Isolation and Identification of Antifungal Metabolites Produced by Rice-Associated Antagonistic *Pseudomonas* spp.

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ABSTRACT

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Antifungal secondary metabolites produced by six strains of rice-associated *Pseudomonas* spp. were determined. Five different media, pigment production medium (PPM), 1/5M523, potato-dextrose agar (PDA), yeast malt (YM), and King's medium B (KMB), were used to determine the best medium for plate assays of these strains against *Rhizoctonia solani* AG1, the rice sheath blight pathogen. Large inhibition zones were obtained on PPM, KMB, and YM using two strains of *P. cepacia*, In-b-6854 and In-b-6858, originating from the rice seed. The other four strains showed varying degrees of inhibition zones in the media tested. Their antifungal metabolites were isolated and compared with metabolites from reference strains and known compounds by regular and reversed-phase thin-layer chromatography. Pyrrolnitrin was present in the cell extracts from the two *P. cepacia* strains. Phenazine-1-carboxylic acid and pyocyanine were produced by two strains of *P. aeruginosa*, In-b-109 and In-b-784. A strain of *P. putida*, In-b-1821, produced 2,4-diacetylphloroglucinol. Several unidentified compounds were produced by a strain of *P. fluorescens*, In-b-7-14. Ten microliters of eluted spots from In-b-6854 and In-b-6858 inhibited the mycelial growth of *R. solani* 20 to 24 mm away.

Bacteria found in rice fields produced fluorescent and nonfluorescent pigments on King's medium B (KMB) and showed antagonism to *Rhizoctonia solani* Kühn AG1, which causes rice sheath blight (20). These bacteria inhibited the mycelial growth of the pathogen, affected sclerotial viability, suppressed the disease, and protected the plant from infection. Their potential role in the reduction of the bakanae disease of rice caused by *Fusarium moniliforme* J. Sheld. was also demonstrated (21).

Several strains of these bacteria were identified by phenotypic tests using the Analytical Profile Index system, morphological and biochemical features, and by comparison of electrophoretic patterns after sodium dodecyl sulfate polyacrylamide gel electrophoresis. These strains were identified as *Bacillus subtilis, B. laterosporus, B. pumilus, Pseudomonas aeruginosa, Pseudomonas* belonging to Section 1, *Erwinia herbicola*-like, and *Serratia marcescens* (22). Additional strains were identified as *P. putida* and *P. cepacia*, which are seed saprophytes. From the total collection, we selected six strains of *Pseudomonas*: *P. cepacia* (In-b-6854 and In-b-6858), *P. putida* (In-b-1821), *P. aeruginosa* (In-b-784 and In-b-109), and *P. fluorescens* (In-b-7-14). We describe in this paper the isolation and characterization of metabolites responsible for antifungal activity, and we determined the optimal media suitable for quantifying inhibition of *R. solani* AG1-1a, the sheath blight pathogen.

MATERIALS AND METHODS

Strains and sources of bacteria. Six strains of rice-associated *Pseudomonas* spp. were used in this study. The identification and spectrum of antifungal activity are shown in Table 1.

Reference strains which are producers of known metabolites were obtained from the culture collection of the Biological Control Research Unit, USDA-ARS, Pullman, WA. Standard chemicals such as pyrrolnitrin and pyoluteorin were provided by J. Roitman through Jennifer Kraus of USDA-ARS, Corvallis, OR. The reference strains and metabolites used are shown in Table 2. Cultures were grown on KMB, transferred into glycerol and maintained at –20°C as stock cultures.

Selection of agar media for antagonism tests against *R. solani*. Five different media were tested. These were: pigment pro-

TABLE 1. Strains and sources of antagonistic bacteria

Strains	Identification	Source	Rice pathogen inhibited
In-b-6854	*Pseudomonas cepacia*	rice seeds	*Rhizoctonia solani* *Fusarium moniliforme* *Sarocladium oryzae* *Pyricularia oryzae*
In-b-6858	*Pseudomonas cepacia*	rice seeds	*R. solani* *F. moniliforme* *S. oryzae* *P. oryzae*
In-b-7-14	*Pseudomonas fluorescens*	*[a]	*R. solani* *F. moniliforme* *P. oryzae*
In-b-1821	*Pseudomonas putida*	rice plants	*F. moniliforme* *R. solani* *S. oryzae* *Gaumannomyces graminis* *S. oryzae* *H. oryzae*
In-b-784	*Pseudomonas aeruginosa*	rice plants	*R. solani* *P. oryzae* *F. moniliforme* *S. oryzae* *G. graminis*
In-b-109	*Pseudomonas aeruginosa*	soil	*R. solani* *G. graminis*

[a] * = Strain was isolated by Dr. S. S. Gnanamanickam, former visiting scientist at the International Rice Research Institute.

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duction media (PPM)—20 g of Difco peptone (Difco Laboratories, Detroit, MI), 20 g of glycerol, 5 g of NaCl, 1 g of KNO_3, 15 g of agar, 7.2 pH, 1,000 ml of distilled water; yeast malt (YM)—3 g of yeast extract, 3 g of malt extract, 5 g of Bacto peptone (Difco), 10 g of glucose, 15 g of agar, 1,000 ml of water; 1/5M523—2 g of sucrose, 1.6 g of casein hydrolysate, 0.8 g of yeast extract, 0.4 g of K_2HPO_4, 0.06 g of $MgSO_4.7H_2O$, 10 g of glucose, 18 g of agar, 200 ml of 0.1 M sodium phosphate (pH 6.5), 800 ml of H_2O, sodium phosphate solution was autoclaved separately and mixed before pouring; KMB—20 g of proteose peptone, 1.908 g of $K_2HPO_4.3H_2O$, 1.5 g of $MgSO_4.7H_2O$, 15 g of Bacto agar (Difco), 15 ml of glycerol, 985 ml of distilled water; and potato-dextrose agar (PDA)—200 g of potato, 20 g of dextrose, 18 g of agar, 1,000 ml of H_2O. *R. solani* AG1, the rice sheath blight pathogen, was grown on PDA for 5 days. Dual culture tests using the media were done as previously described (20).

Extraction and isolation of phenazine, pyocyanine, and oxychlororaphine. Inoculum was prepared from cells harvested from 48-h-old cultures of two strains of *P. aeruginosa* grown in KMB broth at 30°C on a rotary shaker. The cells were collected by centrifugation at 3,500 rpm (5,500 × *g*) for 7 min. The pellet was suspended in 5 ml of PPM broth and then incubated on a rotary shaker at 30°C for 4 days.

The pigments pyocyanine, phenazine-1-carboxylic acid (PCA), and oxychlororaphine were separated by modifications of the method described by Chang and Blackwood (5). The three pigments were separated into their respective fractions after first extraction by acidifying the cultures with an equal volume of benzene (phenazine and oxychlororaphine in the benzene layer) and then extraction of the benzene phase with 5% $NaHCO_3$. PCA was recovered from the bicarbonate layer, while oxychlororaphine remained in the benzene layer. The bicarbonate fraction was extracted once with benzene to recover the phenazine. Pyocyanine which remained in the aqueous fraction was extracted with an equal volume of chloroform. The pigments were air dried, dissolved in methanol, and purified by thin-layer chromatography (TLC) on silica gel with a 250-µm layer thickness. The solvents used were: chloroform/acetone (9:1), toluene/acetone (4:1), isopropanol/ammonia/water (8:1:1), and ethyl acetate/chloroform (9:1). Plates were viewed under UV light at 254 nm and spots were traced on clear plastic. The plates were then sprayed with diazotized sulfanilic acid (DSA). R_f values of the spots were compared with metabolites extracted from reference strains and phenazine (PCA derivative).

Extraction of 2,4-diacetylphloroglucinol. A strain of *P. putida*, In-b-1821, was grown in 5 ml of PPM broth for 4 days on a rotary shaker at 30°C. The fermentation broth was centrifuged at 3,500 rpm (5,500 × *g*) for 5 min in a tabletop centrifuge and the supernatant was collected. It was acidified to pH 2 with 1 N HCl and was then extracted with an equal volume of ethyl acetate. The ethyl acetate extracts were reduced to dryness in vacuo. The residue was dissolved in methanol. Ten-microliter samples were chromatographed on reversed-phase C18 glass TLC plates (Whatman, Inc., Clifton, NJ), developed in acetonitrile/methanol/water (1:1:1), visualized by short wavelength (254 nm), and sprayed with DSA.

Extraction of pyoluteorin and pyrrolnitrin. Pyoluteorin and pyrrolnitrin were extracted from bacterial cells of In-b-6854 and In-b-6858 grown in PPM broth, PPM agar, and spent agar media of cultures by a modification of published procedures (8) and as suggested by Jennifer Kraus (*personal communication*). Bacteria were spread on the surface of PPM medium contained in a petri plate and incubated at 28°C for 5 days. Cells on the agar surface of each plate were suspended in sterile deionized water and collected by centrifugation. Antibiotics were extracted from pelleted cells with 6 ml of 80% acetone. The spent agar was diced and extracted with an equal volume of acetone. The acetone was removed under vacuum and the residual aqueous phase was extracted with 20 ml of ethyl acetate. The acetate phase was dried under vacuum (Speed Vac Savant, Farmingdale, NY) and the residue was dissolved in 50 µl of methanol. Extraction of antibiotics from cells grown in liquid cultures was also done as described by Kraus and Loper (18). Cells were pelleted by centrifugation and the supernatant was discarded. Cells were collected and then transferred to microcentrifuge tubes, vortexed for 30 s with 500 µl of ethyl acetate, and centrifuged. The ethyl acetate phase was collected and dried under vacuum (Speed Vac Savant). The residue was dissolved in 10 µl of methanol and spotted on silica gel reversed-phase C18 glass TLC plates, and then developed with acetonitrile/methanol/water (1:1:1). Spots were visualized by short wavelength UV light and sprayed with DSA.

Assay of metabolites produced by *P. cepacia* strains In-b-6854 and In-b-6858 against *R. solani*. Developed TLC plates were viewed under UV light and then absorbing spots were traced on clear plastic. Activity was eluted from the silica scraped at the corresponding active spots from the developed plates with 100 µl of methanol, and then centrifuged to separate the silica from the eluant. The eluant was assayed against *R. solani* by the dual culture test as previously described. Ten microliters of methanol was used as check. Inhibition zone was measured 48 h after incubation of plates at 28°C.

TABLE 2. Reference strains and antibiotic and metabolite

Strains	Antibiotic	Metabolite
Pseudomonas fluorescens	2-79	phenazine
	Q2-87	phloroglucinol
	Pf-5	pyrrolnitrin
		pyoluteorin
		phloroglucinol
	CHAO	pyrrolnitrin
		pyoluteorin
		phloroglucinol
	30-84	phenazine
Pseudomonas chlororaphis	944b	chlororaphine
	17411	chlororaphine
	17809	chlororaphine
Pseudomonas aeruginosa	PAIO Holloway	pyocyanine

TABLE 3. Inhibition of *Rhizoctonia solani* by six strains of antagonistic bacteria on different media

	Inhibition zone (mm)[a]				
Strains	Potato-dextrose agar	Pigment production medium	1/5M523	Yeast malt	King's medium B
In-b-7-14	22.3 ± 1.25	*[b]	18.0 ± 2.94	*	25.6 ± 1.88
In-b-1821	15.3 ± 2.35	*	0	12.0 ± 0	0
In-b-109	0	12.6 ± 0.94	0	0	0
In-b-784	0	13.0 ± 1.41	0	0	0
In-b-6858	8 ± 0	21.0 ± 2.94	14.6 ± 2.04	17.6 ± 2.05	17.6 ± 2.05
In-b-6854	8 ± 0	21.3 ± 2.35	16.0 ± 2.16	18.3 ± 2.35	16.0 ± 2.16
2-79	0	12.0 ± 0	0	0	13.5 ± 1.5
CHAO	8 ± 0	25.6 ± 0.94	14.3 ± 0.47	0	19.0 ± 1

[a] Means of three replications.

[b] * = No inhibition due to the presence of contaminants.

TABLE 4. Thin-layer chromatographic characteristics of antifungal metabolites from two strains of *P. aeruginosa*

Strains (solvent)	R_f values	Description
In-b-109 (chloroform)	0.53	blue at room temperature, moss green after diazotized sulfanilic acid spray
In-b-784 (chloroform)	0.53	same
P. aeruginosa (chloroform)	0.53	same
In-b-109 (benzene)	0.50	absorb UV
In-b-784 (benzene)	0.50	absorb UV
2-79 (benzene)	0.50	absorb UV
P. aeruginosa (benzene)	0.52	absorb UV
Phenazine	0.51	absorb UV

RESULTS

Selection of agar media for antagonism tests against *R. solani*. Examination of the plates containing five different media inoculated with six strains of *Pseudomonas* and *R. solani* showed that the bacterial colonies were clearly inhibitory to the fungus. Wide zones devoid of mycelial growth were observed around bacterial colonies, while areas without colonies were overgrown. The two strains of *P. cepacia* had large inhibition zones on PPM, KMB, and YM, but not on 1/5M523 and PDA. *P. aeruginosa* had a large inhibition zone on PPM, whereas *P. putida* had large inhibition zones on PDA and YM (Table 3).

Production of phenazines and pyocyanine. The pigments were produced by two strains of *P. aeruginosa*, In-b-109 and In-b784, and isolated according to the scheme previously described. The chromatographic properties of these metabolites were compared with those of known compounds and with those from reference strains. Data are summarized in Table 4. Both the reference and isolated compounds behaved similarly when compared by TLC. It also was evident that *P. aeruginosa* produced diffusible greenish pigment on PPM. Among the four solvent systems used, isopropanol/ammonia/water (8:1:1) was the most effective in separating the active spots from the extracts. In this solvent system, extracted phenazine from strains In-b-109, In-b-784, and *P. fluorescens* 2-79 absorbed UV and appeared as bright yellow spots at $R_f = 0.50$, whereas the synthetic phenazine has a position $R_f = 0.51$. Pyocyanine was detected on plates spotted with chloroform extracts at an R_f value of 0.53. The spot appeared blue at room temperature and turned moss green after DSA spray. At least two active metabolites were produced by *P. aeruginosa*: pyocyanine and PCA.

Production and isolation of 2,4-diacetylphloroglucinol (Phl). *P. putida* strain In-b-1821 grown on PPM produced a metabolite which could be identified in ethyl acetate extracts as Phl by reversed-phase TLC. Reversed-phase TLC plates developed in acetonitrile/ammonia/water (1:1:1) worked well in separating the metabolite from the extract. Distinct spots at R_f of 0.88 that appeared yellow to orange immediately after DSA spray were observed on developed TLC plates.

Production and isolation of pyrrolnitrin from two strains of *P. cepacia*. Cell extracts of two strains of *P. cepacia*, In-b-6854 and In-b-6858, yielded an active metabolite on reversed-phase TLC plates. The chromatographic characteristics were similar to those of pyrrolnitrin. When thin-layer chromatograms of these metabolites were sprayed with DSA, pink spots were produced at the corresponding R_f value of 0.28 for In-b-6854 (Fig. 1) and 0.23 to 0.28 for various extracts of In-b-6858 (Fig. 2). Examination of the chromatograms made from cells grown on agar and in liquid broth showed that cells grown on agar plates yielded more pyrrolnitrin than those grown in liquid cultures. This was evident by darker and bigger spots compared with other extracts, using 10 µl as the initial concentration.

Assay of metabolites produced by *P. cepacia* strains In-b-6854 and In-b-6858 against *R. solani*. Bioassay of the compound eluted from these spots against *R. solani* showed that 10 µl of the eluted spot inhibited the mycelial growth of *R. solani* by 20 to 24 mm.

Production of unidentified compounds by *P. fluorescens* In-b-7-14. Chromatograms of extracts from isolate In-b-7-14 yielded several unidentified active compounds (Fig. 3). Further extraction and purification of these compounds are necessary to identify the metabolite responsible for antifungal activity.

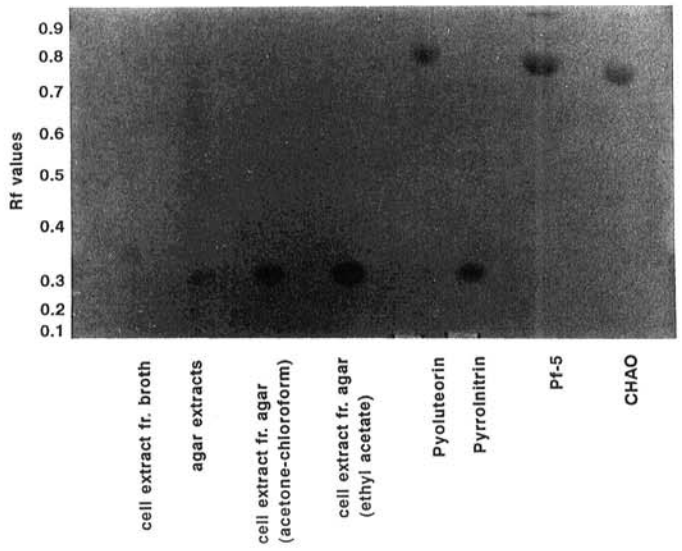

Fig. 1. R_f value of extracts of *Pseudomonas cepacia* strain In-b-6854 detected by reversed-phase thin-layer chromatography.

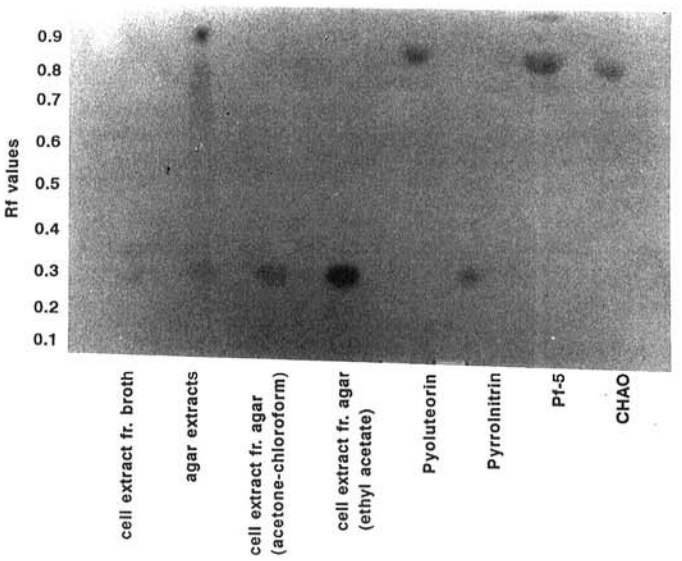

Fig. 2. R_f value of extracts of *Pseudomonas cepacia* strain In-b-6858 detected by reversed-phase thin-layer chromatography.

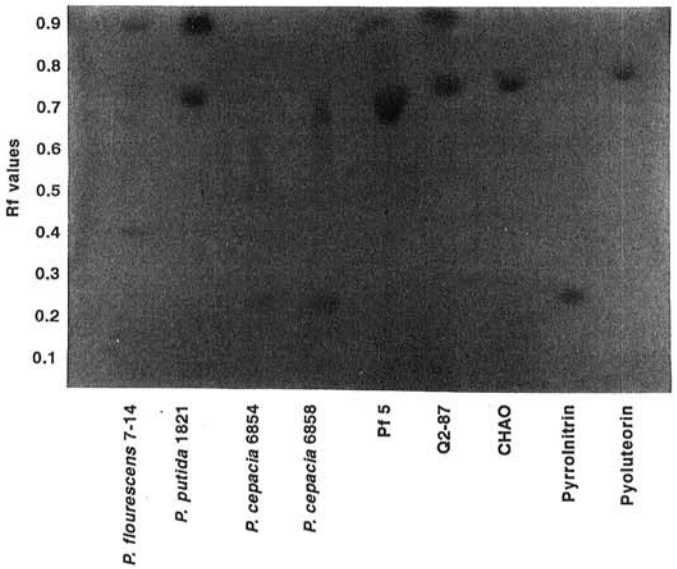

Fig. 3. R_f value of extracts of four strains of *Pseudomonas* spp. detected by reversed-phase thin-layer chromatography.

DISCUSSION

The optimal media for quantifying inhibition of *R. solani* by six strains of antagonistic bacteria were determined. Strains of *P. cepacia* had large inhibition zones on PPM, KMB, and YM, while *P. aeruginosa* had large zones on PPM, and *P. putida* on PDA and YM. The components present in PPM, KMB, and YM enhanced the antagonistic activity of *P. cepacia*. Peptone is present in these three media. These results fit with the results obtained by James and Gutterson (9), which shows that the constituents of the medium exerted a control over the yield of antibiotics produced by *P. fluorescens*. Kanner et al. (13) also reported that in addition to temperature and aeration, the added carbon source has a marked influence on pigment production of *P. aeruginosa*.

The metabolites responsible for the antifungal activity of six strains of rice-associated *Pseudomonas* spp. were isolated and identified, using standard chromatographic methods. Two strains of *P. aeruginosa* (In-b-784 and In-b-109) produced PCA and pyocyanine. This finding corroborates that of Chang and Blackwood (5) who indicate that certain strains produce more than one pigment. *P. aeruginosa* also has been reported to produce, besides pyocyanine, other phenazines such as dihydroxy-phenazine-1-carboxylic acid, PCA, chlororaphine, oxychlororaphine, and aeruginosin B.

P. putida strain In-b-1821 produced Phl. This metabolite could be responsible for the inhibition of several fungal pathogens. A number of *Pseudomonas* strains have been shown to produce phloroglucinol (4,7,15). This compound is not only toxic to fungi and bacteria, but also exhibits herbicidal activity resembling that of 2,4-dichlorophenoxyacetate (2,4-D) (14), and has anthelmintic (3) and antiviral properties (26). It has been shown that the addition of 40 µg of synthetic Phl per gram of artificial soil drastically reduces the severity of tobacco black root rot (16). Interestingly, Tomas-Lorente et al. (27) found that certain plants produce antibiotic phloroglucinol, possibly as a defense mechanism against fungi.

Pyrrolnitrin (3-chloro-4-(2′-nitro-3′-chlorophenyl)pyrrole), a potent antifungal compound, was isolated from two strains of *P. cepacia* originally isolated from rice seeds. *P. cepacia* is one of the most nutritionally versatile bacteria (2), a factor which makes it very attractive for biocontrol use. The bacterium has been reported to reduce southern maize leaf blight (23), and decrease Cercospora leaf spot of peanut (24,25) and Alternaria leaf spot of tobacco in the field. *P. cepacia* was reported to be an important member of the rice rhizoplane, because it converts nitrate to nitrite (1) and enhances nodulation of roots of *Alnus rubra* by *Frankia* sp. (17). This bacterium also was shown to be antagonistic toward several prominent fruit spoilage pathogens (10,11,12). Since *P. cepacia* strains are natural inhabitants of rice seeds, their potential as a biocontrol agent through seed bacterization could have an impact on rice seedborne disease management.

Elander et al. (6) surveyed 29 *Pseudomonas* strains classified as *P. chlororaphis*, *P. aureofaciens*, and *P. cepacia* for the production of pyrrolnitrin. Four strains of *P. cepacia* and four strains belonging to the two fluorescent species excreted pyrrolnitrin into the medium. Contrary to this, Mahoney and Roitman (19) reported that 98% of the phenylpyrroles are contained in the cell extracts, and the broth contains only 1% of the pyrroles produced by the cells during fermentation. Our results are in agreement with these findings, wherein we observed that larger and darker spots were obtained from extracts of cells grown on agar. This was a good indication that a sufficient dosage of bacterial cells are needed to enhance the biocontrol efficiency of such antagonists. According to Howell and Stipanovic (8), pyrrolnitrin persists actively in soil for at least 30 days. It is not readily diffusible and is only released upon cell lysis. Protection against *R. solani* was due to the slow release of antibiotics as cells died.

Thus, our study revealed that at least several active antifungal compounds are produced by rice-associated *Pseudomonas*. As some of these bacterial strains, the *P. cepacia* strains, were isolated from seeds, and others from tillers of rice plants, there was indication that the distribution of these bacteria varied at different plant growth stages (T. W. Mew, *unpublished*). The present study may suggest a deployment strategy of the biological control agent based on the antibiotics they produced. Currently, we are conducting field testing of these strains in various mixture combinations and at different growth stages. The role of these metabolites in disease suppression needs to be investigated further.

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