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Differential Ability of Genotypes of 2,4-Diacetylphloroglucinol-Producing *Pseudomonas fluorescens* Strains To Colonize the Roots of Pea Plants

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Indigenous populations of 2,4-diacetylphloroglucinol (2,4-DAPG)-producing fluorescent *Pseudomonas* spp. that occur naturally in suppressive soils are an enormous resource for improving biological control of plant diseases. Over 300 isolates of 2,4-DAPG-producing fluorescent *Pseudomonas* spp. were isolated from the rhizosphere of pea plants grown in soils that had undergone pea or wheat monoculture and were suppressive to *Fusarium* wilt or take-all, respectively. Representatives of seven genotypes, A, D, E, L, O, P, and Q, were isolated from both soils and identified by whole-cell repetitive sequence-based PCR (rep-PCR) with the BOXA1R primer, increasing by three (O, P, and Q) the number of genotypes identified previously among a worldwide collection of 2,4-DAPG producers. Fourteen isolates representing eight different genotypes were tested for their ability to colonize the rhizosphere of pea plants. Population densities of strains belonging to genotypes D and P were significantly greater than the densities of other genotypes and remained above log 6.0 CFU (g of root)$^{-1}$ over the entire 15-week experiment. Genetic profiles generated by rep-PCR or restriction fragment length polymorphism analysis of the 2,4-DAPG biosynthetic gene *phlD* were predictive of the rhizosphere competence of the introduced 2,4-DAPG-producing strains.

Root colonization is a process by which bacteria introduced on seeds, vegetatively propagated plant parts, or into soil become distributed along roots (61). This process includes colonization of any or all parts of the rhizosphere environment: inside the root, on the root surface (rhizoplane), and in the rhizosphere soil. Plant-associated bacteria that are able to colonize and persist in the rhizosphere are known collectively as rhizobacteria, and those that also improve plant growth are called plant growth-promoting rhizobacteria (PGPR) (21). Growth promotion occurs as a result of direct stimulation of the plant, inhibition of plant pathogens, and/or induction of host defense mechanisms against pathogens (54, 56).

Root colonization has been the subject of intense research during the past two decades because variable colonization remains one of the major impediments to the widespread use of PGPR in agriculture (59). In order to be effective, PGPR must establish and maintain threshold population densities in the rhizosphere environment (4, 17, 39, 41). However, in most cases, the high population densities initially established on roots decline with time and distance from the inoculum source, and the introduced strain comprises a progressively smaller proportion of the total rhizosphere microflora (2, 22, 28, 58, 61). Threshold population densities can be maintained longer by applying larger initial doses of bacteria to the seed or soil (4), but this usually is not economically feasible. Studies designed to identify approaches that enhance PGPR establishment, spread, and survival in the rhizosphere have focused on the impact of biotic and abiotic soil factors (15, 35) and host genotype (50, 61) on colonization. The traits and genes that contribute to root-colonizing ability have also been the subject of intense study (10, 26, 31).

Strains of *Pseudomonas fluorescens* that produce the polyketide antibiotic 2,4-diacetylphloroglucinol (2,4-DAPG) are some of the most effective PGPR controlling root and seedling diseases. For example, *P. fluorescens* CHA0 suppresses black root rot of tobacco, crown and root rot of tomato, *Pythium* damping-off of cucumber, and take-all of wheat (12, 18, 47, 53); *P. fluorescens* F113 suppresses *Pythium* damping-off of sugar beet and cyst nematode and soft rot of potato (6, 7, 48); and *P. fluorescens* Q2-87 and Q8r1-96 suppress take-all of wheat (37, 40, 41). In some soils, 2,4-DAPG producers also play a key role in the natural suppression of take-all disease of wheat, known as take-all decline (38, 40, 42).

The 2,4-DAPG biosynthesis locus contains six genes, *phlA*, *phlB*, *phlC*, *phlD*, *phlE*, and *phlF*, coding for the regulation, synthesis, and putative export of 2,4-DAPG (3). The genes are conserved among 2,4-DAPG-producing pseudomonads isolated from soils worldwide (11, 19, 29, 36, 42). Whole-cell repetitive sequence-based (rep)-PCR with the BOXA1R primer (BOX-PCR) distinguished over a dozen distinct genotypes (A through N) within a worldwide collection of 2,4-DAPG-producing fluorescent *Pseudomonas* spp. (33), and ran-

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dom amplified polymorphic DNA (RAPD) analyses with a variety of primers revealed even greater genetic diversity than found by rep-PCR or amplified 16S ribosomal DNA (rDNA) restriction analysis (ARDRA) (19, 29, 41, 49). However, restriction fragment length polymorphism (RFLP) analysis of *phlD* resulted in genotypic groupings that correlated very well with those established by other methods, including rep-PCR, ARDRA, RAPD analysis, and partial sequencing of *phlD* (29, 44, 57).

Producers of 2,4-DAPG differ considerably in biocontrol activity and rhizosphere competence, and only now is the relationship between the genotype of an isolate and its biological activity being explored. Sharifi-Tehrani et al. (49) compared the biocontrol activity of a collection of 2,4-DAPG-producing fluorescent *Pseudomonas* spp. belonging to either group 1 (produces pyoluteorin and 2,4-DAPG) or group 2 (produces only 2,4-DAPG) as defined by ARDRA (19). As a group, ARDRA group 2 strains were more effective than ARDRA group 1 strains against *Fusarium* crown and root rot of tomato and *Pythium* damping-off of cucumber. Raaijmakers and Weller (41) examined the relationship between genotype and root colonization by natural indigenous populations of 2,4-DAPG-producing fluorescent *Pseudomonas* spp. isolated from roots of wheat grown in a take-all decline soil from Quincy, Wash. Although the isolates belonged to 16 different RAPD groups, representatives of one group accounted for 50% of the 2,4-DAPG producers recovered. When individual isolates were introduced into a virgin soil, the rhizosphere competence of *P. fluorescens* Q8r1-96, a member of the dominant RAPD group, was significantly greater than that of two other *P. fluorescens* isolates belonging to other genotypes. On the basis of these studies, they hypothesized that particular genotypes of 2,4-DAPG producers preferentially colonize the rhizosphere of certain crop plants.

The objective of this study was to determine the relationship between the genotype of a 2,4-DAPG producer and its ability to colonize pea plant roots. We show that isolates belonging to BOX-PCR genotypes D and P are significantly more aggressive colonists of the rhizosphere of pea plants than isolates of other genotypes. Furthermore, genetic profiles generated by rep-PCR or RFLP analysis of *phlD* were shown to be predictive of the rhizosphere competence of introduced 2,4-DAPG-producing strains on pea plants.

MATERIALS AND METHODS

Soils and plants. Soils were obtained from a field located at the Washington State University Research and Extension Center, Mount Vernon, Wash., and from an agricultural field and a noncropped site near Quincy, Wash. The Mount Vernon field (Puget silt loam) is suppressive to *Fusarium* wilt of pea plants (FWS) caused by *Fusarium oxysporum* f. sp. *pisi* (16), and by 1998 the field had been planted for over 30 continuous years with pea varieties as part of a breeding program. The Shano loam agricultural soil from Quincy, designated the Quincy take-all decline (TAD) soil, is suppressive to take-all of wheat, and the Shano sandy loam, designated the Quincy virgin soil, supports native vegetation (41, 42). The FWS and Quincy TAD soils were collected in March 1995, and the Quincy virgin soil was collected in May of 2000. Soils were collected from the upper 30 cm of the soil profile, air dried for 1 week, passed through a 0.5-cm-mesh screen, and stored at room temperature.

Pea (*Pisum sativum* L.) cultivars used in this study included Charo, Little Marvel, Rex, and WSU 31. 'Charo' is resistant to races 5 and 6 of *F. oxysporum* f. sp. *pisi*, 'Little Marvel' is susceptible to races 1, 2, 5, and 6, 'Rex' is susceptible to race 1 and moderately resistant to race 2, and 'WSU 31' is completely resistant to races 1, 2, 5, and 6 (13, 55). Initial studies were conducted with 'Little Marvel' and 'WSU 31', but the seed became difficult to obtain, so the commercial cultivars Charo and Rex were used in field experiments. The spring wheat (*Triticum aestivum* L.) cultivar Alpowa was used throughout the study.

Bacterial strains and growth media. The fluorescent *Pseudomonas* strains used in this study are listed in Table 1. Reference strains were well-characterized biocontrol agents or were characterized previously in studies of the genotypic and phenotypic diversity of 2,4-DAPG producers (29, 33). Our collection also includes strain STAD384-97 (BOX genotype C), a reference strain isolated from roots of wheat grown in a long-term monoculture wheat field in Stillwater, Okla., in 1997. All of the strains harbor *phlD*, a key gene in the biosynthesis of 2,4-DAPG. The presence of *phlD* in a strain correlates with the ability to produce 2,4-DAPG, and thus the terms *phlD*$^+$ and 2,4-DAPG producer are used interchangeably. *P. fluorescens* 2-79 (60), which does not contain genes for the biosynthesis of 2,4-DAPG, was used as a negative control in some PCR assays.

All strains were routinely cultured on a modified semiselective medium for fluorescent *Pseudomonas* spp., consisting of one-third-strength King's B medium (1/3× KMB) (20) supplemented with ampicillin (40 μg/ml), chloramphenicol (13 μg/ml), and cycloheximide (100 μg/ml) (1/3× KMB^{+++}) (32) at 25°C. Indigenous *phlD*$^+$ fluorescent pseudomonads were isolated from the rhizosphere on 1/3× KMB^{+++} agar. Introduced *Pseudomonas* strains used in the colonization studies were spontaneous rifampin-resistant mutants selected by plating wild-type strains on 1/3× KMB^{+++} agar supplemented with rifampin (100 μg/ml) as previously described (30). Wild-type strains and their respective rifampin-resistant mutant derivatives were similar phenotypically. Introduced strains were recovered and quantified in 1/3× KMB^{+++} broth supplemented with rifampin (100 μg/ml) as described below. Frozen stock cultures of all strains were stored in 1/3× KMB^{+++} plus 18% glycerol at −80°C.

Isolation of indigenous 2,4-DAPG-producing *Pseudomonas* spp. from roots of pea and wheat plants. The majority of indigenous *phlD*$^+$ fluorescent *Pseudomonas* isolates from the rhizosphere of growth chamber- or field-grown pea plants were obtained by procedures similar to those described by Raaijmakers et al. (42) and McSpadden Gardener et al. (32). In growth chamber studies, 'Little Marvel' and 'WSU 31' seeds were pregerminated at 20°C on moist filter paper for 5 days before sowing. Four pregerminated seeds were planted in square polyvinyl chloride pots (8 cm high, 7.5 cm wide) containing 200 g of sieved soil. Both cultivars were planted in FWS soil, but only 'Little Marvel' was planted in Quincy TAD soil. Each pot received 50 ml of water supplemented with metalaxyl (Novartis, Greensboro, N.C.) at 2.5 mg of active ingredient ml^{-1} to control *Pythium* root rot. *Pythium* spp. are indigenous in these soils and cause damping-off if not controlled. A 1.5-cm layer of soil was spread over the pregerminated seeds. Each cultivar was replicated four times, and each pot served as a replicate.

Plants were grown in a growth chamber at 15 ± 1°C and 40 to 60% relative humidity (RH), with a 12-h photoperiod. Pots were watered as needed and received 1/3-strength Hoagland solution (macroelements only) twice weekly. After 4 weeks, four plants were harvested from each pot and pooled, and samples of approximately 1 g of root (fresh weight) plus tightly adhering soil were prepared as described below to determine the population size of indigenous *phlD*$^+$ fluorescent *Pseudomonas* spp. The soil and roots not assayed from each pot were decanted into a plastic bag, mixed by shaking, returned to the same pot, and again planted with four pregerminated pea seeds. This process of growth, harvesting, and determination of population sizes was repeated for a total of eight cycles for plants in FWS soil or five cycles for plants in Quincy TAD soil.

In field experiments, strips (3.6 m by 15 m) of pea cv. Charo (168 kg/ha) and wheat cv. Alpowa (112 kg/ha) were sown in 1999 in a section of the FWS field, and the plots were resown to the same crop in 2000. Seed was sown in mid-April, and plants were sampled at approximately 6-week intervals. Weeds were removed by hand, and insecticides and fertilizers were applied in accordance with local recommendations.

To determine the population densities of indigenous *phlD*$^+$ fluorescent *Pseudomonas* spp. in the rhizospheres of growth chamber- or field grown-plants, 1 g (fresh weight) of roots with associated rhizosphere soil was placed in a 50-ml screw-cap centrifuge tube containing 10 ml of sterile water. The tube was agitated vigorously for 1 min on a Vortex mixer and then sonicated in an ultrasonic bath for 1 min. In growth chamber studies, population densities of *phlD*$^+$ fluorescent *Pseudomonas* spp. in root washings were determined by dilution plating on 1/3× KMB^{+++} agar and colony hybridization as described by Raaijmakers et al. (42). Population densities of *phlD*$^+$ *Pseudomonas* spp. in root washings from field samples collected in 1999 and 2000 were determined by dilution plating on 1/3× KMB^{+++} and colony hybridization (42) and the *phlD*-specific PCR-based dilution endpoint method described by McSpadden Gardener et al. (32), respectively (see details below). Both methods detect similar population densities, but the PCR-based method allows more rapid analysis of samples (23).

TABLE 1. Bacterial strains and isolates used in this study

Prefix or isolate[a]	Isolate no(s).	BOX-PCR genotype[b]	Source	Sample[c]	Reference(s)
Prefix					
1MA	1, 2	L	Pea cv. Little Marvel	1997, GC, FWSS	This study
4MA	30, 32	D	Pea cv. Little Marvel	1997, GC, FWSS	This study
	1, 2, 3, 6, 10, 12, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 31, 33, 34	P	Pea cv. Little Marvel		
5MA	1, 2, 3, 4	D	Pea cv. Little Marvel	1997, GC, FWSS	This study
6MA	14	D	Pea cv. Little Marvel	1997, GC, FWSS	This study
	2, 4, 9, 11, 12, 16, 17, 18, 20	P	Pea cv. Little Marvel		
7MA	12, 13, 17, 20, 23	O	Pea cv. Little Marvel	1997, GC, FWSS	This study
	1, 6, 7, 8, 9, 10, 11, 14, 15, 16, 18, 21, 24, 25, 26, 27	P	Pea cv. Little Marvel		
8MA	3, 8, 15, 16, 17, 18, 19, 20, 21, 22	P	Pea cv. Little Marvel	1997, GC, FWSS	This study
1WSU	1, 3, 4, 5, 6, 7	D	Pea cv. WSU 31	1997, GC, FWSS	This study
2WSU	1, 2, 3, 4, 5, 7, 10, 11, 12, 13, 14, 15, 17, 18	D	Pea cv. WSU 31	1997, GC, FWSS	This study
	6	P	Pea cv. WSU 31		
3WSU	1, 2, 3, 7, 10, 12, 13, 14, 15, 16, 18	D	Pea cv. WSU 31	1997, GC, FWSS	This study
	5, 9, 11, 19	P	Pea cv. WSU 31		
5WSU	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 16, 17, 18, 19, 20, 21, 22, 23, 24, 26, 28	D	Pea cv. WSU 31	1997, GC, FWSS	This study
6WSU	18	D	Pea cv. WSU 31	1997, GC, FWSS	This study
	1, 2, 3, 4, 5, 7, 10	P	Pea cv. WSU 31		
7WSU	6, 7, 8	D	Pea cv. WSU 31	1997, GC, FWSS	This study
8WSU	15	D	Pea cv. WSU 31	1997, GC, FWSS	This study
	5, 6	P	Pea cv. WSU 31		
2RP	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29	D	Pea cv. L. Marvel	1997, GC, QTADS	This study
4RP	5, 6, 7, 8	D	Pea cv. L. Marvel	1997, GC, QTADS	This study
5MR	1, 3, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 21, 22, 24, 25, 26, 27, 29, 30	D	Pea cv. L. Marvel	1997, GC, QTADS	This study
	2, 4, 28	E	Pea cv. L. Marvel	1997, GC, QTADS	This study
MVP	1-3	A	Pea cv. Charo	1999, F, FWSS	This study
	1–6, 4-1, 4-2, 4-3, 4-4, 4-5, 4-6, 4-7, 4-8, 4-9, 4-10, 4-11, 4-12, 4-13, 4-14, 4-15, 4-16, 4-19, 4-25, 4-26, 4-27, 4-28, 4-29, 4-30, 4-31, 4-32, 4-33, 4-34, 4-35, 4-37, 4-38, 4-39, 4-40, 4-44, 4-45, 4-46, 4-47, 4-48, 4-49, 4-50, 4-51, 4-52, 4-53, 4-54, 4-55, 4-56, 4-57, 4-58, 4-59, 4-60, 4-61, 4-62, 4-63, 4-64, 4-65, 4-66, 4-67, 4-68, 4-69, 4-70, 4-71, 4-72, 4-73, 4-74, 4-75, 4-76, 4-77, 4-78, 4-79, 4-80	D	Pea cv. Charo	1999, F, FWSS	This study
	6-1	D	Pea cv. Charo	2000, F, FWSS	This study
	1-4, 1-5	P	Pea cv. Charo	1999, F, FWSS	This study
MVW	1-2, 1-4, 2-1, 2-2, 2-3, 2-4, 2-5, 2-6, 2-7, 2-8, 2-9, 2-10, 2-11, 2-12, 2-14, 2-15, 2-16, 2-17, 2-18, 2-19, 2-20, 2-21, 2-22, 2-24, 4-5, 4-6, 4-7, 4-8	D	Wheat cv. Alpowa	1999, F, FWSS	This study
	6-1, 6-2, 6-3, 6-4, 6-5, 6-6, 6-7, 7-4, 7-5, 7-6, 7-9, 7-11, 7-12, 7-13, 7-14, 7-15, 7-16		Wheat cv. Alpowa	2000, F, FWSS	This study
	1-1, 1-3	P	Wheat cv. Alpowa	1999, F, FWSS	This study
	4-2, 4-3, 4-4	Q	Wheat cv. Alpowa	1999, F, FWSS	This study
Reference strains					
Pf-5		A			14
CHA0		A			19, 33
Q2-87, Q2-1		B			33
STAD384-97		C			This study
FFL1R9, FTAD1R34, OC4-1, Q2-5, Q8V8, Q8r1-96, Q8r10-96, Q128-87, QT1-5, W2-6		D			33

Continued on following page

TABLE 1—*Continued*

Prefix or isolate[a]	Isolate no(s).	BOX-PCR genotype[b]	Source	Sample[c]	Reference(s)
Q2-2, Q37-87, QT1-6		E			19, 33
JMP6, JMP7		F			33
FFL1R18		G			33
CV1-1		H			33
FTAD1R36		I			33
FFL1R22, CC3-1		J			33
F113		K			48
W4-4		L			33
PILH1, D27B1		M			33
HT5-1		N			33

[a] Fluorescent *Pseudomonas* spp. from pea cvs. Little Marvel WSU 31 grown in Mount Vernon FWS soil in growth chamber experiments were designated *x*MA*y* and *x*WSU*y*, respectively. Isolates from pea cv. Little Marvel grown in Quincy TAD soil in growth chamber experiments were designated *x*RP*y* or *x*MR*y*. Isolates from pea cv. Charo or wheat cv. Alpowa grown in the field were designated MVP*z-y* and MVW*z-y*, respectively. *x* represents the cycle number, *z* represents the sampling time, and *y* represents the isolate number.

[b] Genotype identified by rep-PCR with the BOXA1R primer.

[c] The year of isolation is given. GC, growth chamber experiments; F, field experiments; FWSS, *Fusarium* wilt-suppressive soil; QTADS, Quincy take-all decline soil.

Representative *phlD*$^+$ *Pseudomonas* isolates from each sample were stored in 35% glycerol at −80°C. Isolates from cvs. Little Marvel and WSU 31 grown in FWS soil in growth chamber experiments were designated *x*MA*y* and *x*WSU*y*, respectively, and isolates from 'Little Marvel' grown in Quincy TAD soil were designated *x*RP*y* and *x*MR*y*, respectively, where *x* represents the cycle number and *y* the isolate number. Isolates from pea cv. Charo or wheat cv. Alpowa grown in the field were designated MVP*z-y* and MVW*z-y*, respectively, where *z* represents a sampling time and *y* the isolate number.

Genotypic fingerprinting of indigenous DAPG-producing *Pseudomonas* spp. A whole-cell rep-PCR fingerprinting method with the BOXA1R primer (BOX-PCR) described previously by McSpadden et al. (33) was used to determine the genotype of over 300 *phlD*$^+$ fluorescent *Pseudomonas* isolates from growth chamber and field experiments. The oligonucleotide primer was synthesized by Operon Technologies, Alameda, Calif. DNA was isolated from whole-cell cultures of fluorescent *Pseudomonas* isolates frozen at −80°C for a minimum of 1 h in sterile distilled water. PCR amplification was performed as described previously (33) in a 20-μl reaction volume containing 5 μl of thawed whole-cell template. Negative control mixtures (no cell lysate) and positive control mixtures (cell lysates from *phlD*$^+$ strains Pf-5, F113, Q2-87, and Q8r1-96) were included in each set of amplification reactions. Analyses were replicated by amplifying at least two independent samples of each strain or isolate on separate occasions.

Amplification was performed with a PTC-200 thermal cycler (MJ Research Inc., Watertown, Mass.), and the resulting PCR products were separated in 1.5% agarose gels at 8°C in 0.5× Tris-borate-EDTA buffer (46) for 6 h at 140 V. A mixture of 1-kb and 0.1-kb DNA ladders (New England Biolabs, Beverly, Mass.) was included in order to normalize the banding pattern of BOX-PCR profiles. DNA banding patterns were visualized by ethidium bromide staining, photographed under UV light, and recorded with a Kodak DC120 digital imaging system (Eastman Kodak Co., Rochester, N.Y.). The digitalized images of BOX-PCR fingerprints were converted, normalized, combined, and analyzed with GelCompar 4.0 software (Applied Maths, Kortrijk, Belgium). To analyze BOX-PCR patterns, a similarity matrix of the whole densitometric curves of the gel tracks was calculated by using the pairwise Pearson's product-moment correlation coefficient (*r* value) (43). Clusters of BOX-PCR patterns were defined by using the 95th percentile (near-minimum) similarity coefficient of replicate assays for identical strains (33). BOX-PCR genotypes of *phlD*$^+$ fluorescent *Pseudomonas* isolates indigenous to Mount Vernon soil were determined by comparison of their fingerprints to the database described by McSpadden et al. (33).

RFLP analyses of the 629-bp *phlD* fragment amplified with primers B2BF and BPR4 from *phlD*$^+$ isolates were performed with restriction enzymes *Hae*III, *Msp*I, and *Taq*I (New England Biolabs, Beverly, Mass.) as described previously (32). RFLP patterns were digitalized, converted, and normalized with a 100-bp molecular size marker and compared with GelCompar software. A band-matching algorithm (band-matching tolerance of 0.75%) was selected to calculate pairwise similarity matrices with the Dice coefficient. Cluster analysis of similarity matrices was performed by the unweighted pair group method using arithmetic averages (UPGMA) (52). Two independent amplifications were done for each strain.

Determination of rhizosphere competence. Fourteen *phlD*$^+$ strains representing eight genotypes and including reference strains and isolates recovered from pea plant roots grown in Mt. Vernon FWS soil and Quincy TAD soil were compared for their ability to colonize the rhizosphere of pea plants. Ten strains from five previously defined (29, 33) BOX-PCR genotypes [Pf-5 (A genotype); Q2-1 and Q2-87 (B genotype); MVP1-6, MVW1-2 and Q8r1-96 (D genotype); 5MR2 and QT1-6 (E genotype); and W4-4 and 1MA1 (L genotype)] and four isolates from three new genotypes [7MA12 (O genotype); MVW1-1 and MVP1-4 (P genotype); and MVW4-2 (Q genotype)] were used (Table 1).

To increase the probability that the 14 *phlD*$^+$ isolates selected were nonclonal, we chose representatives collected from different years, soils, or host crops and from field and greenhouse studies. Strains Q2-1, Q2-87, Q8r1-96, and QT1-6 were isolated from roots of wheat and 5MR2 from roots of pea plants grown in Quincy TAD soil in different years; MVP1-4, MVP1-6, MVW1-2, 1MA1, 7MA12, MVW1-1, and MVW4-2 were isolated from roots of pea or wheat plants grown in the FWS soil; W4-4 was isolated from a TAD soil near Lind, Wash.; and Pf-5 (14) was from the roots of cotton grown in Texas (Table 1). Bacteria were grown on 1/3× KMB^{+++} agar for 48 h at 25°C. Cells were washed in water by centrifugation (8,000 × *g* for 10 min) and suspended in sterile distilled water. Cell densities were determined by turbidity at A_{600} with a microplate spectrophotometer (Dynatech MR5000; Dynatech Laboratories, Burlington, Mass.), diluted in water, and added to sieved Quincy virgin soil in a 1% methylcellulose suspension (100 ml of suspension per kg of soil) to give approximately 10^4 CFU per g (fresh weight) of soil. The actual density of each strain was determined by assaying 0.5-g portions of inoculated soil as described below.

Five seeds of pea cv. Rex were sown in pots containing 200 g of soil inoculated with one of the bacterial strains. Seeds were covered with a 1-cm layer of noninoculated soil. The control treatment consisted of soil amended with a 1% methylcellulose suspension. Each treatment was replicated six times, with one pot serving as a replicate. Peas were grown for five successive 3-week cycles in a growth chamber at 24 ± 1°C and 40 to 60% RH, with a 12-h photoperiod, and pots were watered and fertilized as described above. Bacteria were introduced into the soil only at the beginning of the first cycle, and one plant was selected randomly from each pot after each cycle to determine the population size of the introduced bacteria. Shoots of the remaining plants were excised, and the soil and associated root systems from all pots of the same treatment were decanted into a plastic bag and mixed by shaking. The soil was returned to the pots and immediately replanted with five pea seeds. At the end of the fifth cycle, the soil from pots of the same treatment was decanted into a plastic bag, stored at 20°C for 3 months, and then added to pots and sown to peas (cv. Rex). Plants were grown in a growth chamber at 24 ± 1°C for 4 weeks, and one plant was selected from each pot to determine the population density of the introduced bacteria.

To enumerate population densities of introduced bacteria, either soil (0.5 g) or roots with tightly adhering soil (loosely adhering soil was removed by gently shaking the roots) were placed in centrifuge tubes with 10 ml of sterile distilled water and vortexed and sonicated as described above. One hundred microliters of the wash solution was serially diluted in a 96-well microtiter plate prefilled with 200 μl of sterile distilled water per well, and then 50 μl of each dilution was

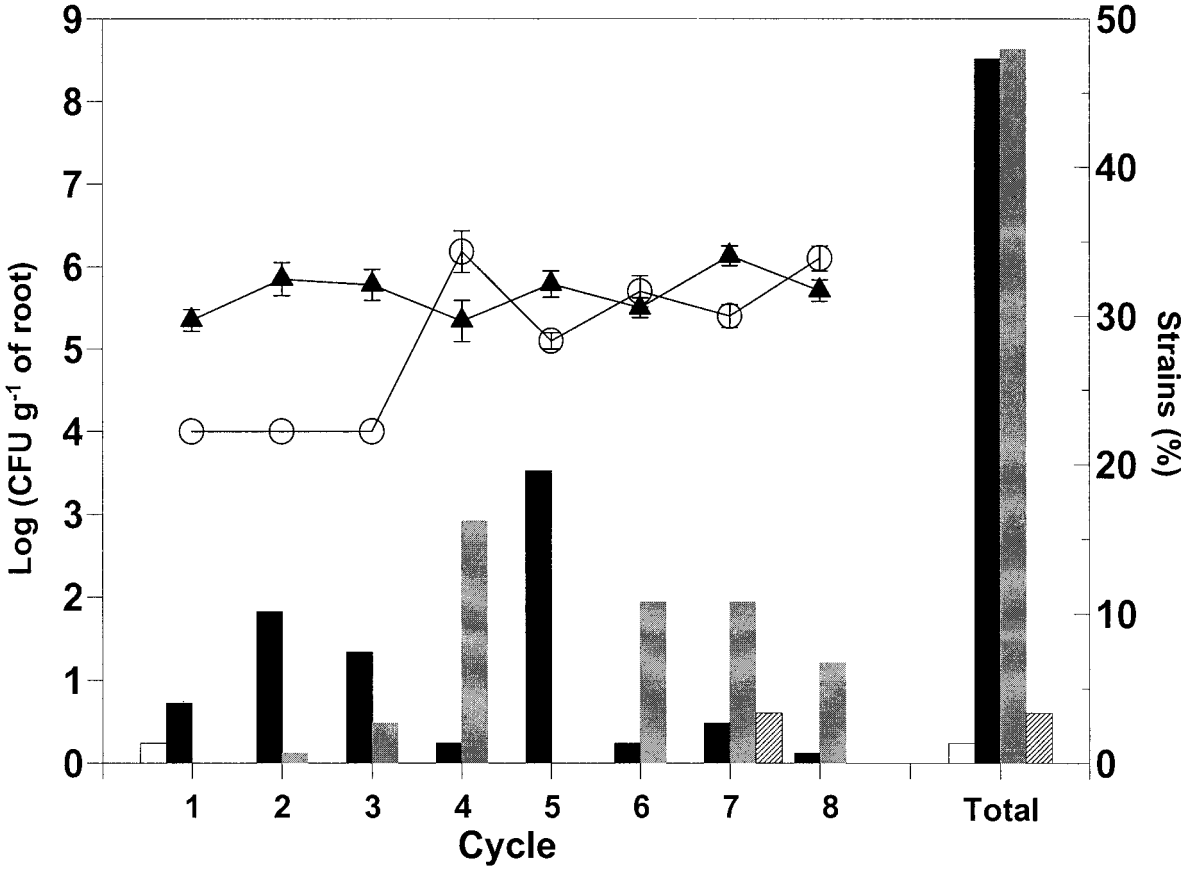

FIG. 1. Mean population densities of different genotypes of indigenous *phlD*$^+$ *Pseudomonas* spp. isolates on the roots of pea cvs. Little Marvel (○) and WSU 31 (■) grown in Mt. Vernon FWS soil for eight successive cycles of 3 weeks each. Mean values and standard deviations are presented. Bars indicate the percentage of *phlD*$^+$ isolates (n = 148) characterized as genotype L (white bars), D (black bars), P (grey bars), and Q (hatched bars) by BOX-PCR.

transferred to a well of a 96-well plate containing 200 μl of fresh 1/3× KMB^{+++} broth with rifampin (100 μg/ml). Microtiter plates were incubated at room temperature in the dark, and bacterial growth was assessed after 72 ± 4 h; an optical density at 600 nm (OD_{600}) of ≥0.05 was scored as positive (32).

Aliquots from the terminal dilution showing growth were confirmed to contain *phlD*$^+$ cells by PCR analysis with primers B2BF and BPR4 (32), and the genotype of the *phlD*$^+$ strain was determined by RFLP analysis of the amplification product after digestion with *Hae*III, *Taq*I, or *Msp*I restriction enzyme as previously described (32). The RFLP patterns generated by digestion with the three enzymes were sufficient to distinguish the previously defined BOX-PCR genotypes A to N (32) and the newly defined genotypes O, P, and Q.

Data analysis. All treatments in the growth chamber experiments were arranged in a randomized complete block design. All population data were converted to log CFU per gram (fresh weight) of soil or root to satisfy the assumptions of the parametric statistical test used. The rhizosphere competence of *phlD*$^+$ *P. fluorescens* isolates in the cycling experiments was determined by calculating four variables indicative of root colonization: (i) mean population density for all cycles; (ii) cycle with the maximum population density; (iii) area under the rhizosphere colonization progress curve (AUCPC), using the trapezoidal integration method (5), which represented the total rhizosphere colonization for all cycles, and (iv) the persistence of the strains, determined as the mean population density on pea plant roots following a 3-month fallow period. Data were analyzed by using STATISTIX (version 7.0; Analytical Software, St. Paul, Minn.). Differences in population densities among treatments were determined by standard analysis of variance, and mean comparisons among treatments were performed by using Fisher's protected least significant difference test at P = 0.05.

RESULTS

Population densities of indigenous *phlD*$^+$ fluorescent *Pseudomonas* spp. in FWS and Quincy TAD soils. The population density of 2,4-DAPG producers in the rhizosphere of 'WSU 31' grown in FWS soil was near or above log 5.5 *phlD*$^+$ CFU (g of root)$^{-1}$ in each of the eight cycles. In contrast, the population density of 2,4-DAPG producers in the rhizosphere of 'Little Marvel' was near or below the detection limit [log 4.0 *phlD*$^+$ CFU (g of root)$^{-1}$] in the first three cycles, but increased to population sizes above log 5.0 *phlD*$^+$ CFU (g of root)$^{-1}$ in subsequent cycles (Fig. 1). 'Little Marvel' grew poorly in the first three cycles due to the presence of an *Aphanomyces* sp. in the soil (data not shown).

When 'Little Marvel' was grown in Quincy TAD soil for five 4-week cycles, the population density of 2,4-DAPG producers detected after the first cycle was log 4.6 *phlD*$^+$ CFU (g of root)$^{-1}$, but increased after subsequent cycles to above log 5.5 *phlD*$^+$ CFU (g of root)$^{-1}$ (data not shown). Population densities of 2,4-DAPG-producing fluorescent *Pseudomonas* spp. on roots of wheat cv. Alpowa and pea cv. Charo averaged log 5.7 and log 5.4 *phlD*$^+$ CFU (g of root)$^{-1}$, respectively, in the field experiment in 1999 and log 5.1 and log 5.8 *phlD*$^+$ CFU (g

of root)$^{-1}$, respectively, in 2000. The population densities of 2,4-DAPG producers on the roots of pea and wheat plants did not differ significantly ($P > 0.17$) in either 1999 or 2000.

Genotypic diversity of 2,4-DAPG producers. One hundred forty-eight *phlD*$^+$ isolates from the cycling experiment in FWS soil (73 from cv. Little Marvel and 75 from cv. WSU 31), 60 isolates from cycling 'Little Marvel' in Quincy TAD soil, and 106 isolates from the 1999 field experiment (33 from wheat cv. Alpowa and 73 from pea cv. Charo) were analyzed by PCR with the BOXA1R primer (Table 1). Amplification products yielded complex genomic fingerprints consisting of fragments ranging in size from 100 to 3,000 bp. From the 314 new *phlD*$^+$ isolates and the 31 *phlD*$^+$ type strains analyzed, 17 distinct genomic clusters were defined based on a 95th percentile similarity coefficient of independent replicates, which was calculated to be 64.6% for this data set. Fourteen of these clusters correlated perfectly with BOX genotypes A through N designated previously by McSpadden et al. (33), and three new BOX genotypes, O, P, and Q, were also identified (Fig. 2) among isolates inhabiting the FWS soil from Mount Vernon, Wash.

The addition of new strains to the cluster analysis caused subtle changes in the topology of the dendrogram shown in Fig. 2 compared to that reported previously (33). For example, more similarity was apparent between clusters A and M and between clusters G and J than was seen in the earlier study (33). The inclusion of genomic fingerprint data from new strains of genotypes A, D, and L, a strain of genotype C, and strains from the new genotypes O, P, and Q could be expected to alter the dendrogram because of the mathematics underlying these types of cluster analyses. However, the consistency with which strains included in both studies were grouped indicates that our approach to defining clusters is particularly robust. Additionally, it should be noted that within the P cluster, two subgroups were identified with a similarity score of 65.1% that was near but still within the value (64.6%) defining identity. Thus, the P genotype may be split into two groups once more representatives of these subgroups are added to our collection.

In previous studies, genotypes defined by DNA polymorphisms within *phlD* correlated well with groupings based on BOX-PCR (29, 32). Similarly, cluster analysis by UPGMA of *phlD* restriction patterns among isolates in this study revealed the same groups (A through Q) identified by analysis of BOX-PCR patterns (Fig. 2 and 3). RFLP analyses of new isolates from the FWS and Quincy TAD soils and belonging to the previously defined BOX-PCR genotypes A, D, E, and L gave the expected RFLP patterns (Fig. 3). However, RFLP analysis of *phlD* fragments from members of the newly defined O, P, and Q BOX-PCR genotypes yielded patterns not observed previously (Fig. 3).

The phylogenetic relationships among genotypes can be inferred to a certain extent from comparisons of the patterns generated by digestion with individual enzymes. For example, BOX genotype O had a unique *Hae*III restriction pattern compared to previously described patterns (32), but digestion with *Taq*I and *Msp*I revealed a number of bands common to members of other genotypes. The *Hae*III restriction patterns of *phlD* from isolates of genotypes Q and I were identical but could be differentiated by digestion with *Taq*I. Members of BOX genotypes P, G, H, J, L, and N had similar *Hae*III restriction patterns. *Taq*I restriction patterns of *phlD* from isolates of those genotypes differentiated all but isolates of BOX genotypes P and J, which required a third digestion with *Msp*I (Fig. 3), as was shown previously to distinguish between isolates of BOX genotypes G and H (32). This lack of polymorphism between certain genotypes indicates a closer phylogenetic relationship than to other distinct genotypes. Interestingly, the patterns of A and B genotype strains, which are known to differ significantly from the other strains based on partial sequencing of the *phlD* gene (32, 44), had the lowest similarity based on this band-based clustering.

Frequency of indigenous *phlD*$^+$ genotypes. Four BOX genotypes (D, L, O, and P) were detected among the 148 *phlD*$^+$ isolates obtained while cycling 'Little Marvel' and 'WSU 31' in FWS soil (Fig. 1, Table 1). The percentage of these genotypes and the mean rhizosphere population sizes of *phlD*$^+$ pseudomonads recovered from each cultivar during the eight growth cycles are presented in Fig. 1. Genotypes D and P occurred at the highest frequency and represented 47.3 and 48.0%, respectively, of the total *phlD*$^+$ isolates collected ($n = 148$). In general, although isolates of genotype D were recovered after each cycle, their frequency diminished after cycle five. In contrast, representatives of genotype P were isolated after the second cycle and subsequently increased in frequency (Fig. 1). Among all the isolates of genotype D ($n = 70$), 10 and 90% were from roots of 'Little Marvel' and 'WSU 31', respectively. In contrast, 83 and 17% of the P isolates ($n = 71$) were from roots of 'Little Marvel' and 'WSU 31', respectively. Genotypes L and O were isolated only from roots of 'Little Marvel', and only in the first and seventh cycle, respectively (Fig. 1).

Although BOX genotypes B, D, and E have been reported in Quincy TAD soil (33), only genotypes D and E were isolated from roots of pea cv. Little Marvel (Table 1). Among 60 isolates analyzed, 95.0 and 5.0% were of genotypes D and E, respectively, and the E genotype isolates were recovered only in the last cycle (Table 1). Raaijmakers and Weller (41) reported that genotype D dominated *phlD*$^+$ populations on wheat plant roots cycled in Quincy TAD soil.

Of 106 *phlD*$^+$ isolates from roots of wheat or pea plants grown in the field in 1999, 92.5, 3.8, 2.8, and 0.9% were of genotypes D, P, Q, and A, respectively. The single genotype A isolate was recovered from pea plants; the three genotype Q isolates were from wheat plants; and of the four genotype P isolates, two (MVP1-4 and MVP1-5) were from pea plants and two (MVW1-1 and MVW1-3) were from wheat plants. D genotype isolates were recovered on all sampling dates and from both crops (Table 1). Samples from the field experiment conducted in 2000 were processed by the *phlD*-specific PCR-based dilution endpoint assay (32). The D genotype was the most abundant among isolates from all samples of both crops (Table 1). Collectively, these results indicate that even when multiple *phlD*$^+$ BOX-PCR genotypes occur in soil, the populations of certain genotypes, in this case D and P, are enriched in the pea plant rhizosphere.

Pea plant root colonization by BOX genotypes. The above findings support our hypothesis, based on the work of Raaijmakers and Weller (41), that particular genotypes of 2,4-DAPG producers preferentially colonize the roots of certain crops. To test this hypothesis further, 14 isolates from eight

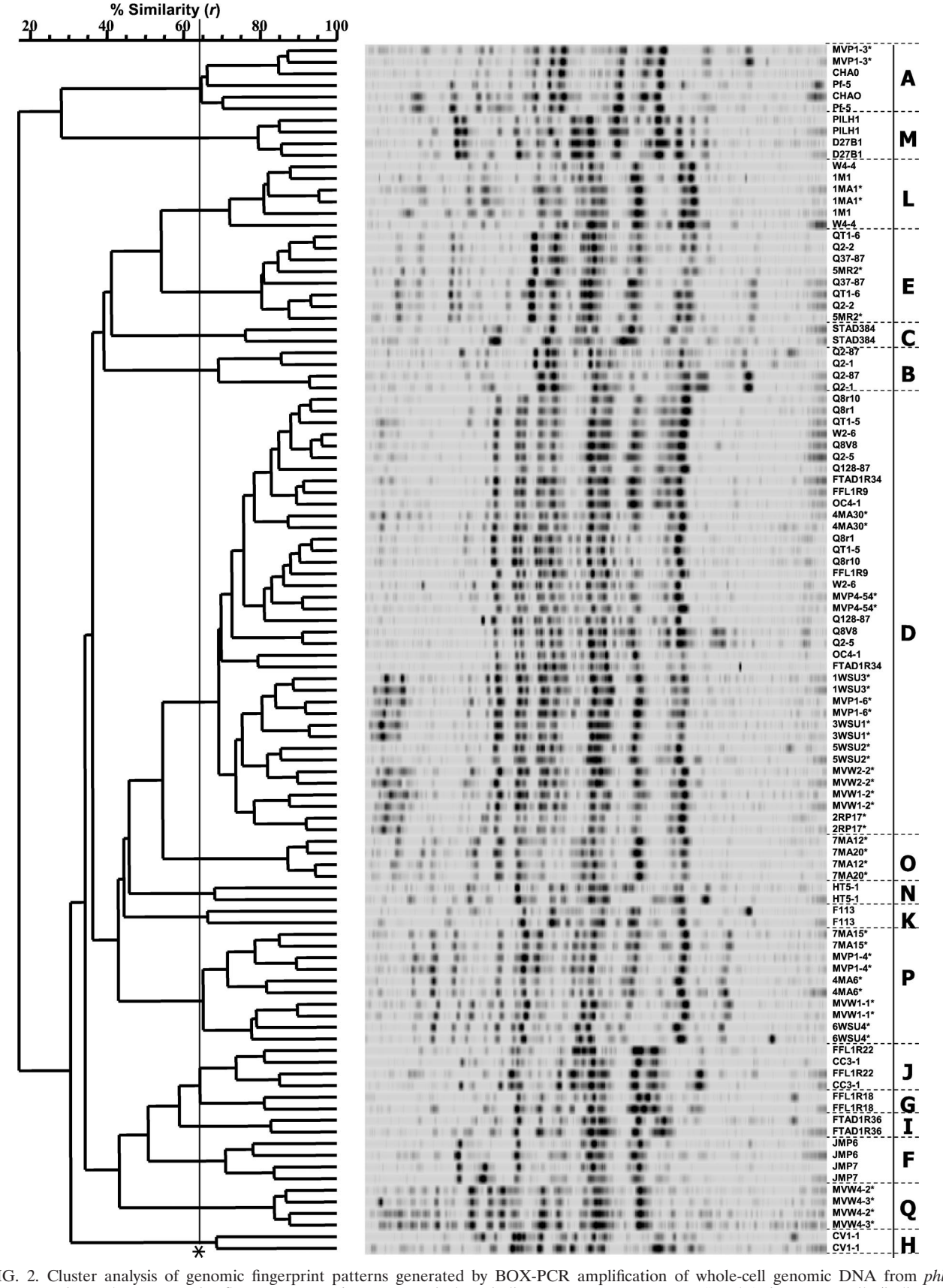

FIG. 2. Cluster analysis of genomic fingerprint patterns generated by BOX-PCR amplification of whole-cell genomic DNA from *phlD*$^+$ reference strains and representative fluorescent *Pseudomonas* spp. isolates indigenous to Mt. Vernon FWS and Quincy TAD soils (indicated by asterisks) (see Table 1). Two independent amplifications were used for each isolate. The UPGMA algorithm was applied to the similarity matrix generated from the tracks of the whole patterns by using the pairwise Pearson's product-moment correlation coefficient (*r* value). The similarity coefficient used to define distinct groups (see Materials and Methods) is noted (*).

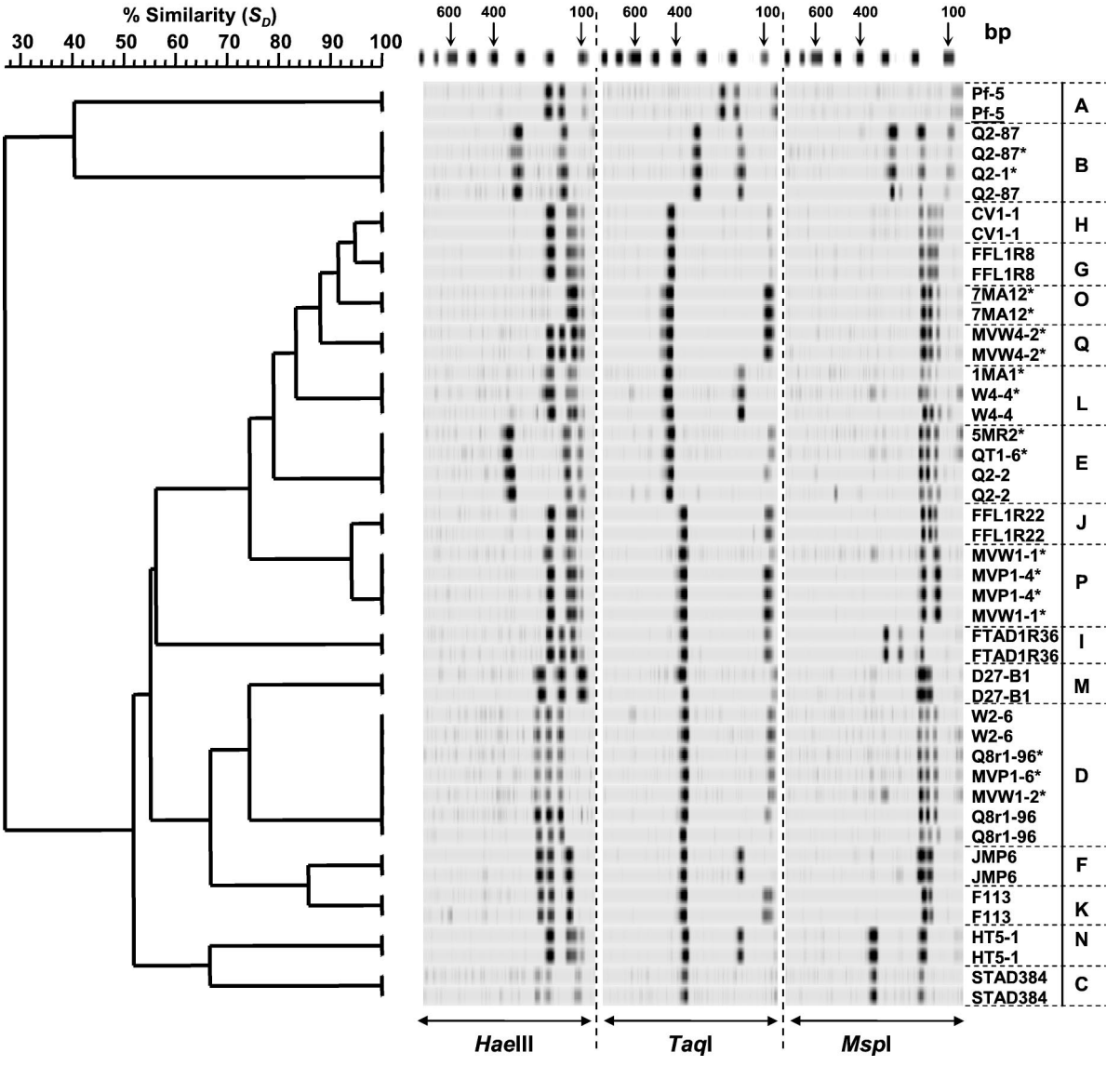

FIG. 3. Cluster analysis of combined *Hae*III, *Taq*I, and *Msp*I RFLP patterns of the 629-bp *phlD* fragment amplified from isolates of fluorescent *Pseudomonas* spp. Patterns include those of reference strains (BOX-PCR genotypes A to N) and isolates used in the cycling experiment (Fig. 4), which are indicated by an asterisks. Two independent amplifications were used for each reference strain. The UPGMA algorithm was applied to the similarity matrix generated with the Dice coefficient. A 100-bp DNA size standard ladder is shown at the top.

different genotypes (A, B, D, E, L, O, P, and Q) were tested in cycling experiments for their ability to colonize the rhizosphere of pea plants. The soil used in these assays contained no or very low frequencies of indigenous *phlD*$^+$ isolates and represents an environment where *phlD*$^+$ isolates will be introduced for biocontrol purposes.

One hour after introduction into the soil (cycle 0), population sizes among the isolates ranged from log 4.35 to 4.03 CFU per g of soil and did not differ significantly ($P \geq 0.05$) (Fig. 4). Rhizosphere colonization by isolates of genotypes D and P was substantially greater than colonization by isolates of genotypes A, B, E, L, O, and Q. Population densities of genotypes D and P increased during the first cycle and subsequently remained above log 6.0 CFU (g of root)$^{-1}$ for the duration of the experiment (15 weeks). Maximum population sizes for members of these two genotypes generally did not occur until cycle 4 or 5 (Fig. 4, Table 2). The mean rhizosphere population density for all D and P genotype isolates was greater than log 6.5 CFU (g of root)$^{-1}$ (Table 2). In contrast, the population densities of isolates representative of genotypes A, B, E, L, O, and Q increased in the first one or two cycles, when maximum population densities occurred, and then declined to log 5.0 CFU (g of root)$^{-1}$ or lower. Isolates Q2-1 and 5MR2 had mean rhizosphere population sizes below log 6.0 CFU (g of root)$^{-1}$, and Pf-5, Q2-87, QT1-6, W4-4, 1MA1, and 7MA12 had mean rhizosphere population sizes below log 5.0 CFU (g of root)$^{-1}$ (Fig. 4, Table 2). Furthermore, all D and P genotype isolates had AUCPC values (used as an indicator of total rhizosphere colonization over all cycles) significantly ($P < 0.0001$) greater than the values for isolates of all the other genotypes. These differences were particularly noticeable after the third cycle.

When the 629-bp *phlD* products amplified from the terminal

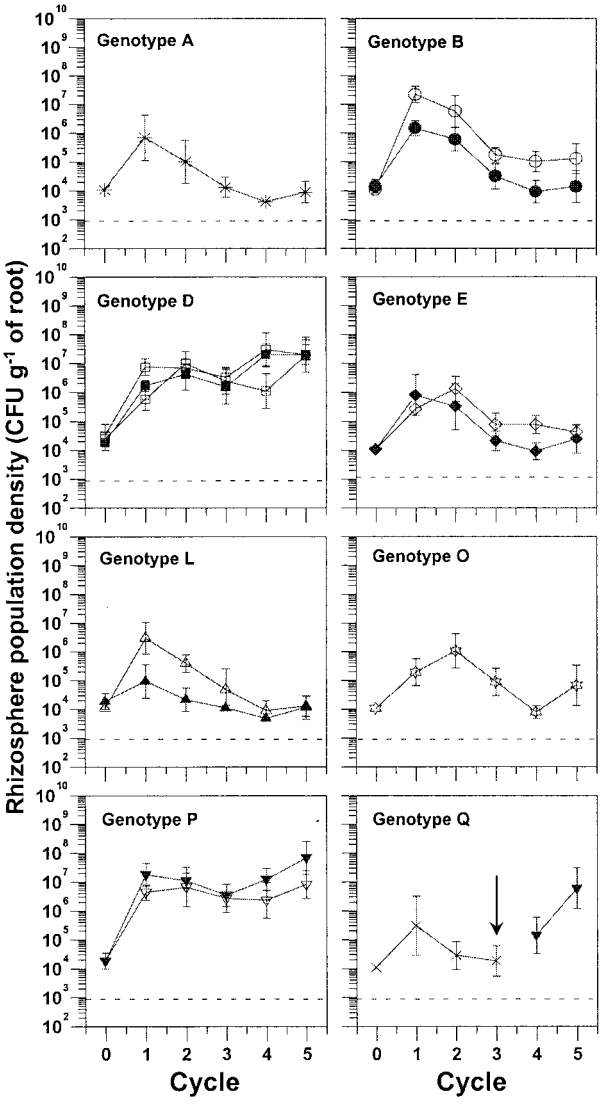

FIG. 4. Population dynamics of *P. fluorescens* Pf-5 (∗), Q2-1 (○), Q2-87 (●), MVP1-6 (⊞), MVW1-2 (□), Q8r1-96 (■), 5MR2 (◇), QT1-6 (◆), W4-4 (▲), 1MA1 (△), 7MA12 (☆), MVW1-1 (▽), MVP1-4 (▼), and MVW4-2 (×) belonging to different genotypes on the roots of pea cv. Rex grown in Quincy virgin soil for five successive cycles of 3 weeks each. Each isolate was introduced into the soil to give a final density of approximately log 4 CFU per g of soil (cycle 0). Mean values and standard deviations are presented. The arrow in the treatment with isolate MVW4-2 (Q genotype) indicates the point at which contamination with the P genotype isolate MVP1-4 occurred during the repotting of the soil (see Results).

dilutions showing growth in the *phlD*-specific PCR-based assay were digested with *Hae*III, *Taq*I, and *Msp*I, the RFLP patterns were identical to those of the isolates originally introduced into the soil (Fig. 3) except in one case. During the repotting of soil containing MVW4-2 (Q genotype) in the third cycle, soil inoculated originally with MVP1-4 (P genotype) was mistakenly used to cover the seed. Most of the top layer of soil was removed with a spoon, and then new seeds were sown and covered with the appropriate soil. However, in the fourth and fifth cycles, only the P genotype was detected by RFLP analysis of $phlD^+$ populations in terminal dilutions of washings of roots grown from these pots (Fig. 4). Furthermore, the density of the P genotype isolate increased over 10-fold from the fourth to the fifth cycle, highlighting further the aggressive colonizing ability of the P genotype on pea plants.

After the fifth cycle, the inoculated soils were stored in plastic bags on a greenhouse bench at 20°C for 3 months. The soils then were placed in pots and again sown to pea plants in order to determine how well each strain persisted in the soil. Four weeks later, the population densities of the D and P genotype representatives were greater than log 6.0 CFU per g of soil, and they were significantly ($P < 0.0001$) greater than those of all of the other strains.

DISCUSSION

Representatives of seven different genotypes (A, D, E, L, O, P, and Q) were identified by BOX-PCR analysis among $phlD^+$ fluorescent *Pseudomonas* spp. isolated from the rhizosphere of pea plants grown in soils that had undergone pea or wheat monoculture and were suppressive to *Fusarium* wilt and take-all, respectively. This increases by three (O, P, and Q) the number of BOX-PCR genotypes identified previously (33) among a worldwide collection of $phlD^+$ isolates. Picard et al. (36) found that the genetic diversity of populations of 2,4-DAPG-producing *Pseudomonas* spp. in the rhizosphere of corn (*Zea mays* L.) at different stages of plant growth was initially low but increased over time and was dominated by members of a single ARDRA group at all stages of plant growth.

Our finding that the majority of $phlD^+$ isolates in the rhizosphere of field-grown plants in the FWS soil are members of the D genotype was especially interesting because D genotype isolates are also predominant in populations from certain field soils that underwent wheat monoculture (33). Furthermore, the observation that $phlD^+$ fluorescent *Pseudomonas* spp. occur at relatively high densities (10^5 to 10^6 CFU g^{-1}) on roots of pea plants grown in FWS soil suggests that they may play a role in the suppressiveness of this soil to *Fusarium* wilt. *F. oxysporum* is sensitive to 2,4-DAPG, and $phlD^+$ *Pseudomonas* spp. were present on pea plant roots at or above the threshold (10^5 CFU g^{-1}) known to be required for 2,4-DAPG-mediated suppression of take-all of wheat (40) and for suppression of *Fusarium* wilt of radish by *Pseudomonas* spp. (39). To date, siderophore-mediated competition for iron by fluorescent *Pseudomonas* spp. and competition for carbon and infection sites as well as induced resistance by nonpathogenic *F. oxysporum* (24) are the primary mechanisms shown to be responsible for the natural suppressiveness of specific soils to *Fusarium* wilt. The role of antibiosis in natural suppressiveness to *Fusarium* wilt of pea plants merits further study.

Raaijmakers and Weller (41) identified 16 RAPD groups among 101 $phlD^+$ isolates from roots of wheat plants cycled in Quincy TAD soil and showed that one group, exemplified by *P. fluorescens* Q8r1-96 (D genotype), comprised up to 50% of the total population of 2,4-DAPG producers. D genotype isolates were also enriched in TAD soils from Moses Lake and Lind, Wash., cycled to wheat (J. M. Raaijmakers and D. M. Weller, unpublished data). When introduced individually into Quincy virgin soil and cycled with wheat for 8 months, Q8r1-96 maintained population densities 10- to 1,000-fold greater than those

TABLE 2. Population dynamics of 2,4-DAPG-producing *P. fluorescens* isolates and reference strains on roots of pea cv. Rex plants grown in Quincy virgin soil for five cycles

Bacterial isolate[a]	BOX genotype[b]	Rhizosphere competence[c]			
		Mean colonization (log CFU/g of root)	Cycle(s) of maximum colonization	AUCPC[d]	Persistence (log CFU/g of root)
Pf-5	A	4.50	1	22.58 g	5.64 cd
Q2-1	B	5.89	1	28.91 d	4.42 e
Q2-87	B	4.91	1, 2	24.53 ef	4.18 e
MVP 1-6	D	6.49	2, 5	30.93 c	6.89 b
MVW 1-2	D	6.99	4, 5	33.50 a	7.70 a
Q8r1-96	D	6.72	4, 5	32.07 b	7.50 ab
5MR2	E	5.18	2	25.63 e	4.05 e
QT1-6	E	4.82	1, 2	23.90 f	n.d.
W4-4	L	4.22	1	21.16 h	4.08 e
1MA1	L	4.96	1	24.77 ef	4.12 e
7MA12	O	5.00	2	24.59 ef	5.35 d
MVW 1-1	P	6.64	n.s.	31.83 bc	6.11 c
MVP 1-4	P	7.15	5	33.95 a	7.74 a
MVW 4-2	Q	4.48*	1	22.34 g*	n.d.*

[a] Bacterial isolates were introduced into the soil to give a final density of approximately 10^4 CFU g^{-1} of soil. Each treatment consisted of six pots with five plants per pot. Plants were grown for five cycles in a growth chamber at 24 ± 1°C and 40 to 60% RH, with a 12-h photoperiod.

[b] Genotypes were defined previously by BOX-PCR genomic fingerprinting (33) or in this study.

[c] The rhizosphere competence of each *phlD*$^+$ isolate was determined by calculating four variables associated with the rhizosphere colonization progress curve: (i) mean colonization for all cycles; (ii) cycle of maximum colonization, (iii) area under the rhizosphere colonization progress curve (AUCPC), using the trapezoidal integration method (5); and (iv) the persistence of the strains, determined as the mean population density on roots of pea plants after 4 weeks of growth following a 3-month fallow period. n.s., not significantly different among the cycles ($P > 0.05$). n.d., nondetectable. One treatment (*) became contaminated with a BOX-PCR genotype P strain after the third cycle; population density data for this strain in the fourth and fifth cycles were considered to be the same as in the third cycle for data analysis.

[d] Means followed by the same letter are not significantly different ($P = 0.05$) according to Fisher's protected least significant difference test.

of strain Q2-87 (B genotype) and 1M1-96 (L genotype). The differences in rhizosphere competence of Q8r1-96 were especially surprising because there were no other obvious phenotypic differences: all three strains belonged to biovar II, produced similar amounts of 2,4-DAPG in situ, and were very similar according to fatty acid methyl ester analysis, substrate utilization profiles, and classical bacteriological tests (41). McSpadden et al. (33) also found that the carbon utilization profiles of 30 *phlD*$^+$ isolates from 13 BOX-PCR genotypes were very similar, with over 72% of the utilizable substrates supporting growth of all 30 isolates. Based on these studies, it was hypothesized that certain genotypes preferentially colonize the roots of specific crops.

Our study with pea plants supports this hypothesis and shows that of the seven genotypes isolated from pea plant roots grown in disease-suppressive soils, only isolates of genotypes D and P demonstrated the unusually high degree of rhizosphere competence described by Raaijmakers and Weller (41). Furthermore, as previously reported (41), a genotype with superior rhizosphere competence emerged (in this case the P genotype) after cycling the target host plant in suppressive soil. We do not yet know why P genotype isolates are not more abundant on roots grown in the field. The effect of the growth stage of the host plant can play an important role in the genotypic composition of *phlD*$^+$ isolates (36) and may, at least in part, explain the discrepancy between the results obtained in growth chamber and field experiments.

Perhaps the most important finding from these studies is the relationship between rhizosphere competence and genotype, as determined by BOX-PCR or RFLP analysis of *phlD*. Until now, the selection of root colonists has been an empirical process involving random screening of isolates. It now appears that this relationship will allow a more directed selection of strains for use in biological control.

Most studies of root colonization in the greenhouse or growth chamber are conducted over a period of days or weeks, whereas the cycling process described in this study allows isolates to be compared for several months. Our results showed that the main differences in colonization among genotypes occurred after the third cycle. We found the area under the rhizosphere colonization progress curve (AUCPC) to be particularly useful to compare the rhizosphere competence of isolates over time. The aggressive colonization by members of the D and P genotypes was most apparent in their ability to maintain populations above 10^6 CFU (g of root)$^{-1}$ throughout the 15-week experiment. This persistence, which was translated into AUCPC values significantly greater than those of the other genotypes, is particularly remarkable in view of the fact that the bacteria were introduced prior to cycling at only 10^4 CFU per g of soil. The significant differences in AUCPC among the D and P genotype isolates tested indicate that it may be possible to determine the relationship between genotype and rhizosphere competence at an even finer genetic scale.

RFLP analysis is a valuable tool in assessing the identity of introduced *phlD*$^+$ isolates during colonization studies; it provides information comparable to that obtained by BOX-PCR analysis, it can be used in the presence of indigenous bacteria that do not produce 2,4-DAPG, and the genotype can be determined without computer-assisted analysis because the fragment patterns generated are comparatively simple. RFLP analyses of *phlD*$^+$ isolates allowed us to confirm that the genotype recovered from the rhizosphere was the same as that introduced into the soil prior to cycling. A quality check is especially

important when working with highly rhizosphere competent bacteria because the possibility of cross contamination between soils during planting or watering is much greater than previously thought (B. B. Landa and D. M. Weller, unpublished data). The aggressiveness of members of genotype P was highlighted when soil treated with MVW4-2 (Q genotype), an isolate with lower rhizosphere competence, accidentally became contaminated with MVP1-4 (P genotype). Although most of the soil containing MVP1-4 was removed from the pot and new seeds were sown, the residual soil (probably less than 0.5%) was sufficient to establish MVP1-4 as the dominant *phlD*$^+$ isolate in the soil, with a population density of log 6.7 CFU (g of root)$^{-1}$ after only two cycles.

The bulk soil is a hostile environment for introducing beneficial bacteria. Biological buffering (9), which is driven by the total amount of metabolically active microbial biomass present in soil, is one explanation for the failure of many inoculants to successfully colonize the habitats into which they are introduced. Competition is even more intense in the rhizosphere because microbial population densities generally are 10 to 20 times greater than in the bulk soil (8, 27). In the cycling study, all of the genotypes established sizable densities on the roots for one or two cycles, but thereafter (Fig. 4), densities of genotypes A, B, E, L, O, and Q declined, which is typical of the population dynamics of most introduced PGPR (2, 22, 28, 58). We speculate that at the outset of the experiment, the indigenous soil microflora were dormant and/or at low population densities, a result of the drying and storage of the tested soils. However, over time, the microbial communities within these soils were reactivated, and some populations outcompeted the introduced strains. The degree to which the decline in the populations of genotypes A, B, E, L, O, and Q was due to loss of viability or lack of competitiveness is uncertain. However, the accidental colonization of pots inoculated with an isolate of the Q genotype by a member of the P genotype indicates that competitiveness for rhizosphere colonization is a major factor in these experiments. How members of the D and P genotypes avoid the adverse effects of biological buffering and maintain stable population densities throughout all cycles is a key question emerging from this study.

Many soil-borne microorganisms, including plant pathogens, rhizobia, and arbuscular mycorrhizal fungi, sidestep constraints on their growth and persistence imposed by biological buffering by occupying niches that require specific recognition mechanisms in order to cause disease or establish symbiotic relationships. The compatibility of these interactions in rhizobia and possibly mycorrhizae is governed by corresponding genes in the plant and the microbe (50). Gene-for-gene interactions between plant hosts and PGPR were not considered likely because the interaction of PGPR with plant roots generally does not require invasion of the plant tissue. However, it is well known that the plant genotype influences both the quantity and the composition of microorganisms in the rhizosphere (1, 25, 34, 45). More recently, Smith et al. (51) reported that the genotype of tomato plays a significant role in supporting the growth of *Bacillus cereus* UW85 and its ability to suppress damping-off caused by *Pythium torulosum.* Genetic analysis of recombinant inbred lines of tomato revealed that three quantitative trait loci associated with biocontrol by *B. cereus* explained 38% of the phenotypic variance found.

Our results showing variation in the ability of different genotypes of 2,4-DAPG producers to colonize the roots of pea plants suggest that specific genes in the bacteria play a role complementary to those in the plant supporting this interaction. A major challenge for the future is the identification of the genes in D and P genotypes and in pea plants that govern such remarkably efficient root colonization.

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