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Frequency of Antibiotic-Producing *Pseudomonas* spp. in Natural Environments

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The antibiotics phenazine-1-carboxylic acid (PCA) and 2,4-diacetylphloroglucinol (Phl) are major determinants of biological control of soilborne plant pathogens by various strains of fluorescent *Pseudomonas* spp. In this study, we described primers and probes that enable specific and efficient detection of a wide variety of fluorescent *Pseudomonas* strains that produce various phenazine antibiotics or Phl. PCR analysis and Southern hybridization demonstrated that specific genes within the biosynthetic loci for Phl and PCA are conserved among various *Pseudomonas* strains of worldwide origin. The frequency of Phl- and PCA-producing fluorescent pseudomonads was determined on roots of wheat grown in three soils suppressive to take-all disease of wheat and four soils conducive to take-all by colony hybridization followed by PCR. Phenazine-producing strains were not detected on roots from any of the soils. However, Phl-producing fluorescent pseudomonads were isolated from all three take-all-suppressive soils at densities ranging from approximately 5×10^5 to 2×10^6 CFU per g of root. In the complementary conducive soils, Phl-producing pseudomonads were not detected or were detected at densities at least 40-fold lower than those in the suppressive soils. We speculate that fluorescent *Pseudomonas* spp. that produce Phl play an important role in the natural suppressiveness of these soils to take-all disease of wheat.

Over the past two decades, numerous strains of antibiotic-producing fluorescent *Pseudomonas* spp. have been isolated from plants grown in soils from diverse geographical regions (7, 8, 15, 24). Antibiotic producers were readily isolated from soils that are naturally suppressive to diseases such as take-all of wheat (39), black root rot of tobacco (15), or fusarium wilt of tomato (31), indicating that they may play an important role in the natural biological control that occurs in these soils. The antibiotics pyoluteorin (Plt), pyrrolnitrin (Prn), phenazine-1-carboxylic acid (PCA), and 2,4-diacetylphloroglucinol (Phl) are currently a major focus of research in biological control. The biosynthetic loci for Plt, Prn, PCA, and Phl have been cloned, and all but Plt have been fully sequenced (1, 3, 11, 18, 23). Results obtained by both the application of molecular techniques and direct isolation have demonstrated unequivocally that these antibiotics are produced in the spermosphere and rhizosphere and play a major role in the suppression of soilborne plant pathogens (9, 18, 20–22, 27, 32, 35, 36, 40). For example, suppression of take-all of wheat, caused by *Gaeumannomyces graminis* var. *tritici*, was correlated, respectively, with the production in situ of PCA by *Pseudomonas fluorescens* 2-79 (33) and with the production of Phl by *P. fluorescens* CHAO (14).

Despite the importance of antibiosis in biological control, little is known about the frequency and ecology of naturally occurring antibiotic-producing fluorescent *Pseudomonas* spp. The availability of cloned and sequenced antibiotic-biosynthetic genes has facilitated the development of specific primers and probes that can be used to detect naturally occurring antibiotic-producing *Pseudomonas* spp. Specific primers and probes can also expedite the search for antibiotic-producing strains that are better adapted to local soil conditions and more effective in specific crop-pathogen systems.

The objective of this study was to develop specific primers and probes for detection and enumeration of naturally occurring fluorescent *Pseudomonas* spp. that produce Phl or PCA. Primers were developed from sequences within the biosynthetic loci for Phl and PCA, and their specificities were tested with Phl- and PCA-producing strains of worldwide origin. The frequency of Phl- and PCA-producing fluorescent pseudomonads was determined on roots of wheat grown in natural soils by colony hybridization followed by PCR.

MATERIALS AND METHODS

Bacterial strains and growth media. Characteristics of the fluorescent *Pseudomonas* strains used in this study are summarized in Tables 1 and 2. Strains Q2-87R and 2-79RN$_{10}$ are spontaneous rifampin-resistant derivatives of *P. fluorescens* Q2-87 and 2-79, respectively. All strains were cultured on King's medium B (KMB) agar (16) at 25°C. Fluorescent *Pseudomonas* spp. were isolated from wheat roots on KMB agar supplemented with cycloheximide (100 μg ml^{-1}), chloramphenicol (13 μg ml^{-1}), and ampicillin (40 μg ml^{-1}) (KMB$^+$) (29).

DNA extraction and cell lysis. Total DNA was isolated from bacterial strains and digested with restriction enzymes by standard protocols (25). Heat-lysed bacterial suspensions used in PCR analysis were prepared from cultures grown on KMB for 48 h at 25°C. Two bacterial colonies (2-mm diameter) were suspended in 100 μl of lysis solution (0.05 M NaOH, 0.25% sodium dodecyl sulfate [SDS]) and incubated for 15 min at 100°C. The suspension was centrifuged for 1 min at 12,000 rpm and diluted 50-fold in sterile distilled water. Five microliters of the diluted suspension was used in each reaction.

Primers and PCR analysis. The oligonucleotide primers listed in Table 3 were developed from sequences within the biosynthetic loci for Phl of *P. fluorescens* Q2-87 (GenBank accession no. U41818) and PCA of *P. fluorescens* 2-79 (GenBank accession no. L48616). Primers were synthesized by Operon Technology Inc. (Alameda, Calif.). Primers Phl2a and Phl2b were developed from sequences within *phlD*, which predicts a protein of 349 amino acids that is homologous to chalcone synthase from plants (2). Primers PCA2a and PCA3b were developed from sequences within *phzC* and *phzD*, respectively. *phzC* includes 400 amino acids with similarity to 2-keto-3-deoxyarabinoheptulosonate-7-phosphate (DAHP) synthase from *Escherichia coli* and plants; *phzD* includes 207 amino acids with similarity to isochorismatase from *E. coli* (3).

PCR amplification was carried out in a 25-μl reaction mixture which contained either approximately 20 ng of total DNA or 5 μl of a diluted heat-lysed cell suspension, 1× GeneAmp PCR buffer (Perkin-Elmer Corp., Norwalk, Conn.), 200 μM each dATP, dTTP, dGTP, and dCTP (Perkin-Elmer), 20 pmol of each

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TABLE 1. Characteristics of *Pseudomonas* strains used to test the specificity of Phl primers

Strain	Phl production[a]	Target organism[b]	Origin	Source or reference
P. fluorescens				
Q2-87	+	*G. graminis* var. *tritici* on wheat	Wheat, Washington	36
Q4-87	+	*G. graminis* var. *tritici* on wheat	Wheat, Washington	15
Q13-87	+	*G. graminis* var. *tritici* on wheat	Wheat, Washington	15
Q55-87	+	*G. graminis* var. *tritici* on wheat	Wheat, Washington	15
Q69c-80	−	*G. graminis* var. *tritici* on wheat	Wheat, Washington	12
Q65c-80	+	*G. graminis* var. *tritici* on wheat	Wheat, Washington	12
M4-80R	−	*G. graminis* var. *tritici* on wheat	Wheat, Washington	10
CHAO	+	*G. graminis* var. *tritici* on wheat, *T. basicola* on tobacco, *P. ultimum* on cucumber	Tobacco, Switzerland	14
Pf1	+	*T. basicola* on tobacco, *P. ultimum* on cucumber	Tobacco, Switzerland	15
F113	+	*P. ultimum* on sugar beet	Sugar beet, Ireland	27
PFM2	+	*S. tritici* on wheat	Wheat, Oklahoma	19
Pf5	+	*P. ultimum* and *R. solani* on cotton	Cotton, Texas	13
Pseudomonas sp.				
PINR2	+	*P. ultimum* on cucumber, *F. oxysporum* f. sp. *radicis-lysopersici* on tomato	Tobacco, Italy	15
PILH1	+	*P. ultimum* on cucumber, *F. oxysporum* f. sp. *radicis-lysopersici* on tomato	Tomato, Italy	15
PGNR1	+	*P. ultimum* on cucumber, *F. oxysporum* f. sp. *radicis-lysopersici* on tomato	Tobacco, Ghana	15
PGNL1	+	*P. ultimum* on cucumber, *F. oxysporum* f. sp. *radicis-lysopersici* on tomato	Tobacco, Ghana	15

[a] +, production of Phl; −, no Phl production.
[b] Biocontrol activity was shown against *G. graminis* var. *tritici*, *Thielaviopsis basicola*, *Pythium ultimum*, *Septoria tritici*, *Rhizoctonia solani*, and *Fusarium oxysporum* f. sp. *radicis-lycopersici*. For many strains, data on biocontrol activity came from unpublished experiments.

primer, and 2.0 U of Ampli*Taq* DNA polymerase (Perkin-Elmer). Each mixture was covered with 1 drop of mineral oil. Amplifications were performed with a Perkin-Elmer thermal cycler 480. The PCR program consisted of an initial denaturation at 94°C for 2 min followed by 30 cycles of 94°C for 60 s, 67°C for 45 s, and 72°C for 60 s. Samples (9 μl) of the PCR products were separated on a 1.2% agarose gel in 1× TBE buffer (90 mM Tris-borate, 2 mM EDTA [pH 8.3]) at 75 V for 3 h. The gel was stained with ethidium bromide for 30 min, and the PCR products were visualized with a UV transilluminator.

Southern and colony hybridization. Transfer of DNA from agarose gels or bacterial colonies to Hybond-N^+ nylon membranes (Amersham) was performed by standard methods (25). After air drying, the membranes were baked for 1 h at 80°C in a vacuum oven. To remove bacterial cell debris from colony blots, membranes were washed for 1.5 h at 42°C in a solution containing 2× SSPE (2× SSPE is 20 mM NaH_2PO_4 [pH 7.4], 0.36 M NaCl, 2 mM EDTA), 0.1% SDS, and pronase (100 μg ml^{-1}) and washed again for 1 h at 56°C in 2× SSPE–0.1% SDS. Hybridizations were performed by standard methods (25). High-stringency conditions consisted of prehybridization for 1.5 h at 65°C, hybridization for 12 h at 65°C, membrane washing twice each for 5 min with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS at room temperature, and membrane washing twice each for 30 min with 0.1× SSC–0.1% SDS at 65°C. Low-stringency conditions consisted of prehybridization and hybridization at 55°C and two membrane washing with 0.5× SSC at 55°C.

TABLE 2. Characteristics of *Pseudomonas* strains used to test specificity of phenazine primers

Pseudomonas strain	Phenazine produced[a]	Target organism[b]	Origin	Source or reference
P. fluorescens				
2-79RN_{10}	PCA	*G. graminis* var. *tritici* on wheat	Wheat, Washington	37
Biotype A	—	U[c]	Water	ATTC 17552
P. aureofaciens				
30-84	PCA, 2OH-PCA, 2OH-Phz	*G. graminis* var. *tritici* on wheat	Wheat, Kansas	22
PGS12	PCA, 2OH-PCA, 2OH-Phz	*F. oxysporum* on celery	Corn, Belgium	9
AP9	PCA, 2OH-PCA	U	Barley, Washington	D. M. Weller
TAMOak81	PCA, 2OH-PCA	U	Oak, Texas	C. Gonzales
13985	PCA, 2OH-PCA	U	River clay, Holland	ATTC
Pseudomonas spp.				
BS1391	PCA, 2OH-PCA	U	Russia	V. Kotchetkov
BS1393	PCA, 2OH-PCA	U	Russia	V. Kotchetkov
P. putida B10	—	*G. graminis* var. *tritici* on wheat	Potato, California	17
P. chlororaphis				
9446	Chlororaphin	U	Plate contaminant	ATTC
17411	Chlororaphin	U	U	ATTC
P. aeruginosa				
PAO1	Pyocyanin	U	Human	B. Holloway
In-b-109	PCA, pyocyanin	*R. solani* and *G. graminis* on rice	Rice, The Philippines	24
In-b-784	PCA, pyocyanin	*R. solani*, *P. oryzae*, *F. moniliforme*, *S. oryzae*, and *G. graminis* on rice	Rice, The Philippines	24
25007	Pyorubin, pyocyanin	U	Human	ATTC
25011	Pyorubin	U	Human	ATTC

[a] —, no production; 2OH-PCA, 2-hydroxy-phenazine-1-carboxylic acid; 2OH-Phz, 2-hydroxyphenazine.
[b] Activity was shown against *G. graminis* var. *tritici*, *Fusarium oxysporum*, *Rhizoctonia solani*, *G. graminis*, *Pyricularia oryzae*, *Fusarium moniliforme*, and *Sarocladium oryzae*.
[c] U, unknown.

TABLE 3. Properties of 20-mer primers used for PCR analysis

Primer	Sequence	G+C (%)	T_m (°C)	Position[a]
Phl2a	GAGGACGTCGAAGACCACCA	60	73	1915
Phl2b	ACCGCAGCATCGTGTATGAG	55	72	2660
PCA2a	TTGCCAAGCCTCGCTCCAAC	60	79	3191
PCA3b	CCGCGTTGTTCCTCGTTCAT	55	76	4341

[a] Position of the 5′ end of the primer in the database sequence.

Probes were generated by randomly primed labeling of PCR fragments by the nonradioactive digoxigenin system (Boehringer Mannheim). The hybridized probes were immunodetected with anti-digoxigenin-alkaline phosphatase-Fab fragments and visualized with the colorometric substrates nitroblue tetrazolium salt and 5-bromo-4-chloro-3-indolylphosphate, as described in the protocols provided by the supplier.

Soils. Seven different soils (Table 4) were obtained from agricultural or virgin fields near Quincy, Lind, Moses Lake, and Mount Vernon, Wash. Soils from the agricultural fields from Quincy, Lind, and Moses Lake are suppressive to take-all of wheat. In 1995, the Lind field had been cropped continuously to wheat for 28 years. In 1980, the fields at Quincy and Moses Lake had been cropped continuously to wheat for 22 years; between 1980 and 1995, other crops besides wheat were also grown. The soil from Mount Vernon was from a field that had been cropped continuously to pea for at least 30 years. The virgin soils from Quincy, Lind, and Moses Lake were collected from sites covered by native vegetation such as sagebrush and bunchgrass and were located near the corresponding agricultural fields. The soils were collected in March 1995 from the upper 30 cm of the soil profile, air dried for 1 week, and passed through a 0.5-cm-mesh screen prior to use. Their physical and chemical properties (Table 4) were determined by the Analytical Sciences Laboratory, University of Idaho.

Seed treatment. Wheat seeds (cultivar Penewawa) were coated with 1% methyl cellulose or with a suspension of *P. fluorescens* Q2-87R in 1% methyl cellulose. The coated seeds were air dried for 5 h in a laminar-flow cabinet. The final density of Q2-87R was approximately 10^3, 10^4, 10^5, 10^6, or 10^7 CFU per seed, as determined by dilution plating on KMB^+ supplemented with rifampin (100 μg ml^{-1}).

Plant cultivation. Twelve wheat seeds were sown in square polyvinyl chloride pots (8 cm high, 7.5 cm wide) containing 200 g of sieved natural soil and 50 ml of water supplemented with metalaxyl (Ciba-Geigy, Greensboro, N.C.) at 2.5 mg of active ingredient ml^{-1} to control pythium root rot. Pseudomonads are not affected by this fungicide (38). A 1-cm layer of soil was spread on top of the seeds. Plants were grown in a controlled-environment chamber at 16°C with a 12-h photoperiod. Pots received 50 ml of dilute (2:3, vol/vol) Hoagland's solution (macroelements only) twice a week. After 21 days of growth, the shoots of the plants were excised at the soil surface, and the soil and associated root system were decanted into a plastic bag and shaken vigorously to aerate and mix. This cultivated soil was stored for 1 week at 15°C, returned to the same pot, and then replanted with 12 wheat seeds. This process of plant growth and harvesting was repeated for a total of four cycles, at which time four randomly selected plants were harvested from each replicate and root samples were prepared to determine the population size of antibiotic-producing fluorescent *Pseudomonas* spp. For each soil, four replicates were used.

Isolation of bacteria from roots of wheat grown in natural soils. Four randomly selected plants were harvested from each replicate, and loosely adhering soil was removed from the roots by gentle shaking. One gram of roots and associated rhizosphere soil was suspended in 5.0 ml of sterile water, and the mixture was shaken vigorously for 1 min on a Vortex mixer. The samples were subsequently sonicated in a ultrasonic cleaner for 1 min, and then serial dilutions of the root wash were plated onto KMB^+. Plates were incubated at 25°C, and colonies were enumerated after 48 h. Colonies of fluorescent pseudomonads were differentiated from nonfluorescent colonies under UV light (wavelength, 366 nm). The number of fluorescent pseudomonads that harbor the genes for Phl or PCA was determined by colony hybridization followed by PCR analysis.

RESULTS

Specificity of Phl primers. Primers Phl2a and Phl2b amplified the predicted 745-bp fragment from DNA of strain Q2-87 (Fig. 1A). A fragment of the same size was amplified from DNA of all other known Phl-producing *Pseudomonas* strains, whereas no PCR products were amplified from DNA of the nonproducing strains Q69c-80, 2-79RN_{10}, M4-80R, and 30-84. All of the PCR products amplified from DNA of the Phl-producing strains hybridized under stringent conditions with a Phl probe consisting of the 745-bp PCR product from strain Q2-87 (Fig. 1B). The detection limit for purified genomic DNA was 1 pg, based on ethidium bromide staining and Southern hybridization. The results obtained by PCR with heat-lysed bacterial cell suspensions were identical to the results obtained with purified genomic DNA.

Specificity of PCA primers. Primers PCA2a and PCA3b amplified the predicted 1,150-bp fragment from DNA of homologous strain 2-79 (Fig. 2A). A fragment of the same size was amplified from DNA of all other phenazine-producing strains tested, except from DNA of the *P. aeruginosa* strains, which produce pyocyanine and other phenazine pigments (Fig. 2A). For the *P. aeruginosa* strains, a fragment of approximately 800 bp was amplified. No products were amplified from DNA of the nonproducing strains Q2-87, Q69c-80, biotype A, and B10. The PCR products amplified from DNA of all of the phenazine-producing strains, except the *P. aeruginosa* strains, hybridized under stringent conditions with the PCA probe which was derived from the PCR product of strain 2-79 (Fig. 2B). The 800-bp fragment amplified from the *P. aeruginosa* strains did not hybridize with the PCA probe from strain 2-79 even at low stringency, indicating that the 800-bp fragment has little or no homology to the PCA probe. In PCR, the detection limit for purified genomic DNA was 10 pg, based on ethidium bromide staining and Southern hybridization. The results obtained by PCR with heat-lysed bacterial cell suspensions were identical to the results obtained with purified genomic DNA.

To further investigate the use of the PCA probe for detection of phenazine-producing strains, Southern hybridization of *Cla*I-digested genomic DNA was performed at low stringency (Fig. 2C). Based on the sequence data for strain 2-79, restriction with *Cla*I should produce two distinct DNA fragments that hybridize with the PCA probe. No hybridization was observed with genomic DNA of the nonproducing strains Q2-87, Q69c-80, biotype A, and B10. Digestion of genomic DNA with *Cla*I produced two distinct fragments of approximately 4,000 and

TABLE 4. Physical, chemical, and biological properties of the natural soils used in this study

Origin	pH	Content (μg)/g of soil				Content (%)				Texture	Suppressive to take-all[b]
		NO_3-N	NH_4-N	P	K	OM[a]	Sand	Clay	Silt		
Quincy	5.3	29.8	0.9	10.3	330	1.01	42.8	10.8	46.4	Loam	+
Quincy virgin	6.8	20.1	1.3	6.8	326	0.98	46.8	4.8	48.4	Sandy loam	−
Lind	5.7	92.8	3.0	4.1	520	1.62	31.2	4.8	64.0	Silt loam	+
Lind virgin	8.0	12.5	0.6	9.1	184	0.98	29.2	4.8	66.0	Silt loam	−
Moses Lake	6.4	78.8	1.5	7.9	346	2.96	49.2	16.8	34.0	Loam	+
Moses Lake virgin	6.8	26.7	0.7	8.1	412	0.59	73.2	2.8	24.0	Sandy loam	−
Mt. Vernon	7.0	16.4	0.8	11.9	273	1.73	17.2	14.8	68.0	Silt loam	−

[a] OM, organic matter (%, wt/wt).
[b] +, suppressive; −, conducive.

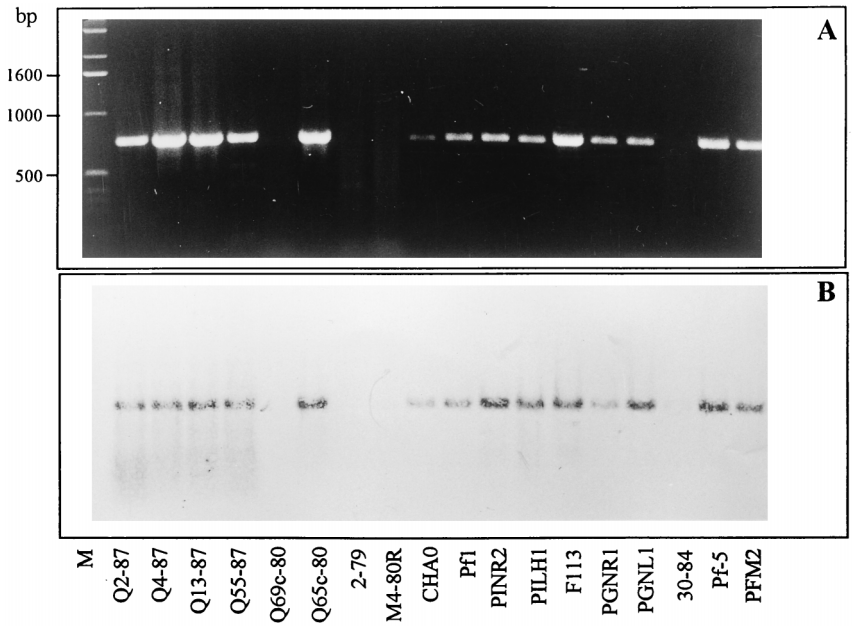

FIG. 1. (A) Agarose gel electrophoresis of the PCR products amplified from genomic DNA of *Pseudomonas* strains with Phl primers Phl2a and Phl2b. Lane M, DNA 1-kb ladder marker (0.2 μg). Characteristics of the *Pseudomonas* strains are listed in Table 1. (B) Southern blot analysis of the PCR fragments of the *Pseudomonas* strains shown in panel A with the Phl probe (745-bp fragment of Q2-87) under stringent conditions. Lanes are identical to those in panel A.

1,500 bp for the homologous strain 2-79 and two distinct fragments of approximately 2,800 and 1,600 bp for all *Pseudomonas aureofaciens* strains. For strains 2-79, 30-84, and AP9, the PCA probe also hybridized with DNA fragments of approximately 6,000 bp, which is probably due to partial digestion of the genomic DNA. Interestingly, the hybridization pattern observed for *Pseudomonas chlororaphis* 9446 was identical to the patterns of the *P. aureofaciens* strains. For all *P. aeruginosa* strains, one fragment of approximately 6,100 bp hybridized with the PCA probe at low stringency (Fig. 2C) but not at high stringency (data not shown).

Efficiency of recovery of antibiotic-producing *Pseudomonas* spp. The protocol to determine the frequency of naturally occurring antibiotic-producing *Pseudomonas* spp. on roots of wheat is described in Fig. 3. Colony hybridizations were performed at high stringency. To determine the efficiency of colony hybridization and PCR, wheat seeds were treated with different densities of Phl-producing strain Q2-87R and sown in natural Quincy virgin soil. Three weeks after planting, population densities of rifampin-resistant strain Q2-87R on wheat roots were determined by colony hybridization followed by PCR and by dilution plating on KMB supplemented with rifampin. Regression analysis demonstrated that enumeration of populations of Q2-87R by colony hybridization and PCR is as effective as enumeration of Q2-87R by dilution plating on KMB supplemented with rifampin (Fig. 4). At population densities of Q2-87R greater than 10^5 CFU/g, 100% of the colonies that were colony hybridization positive were PCR positive with the Phl primers and were rifampin resistant. At population densities of Q2-87R lower than 10^5 CFU/g, colony hybridization alone overestimated the actual population density of Q2-87R; 10 to 20% of the colonies that were colony hybridization positive were PCR negative with the Phl primers and were not rifampin resistant. The detection limit of colony hybridization followed by PCR was 10^4 CFU per g of root. Similar results were obtained when the experiment was repeated.

Frequency of naturally occurring Phl- and PCA-producing pseudomonads. The protocol described in Fig. 3 was used to determine population densities of Phl- and PCA-producing fluorescent pseudomonads on roots of wheat grown in each of seven different natural soils. A common biological characteristic of the agricultural soils from Quincy, Lind, and Moses Lake is their natural suppressiveness to take-all of wheat, whereas the complementary virgin soils and the soil from Mount Vernon are not suppressive to take-all (Table 4). After four successive growth cycles, PCA-producing fluorescent pseudomonads were not detected on roots of wheat grown in any of the soils (Table 5). However, fluorescent pseudomonads that harbor Phl genes were isolated from roots of wheat grown in Quincy, Lind, and Moses Lake agricultural soils at densities of 1.3×10^6, 1.6×10^6, and 5.1×10^5 CFU/g of root (fresh weight), respectively (Table 5). These populations represented, respectively, 11.8, 6.9, and 3.1% of the total population of fluorescent pseudomonads isolated from wheat roots. In the Moses Lake virgin soil, the population density of pseudomonads harboring Phl genes was 1.2×10^4 CFU/g, which was approximately 40-fold less than the density of Phl producers in the Moses Lake suppressive soil. In the other two virgin soils and in Mount Vernon soil, Phl-producing pseudomonads were not detected. Subsequent phenotypic characterization of 90 random isolates with Phl genes demonstrated that when grown for 7 days on KMB agar, all produced the red pigment characteristic of Phl production (15). Similar results were obtained when the experiment was repeated.

DISCUSSION

The antibiotics PCA and Phl are major determinants of biological control of soilborne plant pathogens by strains of fluorescent *Pseudomonas* spp. (34). In this study, we described primers and probes that enable specific detection of a wide variety of strains that produce various phenazine antibiotics or

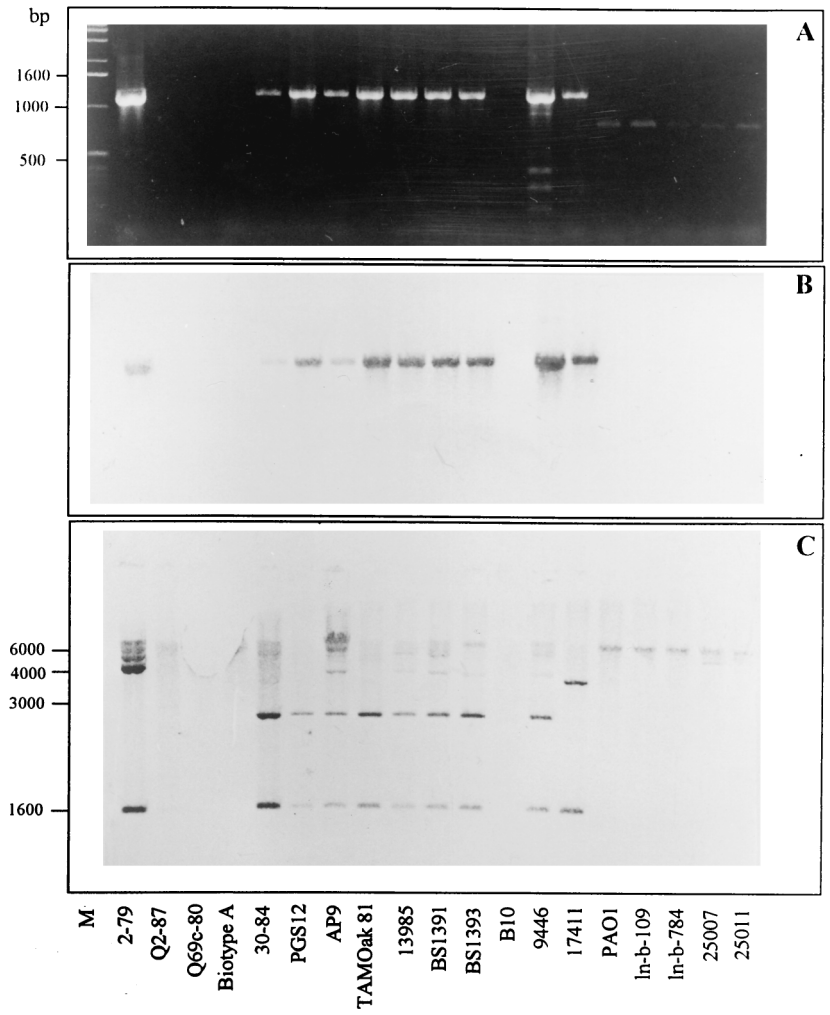

FIG. 2. (A) Agarose gel electrophoresis of the PCR products amplified from genomic DNA of *Pseudomonas* strains with PCA primers PCA2a and PCA3b. Lane M, DNA 1-kb ladder marker (0.2 μg). Characteristics of the *Pseudomonas* strains are listed in Table 2. (B) Southern blot analysis of the PCR fragments of the *Pseudomonas* strains shown in panel A with the PCA probe (1,150-bp fragment of 2-79) under stringent conditions. Lanes are identical to those of panel A. (C) Southern blot analysis of *Cla*I-digested genomic DNA (1 μg) from the *Pseudomonas* strains shown in panel A with the PCA probe (1,150-bp fragment of 2-79) at low stringency. Lanes are identical to those in panel A.

Phl. These primers and probes also allowed specific and efficient detection of Phl- or phenazine-producing strains from natural environments. Gene probe techniques and PCR have been used extensively in environmental microbiology to detect pathogenic bacteria, fungi, and viruses, nitrogen-fixing microorganisms, and water and soil bacteria that degrade xenobiotics (26). To our knowledge, this study is the first to apply these techniques in biocontrol research to isolate naturally occurring microorganisms with specific biocontrol traits. Colony hybridization was chosen over direct PCR of DNA extracted from rhizosphere soil (30) because it allows further analysis of the phenotypic and genetic diversity of the isolated antibiotic-producing pseudomonads and the potential development of these strains for biological control of soilborne plant pathogens. In the method described in this study (Fig. 3), PCR functions as a quality check of the colony hybridization rather than as a direct detection method. The use of direct PCR is currently under investigation to provide a more rapid analysis of the presence of these antibiotic genes in environmental samples such as rhizosphere soil. It may also provide information about

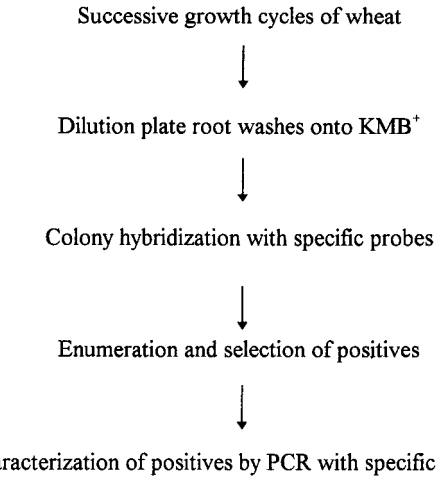

FIG. 3. Protocol to determine the frequency of Phl- and PCA-producing fluorescent *Pseudomonas* spp. on roots of wheat grown in natural soils.

TABLE 5. Frequency of Phl- and PCA-producing fluorescent *Pseudomonas* spp. on roots of wheat grown in natural soils[a]

Soil	Flu pseudomonads[b] (CFU/g of root)	Phl[c] (CFU/g of root)	Phl/Flu pseudomonads (%)	PCA[d] (CFU/g of root)	PCA/Flu pseudomonads (%)
Quincy	9.9×10^6	1.3×10^6	11.8	$<10^4$	<0.1
Quincy virgin	1.0×10^7	$<10^4$	<0.1	$<10^4$	<0.1
Lind	2.4×10^7	1.6×10^6	6.9	$<10^4$	<0.1
Lind virgin	1.4×10^7	$<10^4$	<0.1	$<10^4$	<0.1
Moses Lake	1.6×10^7	5.1×10^5	3.1	$<10^4$	<0.1
Moses Lake virgin	5.6×10^6	1.2×10^4	0.2	$<10^4$	<0.1
Mt. Vernon	1.3×10^7	$<10^4$	<0.1	$<10^4$	<0.1

[a] Mean values of four replicates are given.
[b] Total population of fluorescent *Pseudomonas* spp.
[c] Population of fluorescent *Pseudomonas* spp. harboring Phl genes.
[d] Population of fluorescent *Pseudomonas* spp. harboring PCA genes.

the existence of nonculturable microorganisms harboring these traits.

The Phl primers described in this study were directed against *phlD*, one of the six biosynthetic genes in the Phl biosynthetic cluster of *P. fluorescens* Q2-87 (2). Based on the results of the PCR analysis and hybridization (Fig. 1), it can be concluded that *phlD* is conserved among Phl producers of worldwide origin. These results confirm and extend the observations of Keel et al. (15), who used a 4.8-kb chromosomal DNA fragment of Q2-87, which included *phlD*, as a probe to demonstrate the conservation of the Phl biosynthetic locus in various strains. The PCA primers were directed against *phzC* and *phzD*, two of the nine genes in the PCA biosynthetic cluster of *P. fluorescens* 2-79 (3). These primers allow detection by PCR (Fig. 2A) of various strains that produce phenazine antibiotics which differ structurally from PCA. Combined with the results obtained by Southern hybridization (Fig. 2B and C), these results indicate that *phzC* and *phzD* are conserved among phenazine-producing strains of *P. fluorescens*, *P. aureofaciens*, and *P. chlororaphis*. Additional experiments will be required to determine in more detail the extent of conservation of phenazine biosynthetic genes in strains that produce structurally different phenazine antibiotics. This information will contribute to the construction of primers and probes that allow detection of a wider variety of phenazine-producing microorganisms, including *P. aeruginosa* strains.

Disease-suppressive soils are defined as soils in which the pathogen does not establish or persist, establishes but causes little or no damage, or establishes and causes disease for a while but thereafter the disease is less important, although the pathogen may persist in the soil (5). The development of natural suppressiveness to take-all disease, known as take-all decline, is associated with monoculture of cereals in the presence of the take-all pathogen (28). One widely held explanation for take-all suppressiveness is based on microbiological interactions between the pathogen and specific root-associated microorganisms (4, 6). Weller (37) suggested that take-all decline occurs because of a gradual buildup over years of antagonistic pseudomonads on infected roots and in infested root debris. Wheat roots grown in the Quincy and Moses Lake suppressive soils collected in 1979 were shown to have a significantly larger number of fluorescent pseudomonads antagonistic to *G. graminis* var. *tritici* than roots from the nonsuppressive Lind virgin and Mt. Vernon soils (39). It was speculated that phenazine antibiotics contribute to take-all decline because of their importance in biological control of take-all by strains 2-79 and 30-84 (22, 32). Surprisingly, phenazine-producing pseudomonads were not detected (detection limit, 10^4 CFU/g root) on roots of wheat grown in any of the seven soils in this study (Table 5). This was especially unexpected because strains 2-79 and 30-84 initially were isolated from take-all disease-suppressive soils (22, 38). Our results suggest that phenazine-producing fluorescent *Pseudomonas* spp. are not enriched in the soils tested and consequently do not seem to contribute to the natural suppressiveness of these soils to take-all. In contrast to the phenazine producers, relatively large populations of Phl-producing fluorescent *Pseudomonas* strains were isolated from all three take-all-suppressive soils but not from their complementary take-all-conducive soils (Table 5), indicating that Phl producers may play an important role in the natural suppressiveness of these soils to take-all of wheat. It is notable that Phl-producing pseudomonads have been also readily isolated from soils suppressive to black root rot of tobacco (15) and from soils suppressive to fusarium wilt of tomato (31). For example, Keel et al. (15) reported that 23% of the *Pseudomonas* isolates from wheat roots grown in a black root rot-suppressive soil contained Phl biosynthetic genes. Collectively,

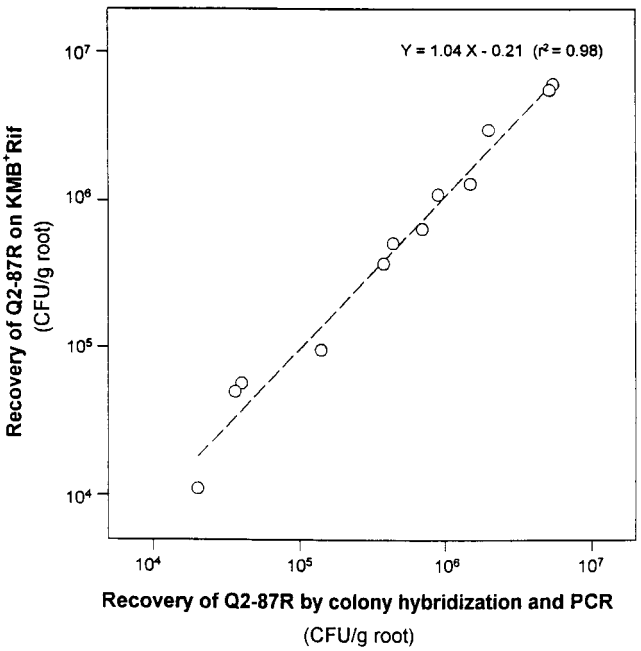

FIG. 4. Efficiency of recovery of *P. fluorescens* Q2-87R from wheat roots grown for 3 weeks in natural Quincy virgin soil. Populations of Q2-87R were determined in root washes by colony hybridization followed by PCR and by dilution plating onto KMB supplemented with rifampin (KMB^+ Rif).

these results indicate the need for a detailed analysis of the population dynamics of Phl producers in take-all- and other disease-suppressive soils and to relate population sizes to disease incidence and severity.

In conclusion, the primers and probes described in this study allow specific detection and isolation of naturally occurring *Pseudomonas* spp. that produce Phl and various phenazine antibiotics, including PCA, 2-hydroxy-phenazine-1-carboxylic acid, and chlororaphin. Knowledge of the ecology of naturally occurring strains that harbor specific biocontrol traits will contribute to improving the efficacy of existing biocontrol agents and may help to identify new strains that are adapted to specific soils and/or host-pathogen systems. Moreover, knowledge of the distribution of antibiotic genes in natural environments could lessen concerns about the environmental release of either nonindigenous strains containing these traits or transgenic biocontrol agents in which these traits have been introduced.

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