APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1994, p. 2031–2039 Vol. 60, No. 6
0099-2240/94/$04.00+0

Pyrrolnitrin Production by Biological Control Agent *Pseudomonas cepacia* B37w in Culture and in Colonized Wounds of Potatoes

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Received 28 October 1993/Accepted 15 March 1994

Bacterial strain B37w (= NRRL B-14858), an isolate noteworthy because it inhibits the growth of the bioherbicide fungus *Colletotrichum truncatum*, was selected for further studies of bacterial antifungal properties. This isolate was identified as a *Pseudomonas cepacia* strain by performing carbohydrate utilization and fatty acid profile analyses, as well as other biochemical and physiological tests. Petri plate assays revealed that strain B37w exhibited antifungal activity against the potato dry rot fungus *Fusarium sambucinum*. Using bioautography, we correlated antifungal activity with production of a specific compound. Isolation from strain B37w and identification of the antifungal antibiotic pyrrolnitrin are described. A whole-potato assay revealed B37w's ability to colonize potato wounds. Wounded potatoes were inoculated with B37w, and pyrrolnitrin was detected in these potatoes by thin-layer chromatography-bioautography at a concentration on the order of nanograms per wound. We performed an assay in which we examined efficacy against *F. sambucinum*-incited potato dry rot and found that B37w inhibited disease development. This is the first report of *P. cepacia* or pyrrolnitrin activity against the economically important potato pathogen *F. sambucinum*.

Bacterial strain B37w (= NRRL B-14858) was originally isolated by Schisler et al. (37) when they were investigating the use of epiphytic bacteria to enhance the efficacy of the fungus *Colletotrichum truncatum* as a mycoherbicide against the weed hemp sesbania (*Sesbania exaltata*). These authors examined microorganisms isolated from leaf surfaces and found that instead of the desired enhancement of bioherbicidal effects, B37w inhibited conidial germination by 75% and completely suppressed appressorium formation. This activity, along with the fact that the bacterium was a plant surface-competent organism, made strain B37w a good candidate to include in a project designed to develop microbial biocontrol of *Fusarium sambucinum*-incited dry rot of potatoes. An initial efficacy assay revealed that B37w was among the best 20 of 350 organisms tested for the ability to protect potatoes from dry rot, reducing disease lesion size by as much as 92% compared with controls. However, B37w was not characterized further.

This study was done to determine the identity of strain B37w and to investigate antibiosis as a potential mechanism for biological control of *F. sambucinum* by B37w. Information gained about antibiotic production as a mode of action was expected to have an impact on process designs for mass production and formulation of an efficient biological control agent. Additional objectives were to isolate and identify the major antifungal antibiotic produced by B37w and to demonstrate the production of this compound in situ. Finally, we wanted to further study strain B37w's efficacy against various strains of *F. sambucinum* in potatoes.

MATERIALS AND METHODS

Taxonomic identification and preparation of B37w mutant B37w rif$^+$. Bacterial isolate B37w (= NRRL B-14858) was characterized by using Biolog GN Log microplates and a microcomputer data base (version 3.0; Biolog, Inc., Hayward, Calif.) and by performing a fatty acid profile analysis (version 3.6 aerobic library; Microbial ID, Inc., Newark, Del.) and other physiological and biochemical tests as described in *Bergey's Manual of Systematic Bacteriology* (31).

In order to facilitate quantitative recovery of B37w in subsequent potato tuber inoculation trials, a spontaneous rifampin-resistant mutant of B37w, designated B37w rif$^+$, was isolated. A 250-μl portion of a suspension of 24-h-old cells of B37w in phosphate buffer (A_{620}, 0.5) was spread over the surface of 0.2× tryptic soy agar (Difco) containing 25 ppm of rifampin. Mutant colonies were picked from the agar surface after the plates were incubated for 48 h at 28°C. A mutant strain selected for additional studies was also identified by using Biolog plates, and this strain had growth and antibiotic production characteristics similar to those of the wild type.

Maintenance of microbial strains. *Pseudomonas* strains were maintained as glycerol stocks (10% glycerine) at −80°C. Before a strain was used, its purity was checked and an inoculum was grown for 24 h at 25°C on Sabouraud maltose agar (Difco) or on 0.2× tryptic soy agar (Difco) (both media contained 1.8% agar). Stock cultures of *F. sambucinum* (Fusarium Research Center accession numbers R-6380, R-9261, and R-9262) were maintained as dried spore suspensions on silica gel crystals (6 to 12 mesh). The inocula used for antifungal bioassays were initially grown on water agar (1.8% Bacto Agar in distilled water), and hyphal tip cultures were then inoculated onto various agar media and grown with a 12-h photoperiod at 25°C for 1 week.

Fermentation. For liquid fermentations we used two stages of growth in Sabouraud maltose broth (Difco). Precultures (25 ml) were grown for 3 days in 125-ml Erlenmeyer flasks at 25°C and 250 rpm on a New Brunswick controlled-environment incubator shaker with 1-in. (2.54-cm) eccentricity, before they were used to inoculate the second-stage flasks. For analytical-scale fermentation, 2.5 ml was used to inoculate each 25 ml of

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second-stage culture. For preparative-scale antibiotic production fermentation, the contents of two preculture flasks (50 ml) were added to each of six Fernbach flasks containing 500 ml of medium. Second-stage fermentation preparations were grown under the same conditions for 168 h (analytical-scale fermentation) or 48 h (preparative-scale fermentation).

Extraction. Analytical samples (6 ml) removed from second-stage cultures after 24, 48, 72, 96, and 168 h were adjusted to a pH value of 3, 7, or 11 by using dilute HCl or NaOH. The samples were centrifuged at 8,000 rpm (7,149 × *g*) for 15 min. Pelleted cells from the samples were extracted with 600 μl of acetone by sonicating the preparations for 1 min and centrifuging them at 4,000 rpm (1,787 × *g*) for 5 min. The acetone supernatant was drawn off, and the pellets were reextracted with an additional 300 μl of acetone. The acetone extracts were combined and were dried in glass vials kept in a heating block at 45°C under a stream of N_2 gas. The extracts were redissolved in ethyl acetate (120 μl) by using a vortex mixer followed by centrifugation. The volume of each ethyl acetate layer used for thin-layer chromatography (TLC) (20 μl) represented the amount of material extracted from the cells in 1 ml of culture.

A total of 3 liters of culture from six 48-h preparative fermentation flasks was centrifuged at 8,000 × *g* for 15 min at 4°C. The resulting cell pellets were sonicated with acetone (approximately 75 ml) and recentrifuged. The acetone solution was reduced in volume on a rotary evaporator with a water bath temperature of 56°C. The remaining water from the acetone solution was extracted with ethyl acetate (approximately 0.33 volume). The entire cell extraction procedure was repeated two more times, until TLC analysis demonstrated that extraction was complete. All ethyl acetate extracts were combined, filtered through anhydrous sodium sulfate kept in a glass funnel lined with filter paper, and dried with a rotary evaporator. After transfer to a tared vial, the crude extract was dried further under a stream of N_2 gas and was kept under a vacuum for 2 days (final weight, 158 mg).

Chromatography. TLC was performed by using precoated 0.25-mm Silica Gel 60 F_{254} plates (catalog no. 5715; Merck) or 0.25-mm reversed-phase plates (type KC18F; Whatman). The plates were activated by heating them at 120°C for 30 min and then were stored in a desiccator. After the distances traveled relative to the solvent front (R_f) were determined for various systems, silica gel plates and chloroform-acetone (9:1) were chosen to optimize the location (R_f, 0.48) of the major antibiotic spot. Developed chromatograms were visualized under UV light (254 nm) and then sprayed with Pauly reagent (diazotized sulfanilic acid in which 5% sodium hydroxide in 50% ethanol is used as the base) or with Erlich reagent (0.1% *para*-dimethyl aminobenzaldehyde in HCl, followed by 0.1% sodium nitrite in water). The plates that were to be used for bioautography (see below) were not sprayed with chemical visualization reagents.

For flash column chromatography we used silica gel 40-μm flash chromatography packing (catalog no. 7024-01; Baker) that was activated by heating it for 1 h at 110°C and then packed with hexane at an N_2 pressure of 8 lb/in^2 in a column that was 35.6 by 1.75 cm. The sample (158 mg of crude B37w extract adsorbed onto 500 mg of silica gel) was added, and then fractions (4.2 ml, 1 min) were collected by elution with benzene-hexane (2:1) at an N_2 pressure of 3.5 lb/in^2. After 1,250 ml of this solvent had eluted the major antibiotic compound followed by a yellow band, the elution solvent was changed sequentially in 500-ml increments to benzene, benzene-acetone (1:1), acetone, and methanol to elute the rest of the sample. A total of 440 fractions and a methanol wash were collected. The fractions were combined on the basis of the TLC analysis results, and bioautography was performed to detect the antifungal activities of the various compounds eluted. Mass spectral and nuclear magnetic resonance (NMR) analyses were performed to identify the purified antibiotic obtained from dried fractions 66 to 95.

Antibiotic mass and NMR spectra. Proton (^{1}H) and carbon (^{13}C) NMR spectra were obtained by using a 50 mM solution of the antibiotic produced by B37w in deuterated chloroform and a Bruker model WM-300 spectrometer operating at 75 and 300 MHz. Electron impact mass spectrometry performed on the antibiotic produced by B37w revealed a base peak for the molecular ion at a mass-to-charge (*m/z*) value of 256.

Petri plate assays. Petri plate assays were performed in triplicate with the following three media: tryptic soy agar, potato dextrose agar, and Sabouraud maltose agar (all three media were obtained from Difco and contained 1.8% agar). Fungal plugs (0.5 cm) were cut from the outer edges of 7-day petri plate cultures of *F. sambucinum* R-6380 with a sterile cork borer and were placed in the centers of petri plates containing the same medium on which the plugs were grown. The bacterial strains to be tested were transferred from 7-day petri plate cultures by using the wooden ends of sterilized cotton swabs and inoculating each test plate with the bacterial culture 1 cm from the outer edge. Control plates were inoculated with fungal plugs only. The plates were incubated at 28°C. The results were expressed as the amount of fungal growth (in millimeters) toward the bacterial colony after 3 and 7 days.

Bioautography. Bioautography was performed by using developed TLC plates sprayed with a suspension of R-6380 spores in Sabouraud maltose broth (approximately 10^6 spores per ml) until the silica gel began to appear translucent. The sprayed plates were incubated at 25°C in plastic bags under white light with a 12-h photoperiod. After 5 days, peach-colored fungal growth was visible; zones of inhibition due to antifungal compounds on the TLC plates appeared as white areas where fungal growth was absent.

Microbial colonization and recovery of antibiotic from inoculated potatoes. A colonization-recovery study was performed in order to determine whether antibiotics were produced by *Pseudomonas cepacia* colonizing wound surfaces. Potatoes were inoculated by using the protocol used to screen antifungal microbial isolates for efficacy against *Fusarium* dry rot (38), except that we used a larger number of wounds per potato (12 versus 4 wounds) to improve our ability to detect antibiotics in extracts obtained from excised potato wounds. Distilled-water-washed potatoes (*Solanum tuberosum* subsp. *tuberosum* cv. Russet Burbank; Wisconsin Seed Potato Certification Agency, Antigo) were wounded with an ethanol-sterilized blunted nail (3 by 2 mm). Twelve wounds were located around the circumference of each tuber, midway between the stem and bud ends.

The inocula used for the assays were prepared by using sterile Fisher Scientific phosphate buffer (pH 7.2 ± 0.2) (phosphate buffer) to dilute 18-h petri plate cultures of bacteria or 7-day petri plate cultures of R-6380. Each wound was inoculated with 5 μl of one of four preparations: (i) *P. cepacia* B37w rif$^+$, with the concentration adjusted to an A_{620} of 0.164, mixed with an equal volume of phosphate buffer (treatment 1); (ii) B37w rif$^+$ (A_{620}, 0.164) mixed with an equal volume of *F. sambucinum* R-6380 (1.1×10^5 viable spores per ml) (treatment 2); (iii) R-6380 (1.1×10^5 viable spores per ml) mixed with an equal volume of phosphate buffer (treatment 3); and (iv) phosphate buffer (treatment 4). Dilution plating revealed that the bacterial stock suspension used contained 1.1×10^8 CFU/ml before it was mixed with phosphate buffer or strain

R-6380 for treatments 1 and 2. Twelve potatoes (144 wounds) were used for each treatment. Potatoes in plastic trays were placed next to dampened cloths in plastic bags and incubated in the dark at 15°C.

After 1 week, an ethanol-sterilized cork borer (diameter, 0.5 cm) was used to excise a cylindrical section of potato from around each wound, and these sections were used for reisolating and counting colonies or for extraction and chromatographic analyses. Each section was cut with a sterile scalpel to a length of 1.25 cm. The average wet weight per section was 0.1791 g (calculated from 10 sections obtained from an uninoculated potato).

For each treatment group, 4 of the 12 potatoes were used for dilution plate analysis by placing the 12 excised wounds obtained from each potato in sterile 50-ml capped tubes containing 5 ml of sterile 0.1% water agar. After agitation for 10 s with a vortex mixer, each water agar suspension was diluted serially with additional water agar. The dilutions were plated onto 0.2× tryptic soy agar containing 25 ppm of rifampin to determine the recoverability of B37w rif$^+$ from the potatoes. The strain reisolated from potatoes that had been inoculated with *P. cepacia* B37w rif$^+$ mixed with phosphate buffer was identified by using the Biolog system.

The wound sections from the remaining eight potatoes in each treatment group were used to obtain extracts for chromatographic analysis. For each treatment group wound sections from 96 wounds were combined and extracted by homogenization with 50 ml of ethyl acetate, followed by centrifugation for 5 min at 7,000 rpm (4,910 × *g*) at 4°C. Dried ethyl acetate layers were redissolved in 500 μl of ethyl acetate, and 30 μl of this preparation was used for TLC. The developed TLC plate was sprayed with *F. sambucinum* R-6380 spores and incubated as described above.

HPLC analysis. To determine whether the antifungal compound pyrrolnitrin [3-chloro-4-(2′-nitro-3′-chlorophenyl)-pyrrole] was present in the extract obtained from potatoes coinoculated with B37w rif$^+$ and *F. sambucinum* R-6380, a high-performance liquid chromatography (HPLC) analysis was done by using a Waters model 6000A solvent delivery system, an Alltech Econosphere C18 5μ column (4.6 mm [inside diameter] by 250 mm), and isocratic acetonitrile-methanol-water (1:1:1.1) (27). Standards and samples (10 μl in methanol) were injected with a Waters model 715 Ultra WISP sample processor. A_{254} was measured with an ABI Spectroflow 757 absorbance detector and was recorded by using a Spectra-Physics ChromJet integrator. A standard curve relating absorbance to concentration of pyrrolnitrin (range, 0.5 to 1,000 μg/ml) was prepared from the data obtained from triplicate injections at each concentration. The concentration of the potato treatment extracts injected was 2.0 mg/ml.

Potato tuber assay to determine biological control efficacy. In addition to the inoculation-recovery study described above, whole potato bioassays to determine the efficacy of B37w for controlling *F. sambucinum* were conducted and evaluated as described elsewhere (38). In these bioassays each potato received four wounds, and inoculated potatoes were incubated for 3 weeks before evaluation. At the end of the incubation period longitudinal cuts that were perpendicular to the tuber surface were made through wounds, and the sum of the depth and width of the darkened, dry-rotted tissue was determined. A one-way analysis of variance and mean comparisons were performed with log-transformed data. We evaluated 16 potato tuber wounds for potatoes treated with *F. sambucinum* alone and four wounds for wound-alone and B37w-*F. sambucinum* coinoculation treatments. In separate experiments, B37w was assayed twice versus *F. sambucinum* R-6380 and once versus strains R-9261 and R-9262.

RESULTS

Taxonomic identification of B37w. B37w was identified as a strain of *P. cepacia* (Burkholder) by using Biolog GN Log microplates (similarity value, 0.836) and by performing a fatty acid profile analysis (similarity value, 0.637). Oxidase-positive, polyhydroxybutyrate accumulation-positive, denitrification-negative, and arginine dihydrolase-negative test results were consistent with identification as *P. cepacia*. After it was reisolated from potato wound surfaces, strain B37w rif$^+$ was also identified as *P. cepacia* by using Biolog plates (similarity value, 0.841).

Identification of pyrrolnitrin. TLC was performed by using extracts from an analytical-scale fermentation of B37w and various solvent systems for previously described *P. cepacia* antibiotics. The color reactions of the isolated antibiotic obtained with spray reagents were consistent with identification as pyrrolnitrin; the antibiotic spot turned maroon with Pauly reagent and blue with Erlich reagent (1, 27). Table 1 shows TLC data obtained in this study and in previous studies of pyrrolnitrin and other *P. cepacia* antibiotics. Although the R_f differed only by 2% from the R_f reported by McLoughlin et al. (29) for pyrrolnitrin, comparisons with data from other studies of pyrrolnitrin revealed differences of 8 to 40%. These variations in TLC R_f values may be explained by the different silica gel adsorbants used and the different methods of preparation used (e.g., commercially prepared plates or hand-poured plates). Our compound also resembled 2-chloropyrrolnitrin; the R_f on reversed-phase TLC plates was within 1% of the value reported by Mahoney and Roitman (27). However, identification as this compound was ruled out by the color reaction with Pauly reagent. Tentative identification of the antibiotic which we isolated as pyrrolnitrin was confirmed by subsequent spectral data.

Preparative-scale fermentation (3 liters) yielded 6.4 mg of light yellow solid material after silica gel flash chromatography. The chromatography profile is shown in Table 2. In Tables 3 and 4 the NMR spectral data obtained for the purified compound are compared with values for the antifungal antibiotic pyrrolnitrin which have been reported previously (28, 36). The numbering of atoms in Tables 3 and 4 refers to the pyrrolnitrin structure (Fig. 1). The *m/z* values in the electron impact mass spectrum and their intensities (levels of relative abundance) are compared in Fig. 2 with previously published electron impact mass spectrometry data for pyrrolnitrin (36). The molecular ion at *m/z* 256 is consistent with the formula $C_{10}H_6Cl_2N_2O_2$, the formula for pyrrolnitrin. The high levels of similarity between our electron impact mass spectrometry, ^{1}H-NMR, and ^{13}C-NMR data and previously published data confirmed that the compound which we isolated was pyrrolnitrin.

Petri plate assays. Petri plate assays were performed to test the ability of bacterial strains to inhibit the growth of potato pathogen *F. sambucinum* R-6380. Of 20 bacterial isolates that exhibited superior biological control of *F. sambucinum* on potatoes, strain B37w exhibited the most potent antifungal activity as determined by petri plate assays (data for other isolates not shown). Fungal growth toward B37w was compared with growth on control plates, and inhibition of R-6380 by B37w was apparent on three different agar media after 3 days and 1 week (Fig. 3). These petri plate assays revealed the ability of strain B37w to inhibit fungal growth on agar media,

TABLE 1. TLC data for pyrrolnitrin and other *P. cepacia* antibiotics

Plate type	Solvent	Compound	R_f	Detection method	Reference
Silica gel	Benzene–*n*-hexane (1:1)	Pyrrolnitrin	0.25	Erlich reagent	2
Silica Gel GF_{254}	Benzene–hexanes (1:1)	Pyrrolnitrin	0.23	Erlich reagent	3
Silica Gel 60 F_{254}	Benzene–*n*-hexane (1:1)	Pyrrolnitrin	0.11	Erlich and Pauly reagents, bioassay	This study
Silica gel	Chloroform	Pyrrolnitrin	0.70	Erlich reagent	2
Silica Gel GF_{254}	Chloroform	Pyrrolnitrin	0.57	Erlich reagent	3
Silica Gel 60	Chloroform	Unknown	0.57	Bioassay	19
Silica Gel 60 F_{254}	Chloroform	Pyrrolnitrin	0.30	Erlich and Pauly reagents, bioassay	This study
Silica gel	Benzene	Pyrrolnitrin	0.65	Erlich reagent	2
Silica Gel GF_{254}	Benzene	Pyrrolnitrin	0.54	Erlich reagent	3
Silica Gel 60 F_{254}	Benzene	Pyrrolnitrin	0.36	Erlich and Pauly reagents, bioassay	This study
Silica Gel 7GF	Chloroform–acetone (9:1)	Pyoluteorin	0.27	UV light	15
Silica Gel 60	Chloroform–acetone (9:1)	Unknown	0.63	Bioassay	19
Silica Gel 60 F_{254}	Chloroform–acetone (9:1)	Pyrrolnitrin	0.48	Erlich and Pauly reagents, bioassay	This study
Silica gel	Methylene chloride	Aminopyrrolnitrin	0.33	Ninhydrin	29
Silica gel	Methylene chloride	Pyrrolnitrin	0.50	Ninhydrin	29
Silica Gel 60 F_{254}	Methylene chloride	Pyrrolnitrin	0.48	Erlich and Pauly reagents, bioassay	This study
Silica Gel 60 F_{254}	Chloroform–ethyl acetate (1:1)	Cepacin B	0.15	Bioassay	33
Silica Gel 60 F_{254}	Chloroform–ethyl acetate (1:1)	Cepacin A	0.20	Bioassay	33
Silica Gel 60 F_{254}	Chloroform–ethyl acetate (1:1)	Pyrrolnitrin	0.57	Pauly and Erlich reagents, bioassay	This study
DC-Fertigplatten sil G-25 u.v.$_{254}$	Benzene–ethyl acetate (1:1)	Pseudanes	0.24	Ferric chloride	12
DC-Fertigplatten sil G-25 u.v.$_{254}$	Benzene–ethyl acetate (1:1)	Pyrrolnitrin	0.82	Sulfuric acid, Erlich reagent	12
Silica Gel 60 F_{254}	Benzene–ethyl acetate (1:1)	Pyrrolnitrin	0.59	Pauly and Erlich reagents, bioassay	This study
Silica Gel 60	Methylene chloride–ethyl acetate (9:1)	Unknown	0.80	Bioassay	19
Silica Gel 60 F_{254}	Methylene chloride–ethyl acetate (9:1)	Pyrrolnitrin	0.63	Pauly and Erlich reagents, bioassay	This study
Silica Gel 60	Methylene chloride–methanol (9:1)	Unknown	0.78	Bioassay	19
Silica Gel 60 F_{254}	Methylene chloride–methanol (9:1)	Pyrrolnitrin	0.66	Pauly and Erlich reagents, bioassay	This study
Silica Gel 7GF	Chloroform–ethyl acetate–formic acid (5:4:1)	Pyoluteorin	0.77	UV light	15
Silica Gel 60	Chloroform–ethyl acetate–formic acid (5:4:1)	Unknown	0.93	Bioassay	19
Silica Gel 60 F_{254}	Chloroform–ethyl acetate–formic acid (5:4:1)	Pyrrolnitrin	0.69	Pauly and Erlich reagents, bioassay	This study
Reversed-phase C18 (Whatman type KC18F)	Acetonitrile–methanol–water (1:1:1)	Aminopyrrolnitrin	0.38	Pauly reagent (maroon)	27
KC18F	Acetonitrile–methanol–water (1:1:1)	2,3-Dichloro-4-(2-amino-3-chloro-phenyl)pyrrole	0.28	Pauly reagent (orange)	27
KC18F	Acetonitrile–methanol–water (1:1:1)	2-Chloropyrrolnitrin	0.22	Pauly reagent (orange)	27
KC18F	Acetonitrile–methanol–water (1:1:1)	3-(2-amino-3-chlorophenyl) pyrrole	0.40	Pauly reagent (maroon)	27
KC18F	Acetonitrile–methanol–water (1:1:1)	Pyrrolnitrin	0.31	Pauly reagent (maroon)	27
KC18F	Acetonitrile–methanol–water (1:1:1)	Pyrrolnitrin	0.23	Pauly reagent (maroon), Erlich reagent (blue)	This study

TABLE 2. Chromatography profile for flash column purification of *P. cepacia* extract

Elution solvent	Vol of elution solvent (ml)	Column fractions	Wt of eluant (mg)	Components detected by TLC-bioautography[a]								
				R_f 0.0	R_f 0.1	R_f 0.2	R_f 0.3	R_f 0.4	R_f 0.5	R_f 0.6	R_f 0.7	R_f 0.8
Benzene-hexane (2:1)	1,250	1–65[b]	2.6									
		66–95	6.4						+			
		96–210	8.5						−			−
		211–250	4.2									−
Benzene	500	251–312	2.9				+[c]		+		−	
Benzene-acetone (1:1)	500	313–314	21.4	−+−				−−		−		
		315–317	7.9	−+−								
		318–323	3.7	−+−								
Acetone	500	324–440	20.7	−								
Methanol	500	NF[d]	76.4	−								

[a] The solvent was chloroform-acetone (9:1). +, active component; −, inactive component; −+− and −−, multiple components eluted.
[b] No compounds were detected.
[c] Detected by bioautography but not by UV light.
[d] NF, not fractionated.

but did not specifically show antibiosis versus other mechanisms of inhibition.

Bioautography. We found that after incubation, extracts of all analytical-fermentation samples of B37w produced inhibition zones on silica gel at the R_f of pyrrolnitrin. These zones were small with 24-h extracts (0.45 cm) and were the same size (1.0 cm) for all later extracts (up to 168 h). From the sizes of the TLC spots and the fungal inhibition zones, it appeared that similar amounts of pyrrolnitrin were extracted at all pHs tested (acidic, neutral, and basic).

Antibiotics other than pyrrolnitrin may be produced by *P. cepacia* B37w. During purification of pyrrolnitrin, the existence of two additional unidentified antibiotics was inferred from zones of *Fusarium* inhibition on bioautography plates containing concentrated column fractions. However, these minor compounds were not consistently detected and were not isolated for identification.

Microbial colonization and pyrrolnitrin recovery from inoculated potatoes. Some potato wounds were inoculated only with *P. cepacia* B37w rif$^+$ (treatment 1), and other wounds were inoculated with a mixture containing the bacterium and *F. sambucinum* R-6380 (treatment 2). Dilution plates prepared from treatment 1 and 2 preparations after 1 week of incubation showed that rifampin-resistant strain B37w was recovered as a pure culture from the potato wounds. The level of B37w rif$^+$ increased from 2.75×10^5 CFU inoculated per wound to 1.72×10^6 CFU reisolated per wound in treatment 1 and to 1.20×10^6 CFU reisolated per wound in treatment 2. Biolog system identification of the strain reisolated from treatment 1 preparations confirmed that *P. cepacia* was recovered from the potatoes. Dilution plates prepared from a control preparation (treatment 3) yielded apparent *F. sambucinum* colonies.

Bioautography performed with extracts obtained from all potato treatment groups (5.57 wounds per extract TLC spot) provided evidence that pyrrolnitrin was present only in the treatment 2 preparation. A small zone of fungal inhibition activity was obtained at an R_f of 0.48 (chloroform-acetone, 9:1) from the treatment 2 extract. This may indicate that a level high enough to be detected by bioautography was produced only when *P. cepacia* was challenged by a high level of fungal spores in the mixed inoculum. When various concentrations of pure pyrrolnitrin obtained for use as a reference standard were chromatographed and sprayed with R-6380, the bioautography results indicated that the lowest level of pyrrolnitrin which created a detectable inhibition zone on TLC plates was between 5 and 50 ng per spot (data not shown); this corresponds to a theoretical TLC-bioautography detection limit of 0.87 to 8.7 ng per wound for potato extracts. Thus, the inhibition zone detected by bioautographic analysis of the

TABLE 3. ^{1}H-NMR data for pyrrolnitrin

Proton(s)	Chemical shift (δ) (multiplicity, integration)	
	Study of Roitman et al.[a]	This study
H-2, H-5	6.81 (m, 2H)	6.82 (m, 2H)
H-6′	7.41 (m, 1H)[b]	
H-5′	7.43 (m, 1H)[b]	7.41–7.53 (m, 3H)
H-4′	7.52 (m, 1H)[b]	
NH	8.38 (br s, 1H)	8.29 (br s, 1H)

[a] See reference 36.
[b] Roitman et al. reproduced the complex 11-line pattern by computer simulation by using J_{ortho} = 8 Hz and J_{meta} = 2 Hz.

TABLE 4. ^{13}C-NMR data for pyrrolnitrin

Carbon	Chemical shift (δ)	
	Study of Martin et al.[a]	This study
C-3	111.7	111.9
C-4	115.3	115.4
C-5	116.7[b]	116.6[b]
C-2	117.5[b]	117.4[b]
C-3′	124.8	124.8
C-1′	127.8	127.6
C-6′	128.5	128.6
C-4′	130.2	130.1[c]
C-5′	130.2	130.3[c]
C-2′	148.3[d]	—[d]

[a] See reference 28.
[b,c] Assignments for values with the same letter may be reversed.
[d] Martin et al. found that this signal was absent in $CHCl_3$ solution.

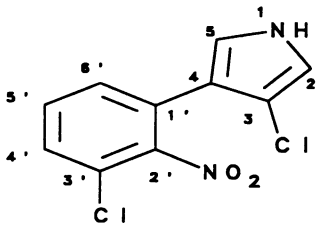

FIG. 1. Pyrrolnitrin [3-chloro-4-(2-nitro-3-chlorophenyl)-pyrrole].

treatment 2 extract indicated that at least 0.87 ng of pyrrolnitrin per wound was present.

Our HPLC analysis of potato wound extracts at a concentration of 2.0 mg/ml revealed no peaks corresponding to the retention volume of the pyrrolnitrin standard. Concentrating extracts more than this was not practical because of interference from other peaks extracted from potatoes. A control study in which we examined recovery of pyrrolnitrin from potatoes revealed that the minimum amount of pyrrolnitrin which gave a detectable peak as determined by HPLC was 60 ng of pyrrolnitrin added per wound (data not shown). The amount that could be extracted at this level after overnight incubation was 4% of the amount applied to wounds. Since pyrrolnitrin was not detected by HPLC, these data indicated that the amount present per wound in treatment 2 potato wounds must have been less than 60 ng.

Potato tuber assay to determine biological control efficacy. In biological control efficacy experiments in which tubers inoculated with the bacterial isolate and the fungal pathogen were incubated for 3 weeks, *P. cepacia* B37w frequently decreased dry rot symptom development compared with control tubers inoculated with *F. sambucinum* R-6380, R-9261, and R-9262 (Table 5). No disease symptoms were observed with wound-alone controls.

DISCUSSION

Bioautography is a useful technique which correlates antimicrobial activity with the presence of antibiotics. It provides direct detection of antifungal compounds in vitro by using paper chromatography (42) or TLC (13, 25). Homma et al. (12) poured molten agar seeded with fungal mycelium or conidia onto the surfaces of TLC plates. In our study, a fungal inoculum was applied by spraying a suspension of conidia in Sabouraud maltose broth. The use of Sabouraud maltose medium and a 12-h photoperiod both contributed to pigment formation by *F. sambucinum*, which aided in detection of inhibition zones. Preliminary bioautography screening of bacterial isolates able to protect potatoes from dry rot revealed that each of the 20 best bacterial strains, including B37w,

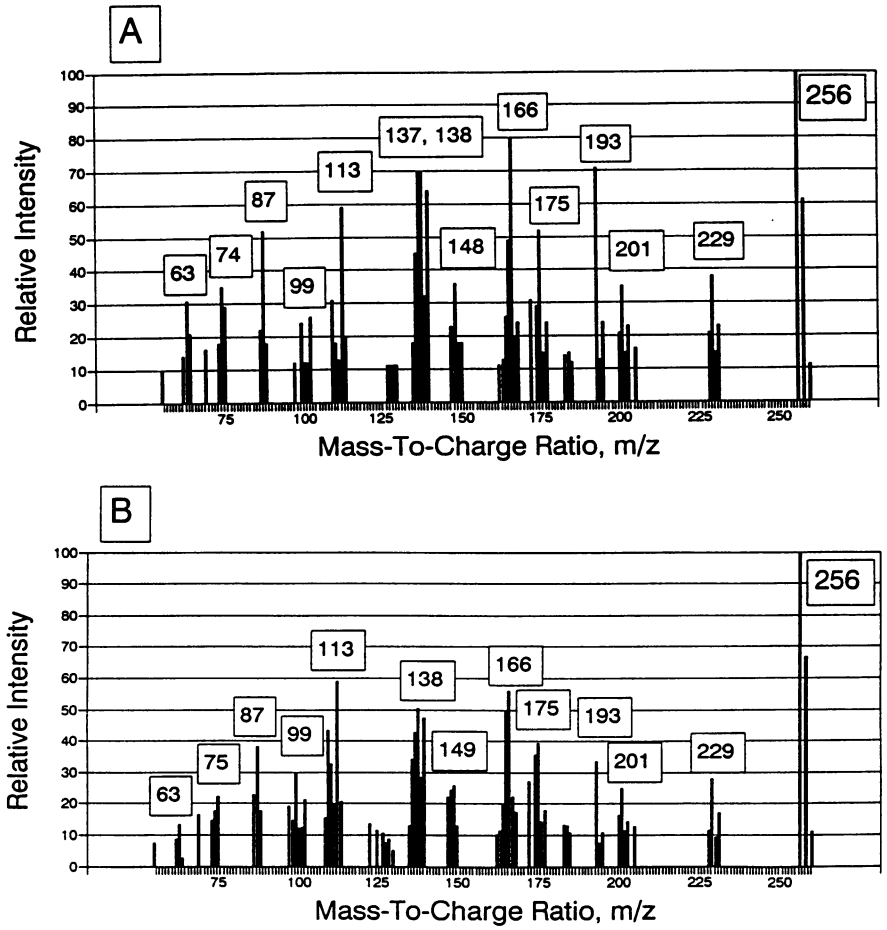

FIG. 2. Similarity of electron impact mass spectrometry data for the antibiotic which we isolated and previously published data, confirming that the compound is pyrrolnitrin. (A) Spectrum constructed from the data of Roitman et al. (36); (B) spectrum constructed from our data.

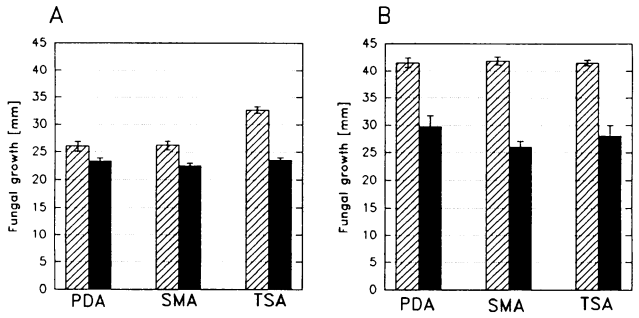

FIG. 3. Growth of *F. sambucinum* R-6380 in the absence and presence of *P. cepacia* B37w on potato dextrose agar (PDA), Sabouraud maltose agar (SMA), and tryptic soy agar (TSA). The amount of fungal growth (in millimeters) was measured from a central agar plug containing inoculum toward the outer edge of the petri plate. A B37w colony, when present, was 1 cm from the outer edge of the petri plate. Each value is the mean ± 1 standard deviation of measurements obtained from three petri plates. (A) Growth measurements after 3 days; (B) growth measurements after 7 days. Cross-hatched bars, control; solid bars, B37w present.

produced at least one antifungal compound in liquid culture (4). In this investigation, bioautography performed with analytical-scale extracts of B37w revealed that pyrrolnitrin was produced in liquid culture; the question of production in planta required demonstration of recovery from inoculated potatoes.

We knew from our colonization-recovery study that pyrrolnitrin was produced in potatoes treated with B37w. On the basis of combined evidence obtained from TLC and HPLC performed with extracts from potato treatment 2 preparations, we determined that the amount of pyrrolnitrin present was between 0.87 and 60 ng per wound. The level of pyrrolnitrin required to control fungal pathogens may be in this range or lower. For example, the in vitro MIC for pyrrolnitrin against *Sclerotinia sclerotiorum* is 0.01 μg/ml; it has been suggested (29) that strains of *P. cepacia* might be able to control the sunflower wilt caused by that fungal pathogen by producing small amounts of pyrrolnitrin in the rhizosphere in response to plant exudates.

The success of *P. cepacia* may be due, at least in part, to production of pyrrolnitrin or other metabolites that exhibit antibiotic activity. Pyrrolnitrin itself has a 30-year history of recorded antifungal activity. First isolated in 1964 (2) from a new species of pseudomonad, *Pseudomonas pyrrocinia* (16), pyrrolnitrin has since been obtained from several *Pseudomonas* species, including *Pseudomonas aureofaciens*, *Pseudomonas fluorescens*, *P. cepacia*, and others (6, 24). Originally, it was suggested that pyrrolnitrin may be useful against dermatophytes, since it exhibited fungistatic activity against *Trichophyton*, *Microsporum*, *Epidermophyton*, and *Penicillium* species. It also exhibited activity against *Aspergillus niger*, *Candida albicans*, *Bacillus subtilis*, *Proteus vulgaris*, and *Staphylococcus aureus* (30). In addition, pyrrolnitrin or the *Pseudomonas* species that produce it have since been shown to be effective against many microbial targets, including members of the genera *Agrobacterium*, *Alternaria*, *Bipolaris*, *Botrytis*, *Cercospora*, *Cochliobolus*, *Colletotrichum*, *Corynebacterium*, *Fusarium*, *Gaeumannomyces*, *Helminthosporium*, *Mucor*, *Neurospora*, *Penicillium*, *Pyricularia*, *Pythium*, *Rhizoctonia*, *Sclerotinia*, *Septoria*, *Thielaviopsis*, *Verticillium*, and *Xanthomonas* (6, 12, 14, 17, 18, 20, 24, 29, 30, 36, 41). The *Fusarium* species that have been reported to be inhibited by pyrrolnitrin or *P. cepacia* include *Fusarium culmorum*, *Fusarium graminearum*, *Fusarium moniliforme*, *Fusarium nivale*, and *Fusarium oxysporum* (12, 20, 24). This paper is the first report that we are aware of which describes activity against *F. sambucinum* by pyrrolnitrin or *P. cepacia.*

TABLE 5. Potato tuber colonization by *F. sambucinum* R-6380, R-9261, and R-9262 as influenced by *P. cepacia* B37w

Treatment	Amt of colonized tissue (mm)[a]			
	R-6380		R-9261[b]	R-9262
	Expt 1	Expt 2		
B37w + *F. sambucinum*	1[c]	14	20[d]	11[d]
F. sambucinum only (control)	12	17	39	25

[a] At 21 days after inoculation, longitudinal cuts that were perpendicular to the tuber surface were made through wounds, and the sum of the depth and width of the darkened, dry-rotted tissue was determined for each wound. A one-way analysis of variance and mean comparisons were performed by using log-transformed data. The values are means based on 4 replicates for each isolate treatment and 16 replicates for each control treatment.

[b] Strain R-9261 is resistant to thiabendazole.

[c] Significantly different ($P = 0.01$) from the control value, as determined by using Fisher's protected least significant difference.

[d] Significantly different ($P = 0.10$) from the control value, as determined by using Fisher's protected least significant difference.

In addition to pyrrolnitrin, other antibiotic metabolites are produced by various strains of *P. cepacia*. These metabolites include other phenylpyrroles (27, 29, 35, 36), the acetylenic antibacterial compounds cepacin A and cepacin B (33), antifungal pseudanes (12), the peptide antifungal compounds altericidin A, altericidin B, and altericidin C (21, 22), bacteriocins (8), pigments and siderophores (40), and unidentified metabolites (19, 24). Some workers have correlated antibiotic production with disease suppression (13) and have demonstrated that disease is suppressed by purified pyrrolnitrin and pyoluteorin (14, 15, 17). Our investigation of recovery of pyrrolnitrin from inoculated potatoes yielded bioautography evidence only for pyrrolnitrin production in planta. Other antibiotics not detected by our experiments may be present at very low levels and may also play a role. Additional unidentified antibiotics were detected in liquid cultures of B37w in a bioautography screening study of 20 potential biocontrol bacteria (4) and during chromatographic purification of crude extract (Table 2).

Whether the control of *Fusarium* growth on the surfaces of potato wounds was due to antibiotic production has still not been proven. We have evidence that pyrrolnitrin is able to inhibit *Fusarium* growth in vitro, as shown by the inhibition zones obtained in bioautography assays performed with extracts obtained from liquid cultures. The detection of pyrrolnitrin in potato wounds supports the hypothesis that antibiosis is a mechanism of biological control in B37w, but does not exclude the possibility that there are other modes of action. The results of McLoughlin et al. (29) obtained with antibiotic-negative mutants of *P. cepacia* suggest that antibiotic production is not the main mechanism of control, since these mutants protected seeds against sunflower wilt as well as the parent strains.

Another important factor that contributes to the effectiveness of *P. cepacia* is success at colonization (23, 32). As determined by our potato wound colonization assay, B37w successfully colonized potatoes. However, the competitiveness of this organism in nature may result from successful competition for nutrients or from production of minute levels of antibiotic(s).

Additional evidence that pyrrolnitrin plays a role in disease

suppression could be obtained from genetic studies. For example, by studying antibiotic-negative mutants and cloning genes involved in pyrrolnitrin synthesis, Hill et al. (10) recently correlated pyrrolnitrin synthesis by *P. fluorescens* BL915 with biological control activity against *Rhizoctonia solani*-incited disease of cotton.

Strain B37w exhibited activity against *F. sambucinum* in the petri plate assay, in the TLC-bioautography assay, and in an initial efficacy experiment in which control of potato dry rot was measured. In later biological control efficacy studies it gave inconsistent performances (Table 5). Some variability in performance is normal in such studies. However, other bacterial strains isolated from soils that suppress *Fusarium* dry rot have since been found to provide more consistent control than the control obtained with strain B37w (38). These organisms are the subjects of continuing investigation.

Although isolate B37w did not consistently effectively control potato dry rot, the general usefulness of *P. cepacia* strains for biological control in itself commands attention. Although *P. cepacia* was first described as a pathogen of onions (5, 7), in many reports on *P. cepacia* workers have since confirmed the antifungal properties of this organism. The antifungal activities of various strains of *P. cepacia* have been demonstrated in petri plate assays (12, 24, 40). *P. cepacia* has also been shown to protect against or decrease the severity of various crop diseases, including onion seedling damping-off caused by *Fusarium oxysporum* f. sp. *cepae* in natural organic soils and petri plates (20), southern maize leaf blight caused by *Bipolaris maydis* in the greenhouse (39, 41), black spot of pear caused by *Alternaria kikuchiana* (21, 22), peanut leaf spot caused by *Cercospora arachidicola* in the field as determined by large-scale tests (23, 41), *Pythium aphanidermatum* damping-off of cucumber when the organism was applied as a seed treatment (26), *Rhizoctonia solani* damping-off of radish, *Fusarium* wilt of tomato and *Verticillium* wilt of eggplant when seed coating or root dip application of bacterial cells was used (13), pea seed preemergence damping-off caused by *Pythium ultimum* and *Pythium sylvaticum* in growth chambers (32), postharvest infections of apples and pears by blue mold (*Penicillium expansum*) and gray mold (*Botrytis cinerea*) (18), and sunflower wilt caused by *Sclerotinia sclerotiorum* in the field (29).

The list given above is not comprehensive, but it highlights the remarkable variety of useful antifungal activities attributed to strains of *P. cepacia*. *Verticillium*, *Sclerotinia*, *Rhizoctonia*, and *Pythium* species are soilborne pathogens which cause disease in potatoes (34). This suggests that the usefulness of *P. cepacia* as a biological control agent for potatoes may extend beyond postharvest protection against *F. sambucinum* dry rot. At a time when synthetic pesticide use is being curtailed (9), biological control agents such as *P. cepacia* and other organisms which can effectively inhibit target organisms by competition or by natural low-level antibiotic production are increasingly commercially attractive.

ACKNOWLEDGMENTS

We thank Holger O. Melzl for excellent technical assistance; Patricia M. Soncasie for preparing the rifampin-resistant strain of *P. cepacia*; Ronald D. Plattner for performing the mass spectral analysis; David P. Labeda for providing the fatty acid profile analysis; Anne E. Desjardins for providing *F. sambucinum* R-6380, R-9261, and R-9262; Noreen E. Mahoney for providing the pyrrolnitrin standard; and Nancy J. Alexander and Gary Armour for critically reviewing the manuscript.

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