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 through-
out 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, 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 22Z-WF-1360 F were most toxic against the two plant pathogens and three plant species. These rhizoxin
analogues 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.

The products synthesized from a hybrid polyketide synthase/nonribosomal peptide synthetase gene cluster in the
gene of Pseudomonas fluorescens Pf-5 were identified using a genomics-guided strategy involving insertion
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 analogues produced by Pf-5, the predominant compound, WF-1360 F, and the newly described
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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.

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_s' ENTR and 2989_3' ENTR (see the supplemental material),
cloned into the gateway entry vector pENTR-DTOPO (Invitrogen), and inte-
grated into the destination vector pLVC-D (32) using the clooney 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

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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.
† Supplemental material for this article may be found at http://aem
.asm.org/.
‡ Published ahead of print on 14 March 2008.
Pt-5 via conjugation, selecting for resistance to streptomycin (100 μg/ml; innate resistance of Pt-5) and tetracycline (200 μg/ml; conferred by the plasmid). Because plVC-D is a suicide plasmid in *Pseudomonas* spp., tetracycline-resistant colonies of Pt-5 were expected to have undergone a single-crossover event between the DNA cloned in plVC-D and the corresponding sequence in the Pt-5 chromosome. Gene disruption and plasmid insertion were confirmed using PCR with primers specific to the plVC-D vector (L attB2 and U attB1) and genomic DNA sequences flanking the 1,394-bp region of *rzxB* (2989_5’ OUT and 2899_3’ OUT) (see the primer table in the supplemental material). A derivative of Pt-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* Pt-5 expressed rzxB. Three replicate cultures of Pt-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 (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 correspond 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 cDNA 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 2899_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 CDCl₃ (Shigemi, Allison Park, PA) or 2.5-mm Match sample tubes (Hilgenberg GmbH, Malsfeld, Germany). Spectra were calibrated to the solvent signal (13C: CDCl₃ δ 77.0 ppm) and the signal contributed by the nondeuterated portion of the solvent (1H: CHCl₃ in CDCl₃ 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 spectrometry (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 Pt-5 and an rzxB mutant. To identify the product(s) of the orphan gene cluster, cultures of Pt-5 and the rzxB mutant, grown under culture conditions conducive to expression of rhizoxin biosynthetic genes, were compared by LC/MS. Pt-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

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FIG. 1. Structures of rhizoxin and the rhizoxin analogs produced by *P. fluorescens* Pt-5. The rhizoxin analogs were isolated at the specified concentrations from 48-h cultures of Pt-5 grown in Davis medium at 21°C. DRR, 2,3-deepoxy-2,3-didehydro-rhizoxin.
TABLE 1. NMR data for 22Z-WF-1360 F in CDCl₃

<table>
<thead>
<tr>
<th>Position</th>
<th>δH[a]</th>
<th>δC[b]</th>
<th>HMBC[c]</th>
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<tr>
<td>1</td>
<td>5.069 (H, d, J = 15.3)</td>
<td>124.9 CH</td>
<td>5, 9a</td>
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<tr>
<td>2</td>
<td>5.808 (H, m)</td>
<td>140.0 CH</td>
<td>5, 9a</td>
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<td>3</td>
<td>1.78 (H, m)</td>
<td>37.9 CH₂</td>
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<tr>
<td>4</td>
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<td>52.7 CH</td>
<td>5, 9a</td>
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<td>5</td>
<td>1.60 (H, m)</td>
<td>29.7 CH</td>
<td>5a</td>
</tr>
<tr>
<td>5a</td>
<td>2.11 (H, m)</td>
<td>36.8 CH₂</td>
<td>5b</td>
</tr>
<tr>
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<td>170.0 qC</td>
<td>5b</td>
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<td>33.8 CH₂</td>
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<td>82.4 CH</td>
<td>6</td>
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<td>16.6 CH₃</td>
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<td>8a</td>
<td>1.17 (H, d, J = 6.4)</td>
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<td>8, 9, 9a</td>
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<td>18</td>
<td>1.36 qC</td>
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<td>130.1 CH</td>
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<td>23</td>
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<td>117.5 CH</td>
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<td>138.4 qC</td>
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<td>7.45 (H, s)</td>
<td>136.3 CH</td>
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<td></td>
</tr>
<tr>
<td>26a</td>
<td>2.47 (H, s)</td>
<td>13.8 CH₂</td>
<td>26</td>
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</tbody>
</table>

[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.

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 yielding final concentrations of the rhizoxin analogs (isolated from cultures of Pf-5) at various concentrations. Seedlings were adapted to a new culture containing 25% of the original concentration of the rhizoxin analogs. The results were normalized to an untreated control. The 50% inhibitory concentration (IC₅₀) was determined using Prism 4.0 software (GraphPad, San Diego, CA).

RESULTS

Characterization of an orphan NRPS/PKS gene cluster in the Pf-5 genome. A 78,871-bp region containing nine biosynthetic genes (PFL2989 to PFL2997) (Fig. 2), six having predicted functions as PKSs or as mixed NRPS/PKSs, was identified previously in this genome (41). The other genes in the cluster have predicted functions as an acyl transferase, a methylenetransferase, and a cytochrome P450 monooxygenase. The closest homolog found for each gene was in the rhizoxin-biosynthetic gene cluster of B. rhizoindica (40), which, along with the cluster in Pf-5 (4), was described during the later stages of this
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 rhiC), acyl transferases (Pfam PF00698 [10]); black arrows (rzxL and rhiL), S-adenosylmethionine-dependent methyltransferases (Pfam PF08241); light-gray arrows (rzxH and rhiH), cytochrome P450 monooxygenases (Pfam PF00067); horizontally striped arrow (rhiD), 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-hydroxacyl-ACP dehydratase; GNAT, GCN5-related N-acetyltransferase (13); HC, modified condensation (heterocyclization) (42); KR, β-ketoacyl-ACP reductase (Pfam PF08659); KS, β-ketoacyl-ACP synthase (Pfam PF08242); OX, oxidase (8); PCP, peptidyl carrier protein (Pfam PF00550); TE, thioesterase (Pfam PF00675). 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).

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 GXXXXXCSS 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 HXXPGX-XXXP 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 rhiD, which is present in *B. rhizoxinica* and absent in the Pf-5 cluster.

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<sub>max</sub> 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, 22Z-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-
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.

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 [α]$_D$ 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, 22Z-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 *B. cinerea* (BC259) was similar to

(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...
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, 22Z-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 rzzB, the first gene in the rhizoxin gene cluster, is essential for the production of rhizoxin analogs by Pf-5. Also, the new compound 22Z-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 22Z-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 22Z-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

### DISCUSSION

<table>
<thead>
<tr>
<th>Compound</th>
<th>Phytotoxicity at concn (µg/ml):</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>WF-1360 F</td>
<td>+</td>
</tr>
<tr>
<td>22Z-WF-1360 F</td>
<td>+</td>
</tr>
<tr>
<td>WF-1360 B</td>
<td>–</td>
</tr>
<tr>
<td>WF-1360 C</td>
<td>–</td>
</tr>
<tr>
<td>Rhizoxin D</td>
<td>–</td>
</tr>
<tr>
<td>Rhizoxin</td>
<td>++</td>
</tr>
</tbody>
</table>

*– 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.*

**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.

![Image of rice seedlings and roots treated with the rhizoxin analog WF-1360 F.](image-url)
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; pKₐ = 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; pKₐ = 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 ß-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 Δ²,³ 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 Δ¹¹,¹² double bond, which is consistent with the data of Scherlach et al. (44), demonstrating that the Δ¹¹,¹² 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 carbino, 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 rhdI, which is present in the rhizoxin-producing B. rhizoxinica but absent in Pf-5, is required for the epoxidation of the Δ²,³ 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 22Z-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 22Z-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 22Z-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 nonselctive cytotoxities. The broad toxicity of rhizoxin is attributed to its binding of ß-tubulin (46), thereby stabilizing microtubule dynamics, blocking cells in the G₂/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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