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Isolation and Identification of Rhizoxin Analogs from *Pseudomonas fluorescens* Pf-5 by Using a Genomic Mining Strategy▽†

Joyce E. Loper,[1] Marcella D. Henkels,[1] Brenda T. Shaffer,[1] Frederick A. Valeriote,[2] and Harald Gross[3]*

Horticultural Crops Research Laboratory, Agricultural Research Service, United States Department of Agriculture, Corvallis, Oregon 97330[1]; Henry Ford Health System, Detroit, Michigan 48202[2]; and Institute for Pharmaceutical Biology, University of Bonn, 53115 Bonn, Germany[3]

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The products synthesized from a hybrid polyketide synthase/nonribosomal peptide synthetase gene cluster in the genome of *Pseudomonas fluorescens* Pf-5 were identified using a genomics-guided strategy involving insertional mutagenesis and subsequent metabolite profiling. Five analogs of rhizoxin, a 16-member macrolide with antifungal, phytotoxic, and antitumor activities, were produced by Pf-5, but not by a mutant with an insertion in the gene cluster. The five rhizoxin analogs, one of which had not been described previously, were differentially toxic to two agriculturally important plant pathogens, *Botrytis cinerea* and *Phytophthora ramorum*. The rhizoxin analogs also caused swelling of rice roots, a symptom characteristic of rhizoxin itself, but were less toxic to pea and cucumber roots. Of the rhizoxin analogs produced by Pf-5, the predominant compound, WF-1360 F, and the newly described compound 22*Z*-WF-1360 F were most toxic against the two plant pathogens and three plant species. These rhizoxin analogs were tested against a panel of human cancer lines, and they exhibited potent but nonselective cytotoxicity. This study highlights the value of the genomic sequence of the soil bacterium *P. fluorescens* Pf-5 in providing leads for the discovery of novel metabolites with significant biological properties.

Secondary-metabolite production is a striking characteristic of *Pseudomonas* spp. (5, 28), and the current availability of genomic sequence data for several *Pseudomonas* spp. further highlights the capacity for secondary-metabolite production in this group of bacteria. For example, at least 6% of the genome of *Pseudomonas fluorescens* Pf-5, a rhizosphere bacterium that suppresses plant diseases, is devoted to secondary metabolism, with gene clusters for the biosynthesis of two siderophores, hydrogen cyanide, and several antibiotics (pyrrolnitrin, 2,4-diacetylphloroglucinol, and pyoluteorin) distributed throughout the genome (30). In addition to the secondary metabolites known to be produced by Pf-5 prior to genomic sequencing, three orphan gene clusters were identified in the genome of this bacterium (41). These three orphan genetic loci contain sequences that are characteristic of polyketide synthases (PKS) or nonribosomal peptide synthetases (NRPS) (51). One of the orphan metabolites has since been identified as orfamide A, the founder of a new group of bioactive cyclic lipopeptides that lyses zoospores of an oomycete plant pathogen and functions in the swarming motility of Pf-5 (11). In our continuing effort to identify the products of orphan pathways from the *P. fluorescens* Pf-5 genome, we describe here the products of a cluster containing genes with characteristic sequences of both PKS and NRPS. Using a screening strategy comparing Pf-5 to a derivative with a mutation in this cluster (12), we isolated and identified several metabolites structurally related to rhizoxin (Fig. 1), a 16-member macrolide first isolated from *Rhizopus chinensis* (17), a fungus causing a disease of rice seedlings. Rhizoxin has since been isolated from *Burkholderia rhizoxinica* sp. nov. (formerly designated *Burkholderia rhizoxina*) (38), an endosymbiont of *Rhizopus microsporus* (39), and rhizoxin analogs have been isolated from strains of *Pseudomonas* spp. that inhabit the rhizosphere (19) and ocean waters (43). Rhizoxin exhibits phytotoxic (36), antifungal (17), and antitumor (49) activities by binding to β-tubulin (46), thereby interfering with microtubule dynamics during mitosis (14).

Here, we report the isolation of five rhizoxin analogs from cultures of *P. fluorescens* Pf-5 and demonstrate their antifungal, cytotoxic, and phytotoxic properties.

MATERIALS AND METHODS

Organisms. *P. fluorescens* Pf-5 was provided by C. Howell, who isolated it from soil in College Station, TX (16). The oomycete *Phytophthora ramorum* Pr-008 was obtained from Niklaus Grunwald, Agriculture Research Service, U.S. Department of Agriculture, Corvallis, OR. Two isolates (BC250 and BC259) of the ascomycete *Botrytis cinerea* (teleomorph, *Botryotinia fuckeliana*) were obtained from Ken Johnson, Oregon State University, Corvallis, OR.

Sequence analysis. NRPS and PKS domains were identified using the Web-based software NRPS-PKS (1) (http://www.nii.res.in/nrps-pks.html), by BLAST comparison with characterized domains from other PKS and NRPS gene clusters and from sequence alignments constructed using ClustalW, available through Vector NTI (Invitrogen, Carlsbad, CA). Specificity prediction of the adenylation domain was performed according to the method of Challis et al. (6).

Allelic-exchange mutagenesis of strain Pf-5. A 1,394-bp PCR product of the rhizoxin-biosynthetic gene *rzxB* was obtained from the genome of Pf-5 using primers 2989_5′ ENTR and 2989_3′ ENTR (see the supplemental material), cloned into the gateway entry vector pENTR/D-TOPO (Invitrogen), and integrated into the destination vector pLVC-D (32) using the clonase protocol described by Invitrogen. The resultant plasmid (pLVC-D, containing 1,394 bp of *rzxB*) was transferred from the mobilizing strain *Escherichia coli* S17-1 (45) to

* Corresponding author. Mailing address: Institute for Pharmaceutical Biology, University of Bonn, Nussallee 6, 53115 Bonn, Germany. Phone: 49 228 73-2676. Fax: 49 228 73-3250. E-mail: harald.gross@uni-bonn.de.

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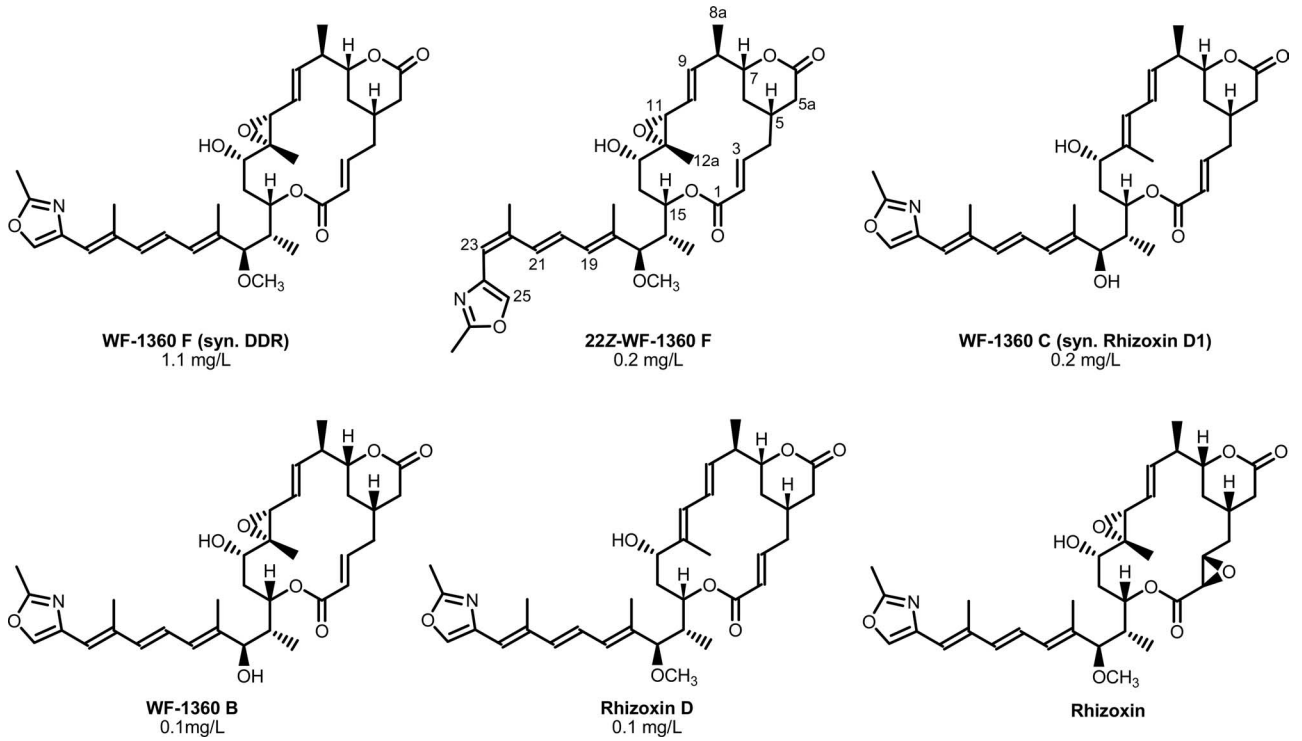


FIG. 1. Structures of rhizoxin and the rhizoxin analogs produced by *P. fluorescens* Pf-5. The rhizoxin analogs were isolated at the specified concentrations from 48-h cultures of Pf-5 grown in Davis medium at 21°C. DDR, 2,3-deepoxy-2,3-didehydro-rhizoxin.

Pf-5 via conjugation, selecting for resistance to streptomycin (100 μg/ml; innate resistance of Pf-5) and tetracycline (200 μg/ml; conferred by the plasmid). Because pLVC-D is a suicide plasmid in *Pseudomonas* spp., tetracycline-resistant colonies of Pf-5 were expected to have undergone a single-crossover event between the DNA cloned in pLVC-D and the corresponding sequence in the Pf-5 chromosome. Gene disruption and plasmid insertion were confirmed using PCR with primers specific to the pLVC-D vector (L *att*B2 and U *att*B1) and genomic DNA sequences flanking the 1,394-bp region of *rzxB* (2989_5′ OUT and 2989_3′ OUT) (see the primer table in the supplemental material). A derivative of Pf-5 having the expected insertion in *rzxB* (designated JL4778) was selected for further analysis.

Identification of culture conditions conducive to expression of rhizoxin biosynthetic genes. Reverse transcriptase PCR was used to identify culture conditions where *P. fluorescens* Pf-5 expressed *rzxB*. Three replicate cultures of Pf-5 were grown in each of six different media: Difco nutrient broth (Becton Dickinson, Sparks, MD) with 0.5% (vol/vol) glycerol (NB-gly), Difco nutrient broth with 1% (wt/vol) glucose (NB-glu), 925 broth with 1% (wt/vol) sucrose (27), King's medium B (KMB) broth (24), Difco minimal broth Davis without dextrose (Becton Dickinson) containing 20 mM glycerol (Davis), and pigment production medium (PPM) broth with 1% (vol/vol) glycerol (29). The cultures were grown with shaking (200 rpm) at 20°C, and cells were harvested at 8 and 24 h after inoculation, which corresponded generally to cultures in exponential and stationary growth phases. The average optical densities (600 nm) of 8-h and 24-h cultures were 1.8 and 3.1 (NB-gly), 1.7 and 3.1 (NB-glu), 0.3 and 2.2 (925), 1.8 and 6.7 (KMB), 0.5 and 1.9 (Davis), and 0.9 and 3.1 (PPM). RNAProtect (Qiagen, Valencia, CA) was added to each culture, RNA was extracted using the RNA/DNA Midi kit (Qiagen), and DNA was removed using an on-column DNase treatment (RNeasy Mini kit with DNase I; Qiagen). PCR was performed on 1 μg of the RNA to determine that detectable DNA had been removed, and RNA samples were analyzed for quality using the BioAnalyzer 2100 (Agilent, Palo Alto, CA) at the Center for Genomic Research and Biocomputing Core Laboratories, Oregon State University. cDNA was generated from 5 μg RNA using SuperScript II (Invitrogen) and random hexamers. To confirm that DNA was removed, samples processed in parallel without reverse transcriptase served as negative controls in quantitative-PCR experiments as described below. Following reverse transcription, the RNA was hydrolyzed with 2.5 M NaOH, and samples were neutralized with 2 M HEPES-free acid.

Quantitative PCR was performed on 1 μg of the cDNA using LightCycler FastStart DNA MasterPlus Sybr green I (Roche, Indianapolis, IN) on a Roche Lightcycler II (Roche, Indianapolis, IN), following the manufacturer's specifications. An external standard curve, generated using a purified *rzxB* PCR product over a dilution range of known concentrations, was used to estimate template concentrations (in pg) of the *rzxB* gene (218-bp product; primers 2989_Fq and 2989_Rq) (see the supplemental material). Melting-curve analysis of products was used to verify the amplification of a specific product. The concentrations of amplification products from negative controls (RNA samples to which no superscript was added) were 100 to 1,000 times less than those of the corresponding cDNA samples in each case, indicating lack of interference from contaminating DNA.

General analytical procedures. Thin-layer chromatography grade (10- to 40-μm) silica gel was used for vacuum liquid chromatography. High-pressure liquid chromatography (HPLC) was carried out using a Waters system consisting of a degasser, a 600 pump, a 996 photodiode array detector, and a 717 plus autosampler. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance 300 DPX spectrometer using 5-mm advanced microtubes matched to $CDCl_3$ (Shigemi, Allison Park, PA) or 2.5-mm Match sample tubes (Hilgenberg GmbH, Malsfeld, Germany). Spectra were calibrated to the solvent signal (^{13}C: $CDCl_3$ δ 77.0 ppm) and the signal contributed by the nondeuterated portion of the solvent (^1H: $CHCl_3$ in $CDCl_3$ δ 7.26 ppm). UV and infrared spectra were taken on Perkin-Elmer Lambda 40 and Perkin-Elmer Spectrum BX instruments, respectively. Optical rotations were measured with a Jasco DIP 140 polarimeter. Liquid chromatography/mass spectroscopy (LC/MS) measurements were obtained by employing an Applied Biosystems LC/MS system consisting of an Agilent 1100 HPLC system and an MDS Sciex API 2000 mass spectrometer equipped with an API-electrospray ionization source. High-resolution electron impact mass spectra (HR-EIMS) were recorded on a ThermoQuest Finnigan Mat 95 XL.

Metabolic profiling of Pf-5 and an *rzxB* mutant. To identify the product(s) of the orphan gene cluster, cultures of Pf-5 and the *rzxB* mutant, grown under culture conditions conducive to expression of rhizoxin biosynthesis genes, were compared by LC/MS. Pf-5 and the *rzxB* mutant were grown with shaking (150 rpm) in 1 liter of Davis medium at 21°C. After 48 h of incubation, the fermentation broth was extracted exhaustively with ethyl acetate and evaporated to dryness. The crude extracts were dissolved in MeCN to a final concentration of

TABLE 1. NMR data for 22*Z*-WF-1360 F in $CDCl_3$

Position	δ_H[a]	δ_C[b]	HMBC[c]
1		165.3 qC	
2	5.69 (1H, d, J = 15.3)	124.9 CH	1, 4
3	6.80 (1H, m)	146.0 CH	1, 4
4	1.78 (1H, m)	37.9 CH_2	5, 5a
	2.56 (1H, m)		
5	1.80 (1H, m)	29.7 CH	5a
5a	2.11 (1H, m)	36.8 CH_2	5b
	2.79 (1H, dd, J = 3.3, 18.0)		
5b		170.0 qC	
6	0.72 (1H, dd, J = 11.8, 14.0)	33.8 CH_2	
	1.96 (1H, m)		
7	3.74 (1H, ddd, J = 3.3, 10.8, 11.8)	82.4 CH	
8	2.32 (1H, ddd, J = 3.3, 6.4, 9.8)	45.0 CH	9, 10
8a	1.17 (3H, d, J = 6.4)	16.6 CH_3	7, 8, 9
9	5.54 (1H, dd, J = 9.8, 15.4)	140.2 CH	10, 11
10	5.34 (1H, dd, J = 9.4, 15.4)	126.3 CH	11, 12
11	3.18 (1H, d, J = 9.4)	64.2 CH	13
12		65.5 qC	
12a	1.42 (3H, s)	12.3 CH_3	11, 12, 13
13	3.07 (1H, d, J = 10.4)	77.1 CH	14
14	1.83 (1H, m)	32.1 CH_2	12, 15
	2.08 (1H, m)		
15	4.57 (1H, dd, J = 3.0, 9.0)	75.3 CH	1
16	2.42 (1H,m)	38.3 CH	17
16a	1.00 (3H, d, J = 6.8)	10.0 CH_3	15, 16, 17
17	3.30 (1H, d, J = 9.4)	90.0 CH	19
17-OCH_3	3.17 (3H, s)	56.3 CH_3	17
18		136.9 qC	
18a	1.89 (3H, s)	11.2 CH_3	17, 18, 19
19	6.21 (1H, d, J = 11.0)	130.1 CH	20, 21
20	6.61 (1H, dd, J = 11.0, 15.4)	125.9 CH	18, 21, 22
21	7.44 (1H, d, J = 15.4)	131.8 CH	22, 23
22		135.2 qC	
22a	2.02 (3H, s)	20.9 CH_3	21, 22, 23
23	6.06 (1H, s)	117.5 CH	24
24		138.4 qC	
25	7.45 (1H, s)	136.3 CH	23, 24, 26
26		161.6 qC	
26a	2.47 (3H, s)	13.8 CH_3	26

[a] δ in ppm; multiplicity: singlet (s), doublet (d), and multiplet (m); J in Hz. Recorded at 300 MHz.

[b] δ in ppm. Recorded at 75 MHz; multiplicity determined by distortionless enhancement by polarization transfer.

[c] Protons showing long-range correlation with the indicated carbon.

5 mg/ml, and 10 μl was evaluated by LC/MS using a 2 mM ammonium acetate-buffered MeOH/H_2O gradient, increasing the MeOH from 10% to 100% over 20 min and holding it at 100% MeOH for 10 min (Macherey-Nagel C_{18} Nucleodur 100-5; 125 by 2 mm; 5-μm column; 0.25 ml/min flow rate, with total ion current and photodiode array monitoring).

Isolation of rhizoxin analogs. Pf-5 was grown with shaking (150 rpm) at 21°C in seven 5,000-ml Erlenmeyer flasks, each containing 1.5 liters Davis medium. After 48 h of incubation, the fermentation broth was extracted exhaustively with ethyl acetate to yield 1.9 g of crude organic extract. The extract was fractionated by vacuum liquid chromatography over silica gel using a stepwise gradient of hexane-ethyl acetate and ethyl acetate-MeCN to give seven fractions, each of 350 ml. Fractions 5 and 6 were further separated by reversed-phase HPLC (Knauer C_{18} Eurospher-100; 250 by 8 mm; 5-μm column; MeCN-H_2O [60:40] containing 1% acetic acid solvent system; 2-ml/min flow rate), yielding 12.0 mg of WF-1360 F, 1.8 mg of 22*Z*-WF-1360 F, 1.5 mg of WF-1360 B, 2.3 mg of WF-1360 C, and 1.5 mg of rhizoxin D (Fig. 1).

Description of 22*Z*-WF-1360 F. 22*Z*-WF-1360 F is a pale-yellow solid (1.8 mg), $[\alpha]_D^{20}$+159° (*c* 0.12, $CHCl_3$) (NMR spectral data are given in Table 1). UV (MeOH) λ_{max} (log ε) 299 sh (4.11), 310 (4.16), 321 sh (4.05) nm; IR (ATR) ν_{max} 3,366, 2,926, 1,715, 1,649, 1,077, 1,018, and 982 cm^{-1}; HR-EIMS *m/z* 609.3284 $[M]^+$ (calculated for $C_{35}H_{47}NO_8$, 609.3302, Δ − 3.0 ppm).

Assays for biological activity. (i) Activities against fungal and oomycete plant pathogens. Rhizoxin (Sigma, St. Louis, MO) and rhizoxin analogs isolated from Pf-5 cultures were tested for their effects on germination and germ tube elongation from encysted zoospores of the oomycete *P. ramorum* Pr-008 and conidia of *B. cinerea* BC250 and BC259, which are sensitive and resistant, respectively, to the benzimidazole fungicide benomyl. Fifty microliters of a suspension containing encysted zoospores or conidia in 20% potato dextrose broth was placed in individual wells of a 96-well tissue culture plate at a concentration of 10^3 propagules/well. Rhizoxin and its analogs were suspended in dimethyl sulfoxide (DMSO) to a concentration of 10 mg/ml and diluted with sterile distilled water (dH_2O). Fifty microliters of the diluted sample was added to each well, bringing the total volume per well to 100 μl and yielding final concentrations of the rhizoxin analogs of 1, 5, 10, or 20 μg/ml. Sterile dH_2O and 0.2% (vol/vol) DMSO (corresponding to the highest concentration of DMSO in samples of rhizoxin analogs) served as controls. The plates were incubated at 27°C for 24 h before microscopic observations were recorded. Four replicates per treatment were conducted, and the experiment was done twice with nearly identical results.

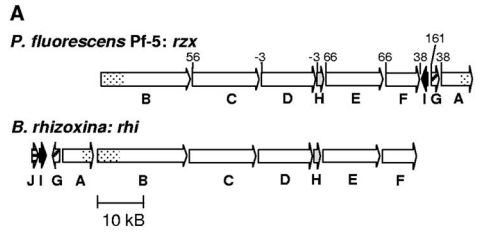
(ii) Phytotoxicities against rice, cucumber, and pea. The seeds used in all experiments were surface sterilized and germinated under aseptic conditions prior to exposure to rhizoxin or rhizoxin analogs. Rice seeds (cv. Koshihikari) were placed in individual wells of a 24-well tissue culture plate containing 275 μl sterile dH_2O for germination. Pea (cv. Sugar Snap) and cucumber (cv. Marketmore 76) were germinated on sterile moist filter paper in petri dishes. After germination, seedlings were selected for uniformity and transferred to individual wells of a 24-well tissue culture plate containing 275 μl rhizoxin or rhizoxin analogs (isolated from cultures of Pf-5) at various concentrations. Seedlings placed in wells containing sterile dH_2O or 0.2% (vol/vol) DMSO served as controls. The plates were incubated at 27°C for 24 h prior to microscopic observation. Each experiment evaluated three replicate seeds of each plant for each concentration of each compound, and the experiment was done twice with nearly identical results.

(iii) Cytotoxicity assays. ***(a)*** **IC_{50} determination.** Human colon carcinoma cells (HCT-116) (3) were grown in 5 ml culture medium (RPMI 1640 plus 15% fetal bovine serum containing 1% penicillin-streptomycin and 1% glutamine) (34) at 37°C and 5% CO_2 from a starting cell density of 5×10^4 cells/T25 flask. On day 3, the cells were exposed to different concentrations of the rhizoxin analogs. The flasks were incubated for 120 h (5 days) at 5% CO_2 and 37°C, and the cells were harvested with trypsin, washed once with Hanks' balanced salt solution, resuspended in Hanks' balanced salt solution, and counted using a hemocytometer. The results were normalized to an untreated control. The 50% inhibitory concentration (IC_{50}) was determined using Prism 4.0 software (GraphPad, San Diego, CA).

(b) **Disk diffusion soft-agar colony formation assay.** An in vitro cell-based assay using murine L1210 (leukemia), C38 (colon), and CFU-GM (normal) cells and human H116 (colon), H125 (lung), and leukemia (CEM) cells assessed the general and differential cytotoxicities of pure compounds. Samples were dissolved in 250 μl of DMSO, and a 15-μl aliquot was applied to a cellulose disk in an agar plate containing cells. After a period of incubation, a zone of cell colony inhibition (*z*) was measured from the edge of the disk to the edge of colony growth and expressed as zone units (zu), where 200 zu was equal to 6 mm. General cytotoxic activity for a given sample was defined as an antiproliferation zone of 300 zu or greater. The differential cytotoxicity (50) of a pure compound was expressed by observing a zone differential of 250 units or greater between any solid-tumor cell (murine colon C38, human colon HCT-116, or human lung H125 cell) and either leukemia cells (murine L1210 or human CEM cells) or normal cells (CFU-GM cells).

RESULTS

Characterization of an orphan NRPS/PKS gene cluster in the Pf-5 genome. A 78,871-bp region containing nine biosynthetic genes (PFL_2989 to PFL_2997) (Fig. 2), six having predicted functions as PKS or as mixed NRPS/PKS, was identified previously in the Pf-5 genome (41). The other genes in the cluster have predicted functions as an acyl transferase, a methyltransferase, and a cytochrome P450 monoxygenase. The closest homolog found for each gene was in the rhizoxin-biosynthetic gene cluster of *B. rhizoxinica* (40), which, along with the cluster in Pf-5 (4), was described during the later stages of this



B

Peptide	Domains Identified						Module
RzxA					GNAT	ACP	Loading
	KS				*HC HC*		PKS/NRPS
RzxB	A-Ser					PCP OX OX	NRPS
	KS					*ACP*	
	KS	*DH*	KR	MT		ACP	1
	KS	*(DH)*	KR			ACP, ACP	2
	KS	DH	KR				3
RzxC				MT		ACP	3
	KS		KR	MT		ACP	4
	KS		KR			ACP	5
	KS		KR	MT		ACP	6
RzxD	KS	*(DH)*	KR			ACP	7
	KS	DH	KR	MT		ACP	8
	KS						
RzxE						*ACP*	
	KS		KR			ACP	9
	KS	*DH*	KR			ACP	10
	KS					ACP	
RzxF	KS	DH	KR			ACP	11
	KS					ACP TE	Download

FIG. 2. Biosynthetic gene clusters for rhizoxin analogs (*rzx*) in *P. fluorescens* Pf-5 and for rhizoxin (*rhi*) in *B. rhizoxinica*. (A) Organization and putative functions of genes in the clusters. Open arrows, PKSs, with shading depicting the locations of NRPS domains; hatched arrows (*rzxG* and *rhiG*), acyl transferases (Pfam PF00698 [10]); black arrows (*rzxI* and *rhiI*), *S*-adenosylmethionine-dependent methyltransferases (Pfam PF08241); light-gray arrows (*rzxH* and *rhiH*), cytochrome P450 monooxygenases (Pfam PF00067); horizontally striped arrow (*rhiJ*), oxygenase (40). The numbers above the *rzx* gene cluster denote the sizes of intergenic regions (nucleotides). (B) NRPS and PKS domains identified in the *rzx* gene cluster. A-Ser, adenylation domain predicted to specify serine (1); ACP, Pfam PF00550; DH, β-hydroxyacyl-thioester dehydratase; GNAT, GCN5-related *N*-acyltransferase (13); HC, modified condensation (heterocyclization) (42); KR, β-ketoacyl-ACP reductase (Pfam PF08659); KS, β-ketoacyl-ACP synthase (2); MT, *S*-adenosylmethionine-dependent methyltransferase (Pfam PF08242); OX, oxidase (8); PCP, peptidyl carrier protein (Pfam PF00550); TE, thioesterase (Pfam PF00975). Italics indicate domains with motifs deviating from consensus sequences of functional domains, and parentheses indicate that the consensus sequence was present in the corresponding *rhi* domain. Module descriptions are according to the model proposed by Partida-Martinez and Hertweck (40).

study. The gene nomenclature (*rzx*) proposed for the rhizoxin-biosynthetic gene cluster in Pf-5 (4) is used here.

Domains of PKS and NRPS were identified by sequence analysis (Fig. 2), and with few exceptions, the deduced amino acid sequences of conserved motifs are identical between corresponding domains in the *rzx* and *rhi* clusters. Sixteen putative β-ketoacyl synthase domains were identified, with 15 having the active-site Cys within the conserved GPXXXXXXXCSS motif (2). Thirteen of the putative β-ketoacyl synthase domains have the essential His residues located ~136 and 175 amino acids downstream of the Cys active site, whereas three domains lack one of these residues (Fig. 2). Seventeen putative acyl carrier proteins (ACPs) were identified, with 15 having the characteristic GXDS motif containing the active-site Ser (2). A tandem ACP doublet is present in RzxB, a feature shared with RhiB; however, the tandem ACP doublet found in RhiC was not found in RzxC. Seven putative dehydratase domains were identified, with three having the characteristic HXXXGXXXP motif (9). Four dehydratase domains have one or two substitutions in this motif, whereas two of the corresponding Rhi domains conform to the conserved motif (Fig. 2). Our analyses of other domains in the region conform to those reported recently by Brendel et al. (4), who also proposed a biosynthetic model based on this domain structure. In addition to the domain differences described here, the most obvious differences between the rhizoxin-biosynthetic gene clusters of *B. rhizoxinica* and *P. fluorescens* Pf-5 are in gene order and in *rhiJ*, which is present in *B. rhizoxinica* and absent in the Pf-5 cluster.

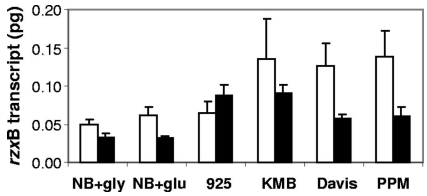
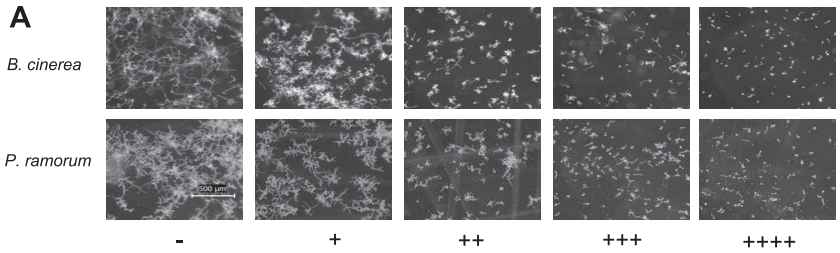


FIG. 3. Expression of *rzxB* by Pf-5 grown in different culture media. *rzxB* transcript levels expressed by Pf-5 at 8 h (open bars) and 24 h (solid bars) after inoculation in various media: NB-gly, NB-glu, 925 broth with 10% (wt/vol) sucrose, KMB broth, Davis minimal broth without dextrose containing 20 mM glycerol, and PPM broth with 1% (vol/vol) glycerol. The transcript levels are the means (plus standard errors) of three replicate cultures grown at 20°C.

Metabolic profiling of Pf-5 and an *rzxB* mutant. Gene inactivation, followed by comparative metabolite profiling, was the approach employed to identify the product of the orphan gene cluster (12). To identify conditions conducive to the expression of the biosynthetic genes in the cluster, we used quantitative reverse transcription-PCR to estimate *rzxB* transcript levels in cultures of Pf-5 grown in a variety of rich and defined media. *rzxB* transcript levels differed among the six media tested but were highest in cultures of Pf-5 grown in Davis, PPM, and KMB broth media (Fig. 3). Davis medium was selected for metabolic profiling due to its defined composition and our previous experience with the medium (11). Comparison of the metabolite profiles of Pf-5 and an *rzxB* mutant, derived by allelic-exchange mutagenesis, indicated that the orphan gene cluster was involved in the biosynthesis of at least two compounds with $[M+H]^+$ peaks at 580.7 and 610.3 *m/z*, respectively (see the supplemental material). From the detected molecular masses and UV spectra, the compounds could be readily dereplicated by a database search as the rhizoxin analogs WF-1360 C and WF-1360 F (25).

Isolation and structure elucidation of the rhizoxin analogs from *P. fluorescens* Pf-5. For the isolation of the rhizoxin analogs, *P. fluorescens* Pf-5 was grown on a large scale (10.5 liter). The culture broth was extracted with ethyl acetate, and the extract was fractionated by silica gel vacuum liquid chromatography. HPLC analysis with photodiode array detection of the fractions revealed the presence of several metabolites featuring the characteristic UV profile of rhizoxins (λ_{max}, 295, 310, and 320 nm). Further purification of two of these fractions by reversed-phase HPLC afforded a suite of rhizoxin analogs. One new rhizoxin congener, 22*Z*-WF-1360 F, and the known metabolites WF-1360 B, WF-1360 C, WF-1360 F (25), and rhizoxin D (18, 52, 53) (Fig. 1) were isolated as major metab-



B

Compound	*B. cinerea*					*P. ramorum*				
	0.1 µg/ml	0.5 µg/ml	1.0 µg/ml	5.0 µg/ml	20.0 µg/ml	0.1 µg/ml	0.5 µg/ml	1.0 µg/ml	5.0 µg/ml	20.0 µg/ml
WF-1360 F	-	+++	+++	++++	++++	-	-	++	+++	++++
22*Z*-WF-1360 F	+	+++	++++	++++	++++	-	+	+	+++	++++
WF-1360 B	-	-	-	+	++++	-	-	+	++	++++
WF-1360 C	-	-	-	+++	++++	-	-	-	++	++++
Rhizoxin D	-	-	-	-	++	-	-	-	-	++
Rhizoxin	+	+++	++++	++++	nt	+	++	+++	+++	nt

FIG. 4. Inhibition of the phytopathogens *B. cinerea* and *P. ramorum* by rhizoxin and rhizoxin analogs. (A) Twenty-four hours after conidia of *B. cinerea* and encysted zoospores of *P. ramorum* were placed in solutions of rhizoxin analogs, their germination and germination tube elongation were assessed under a dissecting microscope. The scale used to assess the toxicities of rhizoxin analogs was as follows: −, no inhibition; +, slight stunting and coiling of hyphae; ++, moderate hyphal stunting; +++, extreme hyphal stunting; ++++, germination inhibited. The blurry circles visible in some photographs are propagules outside the depth of field of the microscope. (B) Five concentrations of each rhizoxin analog were tested for toxicity against the two phytopathogens. Four replicate conidial suspensions were evaluated for each concentration using the scale in the legend to panel A, and the experiment was done twice with nearly identical results. nt, not tested.

olites and elucidated by NMR and MS spectroscopic experiments.

HR-EIMS data for 22*Z*-WF-1360 F (Fig. 1) gave a molecular formula of $C_{35}H_{47}NO_8$. It required 13 double-bond equivalents, 2 of them accounted for by the presence of two ester functionalities, one carbon-nitrogen double bond, and six carbon-carbon double bonds, indicating the tetracyclic nature of the new compound. ^{1}H,^{13}C NMR data and the UV spectrum suggested that 22*Z*-WF-1360 F is an analog of rhizoxin. The typical rhizoxin scaffold, consisting of a 16-member lactone ring system and a branching conjugated linear side chain bearing a terminal methyl-oxazole ring, was delineated by interpretation of the ^{1}H-^{1}H-correlation spectroscopy and heteronuclear multiple-bond coherence NMR spectra (see the supplemental material). The chemical shift of carbon C-7 (δ 82.4 ppm) and the required ring double-bond equivalents indicated the ring closure between C-5b and C-7. Hence, 22*Z*-WF-1360 F possessed the same basic structure as the known compound WF-1360 F (Fig. 1), and the two molecules must differ in either the absolute configuration of the nine chiral centers or the geometry of the five carbon-carbon double bonds. One-dimensional selective-gradient and two-dimensional nuclear Overhauser enhancement spectroscopy NMR experiments proved the relative configuration at all chiral centers of 22*Z*-WF-1360 F to be identical with that of WF-1360 F. Large coupling constants (Table 1) and the ^{13}C NMR chemical shift for C-18a (<20 ppm) showed the carbon-carbon double bonds $\Delta^{2,3}$, $\Delta^{9,10}$, $\Delta^{18,19}$, and $\Delta^{20,21}$ of 22*Z*-WF-1360 F to have *E* geometries as given in WF-1360 F. However, through-space interactions, observed between H_3-22a/H-23 and H-25/H-19 and the downfield shift of C-22a (>20 ppm) in the ^{13}C NMR spectrum identified 22*Z*-WF-1360 F as the 22*Z* isomer of WF-1360 F.

The identities of the previously reported compounds WF-1360 B, WF-1360 C, WF-1360 F, and rhizoxin D were established by direct comparison of ^{13}C NMR, HR-EIMS, and $[\alpha]_D^{20}$ data with the literature (see the supplemental material). The ^{13}C NMR data for rhizoxin D, which are not in the published literature, were compared to those for synthetic derived rhizoxin D (see Table S5 in the supplemental material).

Biological activity. (i) Activity against fungal and oomycete plant pathogens. At concentrations of 20 μg/ml, all rhizoxin analogs produced by Pf-5 were toxic to the fungal plant pathogen *B. cinerea*, inhibiting germination and germ tube elongation from conidia, and to the oomycete plant pathogen *P. ramorum*, inhibiting mycelial growth from encysted zoospores (Fig. 4). Of the five rhizoxin analogs, WF-1360 F and its *Z*-configured isomer, 22*Z*-WF-1360 F, were most toxic against *B. cinerea*, causing stunting of the hyphae at concentrations as low as 0.1 to 0.5 μg/ml. These compounds and WF-1360 B were also toxic against *P. ramorum* at concentrations as low as 0.5 to 1.0 μg/ml (Fig. 4). Rhizoxin D was the least toxic rhizoxin analog tested, exhibiting inhibition only at 20 μg/ml. WF-1360 C exhibited intermediate levels of toxicity, inhibiting both pathogens at 5.0 μg/ml. None of the five compounds produced by Pf-5 was as toxic as rhizoxin itself, which inhibited both pathogens at concentrations as low as 0.1 μg/ml.

Both rhizoxin and the fungicide benomyl inhibited fungi by inhibiting β-tubulin (7, 46); therefore, we compared the toxicity of WF-1360 F against a fungus resistant to benomyl. A benomyl-resistant isolate of *B. cinerea* (BC259) was similar to a benomyl-sensitive isolate (BC250) in its sensitivity to the rhizoxin analog WF-1360 F at all concentrations tested (data not shown).

(ii) Phytotoxicities against rice, cucumber, and pea. At concentrations of 20 μg/ml, four of the five rhizoxin analogs produced by Pf-5 were phytotoxic to rice (Table 2), inducing the thickened and shortened root morphology (Fig. 5 and 6) that is

TABLE 2. Phytotoxicities of rhizoxin analogs on rice[a]

Compound	Phytotoxicity at concn (μg/ml):		
	1	10	20
WF-1360 F	+	++	++
22*Z*-WF-1360 F	+	++	++
WF-1360 B	−	+	++
WF-1360 C	−	++	++
Rhizoxin D	−	−	−
Rhizoxin	++	++	++

[a] −, no phytotoxicity observed; +, slight thickening of the root observed (Fig. 5E); ++, considerable thickening of the root observed (Fig. 5F). The toxicity of each concentration was evaluated on three seeds of each plant, and the experiment was done twice with nearly identical results.

typical of rhizoxin toxicity (36). Rhizoxin D had no detectable effect on root morphology at any of the concentrations tested. WF-1360 F and its *Z*-configured isomer (22*Z*-WF-1360 F) were most phytotoxic, causing some thickening of rice roots at concentrations as low as 1 μg/ml. Rhizoxin and the five analogs were also tested for phytotoxicity on cucumber and pea, plants on which Pf-5 exhibits beneficial effects due to suppression of soil-borne plant pathogens (Fig. 6). Rhizoxin induced root thickening on both plants, whereas rhizoxin D had no visible effect on the root morphology of either plant. Pea roots exhibited only minor symptoms of root thickening when exposed to the other four rhizoxin analogs tested (Fig. 6). The sensitivity of cucumber was intermediate to those of rice and pea, exhibiting some root thickening when exposed to 20 μg/ml of each of four rhizoxin analogs.

(iii) Activities against human tumor cell lines. The two rhizoxin analogs (WF-1360 F and 22*Z*-WF-1360 F) tested for cytotoxicity to HCT-116 cells showed IC_{50}s of 0.8 and 0.2 ng/ml, respectively. The cytotoxicity 22*Z*-WF-1360 F was similar to that observed for rhizoxin itself (IC_{50} = 0.2 ng/ml). In the disk diffusion assay, both compounds were, like rhizoxin itself, active against all cell lines tested but showed no selectivity toward solid-tumor cell lines (see Table S6 in the supplemental material).

DISCUSSION

This study demonstrates that the well-characterized biological-control bacterium *P. fluorescens* Pf-5 produces five analogs of rhizoxin that are differentially toxic to a phytopathogenic fungus, an oomycete, and three plant species. The production of rhizoxin analogs by Pf-5 was discovered through a genomic mining strategy, by comparative metabolic profiling of Pf-5 and a derivative with a mutation in an orphan gene cluster identified from the genomic sequence of Pf-5 (41). Using a similar approach, another group working independently also discovered the rhizoxin-biosynthetic gene cluster in *B. rhizoxinica* (40) and *P. fluorescens* Pf-5 (4). This study extends the previous reports in (i) identifying a new rhizoxin analog, 22*Z*-WF-1360 F; (ii) demonstrating the spectra and different degrees of toxicity exhibited by the rhizoxin analogs; (iii) demonstrating that the sensitivity of the fungus *B. cinerea* to WF-1360 F, the predominant rhizoxin analog produced by Pf-5, is independent of its sensitivity to benomyl, a fungicide like rhizoxin, whose mode of action involves binding of β-tubulin; and (iv) demonstrating that *rzxB*, the first gene in the rhizoxin gene cluster, is essential for the production of rhizoxin analogs by Pf-5. Also, the new compound 22*Z*-WF-1360 F was characterized structurally and found to be among the most toxic of the rhizoxin analogs produced by Pf-5.

Three (WF-1360 F, WF-1360 C, and rhizoxin D) of the five rhizoxin analogs found to be produced by Pf-5 in this study (Fig. 1) were also reported recently as metabolites of Pf-5 (4). However, WF-1360 B and the new compound 22*Z*-WF-1360 F, which were detected in Pf-5 culture supernatants in this study, were not reported previously. Instead, Brendel et al. (4) isolated from cultures of Pf-5 several *seco*-rhizoxins, derivatives with an open δ-lactone ring. These *seco*-rhizoxins included rhizoxin D3, S1, S2, and Z1, which are the corresponding *seco* forms of WF-1360 C, WF-1360B, WF-1360 F, and 22*Z*-WF-1360 F, respectively. At 1 mg/liter culture medium, rhizoxin S2 was the most prevalent rhizoxin analog detected by Brendel et al. (4), whereas the corresponding closed-ring structure, WF-1360 F, at 1.1 mg/liter culture medium, was the most prevalent

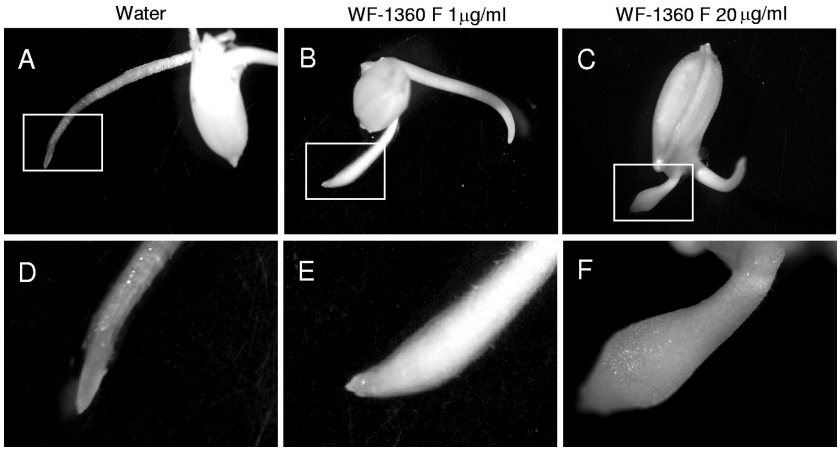


FIG. 5. Rice seedlings and roots treated with the rhizoxin analog WF-1360 F. The roots in the boxed areas (A to C) (×2.5) are shown at higher magnification (×10) in the frames below (D to F). Seedlings exposed to WF-1360 F, especially at the higher concentration, exhibit swollen and shortened root morphology. Three seedlings were assessed for each treatment, and the experiment was done twice with nearly identical results.

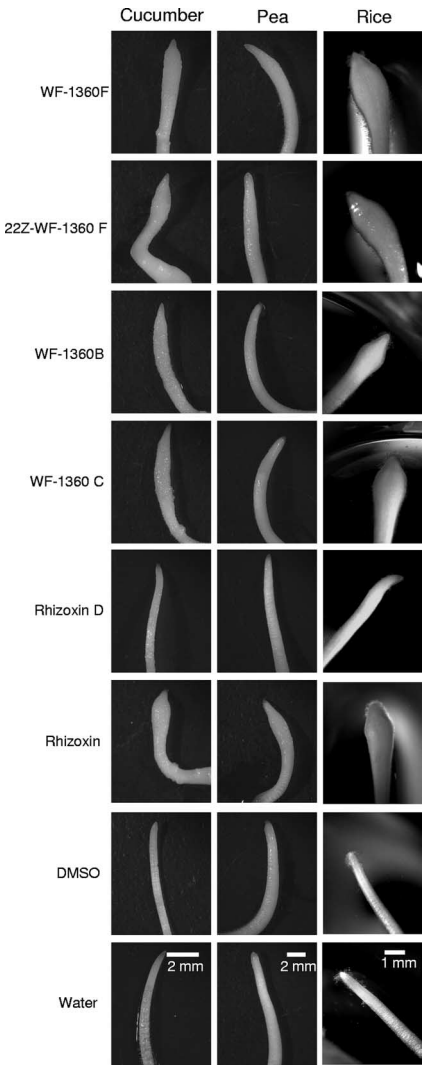


FIG. 6. Effects of rhizoxin and rhizoxin analogs on root morphologies of cucumber, pea, and rice. Seedlings were placed in solutions containing 20 μg/ml of the specified compound for 24 h prior to these observations. Control seedlings were placed in solution containing the same concentration of DMSO present in the other treatments. The photographs are representative of three replicate seedlings assessed for each treatment, and the experiment was done twice with nearly identical results.

rhizoxin analog detected in this study. Considering these coherences, our results parallel the findings of the previous study (4). Assuming that the closed-ring molecules are the correct products of the biosynthetic pathway, differences among the detected rhizoxin analogs could be explained by an inactive cyclase in the isolate of Pf-5 used by Brendel et al. (4) or by isolation of artifacts, possibly caused by fermentation, isolation, or analytical conditions used in one of the two studies. Because lactone rings can be cleaved by strongly acidic or basic conditions, pH could be a crucial factor in these experiments. We used a weakly acidic, ammonium acetate-buffered (pH = 6) flow system instead of the strongly acidic (0.1% trifluoroacetic acid; $pK_a = 0.2$) flow system used by Brendel et al. (4), during the LC-MS analysis of the crude extract and a weak acid (1% acetic acid; $pK_a = 4.8$) for the subsequent purification by reversed-phase HPLC. These experimental protocols allowed the isolation of rhizoxin analogs in their closed-ring forms.

The five rhizoxin analogs produced by Pf-5 (Fig. 1) share a 16-member macrolide core, including a ring-fused δ-lactone and a triene oxazole-containing side chain, but differ at positions C-11/C-12 (double bond or epoxide functionalization) and C-17 (hydroxy or methoxy group) or in the $\Delta^{22,23}$ double-bond geometry. An *rzxB* mutant of Pf-5 did not produce any of the five analogs, indicating that the *rzx* gene cluster is responsible for the biosynthesis of all of them. Correspondingly, the rhizoxin analogs are considered to be intermediates or branch products of the WF-1360 F-biosynthetic pathway. We speculate that the cytochrome P450 monooxygenase RzxH may be involved in the epoxidation of the $\Delta^{11,12}$ double bond, which is consistent with the data of Scherlach et al. (44), demonstrating that the $\Delta^{11,12}$ double bond is blocked by cytochrome P450 monooxygenase inhibition. The *S*-adenosylmethionine-dependent methyltransferase RzxI is likely to be required for *O*-methylation at the C-17 carbinol, as proposed by Partida-Martinez and Hertweck (40). It is noteworthy that neither rhizoxin nor any other bis-epoxidated metabolite was isolated from *P. fluorescens* Pf-5. Instead, the rhizoxin analogs obtained lacked a second epoxide ring at C-2. We speculate that *rhiJ*, which is present in the rhizoxin-producing *B. rhizoxinica* but absent in Pf-5, is required for the epoxidation of the $\Delta^{2,3}$ double bond in the final step of rhizoxin biosynthesis.

The toxicities of the five rhizoxin analogs produced by Pf-5 were evaluated against the fungus *B. cinerea*, the oomycete *P. ramorum*, and three plant species. WF-1360 F and the new compound 22*Z*-WF-1360 F were the most toxic of the five analogs, WF-1360 B and WF-1360 C exhibited moderate toxicity, and rhizoxin D showed the weakest activity. The superior toxicities of 22*Z*-WF-1360 F and WF-1360 F versus the other three rhizoxin analogs argue for the importance of the epoxide at C-11/C-12 in biological activity. *Z*-configured rhizoxin isomers have been reported as less potent analogs (21, 44), possibly associated with photoisomerization artifacts (21), but substantiated biochemical or structure-activity relationship studies have been lacking. In this study, there was no observable loss of toxicity associated with the *Z* conformation of 22*Z*-WF-1360 F. The relatively weak toxicity of rhizoxin D suggests that the methyl group at the hydroxyl group of C-17, which is also present in the most toxic analogs and rhizoxin itself, is not sufficient for the toxicities of these molecules. Nevertheless, the C-17 methoxy group may contribute to toxicity in compounds also having an epoxide at C-11/C-12, which could explain the greater toxicity of WF-1360 F than WF-1360 B. While all five of the rhizoxin analogs produced by Pf-5 exhibited toxicity, none were as toxic as rhizoxin itself, which suggests that the epoxide at C-2/C-3 plays a major role in the biological activities of this class of compounds.

Two analogs of rhizoxin produced by Pf-5 were tested for toxicity against human cancer cell lines, and they exhibited potent but nonselective cytotoxicities. The broad toxicity of rhizoxin is attributed to its binding of β-tubulin (46), thereby stabilizing microtubule dynamics, blocking cells in the G_2/M stage of the cell cycle, and ultimately resulting in apoptosis (14). Inhibitory effects on angiogenesis are also proposed as a mode of action (37). At one time, the striking antitumoral

activity of rhizoxin attracted considerable interest in the synthetic and pharmacologic community, and consequently, it went through extensive clinical trials as an anticancer drug in the 1990s. Due to its moderate in vivo activity (15, 23, 48), however, rhizoxin was never taken into phase III clinical trials (35), shifting the focus of the biological significance toward its antifungal properties (17).

Strains of *Pseudomonas* spp. that produce the rhizoxin analog WF-1360 F (synonym, 2,3-deepoxy-2,3-didehydro-rhizoxin) are known to suppress a number of plant-pathogenic fungi and an oomycete in culture (19). *Pseudomonas chlororaphis* MA 342 (20), a commercial biological-control agent (Cedomon) that has been used for management of seed-borne pathogens of barley in Europe since 1997, is known to produce WF-1360 F. This study establishes the toxicity of the compound WF-1360 F and other rhizoxin analogs produced by Pf-5 against a fungal and an oomycete phytopathogen. A benzimidazole-resistant isolate of *B. cinerea* retained sensitivity to WF-1360 F, the prevalent rhizoxin analog produced by Pf-5, despite the shared mechanism of β-tubulin interference of the two compounds (7, 46). This result is consistent with those of a previous study demonstrating that a benzimidazole-resistant mutant of *Aspergillus nidulans* retains sensitivity to rhizoxin (47). Different amino acid substitutions in β-tubulin result in resistance to rhizoxin (46) versus benzimidazole fungicides (31). Therefore, we expect that the efficacies of rhizoxin derivatives will be retained even if the occurrence of benzimidazole resistance in populations of phytopathogenic fungi increases with continued use of this fungicide in agriculture.

We also demonstrated that the rhizoxin analogs produced by Pf-5, including WF-1360 F, exhibit phytotoxicity against rice, a plant known to be very sensitive to rhizoxin (36). Other secondary metabolites that contribute to the plant disease-suppressive properties of *Pseudomonas* spp., such as pyoluteorin and 2,4-diacetylphloroglucinol, also exhibit phytotoxicity when applied to plants at high concentrations (22, 33). Because plant species typically differ in their sensitivities to these bacterial metabolites (22, 33), we tested the rhizoxin analogs for phytotoxicity against pea and cucumber, plants known to benefit from seed inoculation with Pf-5 (26; M. D. Henkels and J. E. Loper, unpublished data). These plants exhibited only minor deformation of developing roots when exposed to rhizoxin analogs at concentrations (20 μg/ml) that were 20- to 40-fold greater than those inhibiting germination or germ tube elongation of the fungal and oomycete plant pathogens. Consequently, it is quite possible that Pf-5 could produce rhizoxin analogs on plant roots in concentrations adequate to inhibit microbial pathogens without deleterious effects on certain plant hosts. To date, however, the production of rhizoxin analogs by Pf-5 on plant roots and the roles of these compounds in biological control have not been established.

The genomic sequence of *P. fluorescens* Pf-5 provides a rich source of information useful in the discovery of novel metabolites with significant biological properties. Approaches from different disciplines, including bioinformatics, natural-product chemistry, and plant and microbial biology, were employed in this study to establish a link between the genome sequences, chemical structures, and biological functions of rhizoxin analogs. In addition to the compounds described here, at least seven other secondary metabolites are produced by Pf-5 (30). These seven compounds exhibit a range of antibiotic, surfactant, and iron-chelating activities (30). How rhizoxin analogs interact with other secondary metabolites to influence the biological-control activity of Pf-5 is an intriguing question for future study. The discovery and structural and toxicological characterization of rhizoxin analogs produced by Pf-5 represent important steps toward our larger goal of identifying factors contributing to the ecology of rhizosphere bacteria and the biological control of plant diseases.

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