Genetic Diversity of *phlD* from 2,4-Diacetylphloroglucinol-Producing Fluorescent *Pseudomonas* spp.

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ABSTRACT

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Fluorescent *Pseudomonas* spp. that produce 2,4-diacetylphloroglucinol (2,4-DAPG) have biocontrol activity against damping-off, root rot, and wilt diseases caused by soilborne fungal pathogens, and play a key role in the natural suppression of *Gaeumannomyces graminis* var. *tritici*, known as take-all decline. Diversity within *phlD*, an essential gene in the biosynthesis of 2,4-DAPG, was studied by restriction fragment length polymorphism (RFLP) analysis of 123 2,4-DAPG-producing isolates from six states in the United States and six other locations worldwide. Clusters defined by RFLP analysis of *phlD* correlated closely with clusters defined previously by BOX-polymerase chain reaction (PCR) genomic fingerprinting, indicating the usefulness of *phlD* as a marker of genetic diversity and population structure among 2,4-DAPG producers. Genotypes defined by RFLP analysis of *phlD* were conserved among isolates from the same site and cropping history. Random amplified polymorphic DNA analyses of genomic DNA revealed a higher degree of polymorphism than RFLP and BOX-PCR analyses. Genotypic diversity in a subset of 30 strains representing all the *phlD* RFLP groups did not correlate with production in vitro of monoacetylphloroglucinol, 2,4-DAPG, or total phloroglucinol compounds. Twenty-seven of the 30 representative strains lacked pyrrolnitrin and pyoluteorin biosynthetic genes as determined by the use of specific primers and probes.

Plant growth-promoting rhizobacteria (PGPR) are rhizobacteria that have the ability to promote the growth of plants following inoculation onto seeds or subterranean plant parts (13). PGPR mediate improved plant growth either directly, by stimulation of the plant (8,15) or indirectly, through biological control of pathogens or induction of host defense mechanisms (20,23,35–37). Fluorescent pseudomonads that produce the polyketide antibiotic 2,4-diacetylphloroglucinol (2,4-DAPG) are an important group of PGPR that suppress root and seedling diseases on a variety of crops. Examples include *Pseudomonas fluorescens* CHA0 that suppresses black root rot of tobacco (33), take-all of wheat (10) and Fusarium wilt and crown and root rot of tomato (6,34), *Pseudomonas* sp. F113 that suppresses damping-off of sugar beet (7,31), and *P. fluorescens* Q2-87 (9,22) and Q8r1-96 (26) that suppress take-all of wheat. Strains of *P. fluorescens* that produce 2,4-DAPG also have a key role in the natural biological control of take-all known as take-all decline (25–27). 2,4-DAPG inhibits a wide range of fungi and bacteria, and its importance in biocontrol activity has been demonstrated conclusively by genetic approaches (35) and direct isolation from the rhizosphere environment (4,6,25,26).

Genes required for the synthesis of 2,4-DAPG by *P. fluorescens* Q2-87 have been cloned (2). The biosynthetic locus includes *phlA*, *phlC*, *phlB*, and *phlD*, which are transcribed as an operon from a promoter upstream of *phlA* (3). PhlD is responsible for the production of monoacetylphloroglucinol (MAPG), and PhlA, PhlC, and PhlB are necessary to convert MAPG to 2,4-DAPG. The biosynthetic operon is flanked on either side by *phlE* and *phlF*, which code respectively for putative efflux and repressor proteins. PhlD is especially interesting because of its homology to members of the highly conserved chalcone and stilbene synthase family of plant enzymes, which is suggestive of a common evolutionary origin (3). Probes and primers specific for sequences in *phlD* have been used in combination with colony hybridization and polymerase chain reaction (PCR) to quantify population sizes of 2,4-DAPG producers in the rhizosphere environment (21,26,27).

Several distinct groups of 2,4-DAPG-producing fluorescent pseudomonads have been identified (11,17,32). Two major phenotypic groups have been distinguished based on the production of antifungal compounds, with one group synthesizing 2,4-DAPG, hydrogen cyanide, and pyoluteorin and the other, only the first two metabolites (11). Some pyoluteorin-producing strains such as Pf-5 (18,19) and CHA0 (10,11,32) also can synthesize pyrrolnitrin, although whether this ability is widespread among this group is unclear. Pyoluteorin-producing strains are comparatively homogeneous, whereas pyoluteorin-negative strains include eight distinct genotypes differentiated by random amplified polymorphic DNA (RAPD) analysis. The results of Sharifi-Tehrani et al. (32) suggest that promising biocontrol pseudomonads may be identified functionally, based on the ability to produce 2,4-DAPG, or taxonomically, based on amplified ribosomal DNA restriction analysis (ARDRA) fingerprints. However, ARDRA distinguishes only three (11,17,32) or four (20) groups of 2,4-DAPG producers, and does not reflect the full range of diversity among isolates. McSpadden Gardener et al. (17) identified 13 and 15 genotypes by BOX-PCR and enterobacterial repetitive intergeneric consensus (ERIC)-PCR, respectively, in a collection of *phlD*-containing strains including reference strains from a previous study (11) and isolates from wheat grown in soils from the United States and the Netherlands. In another study, Picard et al. (21) differentiated 64 RAPD genotypes among 150 representatives of a single ARDRA group of *phlD*-containing isolates from the roots and rhizosphere

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of maize. Such data, which relate population structure to the presence of *phlD*, reveal a high level of genetic diversity among *phlD*-containing strains but provide little insight into whether this diversity is paralleled by diversity within *phlD* itself, or if 2,4-DAPG production and biocontrol efficacy are related to the physical structure of *phlD* or the 2,4-DAPG biosynthetic operon. Knowledge of the diversity within *phlD* is a necessary prerequisite to assessing the potential or frequency of horizontal transfer of the biosynthetic genes between members of the rhizosphere microbial community and might provide a basis for developing a rapid genetic screen to identify strains with superior biocontrol activity. The objectives of this research were to define the degree of heterogeneity in *phlD* among members of a large, genetically diverse collection of 2,4-DAPG-producing fluorescent *Pseudomonas* spp.; and to determine whether diversity in *phlD* correlates with whole genome diversity as defined by BOX-PCR and RAPD analyses, or with the amount of 2,4-DAPG produced in vitro. In addition, because 2,4-DAPG producers can be differentiated phenotypically by their ability to produce pyoluteorin and under appropriate culture conditions some also produce pyrrolnitrin, we assessed the frequency of co-occurrence of these biosynthetic genes among the various *phlD* genotypes.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and media. A total of 123 *phlD*$^+$ strains of fluorescent *Pseudomonas* spp. isolated from six states in the United States and six other locations worldwide were included in this study (Table 1). The sources and characteristics of most of the strains were described previously (11,17). Those designated STAD were isolated from the rhizosphere of wheat grown in soil from Stillwater, OK, with a history of continuous cropping to wheat. All strains were cultured on Pseudomonas agar F (Difco Laboratories, Detroit) or King's medium B (KMB) broth (20 g of Bacto proteose peptone, 1.2 g of KH_2PO_4, 1.5 g of $MgSO_4 \cdot 7H_2O$, and 10 ml of glycerol per liter) at 25°C. Stock cultures were stored frozen in Luria-Bertani broth plus 40% glycerol at –80°C.

TABLE 1. Bacterial strains used in the study

Prefix or strain[w]	Isolate numbers	RFLP[x]	Source	Ref.[y]
CV	1-1, 2-3, 2-4, 2-8, 3-3, 3-6, 4-1, 4-3, 4-5, 4-7	H	Caldwell, KS	17
CC	1-1, 1-3, 2-1, 2-8, 3-6, 4-1, 5-1	J	Caldwell, KS	17
CC	3-1	J1	Caldwell, KS	17
FTAD1R	5, 25, 26, 27, 33, 34, 35, 37, 38	D	Fargo, ND	17, TS
FTAD1R	36	J	Fargo, ND	17
FFL1R	8, 10, 13, 14, 17, 18, 21, 22, 25	J	Fargo, ND	17
FFL1R	9	D	Fargo, ND	17
HT	5-1, 5-5, 5-8, 5-10, 5-12, 5-15, 6-2, 6-4, 6-7	N	Hallock, MN	17
OC	4-1, 4-2	D	Ithaca, NY	17
W	2-4, 2-6, 2-9	D	Lind, WA	17, TS
W	4-4	L	Lind, WA	17
QT	1-5, 5-1, 5-2	D	Quincy, WA	17
QT	1-6, 2-1, 2-2, 3-1, 3-2, 4-2, 6-1	E	Quincy, WA	17, TS
Q	1-3, 1-4, 2-1, 2-6, 2-10, 2-12	B	Quincy, WA	17
Q	2-5, 2-18	D	Quincy, WA	17, TS
Q	2-2, 2-19	E	Quincy, WA	17
QX-87[z]	1, 2, 4, 5, 9, 12, 13, 88	B	Quincy, WA	9
QX-87	37	E1	Quincy, WA	10
QX-87	128	D	Quincy, WA	9
Q8rX-96	1	D	Quincy, WA	26
STADX-97	375, 376, 377, 378, 379, 384, 385, 387, 388 389	C	Stillwater, OK	TS
D27B	1, 2, 3, 4, 5, 6, 7, 8, 9, 10	M	Woensdrecht1	17
JMP	6, 7, 9, 10, 11, 12, 16, 17, 18, 22	F	Woensdrecht2	17
CHA0		A	Switzerland	33
Pf1		A	Switzerland	11
F113		K	Ireland	31
Pf-5		A	Texas	10, 19
PGNR1	1, 2, 3	A	Ghana	11
PINR2	2, 3	A	Italy	11

[w] A complete description of the source of each strain is provided in the referenced publications.

[x] Restriction fragment length polymorphism-defined *phlD*$^+$ genotype, based on combined patterns obtained by digestions of 745 bp of *phlD* with *Hae*III, *Rsa*I, and *Taq*I (Table 3).

[y] TS = this study.

[z] To obtain the strain designation, substitute X with the isolate number.

RAPD analyses. Total genomic DNA was isolated from bacterial strains by a cetyltrimethylammoniumbromide (CTAB)-based miniprep protocol (1) and amplified with the RAPD primer M13 (5′-GGTGGTCAAG-3′). PCR was conducted in a total volume of 25 µl containing 1× Stoffel fragment buffer, 80 pmol of M13 primer, 0.08 units of *Taq* polymerase Stoffel fragment (Perkin-Elmer, Norwalk, CT), 4 mM MgCl2, 200 µmol each of dATP, dTTP, dGTP, and dCTP (Perkin-Elmer), and 40 ng of genomic DNA. The amplification was performed with a thermocycler (PTC-200; MJ Research, Watertown, MA) and a cycling program that included initial denaturation at 94°C for 1.5 min followed by 2 cycles at 94°C for 30 s, 36°C for 30 s, and 72°C for 2 min, and 29 cycles at 94°C for 30 s, 36°C for 15 s, 45°C for 15 s, and 72°C for 1.5 min, and a final extension at 72°C for 30 s. All RAPD PCR reactions were run at least twice with similar results. The amplification products were separated on 1.5% agarose gels in 0.5× Tris-borate-EDTA (TBE) buffer for 6 h at 140 V at 10°C, stained with ethidium bromide, and photographed under UV light. Images were recorded with a digital imaging system (Kodak DC120; Eastman Kodak Co., Rochester, NY) and analyzed by GelCompar (version 4.0; Applied Maths, Kortrijk, Belgium).

Restriction fragment length polymorphism analyses. Restriction fragment length polymorphism (RFLP)-PCR analyses were performed with whole or heat-lysed cells from 1-day-old cultures grown on Pseudomonas agar F. To lyse cells, two bacterial colonies (2-mm diameter) were suspended in 100 µl of lysis solution (0.05 M NaOH and 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 in a microcentrifuge to remove cell debris, diluted 50-fold in sterile distilled water, and 5 µl was used as template for PCR amplification.

The gene-specific primers Phl2a (5′-GAGGACGTCGAAGACCACCA-3′) and Phl2b (5′-ACCGCAGCATCGTGTATGAG-3′) (27) were used to amplify a 745-bp fragment of *phlD*. For most strains, the cycling program was as described by Raaijmakers et al. (27). For 25 of 40 strains, including representatives from Caldwell, KS (CV and CC), Hallock, MN (HT), Ithaca, NY (OC), Lind, WA (W), and Quincy, WA (QT), a modified cycling program consisting of initial denaturation at 94°C for 90 s followed by 35 cycles at 94°C for 35 s, 53°C for 30 s, and 72°C for 45 s was used to eliminate additional fragments resulting from nonspecific amplification. The cycling program was followed by final extension at 72°C for 30 s. For the remaining 15 strains from Caldwell (CC and CV), the 745-bp *phlD* fragment was isolated from 0.8% agarose gels and re-amplified prior to RFLP analysis.

For all strains, amplification products were precipitated with ethanol, resuspended in sterile distilled water, and the DNA concentration was measured spectrophotometrically at 260 nm (30). Equal volumes (6 µl) of the PCR products were digested (30) with *Hae*III or *Rsa*I at 37°C or *Taq*I at 65°C for 1.5 h and stored at –20°C. Banding patterns were resolved by electrophoresis in 3.5% agarose gels (MetaPhor; FMC BioProducts, Rockland, ME) at room temperature in 1× TBE buffer for 3 h at 98 V. Bands were visualized with ethidium bromide and photographed under UV light. Images were recorded and analyzed as described above. PCR reactions were conducted at least twice, with similar results, for a subset of 38 strains containing all of the distinct genotypes found in different soils (as defined by RAPD analyses, described below), and only once for the rest of the strains.

Banding pattern analysis. The digitized images of RFLP and RAPD bands were converted, normalized, analyzed, and combined with GelCompar version 4.0 software by correlation-based clustering (28,29). A 20-bp molecular ruler (Bio-Rad Laboratories, Hercules, CA) was included in every fifteenth lane in order to normalize the banding pattern of RFLP profiles. Banding patterns of RAPD profiles were normalized with a mixture of 1- and 0.1-kb DNA ladders (New England Biolabs, Beverly, MA). The minimum similarity coefficient of the replicate RAPD PCR assays of all 123 individual strains was used to define distinct clusters of banding patterns. Based on this analysis, 38 representative strains from distinct clusters were chosen for RFLP analysis, which was conducted at least twice. Clusters of the RFLP patterns for the 38 strains were defined by the 95th percentile (near-minimum) similarity coefficient of replicate assays (17) and applied to the complete set of 123 strains, which was analyzed once.

TABLE 2. Oligonucleotide primers used in the study

Primer	Sequence	Target[x]	GenBank Accession No.	Position[y]	T_m[z]
PltBf	CGG AGC ATG GAC CCC CAG C	*PltB*	AF081920	8160–8178	64.0°C
PltBr	GTG CCC GAT ATT GGT CTT GAC CGA G	*PltB*	AF081920	8927–8951 (complement)	63.8°C
plt1	ACT AAA CAC CCA GTC GAA GG	*PltB*	AF081920	4812–4831	50.2°C
plt2	AGG TAA TCC ATG CCC AGC	*PltB*	AF081920	5234–5251 (complement)	57.9°C
PrnCf	CCA CAA GCC CGG CCA GGA GC	*prnC*	U74493	3478–3497	66.9°C
PrnCr	GAG AAG AGC GGG TCG ATG AAG CC	*prnC*	U74493	4175–4197 (complement)	62.9°C

[x] *pltB* encodes a protein similar to type I polyketide synthase in *Pseudomonas fluorescens* Pf-5 (18). *prnC* encodes a halogenase catalyzing chlorination of monodechloroaminopyrrolnitrin to aminopyrrolnitrin in *P. fluorescens* BL915 (12).
[y] Position of the primer in the database sequence.
[z] T_m, melting temperature calculated by Omiga 2.0.

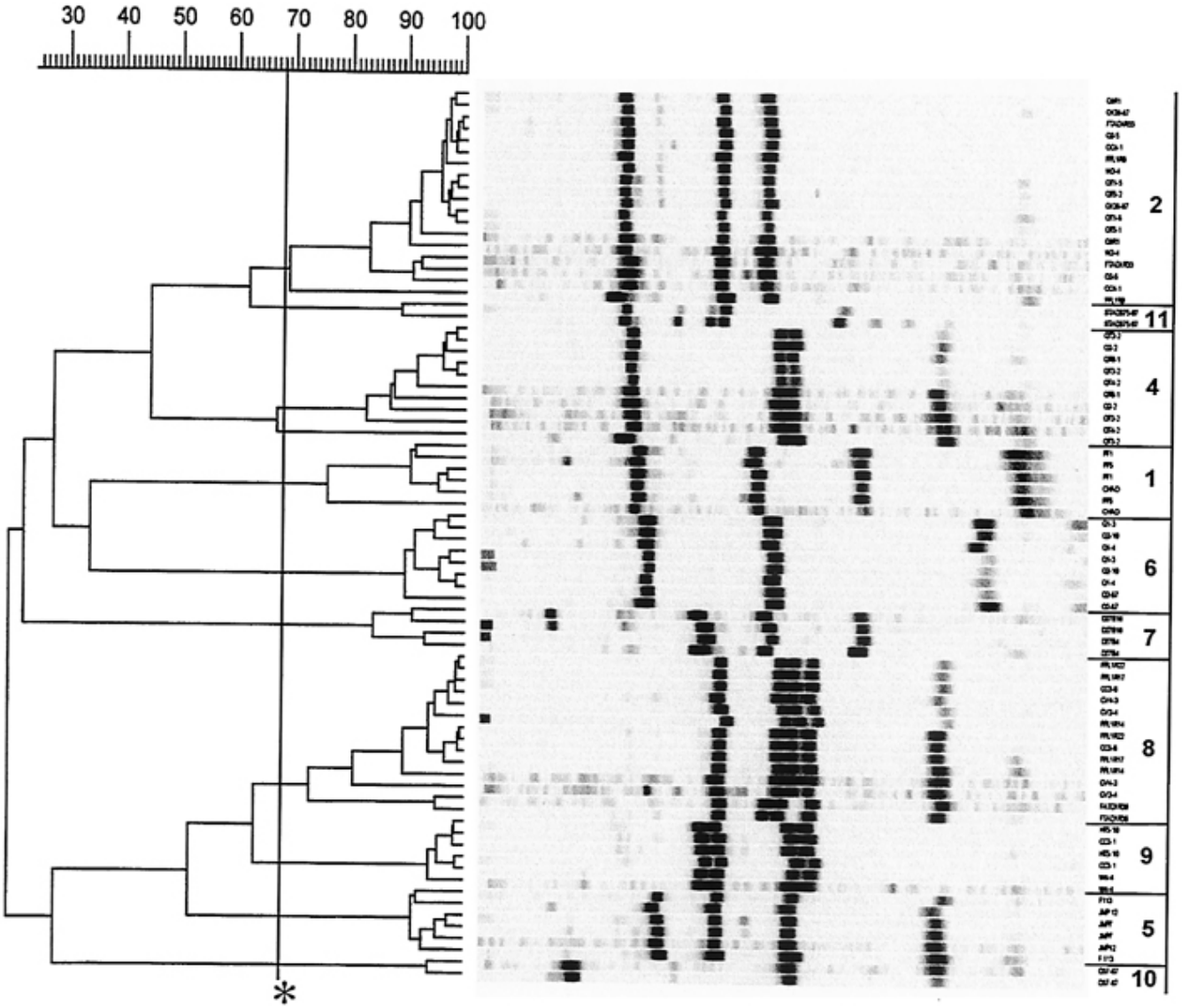

Fig. 1. Random amplified polymorphic DNA cluster analysis of fingerprint patterns generated with the M13 primer from genomic DNA of *phlD*-containing *Pseudomonas* spp. strains. Genomic fingerprints patterns for 38 isolates representing all of the unique genotypes present in the collection of 123 isolates are shown. Two independent amplifications were used for each strain. Using GelCompar 4.0, the unweighted pair-group method, arithmetic mean (UPGMA) algorithm was applied to the similarity matrix generated by Pearson's correlation coefficient from the tracks of the whole patterns. The similarity coefficient used to define distinct genotypic groups is noted (*). Strain designations are listed and distinct clusters of the genomic fingerprints are labeled alphabetically.

Screening for pyoluteorin and pyrrolnitrin biosynthetic loci. The oligonucleotide primers listed in Table 2 were developed by Omiga 2.0 Software (Oxford Molecular Ltd., Oxford, U.K.). Primers PltBf2 and PltBr for the pyoluteorin (Plt) biosynthetic locus were developed from sequences within the module II ketoacyl synthase domain of *pltB* (GenBank Accession No. AF003370), which encodes a protein in *P. fluorescens* Pf-5 similar to type I polyketide synthases (18). Primers PrnCf and PrnCr for the pyrrolnitrin (Prn) biosynthetic locus were developed from sequences within *prn*C (GenBank Accession No. U74493) of *P. fluorescens* BL915, which encodes a halogenase that catalyzes chlorination of monodechloroaminopyrrolnitrin to aminopyrrolnitrin (12). PCR amplification was carried out in 25-µl reaction mixture containing 1× *Taq* DNA polymerase buffer, 200 µM each of dATP, dTTP, dGTP, and dCTP, 20 pmol of each primer, 1.5 mM $MgCl_2$, and 0.06 units of Ampli*Taq* DNA polymerase (Perkin-Elmer). The PCR cycling program consisted of initial denaturation at 94°C for 2 min followed by 29 cycles of 94°C for 1 min, 58°C for 45 s, and 72°C for 1 min. The amplification products were electrophoresed in 0.8% agarose gels in 1× TBE buffer for 1 h at 100 V at room temperature, stained with ethidium bromide, and photographed under UV light.

For Southern blotting and hybridization, total DNA samples were digested with *Eco*RI and *Pst*I restriction endonucleases, separated by electrophoresis in a 0.8% agarose gel, and transferred onto nylon membrane (BrightStar-Plus; Ambion Inc., Austin, TX) in 0.4 M NaOH with subsequent crosslinking of DNA by exposure of membranes to UV (254 nm) (1). Membranes were prehybridized for 2 h at 55 to 60°C in a solution containing 4× SSC (1× SSC is 150 mM NaCl plus 15 mM sodium citrate, pH 7.0) (30), 4× Denhardt's solution (30), 0.1% SDS, and 100 µg of denatured salmon sperm DNA (Sigma Chemical Co., St. Louis) per ml. Prehybridized membranes were incubated with probes overnight under the same conditions and washed with 2× SSC, 0.1% SDS at room temperature (twice), 0.2× SSC, 0.1% SDS at room temperature (twice), 0.2× SSC, 0.1% SDS at 55 to 60°C (twice), and 0.1× SSC, 0.1% SDS at 55 to 60°C (once). DNA-DNA hybrids were detected with a nonisotopic detection kit (BrightStar; Ambion) according to the manufacturer's protocol. The *plt* and *prn* gene probes were amplified from genomic DNA of *P. fluorescens* Pf-5 with the primer pairs Plt1 and Plt2 (which anneal to the 5′ end and internally to *pltB*) or PrnCf and PrnCr (Table 2), respectively, and labeled with a random primer biotin labeling kit (NEN Life Science Products, Boston, MA).

Extraction and detection of 2,4-DAPG and related metabolites. Strains were grown in KMB broth for 72 h at 25°C. Samples (400 µl) were acidified with 4.5 µl of 10% trifluoroacetic acid (TFA) and extracted twice with 1 ml of ethyl acetate (90% extraction efficiency). The organic phase containing phloroglucinol derivatives was evaporated to dryness and suspended in 100 µl of 35% acetonitrile (ACN) containing 1% TFA. Thirty-microliter volumes of the extracts, or dilutions thereof, were fractionated by C_{18} reverse phase high-performance chromatography (HPLC) on a Waters NOVA-PAK C_{18} Radial-PAK cartridge (4 µm, 8 × 100 mm, Waters Corp., Milford, MA) (4). Solvent conditions included a flow rate of 1.0 ml/min with a 2-min initial condition at 10% ACN-0.1% TFA, followed by a 20-min linear gradient to 100% ACN-0.1% TFA. HPLC gradient profiles were monitored at the spectral peak maxima (270 and 330 nm) characteristic of phloroglucinol compounds in the designated solvent system. The HPLC system included a 710B WISP, 510 pumps, and a 680 automated gradient controller with 990 photodiode array detector. 2,4-DAPG and MAPG were identified by retention time and ultraviolet spectra (4). Each strain was grown and assayed three separate times.

Statistics. The statistics related to the genotypic diversity (i.e., Pearson's correlation coefficient, unweighted pair-group method, arithmetic mean [UPGMA] clustering) were calculated with GelCompar 4.0. When analyzing the HPLC data, all statistics were calculated using the JMP software release 3.2 (SAS Institute, Cary, NC). The peak areas corresponding to each of the phloroglucinol compounds were compared using the Tukey-Kramer test. Additionally, the correlation between MAPG and 2,4-DAPG production was calculated by Kendall's tau.

TABLE 3. Comparison of the genotypic diversity of *phlD*-containing *Pseudomonas* spp. defined by restriction fragment length polymorphism (RFLP) analysis of *phlD* and genomic fingerprints

	RFLP genotype[y]			Genotype[z]		
Strains[x]	*Rsa*I	*Taq*I	*Hae*III	Combined	RAPD M13	BOX-PCR
Pf1	1	1	1	A	A	A
Pf-5	1	1	1	A	A	A
CHA0	1	1	1	A	A	A
Q1-3	2	6	6	B	B	B
Q2-10	2	6	6	B	B	B
Q1-4	2	6	6	B	B1	B
Q2-87	2	6	6	B	B2	B
STAD375-97	2	2	11	C	C	C*
Q8R1-96	2	2	2	D	D	D
Q128-87	2	2	2	D	D	D
W2-4	2	2	2	D	D	D
QT1-5	2	2	2	D	D	D
QT5-2	2	2	2	D	D	D
FTAD1R33	2	2	2	D	D1	D
Q2-5	2	2	2	D	D1	D
OC4-1	2	2	2	D	D2	D
FFL1R9	2	2	2	D	D3	D
Q37-87	2	4	10	E1	E	E
QT3-2	2	4	4	E	E	E
QT2-2	2	4	4	E	E	nd
Q2-2	2	4	4	E	E1	E
QT6-1	2	4	4	E	E2	E
QT4-2	2	4	4	E	E3	E
JMP7	2	5	5	F	F1	F
JMP12	2	5	5	F	F	F
CV2-4	2	4	8	H	H	H
CV4-3	2	4	8	H	H	H
FTAD1R36	2	2	8	J	I	I
CC3-1	2	2	9	J1	J1	J
FFL1R22	2	2	8	J	J2	J
FFL1R17	2	2	8	J	J3	J
FFL1R14	2	2	8	J	J	J
CC3-6	2	2	8	J	J4	J
F113	2	2	5	K	K	K
W4-4	3	3	9	L	D3	L
D27B4	2	2	7	M	M	M
D27B10	2	2	7	M	M	M
HT5-10	2	5	9	N	N	N
N	3	6	10	14	25	13

[x] Typical representatives from all unique genotypes. N indicates total number of distinct genomic groups.

[y] Genotypes were defined using RFLP patterns of *phlD* gene generated with restrictions enzymes *Rsa*I, *Taq*I, and *Hae*III.

[z] Combined = combined patterns obtained with the three restriction enzymes. RAPD = random amplified polymorphic DNA. Genotypes were defined by banding patterns amplified with M13 primer. BOX-PCR = BOX-polymerase chain reaction, result from McSpadden Gardener et al. (17). Asterisk indicates unpublished data and nd = not determined.

RESULTS

RAPD analysis of *phlD*$^+$ strains. Based upon the presence or absence of amplification products ranging in size from 200 to 3,500 bp, 25 different M13 RAPD patterns (Fig. 1) were distinguished among the 123 *phlD*$^+$ isolates tested. Isolates from Quincy, Lind, Fargo (ND), and Ithaca, previously described by McSpadden Gardener et al. (17) as BOX group D, were separated into four RAPD groups designated D (Q8r1-96 and W2-4), D1 (FTAD1R33, Q2-5), D2 (OC4-1), and D3 (FFL1R9) (Table 3). Similarly, BOX groups B, E, F, and J formed three, four, two, and five additional subgroups, respectively (Table 3). Eighty-four percent of the distinct RAPD groups defined in this collection con-

tained isolates from single geographic locations. However, groups A, D, D1, and D3 included isolates from more than one location. Multiple RAPD genotypes were detected among isolates obtained from Caldwell, Fargo, Lind, and Quincy. For example, FTAD1R isolates from long-term wheat soil at Fargo included members of RAPD groups D1 and I. FFL1R isolates from long-term flax soil were distributed among RAPD groups D3, J, J2, and J3. Single genotypes occurred among HT, OC, STAD, and D27B isolates from Hallock, Ithaca, Stillwater, and Woensdrecht1, respectively. Comparison of the RAPD and BOX-PCR clustering patterns indicated seven identical groups (A, C, H, I, K, M, and N), with the remaining six BOX groups separated into 18 groups by RAPD analysis (Table 3).

RFLP analyses of *phlD*. The results of the RFLP analyses are summarized in Table 3 for a set of 38 *phlD* strains that includes representatives of each of the 25 distinct groups identified by RAPD analysis. Digestion with *Rsa*I and *Taq*I identified only three and six groups, respectively, whereas digestion with *Hae*III distinguished 10 groups of *phlD* strains. Group one was distinct by all three digests. Group two, as defined by RFLP-*Rsa*I analysis, was separated into four groups by *Taq*I analysis and into eight groups by RFLP-*Hae*III analysis. Figure 2 shows the RFLP for the 38 strains listed in Table 3, digested with *Hae*III restriction endonuclease. The combined patterns obtained with the three restriction endonucleases defined 14 distinct genomic groups among the reduced set of 38 representative strains (Table 3) and the full set of 123 different isolates (Table 1). Among the 14 groups, only groups A, D, and J included isolates from more than one location. Of these, the D group was the largest and contained 22 isolates (18% of the total) from Quincy, Lind, Ithaca, and Fargo. The second largest group, J, included 17 isolates (14% of the total) from two different soils, one from Caldwell and the other from Fargo. Group A contained strains CHA0, Pf1, Pf-5, PINR2, PINR3, PGNR1, PGNR2, and PGNR3, isolated from Swiss, Italian, Texas, and Ghanian soils (11). All of these strains were reported to produce 2,4-DAPG and pyoluteorin (11). The other 11 genotypes each contained isolates from a single location. The JMP and D27B isolates, from two different fields in the Netherlands, clustered in groups F and M, respectively. Group B contained isolates from Quincy, with the QX-87 isolates obtained from a

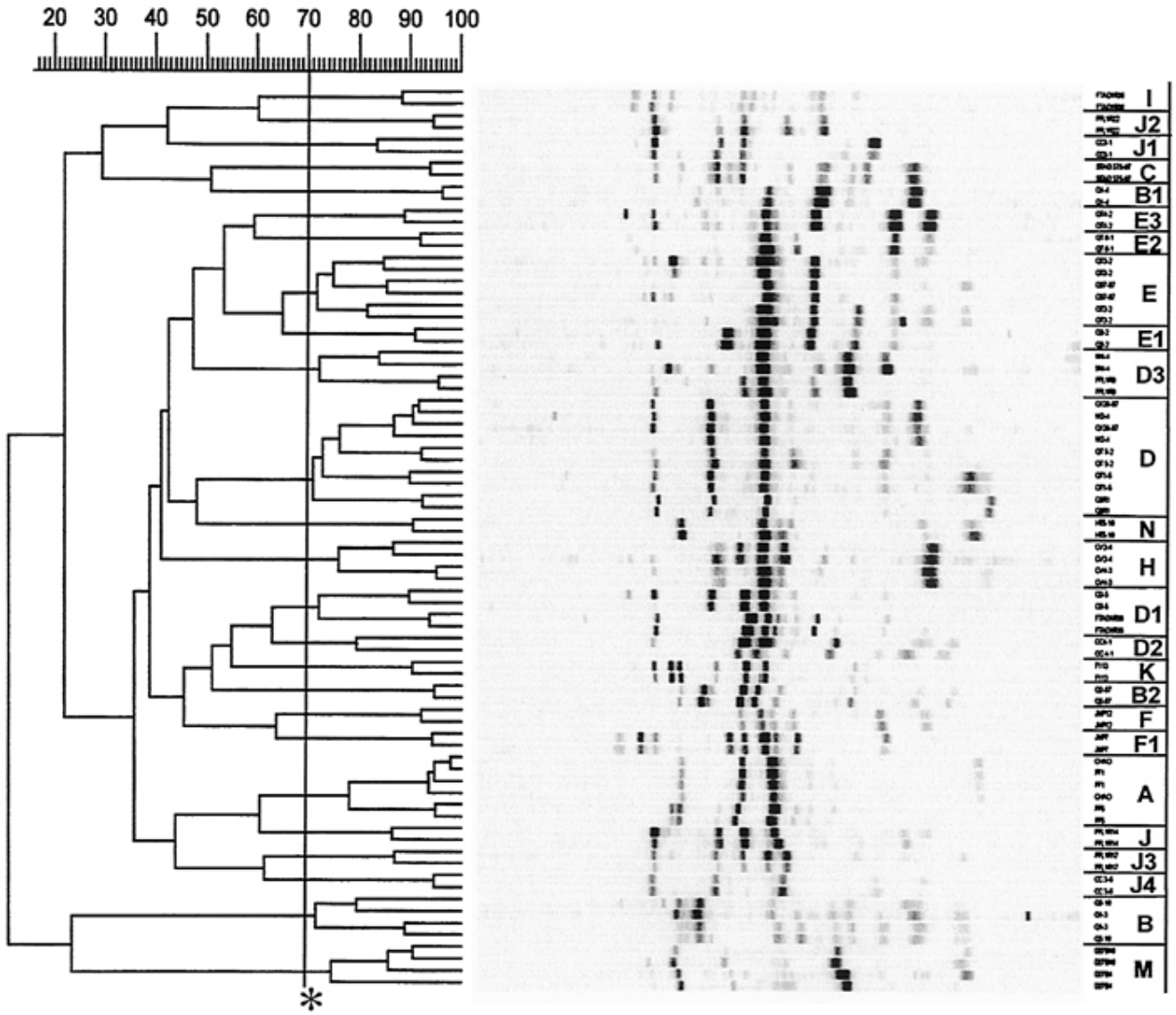

Fig. 2. Cluster analysis of restricted fragment length polymorphism (RFLP) patterns obtained from the digestion of the 745-bp *phlD* fragment with *Hae*III. Patterns for 38 isolates representing all of the unique random amplified polymorphic DNA-defined genotypes are shown. Two independent amplifications were used for each strain. Using GelCompar 4.0, the unweighted pair-group method, arithmetic mean algorithm was applied to the similarity matrix generated from the tracks of the whole patterns by Pearson's correlation coefficient. The similarity coefficient used to define distinct groups is noted (*). Strain designations are listed, and distinct clusters of RFLP patterns are labeled numerically.

growth chamber experiment in 1987 and the Q isolates isolated from the field in 1998. Clusters E1, K, and L each contained a single representative: Q37-87 from Quincy, F113 from Ireland, and W4-4 from Lind, respectively.

Interestingly, the genomic groups identified by the combined RFLP analysis were nearly identical to the groups defined by BOX-PCR analysis. In the reduced set, 35 of the 38 strains (92%) clustered in the same genotypic groups by both methods. The only exceptions were strains Q37-87 and CC3-1, which were separated by RFLP analysis into groups E1 and J1, respectively. A comparison of the RFLP and RAPD fingerprinting techniques showed seven identical groups (A, C, H, J1, K, M, and N), with the remaining seven RFLP groups separated by RAPD into 18 groups (Table 3).

Production of phloroglucinol compounds in vitro. Thirty strains representative of 13 RFLP genotypes were analyzed by HPLC for phloroglucinol production in KMB broth, which supported the synthesis of larger amounts of 2,4-DAPG by a greater number of strains than did yeast malt or *Pseudomonas* F broth (data not shown). Among the 30 strains, 24 produced detectable amounts of 2,4-DAPG as well as MAPG and three other uncharacterized phloroglucinol derivatives (Table 4). All three of the RFLP-defined group A strains tested (CHA0, Pf1, and Pf-5) and three other strains (CC3-6, HT5-10, and W4-4), failed to produce detectable phloroglucinol compounds under these assay conditions. Among the 24 strains where phloroglucinol compounds were detected, production of DAPG and MAPG were positively correlated (Kendall's tau = 0.46; $P < 0.001$). Strain CV4-3 produced more MAPG and 2,4-DAPG than the other strains, but in general, the 24 isolates did not produce significantly different amounts of either MAPG or 2,4-DAPG under our assay conditions. Because MAPG is a precursor of 2,4-DAPG (3,31), and the uncharacterized phloroglucinol compounds detected in these analyses are likely to include condensation products of either or both compounds (R. F. Bonsall, O. V. Mavrodi, D. V. Mavrodi, and D. M. Weller, *unpublished data*), we also assayed total phloroglucinol production. The data indicated that except for RFLP genotypes H and J, representatives within an RFLP genotype (i.e., A, B, D, E, and M) did not differ in the amounts of phloroglucinol compounds produced (Table 4). Only two strains, CV4-3 (group H) and CC3-1 (group J1) produced significantly more phloroglucinol-containing compounds than most of the other strains tested (Table 4).

Screening of *phlD Pseudomonas* spp. for pyoluteorin and pyrrolnitrin biosynthetic loci. Thirty strains representative of 13 *phlD* RFLP groups were screened for the presence of pyoluteorin and pyrrolnitrin biosynthetic loci by PCR with the PltBf2 and PltBr or PrnCf and PrnCr primers, respectively (Table 2). Primers PltBf2 and PltBr amplified the predicted 773-bp fragment of *pltB* from the DNA of *P. fluorescens* strains CHA0, Pf-5, and Pf1. All of these strains are members of RFLP genotype A. No PCR product was amplified from any of the other *phlD* strains (data not shown). Similar results were obtained with the PrnCf and PrnCr primers that amplified the predicted 719-bp fragment of *prnC* from the same three strains (CHA0, Pf-5, and Pf1) but not from the other 27 strains (data not shown). Southern hybridization of digested total genomic DNA from the same 30 strains with *plt* and *prn* gene probes yielded results identical to those obtained by PCR screening (Fig. 3). Under stringent conditions the *plt* and *prn* probes hybridized only with genomic DNA obtained from *P. fluorescens* strains CHA0, Pf5, and Pf1 (Fig. 3).

DISCUSSION

Antibiotic-producing PGPR have been studied intensively during the last decade, and special attention has been given to 2,4-

TABLE 4. Production of phloroglucinol compounds by fluorescent *Pseudomonas* spp. in King's medium B broth[z]

Strain	Genotype	MAPG	DAPG	MAPG + 2,4-DAPG	PHL compounds
CHA0	A	ND	ND	ND	ND
PF1	A	ND	ND	ND	ND
Pf-5	A	ND	ND	ND	ND
Q1-3	B	0.17 (0.05) a–c	0.11 (0.13) a	0.23 (0.13) ab	0.57 (0.15) ab
Q1-4	B	0.16 (0.05) a–c	0.18 (0.13) ab	0.34 (0.13) ab	0.78 (0.15) ab
Q2-87	B	0.09 (0.05) ab	0.08 (0.13) a	0.17 (0.13) ab	0.32 (0.15) a
STAD375-97	C	0.10 (0.05) ab	0.11 (0.13) a	0.21 (0.13) ab	0.51 (0.15) ab
Q8R1-96	D	0.16 (0.05) ab	0.10 (0.13) a	0.26 (0.13) ab	0.67 (0.15) ab
Q128-87	D	0.11 (0.05) ab	0.07 (0.13) a	0.18 (0.13) ab	0.53 (0.15) ab
W2-4	D	0.16 (0.05) a–c	0.05 (0.13) a	0.21 (0.13) ab	0.75 (0.15) ab
QT5-2	D	0.17 (0.05) a–c	0.41 (0.13) a–c	0.57 (0.13) abc	0.98 (0.15) ab
FTAD1R33	D	0.17 (0.05) a–c	0.07 (0.13) a	0.23 (0.13) ab	0.71 (0.15) ab
OC4-1	D	0.18 (0.05) abc	0.08 (0.13) a	0.26 (0.13) ab	0.78 (0.15) ab
Q37-87	E1	0.33 (0.05) abc	0.15 (0.13) ab	0.48 (0.13) a–c	0.92 (0.15) ab
QT2-2	E	0.12 (0.05) ab	0.08 (0.13) a	0.21 (0.13) ab	0.50 (0.15) ab
Q2-2	E	0.11 (0.06) ab	0.07 (0.16) ab	0.18 (0.16) ab	0.47 (0.19) ab
JMP12	F	0.44 (0.05) b–d	0.43 (0.13) a–c	0.88 (0.13) bc	1.33 (0.15) b
CV2-4	H	0.68 (0.05) b–d	0.39 (0.13) a–c	1.07 (0.13) bc	1.51 (0.15) b
CV4-3	H	1.98 (0.05) f	2.16 (0.13) d	4.14 (0.13) e	4.36 (0.15) d
FFL1R25	J	0.25 (0.05) a–c	0.03 (0.13) a	0.28 (0.13) ab	0.82 (0.15) ab
CC3-6	J	ND	ND	ND	ND
FTAD1R36	J	0.22 (0.05) a–c	0.04 (0.13) a	0.26 (0.13) ab	0.57 (0.15) ab
FFL1R14	J	0.38 (0.06) b–d	0.34 (0.16) a–c	0.72 (0.16) a–c	1.01 (0.19) ab
FFL1R17	J	0.14 (0.05) ab	0.04 (0.13) a	0.17 (0.13) ab	0.85 (0.16) ab
FFL1R22	J	0.06 (0.05) a	0.01 (0.13) a	0.07 (0.13) a	0.51 (0.15) ab
CC3-1	J1	1.29 (0.05) c	0.82 (0.13) bc	2.11 (0.13) d	2.65 (0.15) c
W4-4	L	ND	ND	ND	ND
D27B4	M	0.15 (0.06) a–c	1.12 (0.16) bc	1.3 (0.16) bc	1.27 (0.19) b
D27B10	M	0.25 (0.05) a–c	0.51 (0.13) a–c	0.76 (0.13) a–c	0.76 (0.16) ab
HT5-10	N	ND	ND	ND	ND

[z] Strains represent all unique genotypes. Prefix designations are the same as those noted in Table 1. Genotype was defined by the combined restriction fragment length polymorphism analyses of *phlD*. All phloroglucinol (PHL) compounds include monoacetylphloroglucinol (MAPG), 2,4-diacetylphloroglucinol (DAPG), and three other uncharacterized PHL derivatives (R. F. Bonsall, O. V. Mavrodi, D. V. Mavrodi, and D. M. Weller, *unpublished data*). Each value is the mean and standard deviation of the peak area (absorbance units at 270 nm) of three replicate assays. ND = not detected. Values followed by the same letter are not significantly different according to the Tukey-Kramer test ($P < 0.05$).

DAPG-producing fluorescent *Pseudomonas* spp. because of their ability to control a wide variety of plant diseases (5–7,22,32–34,37). At the same time, the application of molecular techniques has significantly changed our capacity to rapidly characterize PGPR, their mechanisms of pathogen suppression and growth promotion, and to track indigenous and introduced rhizobacteria in the field. For example, probes and primers specific for sequences within *phlD* have been used to monitor the population dynamics of 2,4-DAPG producers in take-all suppressive and conducive soils (26,27) and in the rhizosphere of maize (21). Studies of the genotypic diversity of 2,4-DAPG producers by different techniques, including BOX-PCR, ERIC-PCR, ARDRA, RAPD, and RFLP analyses, have revealed a substantial number of distinct groups (11,17,21,32). Genomic fingerprinting with repetitive sequence-based (rep)-PCR, which has been used to analyze the diversity of a wide variety of bacterial taxa (14), revealed 13 and 15 distinct groups of *phlD* isolates by the BOXAIR and ERIC primers, respectively (17). Our present study extended the genotypic analysis of a large collection of 2,4-DAPG producers to include RAPD analysis of the whole genome and RFLP analyses of the *phlD* gene, which encodes a protein necessary for the biosynthesis of phloroglucinol compounds (2,3). It was not unexpected that the diversity among *phlD* strains, as detected by RAPD analysis, was consistent with that defined by rep-PCR because both are measures of the overall genomic structure. However, we were surprised to find that the genotypic groups identified by RFLP analysis of *phlD* correlated nearly perfectly with those identified by BOX-PCR (17) (Table 3). These two types of analysis, one focused on the entire genome and the other on a single gene, strongly suggest that the *phlD* gene evolved in concert with the rest of the bacterial genome. This observation argues against frequent horizontal transfers of the *phl* locus between strains and should lessen concern about the potential for transfer of the locus to other rhizosphere bacteria when 2,4-DAPG producers are applied as biocontrol agents.

RAPD analysis revealed the highest degree of polymorphism among the strains studied. Five of the groups (B, D, E, F, and J) defined by *phlD*$^+$ RFLP and BOX-PCR were divided into 17

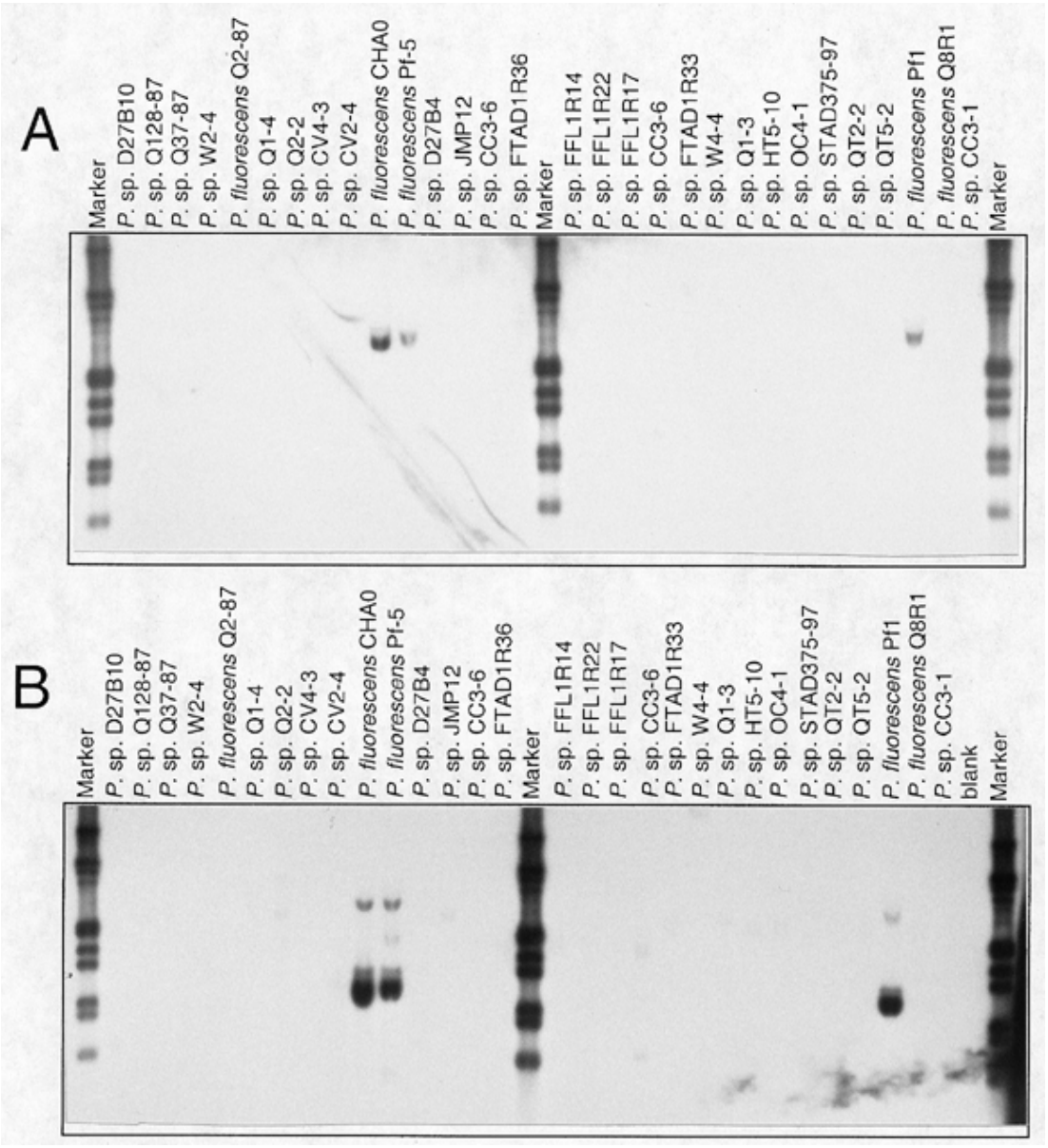

Fig. 3. Southern hybridization of DNA from *phlD* strains with probes for pyoluteorin and pyrrolnitrin biosynthetic loci. Total genomic DNA from each strain was digested with *Eco*RI and *Pst*I restriction endonucleases, separated by electrophoresis in a 0.8% agarose gel, and transferred onto a BrightStar-Plus nylon membrane. The blots were hybridized with biotin-labeled probes targeting **A,** *pltB* or **B,** *prnC*. The *plt* and *prn* probes were prepared, respectively, from the 440-bp polymerase chain reaction (PCR) amplified with Plt1 and Plt2 or the 719-bp PCR product amplified with PrnCf and PrnCr from *Pseudomonas fluorescens* strain Pf-5.

groups by RAPD analysis. The larger number of genotypes distinguished by the RAPD assay as compared with the two other assays was somewhat unexpected. However, Pooler et al. (24) observed a similar situation when studying the genetic diversity of 25 isolates of *Xanthomonas fragariae* by RAPD, ERIC-PCR, and REP-PCR. In that study, RAPD assays also revealed more genotypic diversity than rep-PCR (24). Both methods clearly provide a high degree of discrimination in analyses of population structure, but based on our experience with *phlD Pseudomonas* spp., we agree with Louws et al. (14) that rep-PCR genomic fingerprinting analyses are more amenable to whole-cell PCR and provide more consistent results than standard RAPD analyses. We anticipate that these genomic fingerprints or the associated *phlD* RFLP profiles will be useful to predict the relative ability of a 2,4-DAPG producer to suppress certain diseases or to colonize certain host crops. By matching the crop or crop variety with the appropriate *phlD*$^+$ genotype, it may be possible to achieve consistent and effective biological control or growth promotion at lower inoculum doses than currently applied.

Previous studies (11,32) have differentiated 2,4-DAPG-producing *Pseudomonas* spp. into two groups based on the number of antifungal metabolites produced. The first group, consisting of strains isolated from tobacco, tomato, cucumber, and cotton, produces 2,4-DAPG, hydrogen cyanide, and pyoluteorin. In contrast, members of the second group produce only 2,4-DAPG and hydrogen cyanide. We screened our collection of *phlD* isolates for the pyoluteorin and pyrrolnitrin biosynthetic genes to determine whether the ability to produce these other metabolites is widely distributed within a diverse spectrum of 2,4-DAPG producers. We used a genetic approach for this purpose, rather than direct analysis of the metabolites themselves, to avoid potential effects of culture conditions on synthesis. We were surprised to find that the distribution of these genes was very limited within our collection of isolates from widely distributed soils and soils with long histories of agricultural cropping. The clear presence of *plt* and *prn* genes only with strains belonging to group A, as defined by *phlD*-RFLP, genomic RAPD, BOX-PCR, and ARDRA analyses, may indicate that the co-occurrence of these biosynthetic pathways is limited to this specific group of 2,4-DAPG producers.

Another important goal of our study was to determine the relationship between genotype and 2,4-DAPG production. Our HPLC analyses of metabolites from 30 strains representing 13 distinct *phlD*-RFLP genotypes demonstrated that except for strains of group A (which consistently did not produce phloroglucinol compounds at detectable levels under the culture conditions used), there was no clear association between *phlD*-RFLP genotype and 2,4-DAPG production, because most strains produced similar amounts of phloroglucinol compounds regardless of genotype. Notable exceptions included strains CV4-3 and CC3-1, which produced significantly more phloroglucinol compounds than other strains. Keel et al. (11) also did not detect production of phloroglucinol compounds when strains CHA0, Pf1, and Pf-5 were grown on KMB agar. This medium may not be optimal for the production of phloroglucinol compounds or the compounds may be degraded. In either case, we think that the amounts detected in vitro are unlikely to be indicative of the biologically active levels produced in nature. For example, in the case of *P. fluorescens* strain CHA0, 2,4-DAPG was not produced in our in vitro assays but it has been recovered from the rhizosphere of tomato plants inoculated with CHA0 (6). In our laboratories, studies are in progress to determine the relationship between production of 2,4-DAPG in situ and the biocontrol activity of different *phlD* genotypes in the rhizosphere of crop plants (B. McSpadden Gardener and D. M. Weller, *unpublished data*).

Our results, like those of McSpadden Gardener et al. (17), indicate that in most cases a single *phlD* genotype predominated among isolates from each soil. Although multiple genotypes were observed among isolates from the Fargo, Quincy, Caldwell, Lind, and Woensdrecht soils, one genotype was still predominant. The presence of multiple genotypes in some soils can be explained, in part, by different isolation techniques for strains obtained from the Quincy site, and by different cropping histories for soils obtained from Fargo and Woensdrecht (17). Because differences in the *phlD* gene reflect the overall genetic diversity among 2,4-DAPG-producing strains from soils of different geographical origins and cropping histories, we conclude that *phlD* itself can be used to study the genetic diversity and population structure of such strains. Recently, a rapid PCR-based technique was developed in our laboratory to quantify population sizes of *phlD* strains in rhizosphere samples (16). With this technique it will be possible to rapidly determine the genotype of the most dominant 2,4-DAPG producers in a sample by PCR-RFLP analyses similar to those described here.

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Erratum

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In the manuscript entitled "Genetic Diversity of phlD from 2,4-Diacetylphloroglucinol-Producing Fluorescent *Pseudomonas* spp." by O. V. Mavrodi, B. B. McSpadden Gardener, D. V. Mavrodi, R. F. Bonsall, D. M. Weller, and L. S. Thomashow (Phytopathology 91:35-43), Figures 1 and 2 are transposed. The correct image of the random amplified polymorphic DNA (RAPD) cluster analysis and fingerprint patterns generated from genomic DNA of phlD-containing *Pseudomonas* spp. strains is shown in Figure 2. An illustration of the cluster analysis of restriction fragment length polymorphism (RFLP) patterns obtained by digestion of a 745-bp phlD fragment is shown in Figure 1.