels of DAPG, MAPG, rhizoxin WF-1360F, and orfamide A than wild-type Pf-5. The mechanism for this is not known, but it could be due in part to the enhanced growth of the Δ*hcnB* mutant relative to the wild type (data not shown), which in turn could be due to the inherent toxicity of HCN to the producing strain. The Δ*pltA* mutant produced enhanced levels of DAPG and MAPG, which is likely due to the known reciprocal regulation of pyoluteorin and the phloroglucinol derivatives (9, 42, 44, 62).

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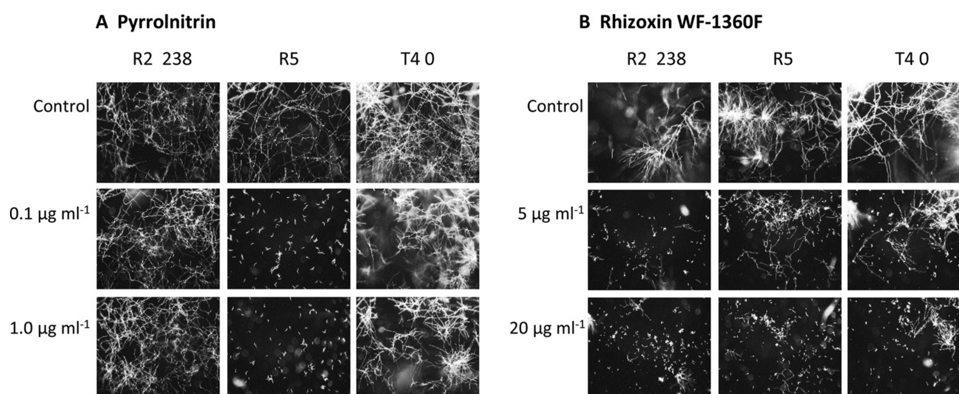

FIG 3 Inhibition of conidial germination and germination tube elongation of *F. oxysporum* f. sp. *pisi* R2-238, *F. oxysporum* f. sp. *pisi* R5, and *F. verticillioides* T4-0 by pyrrolnitrin (A) and rhizoxin WF-1360F (B). Twenty hours after conidia of the *Fusarium* spp. were placed in antibiotic solutions, germination and germination tube elongation were observed under a dissecting microscope and photographed. The blurred regions visible in some photographs are propagules outside the depth of field of the microscope. Four replicate conidial suspensions were assessed for each antibiotic concentration, and the experiment was done three times with nearly identical results.

To identify the specific metabolite(s) contributing to inhibition of the *Fusarium* spp. on PDA-Fe, we first compared the 5-fold mutant (Δ*phlD* Δ*rzxB* Δ*prnC* Δ*hcnB* Δ*pltA*) and the 7-fold mutant (Δ*phlD* Δ*rzxB* Δ*prnC* Δ*hcnB* Δ*pltA* Δ*ofaA* Δ*toxB*) to the Δ*gacA* mutant and Pf-5. Compared to wild-type Pf-5, both the 5-fold and 7-fold mutants showed reduced inhibition of *Fusarium* spp., indicating that one or more of the mutagenized genes had a role in inhibition (Fig. 2). The percent inhibition of *Fusarium* spp. by both mutants was similar to that by the Δ*gacA* mutant (see Table S1 in the supplemental material). In contrast, all mutants deficient in only one compound suppressed the *Fusarium* spp., as the percent inhibition exhibited by each single mutant was greater than that by the Δ*gacA* mutant (Fig. 2; see Table S1 in the supplemental material). These results suggest that more than one compound contributes to fungal inhibition by Pf-5; consequently, antibiosis was not lost when the function of a single biosynthetic gene cluster was lost due to mutation. To determine the mixture of compounds responsible for inhibition, we then tested the 3-fold Δ*phlD* Δ*rzxB* Δ*prnC* mutant, which did not differ significantly from the Δ*gacA*, 5-fold, or 7-fold mutant in suppression of *Fusarium* spp. on PDA-Fe (Fig. 2; see Table S1 in the supplemental material).Because *phlD* is required for production of both DAPG and pyoluteorin, as described above, we repeated the antibiosis tests with a set of mutants having a *phlA* mutation, which is required for DAPG but not pyoluteorin production by Pf-5 (9). The Δ*phlA* Δ*rzxB* Δ*prnC* mutant, like the Δ*phlD* Δ*rzxB* Δ*prnC* mutant, did not differ significantly from the Δ*gacA* mutant in antibiosis against the two *Fusarium* spp. tested (see Fig. S1 in the supplemental material). These results indicate that DAPG, rhizoxin derivatives, and pyrrolnitrin are the primary metabolites responsible for suppression of the isolates of *Fusarium* spp. This finding is not surprising, as the toxicities of DAPG (63), rhizoxin WF-1360F (5, 64), and pyrrolnitrin (65) against *Fusarium* spp. are known. Pf-5 mutants deficient in the production of any two of the three compounds varied in inhibition of *Fusarium* spp., however, suggesting variation in the relative roles of the three compounds in antibiosis. For example, the Δ*phlD* Δ*rzxB* double mutant did not suppress mycelial growth of isolate R2-238 or T4-0 but did suppress mycelial growth of isolate R5 by approximately 30%. These results indicate that pyrrolnitrin production contributed to Pf-5's suppression of isolate R5 but did not contribute to Pf-5's suppression of isolate R2-238 or T4-0. One possible explanation for this result is that the isolates of *Fusarium* spp. differ in sensitivity to the antifungal compounds produced by Pf-5.

Isolates of *Fusarium* spp. differ in sensitivity to pyrrolnitrin and rhizoxin. To determine the relative sensitivities of the *Fusarium* spp. to the Pf-5 metabolites, the three *Fusarium* isolates were exposed to purified pyrrolnitrin and WF-1360F, the predominant rhizoxin analog produced by Pf-5, and hyphal growth was assessed. Of the three isolates, *F. oxysporum* f. sp. *pisi* R5 was most sensitive to pyrrolnitrin (Fig. 3A). At 0.1 μg ml^{-1} pyrrolnitrin, hyphal growth of R5 was severely inhibited, whereas isolates R2-238 and T4-0 grew even at a 10-fold-higher concentration of pyrrolnitrin. Of the three isolates, R2-238 was the most sensitive to the rhizoxin analog WF-1360F: 5 μg ml^{-1} WF-1360F reduced hyphal growth of R2-238, whereas 20 μg ml^{-1} of the compound was required to cause a similar level of inhibition for isolates R5 and T4-0 (Fig. 3B). Therefore, as expected from the experiments evaluating the antibiosis of mutants of Pf-5, isolate R5 was more sensitive than isolate R2-238 to pyrrolnitrin and was less sensitive to WF-1360F (Fig. 3). The differential sensitivities of the two isolates to these compounds correlates to their inhibition by the Δ*phlD* Δ*rzxB* double mutant versus the Δ*phlD* Δ*rzxB* Δ*prnC* triple mutant of Pf-5 (Fig. 2).

FA influences antibiotic production by Pf-5. Having identified the primary metabolites responsible for inhibition of *Fusarium* spp. by Pf-5, we then set out to determine the influence of the signaling compound FA on antibiotic production by Pf-5. Antibiotic production profiles were obtained from cultures of Pf-5 grown in NYBGly-Zn to correspond to our HPLC profiling of Pf-5 mutants and in PDB-Fe to correspond with assays evaluating inhibition of *Fusarium* spp. Amendment of the media with 0.5 mM FA did not alter growth rate of Pf-5 (data not shown) but decreased the production of DAPG and MAPG by Pf-5 by 75% to 99% (Table 4), as reported previously for *P. protegens* strain CHA0 (31) and other *Pseudomonas* spp. (66). Amendment of the media with 0.5 mM FA increased pyoluteorin production in NYBGly-Zn, but pyoluteorin production in PDB-Fe was near or below the detection limits regardless of FA amendment. In a previous study, 100 μg ml^{-1} FA decreased pyoluteorin production by *P. protegens*

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TABLE 4 Influence of fusaric acid on antibiotic production by *Pseudomonas protegens* Pf-5[a]

Antibiotic	Concn, μg ml^{-1} (mean ± SE) in[b]: NYBGly-Zn Without FA	NYBGly-Zn With FA	PDB-Fe Without FA	PDB-Fe With FA
DAPG	9.8 ± 0.9	0.06 ± 0.02**	2.2 ± 0.3	0.56 ± 0.13**
MAPG	6.6 ± 0.9	0.07 ± 0.03**	15 ± 1	0.93 ± 0.05**
Orfamide A	95 ± 9	78 ± 11	15 ± 1	18 ± 1*
Pyoluteorin	0.87 ± 0.04	1.4 ± 0.1**	BD	0.02 ± 0.01
Pyrrolnitrin	0.36 ± 0.06	0.31 ± 0.06	1.2 ± 0.1	1.4 ± 0.1*

[a] Strain Pf-5 was grown in 5 ml of NYBGly supplemented with 0.35 mM zinc sulfate (NYBGly-Zn) or in 5 ml of PDB with 0.1 mM $FeCl_3$ (PDB-Fe). Both media were amended with fusaric acid (FA) at 0 or 0.5 mM. Cultures were incubated for 48 h at 27°C with shaking at 200 rpm. Prior to extraction, culture supernatants were acidified to pH 2.0, which enhances extraction of some antibiotics but causes degradation of rhizoxin (6, 55). Consequently, rhizoxin WF-1360F concentrations were not assessed in this experiment. Concentrations of antibiotics listed were quantified by HPLC.

[b] Values represent the mean and standard error for four replicate broth cultures. Values were rounded to two significant digits. BD, below detection, with detection limits being approximately 0.02 μg/ml for all compounds. For each medium, asterisks (* and **) in the same row indicate values that are significantly different from the control value (α = 0.05 and 0.01, respectively) according to Student's *t* test.

TABLE 5 Influence of fusaric acid on the suppression of *Fusarium verticillioides* T4-0 by Pf-5 and derivative strains on PDA-Fe[a]

Strain	Inhibition, %[b] Without FA	With FA
Wild type	30	16**
Δ*gacA*	6	1**
Δ*phlA*	21	15
Δ*rzxB*	21	9**
Δ*prnC*	28	19**
Δ*prnC* Δ*rzxB*	13	6**
Δ*phlA* Δ*prnC*	15	17
Δ*phlA* Δ*rzxB*	9	7
Δ*phlA* Δ*rzxB* Δ*prnC*	2	1

[a] Inhibition of radial growth of *F. verticillioides* on PDA-Fe was measured after 5 days of incubation at 2 °C.

[b] The results are means for four replicate agar plates. Asterisks (* and **) in the same row indicate values that are significantly different from the control value (α = 0.05 and 0.01, respectively) according to Student's *t* test.

strain CHA0 grown in PCG medium (28). The different effects of FA on pyoluteorin production in the two studies could be due to different FA concentrations, strains, or media evaluated. Amendment of the media with 0.5 mM FA slightly increased pyrrolnitrin and orfamide A production in PDB-Fe but had no significant influence on the production of either antibiotic in NYBGly-Zn (Table 4). Although we did not assess the influence of FA on siderophore production by Pf-5, FA is known to increase pyoverdine production by Pf-5 (41). These results extend those from previous studies demonstrating that FA reduces the production of DAPG (31, 44), pyoluteorin (28), and phenazines (39, 67) by *Pseudomonas* spp. Our results show that FA can enhance the production of antibiotics, such as pyoluteorin, and that the effect of FA on secondary metabolite production can vary with the growth medium.

FA influences antibiotic and siderophore gene expression. The influence of FA on transcription of secondary metabolite biosynthetic genes was assessed by RT-qPCR of cells harvested from late-exponential- and stationary-phase cultures of Pf-5 in NBGly-Zn. FA decreased the transcript abundance of *phlD* by 10-fold in late-exponential-phase cells and by approximately 50-fold in stationary-phase cells of Pf-5 (Fig. 4), in agreement with previous studies demonstrating that FA decreased the expression of DAPG biosynthesis genes of *P. protegens* CHA0 (43, 44). No significant effect of FA on the transcript abundance of *hcnA* or *pchC* was detected. The effects of FA on expression of other metabolic biosynthesis genes were smaller in magnitude and dependent on the growth phase of Pf-5. The transcript abundances of *pltA*, *rzxB*, and *pvdA* were significantly higher in late-exponential-phase cultures grown in FA-amended medium than in nonamended medium, but no significant effect was observed in stationary-phase cells. In contrast, the transcript abundance of *prnC* was enhanced by FA only in stationary-phase cells.

In this study, FA had a parallel influence on the transcription of *phlD* and production of DAPG. Similarly, the influence of FA on *pltA* transcription by cells in late-exponential phase (i.e., 24-h cultures) correlated to its influence on pyoluteorin production, assessed from 48-h cultures. These results align with those from previous studies where the expression of genes for DAPG and pyoluteorin biosynthesis by cells from late-exponential or stationary-phase cultures correlated with the levels of these metabolites that subsequently accumulated (16, 17, 56). In contrast, the influence of FA on transcription of biosynthesis genes for pyrrolnitrin did not correlate with the levels of this metabolite quantified from 48-h cultures. Secondary metabolite production is regulated at many levels, and in this example, the transcription of a single biosynthesis gene was not predictive of subsequent levels of the metabolite that accumulated in cultures of Pf-5.

FA influences antibiosis of Pf-5 against *F. verticillioides*. We then evaluated the influence of FA on the antibiosis of Pf-5 and derivative strains against *F. verticillioides* T4-0 on PDA-Fe. FA decreased the inhibition of *F. verticillioides* T4-0 by Pf-5 and all derivative strains that produced DAPG (i.e., the Δ*rzxB*, Δ*prnC*, and Δ*rzxB* Δ*prnC* mutants) (Table 5). Accordingly, FA reduced the production of DAPG by Pf-5 in this medium, as determined by HPLC (Table 4). In contrast, FA did not alter the antibiosis exhibited by the

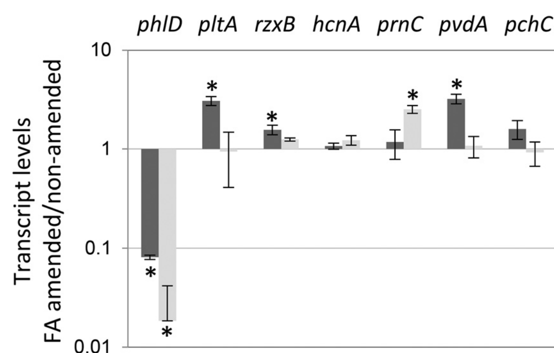

FIG 4 Influence of fusaric acid on the expression of selected genes for secondary metabolite and siderophore biosynthesis by *P. protegens* Pf-5. RNA was extracted from late-exponential-phase (OD_{600} of 3.0) (black bars) and stationary-phase (OD_{600} of 7.2) (gray bars) cultures of Pf-5 grown in NBGly-Zn amended with 0 or 0.5 mM fusaric acid. Values represent the expression ratio of the target gene in cultures of Pf-5 grown in NBGly-Zn amended with fusaric acid versus nonamended medium. Bars represent the mean for four replicate cultures, and error bars show the standard error of the mean. Asterisks indicate values that differ significantly ($P \leq 0.05$) between cultures grown in fusaric acid-amended versus nonamended medium, as determined by the method of Pfaffl et al. (60).

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Δ*phlA*, Δ*phl* Δ*prnC*, and Δ*phlA* Δ*rzxB* mutants (Table 5), which relied on pyrrolnitrin and/or rhizoxin to inhibit *Fusarium* spp. With the exception of rhizoxin, which we were unable to quantify in the experiment described in Table 4, the data show that FA has distinct effects on the production of different antibiotics by Pf-5 that influence the antifungal activity of the bacterium.

Conclusions. *P. protegens* Pf-5 produces an array of antifungal compounds toxic to phytopathogenic fungi. Here, we assembled a set of mutants of Pf-5 having individual or multiple mutations in gene clusters for each of the known antifungal compounds and used the mutant set to pinpoint the specific compounds contributing to inhibition of *F. oxysporum* f. sp. *pisi* and *F. verticillioides*. Three metabolites—DAPG, rhizoxin derivatives, and pyrrolnitrin—were the primary determinants of antibiosis exhibited by Pf-5 against these *Fusarium* spp. Isolates of *Fusarium* spp. varied in their sensitivities to the three compounds, and their sensitivities determined the relative importance of the three compounds in antibiosis by Pf-5. FA, a mycotoxin produced by *Fusarium* spp., had a differential effect on the production of Pf-5's metabolites, greatly decreasing the production of DAPG and moderately increasing the production of pyoluteorin, pyrrolnitrin, and orfamide A by Pf-5 in one of two media evaluated. We observed parallel influences of FA on the expression of the pyoluteorin biosynthetic gene *pltA* and pyoluteorin production and on the expression of *phlD* and DAPG production. In accordance with a recent report showing that FA enhanced pyoverdine production by Pf-5 (41), we observed that FA increased the expression of *pvdA*, a biosynthesis gene for pyoverdine production. Finally, we assessed the influence of FA on the inhibition of *F. verticillioides* by Pf-5 and selected derivative strains. FA diminished antibiosis exhibited by Pf-5 and derivative strains that produced DAPG but had no significant influence on antibiosis exhibited by *phlA* mutants, which do not produce DAPG but suppress *F. verticillioides* due to rhizoxin and pyrrolnitrin production. In summary, using a systematic approach employing a set of mutants representing the known antifungal metabolites of Pf-5, we demonstrated the roles of three compounds in antibiosis of *Fusarium* spp. and the parallel influences of the signaling molecule FA on antifungal metabolite production and antibiosis of this phytopathogenic fungus.

ACKNOWLEDGMENTS

We thank the following people for their generosity in providing materials for this work: Cynthia Ocamb, Lyndon Porter, and Walter Maccheroni, Jr., for strains of *Fusarium* spp., Herbert Schweizer for plasmid pEX18Tc, Harald Gross for purified orfamide A and rhizoxin WF-1360F, Brian Nowak-Thompson for purified pyoluteorin, and Christoph Keel for purified MAPG and DAPG. We are grateful to Charles Bacon for valuable advice on assessing the influence of FA on metabolite production by Pf-5.

FUNDING INFORMATION

USDA | National Institute of Food and Agriculture (NIFA) provided funding to Joyce E. Loper under grant number 2006-35319-17427. USDA | National Institute of Food and Agriculture (NIFA) provided funding to T. Mark Zabriskie and Joyce E. Loper under grant number 2011-67019-30192. Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) provided funding to Maria Carolina Quecine under grant number 2005-53748-6.

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