APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 2005, p. 993–1003 Vol. 71, No. 2
0099-2240/05/$08.00+0 doi:10.1128/AEM.71.2.993–1003.2005

Assessment of Genotypic Diversity of Antibiotic-Producing *Pseudomonas* Species in the Rhizosphere by Denaturing Gradient Gel Electrophoresis

M. Bergsma-Vlami, M. E. Prins, M. Staats, and J. M. Raaijmakers*

Laboratory of Phytopathology, Wageningen University, Wageningen, The Netherlands

Received 6 July 2004/Accepted 13 September 2004

The genotypic diversity of antibiotic-producing *Pseudomonas* spp. provides an enormous resource for identifying strains that are highly rhizosphere competent and superior for biological control of plant diseases. In this study, a simple and rapid method was developed to determine the presence and genotypic diversity of 2,4-diacetylphloroglucinol (DAPG)-producing *Pseudomonas* strains in rhizosphere samples. Denaturing gradient gel electrophoresis (DGGE) of 350-bp fragments of *phlD*, a key gene involved in DAPG biosynthesis, allowed discrimination between genotypically different *phlD*$^+$ reference strains and indigenous isolates. DGGE analysis of the *phlD* fragments provided a level of discrimination between *phlD*$^+$ genotypes that was higher than the level obtained by currently used techniques and enabled detection of specific *phlD*$^+$ genotypes directly in rhizosphere samples with a detection limit of approximately 5×10^3 CFU/g of root. DGGE also allowed simultaneous detection of multiple *phlD*$^+$ genotypes present in mixtures in rhizosphere samples. DGGE analysis of 184 indigenous *phlD*$^+$ isolates obtained from the rhizospheres of wheat, sugar beet, and potato plants resulted in the identification of seven *phlD*$^+$ genotypes, five of which were not described previously based on sequence and phylogenetic analyses. Subsequent bioassays demonstrated that eight genotypically different *phlD*$^+$ genotypes differed substantially in the ability to colonize the rhizosphere of sugar beet seedlings. Collectively, these results demonstrated that DGGE analysis of the *phlD* gene allows identification of new genotypic groups of specific antibiotic-producing *Pseudomonas* with different abilities to colonize the rhizosphere of sugar beet seedlings.

Antibiotic compounds produced by fluorescent *Pseudomonas* strains play key roles in the suppression of various soilborne plant pathogens (41, 47, 52). 2,4-Diacetylphloroglucinol (DAPG) produced by *Pseudomonas fluorescens* has activity against a range of plant pathogens, including bacteria, fungi, and nematodes (reviewed in reference 41). Recently, the broad-spectrum activity of DAPG also has drawn attention in the medical area because of the bacteriolytic activity of DAPG against multidrug-resistant *Staphylococcus aureus* (18). DAPG-producing *Pseudomonas* spp. have been isolated from the rhizospheres of different crops grown in soils from diverse geographic regions (19), and they are predominant constituents of the rhizosphere of wheat plants grown in soils that naturally suppress take-all disease (4, 30, 39, 58). They also have been isolated from soils that naturally suppress black root rot of tobacco (19, 43) or *Fusarium* wilt disease (23).

Multiple genes are involved in biosynthesis and regulation of DAPG production in *P. fluorescens* (reviewed in reference 12). One of these genes, the polyketide synthase gene *phlD*, is essential for synthesis of the DAPG precursor monoacetylphloroglucinol (1). It has been well documented that the *phlD* gene is conserved among DAPG-producing *Pseudomonas* strains found worldwide (19, 39) but displays a certain degree of polymorphism (23, 28, 42). Given that the genotypic diversity among DAPG-producing *Pseudomonas* strains provides an enormous resource for identifying strains that are highly rhizosphere competent and superior for biological control of plant diseases (40, 47), the sequence heterogeneity of the *phlD* gene is now routinely used to assess the diversity of this group of antagonistic bacteria (24, 28, 31, 42, 56). A range of other methods have been used to determine the genotypic diversity of DAPG-producing *Pseudomonas* strains; these methods include amplified ribosomal DNA restriction analysis (19, 37), random amplified polymorphic DNA (RAPD) analysis (28, 40), and BOX-PCR (31). A notable difficulty with all of these methods is the requirement for isolation and cultivation of *phlD*$^+$ pseudomonads from soil and rhizosphere environments prior to genotypic characterization of the organisms. Isolation of *phlD*$^+$ pseudomonads can be achieved by plating on semiselective media, followed by colony hybridization (39), a time-consuming method. Alternatively, direct characterization of *phlD*$^+$ *Pseudomonas* isolates in rhizosphere samples can be performed by a rapid PCR assay (32). However, this method also requires cultivation of a rhizosphere sample in semiselective nutrient broth prior to characterization of the *phlD*$^+$ genotype and may be biased toward detecting the most predominant genotype.

The aim of this work was to develop a simple and rapid method to study the presence and genotypic diversity of *phlD*$^+$ *Pseudomonas* strains directly in rhizosphere samples without prior isolation or enrichment on nutrient media. New *phlD*-specific primers were developed, and their specificity was tested with a range of different *phlD*$^+$ genotypes, alone and in mixtures. Polymorphisms within the amplified 350-bp *phlD* fragments were assessed by denaturing gradient gel electro-

* Corresponding author. Mailing address: Laboratory of Phytopathology, Wageningen University, Binnenhaven 5, 6709 PD Wageningen, The Netherlands. Phone: 31 317 483427. Fax: 31 317 483412. E-mail: jos.raaijmakers@wur.nl.

TABLE 1. *phlD*$^+$ *Pseudomonas* strains used in this study

Strain or isolate	Code	Accession no. for *phlD*	Reference
Q2-87	PfQ287	U41818	1
F113	PfF113	AJ278811	8
CHA0	PfCHA0	AJ278806	49
Pf-5	PfPf5	AF214457	16
Q8R1-96	PfQ8R196	AF207693	40
Q65C-80	PfQ65c80	AJ278807	13
CMIA2	PfCMIA2	AJ278808	9
MI-96	PfMI96	AF207692	40
PILH1	PfPILH1	AJ278810	19
PITR2	PfPITR2	AJ278809	19
HR3-A13	PfY	AY391780	29
PR3-A52	PfZ	AY391779	29
42-36	Pf4236	AF396857	38
42-27	Pf4227	AF396856	38
39-8	Pf398	AF396855	38
37-27	Pf3727	AF396854	38
22-27	Pf2227	AF396853	38
19-41	Pf1941	AF396852	38
19-30	Pf1930	AF396851	38
19-7	Pf197	AF396850	38
18-33	Pf1833	AF396849	38
11-18	Pf1118	AF396848	38
7-37	Pf737	AF396847	38
6-28	Pf628	AF396846	38
3-1	Pf31	AF396845	38
D27B1	D27B1	NA[a]	23
PSB459	Psp A1	AY486314	This study
PWB152	Psp A2	AY486317	This study
PWB134	Psp B	AY486316	This study
PSB516	Psp C	AY486315	This study
PSB211	Psp D	AY486313	This study
PSB113	Psp E1	AY486312	This study
PPB433	Psp E2	AY486310	This study
PWB522	Psp E3	AY486319	This study
PPB239	Psp E4	AY486309	This study
PWB257	Psp E5	AY486318	This study
PPB617	Psp F	AY486311	This study
PWB5516	Psp Z	AY486320	This study

[a] NA, not available.

phoresis (DGGE) analysis, sequencing, and phylogenetic analysis. The specificity and resolving capacity of the PCR-DGGE system were compared to the specificity and resolving capacity of currently used techniques, including *phlD* restriction fragment length polymorphism (RFLP) (31), RAPD analysis (19), and the rapid PCR assay (32). Finally, the biological significance of the newly developed PCR-DGGE classification was tested in root colonization assays with sugar beet seedlings treated with eight genotypically different *phlD*$^+$ genotypes of *Pseudomonas*.

MATERIALS AND METHODS

***Pseudomonas* strains and culture conditions.** All *Pseudomonas* strains used in this study were cultured on King's medium B (KMB) agar (20) at 25°C for 48 h. To determine the specificity and resolving capacity of the PCR-DGGE method developed in this study, we tested multiple *phlD*$^+$ reference strains (Table 1), as well as 184 indigenous *phlD*$^+$ *Pseudomonas* isolates obtained from the rhizospheres of three plant species (wheat, sugar beet, and potato) by colony hybridization with a *phlD*-specific probe.

PCR-DGGE analysis. PCR amplification was carried out in a 25-μl reaction mixture which contained 3 μl of a 40-fold-diluted heat-lysed cell suspension (39), 1× GeneAmp PCR buffer (Perkin-Elmer Corp., Norwalk, Conn.), each deoxynucleoside triphosphate (Promega) at a concentration of 500 μM, 40 pmol of the reverse primer, 40 pmol of the forward primer (Amersham Pharmacia Biotech), 1.5 mM $MgCl_2$, and 1.0 U of AmpliTaq DNA polymerase (Perkin-Elmer). The PCR program consisted of an initial denaturation at 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 67°C for 30 s, and 72°C for 60 s. The reactions were performed by using a Peltier Thermal Cycler-200 (Biozym, Landgraaf, The Netherlands). Fifteen microliters of the PCR product was used for analysis by DGGE with the Dcode universal mutation detection system (Bio-Rad Laboratories, Hercules, Calif.). The DGGE analysis protocol was based on the initial protocol of Muyzer et al. (35) and was performed by using an 8% (wt/vol) acrylamide gel with a linear denaturing gradient (100% denaturant contained 7 M urea plus 40% [vol/vol] deionized formamide). In almost all cases a gradient from 32% denaturant at the top to 41% denaturant at the bottom gave optimal separation of the amplified products, and this gradient was routinely used. Gels were run for 10 min at 200 V and subsequently for 16 h at 85 V (60°C), stained with ethidium bromide (0.5 μg/ml in 1× Tris-acetate-EDTA [TAE] [pH 8.3]) for 30 min, and visualized on an UV transilluminator.

DNA sequence analysis. *phlD* fragments from multiple representative strains were amplified by using the Expand High Fidelity *Taq* polymerase (Roche, Almere, The Netherlands) and were subsequently sequenced by BaseClear (Leiden, The Netherlands). Alignment of *phlD* sequences obtained in this study and *phlD* sequences present in the databases was performed with Clustal W (53). Distance matrices were computed with MEGA, and phylogenetic trees were constructed by using the neighbor-joining method (44); the topology was checked by bootstrap analysis (1,000 data sets).

RFLP and RAPD analyses. To determine the resolving capacity of the classifications assessed by PCR-DGGE analysis, multiple *phlD*$^+$ strains were also subjected to *phlD* RFLP and RAPD analyses, two techniques that are currently used to determine the genotypic diversity of DAPG-producing *Pseudomonas* spp. For the *phlD* RFLP analysis, 629-bp fragments of the *phlD* gene were amplified with primers B2BF and BPR4, and this was followed by restriction with HaeIII, MspI, or TaqI (32). Restriction fragments were separated on a 2% agarose gel in 1× TAE for 2 to 3 h at 120 V. RAPD analysis with the 10-mer primers M12, M13 and D7 was performed according to protocols described previously (19, 40). The amplification products were separated on a 2% agarose gel in 1× TAE at 120 V for 3 h. *phlD* RFLP patterns and RAPD markers were visualized with a UV transilluminator and were photographed by using a digital camera. All PCR-RAPD amplifications were repeated at least two times, and only the consistent RAPD markers were included in the evaluation. The sizes of the restriction fragments obtained in the *phlD* RFLP analysis and the RAPD markers were determined with the Phoretix 1D software (version 3.0; Phoretix International, Newcastle, England). Band positions were converted to R_f values (0 and 1), and profile similarities were calculated by determining the pairwise coefficients of similarity (Nei-Li distances) for the total number of lane patterns. Cluster analysis with neighbor joining (44) and the corresponding bootstrap analysis (1,000 data sets) were performed with the Treecon software (version 1.3b) for Windows (54).

Plant cultivation and DNA extraction from the rhizosphere. Wheat plants (*Triticum aestivum* L. cv. Bussard) were grown in a soil consisting of agricultural CB soil (4) mixed at a 1:1 ratio (wt/wt) with quartz sand. Sixteen wheat seeds were sown in square polyvinyl chloride pots containing 250 g (dry weight) of soil. A spontaneous rifampin-resistant derivative of *phlD*$^+$ *Pseudomonas* isolate PWB532, representing DGGE group E, was introduced into soil at densities of 0, 10, 10^2, 10^3, 10^4, and 10^6 cells/g with an initial water content of 20% (vol/wt). One additional control treatment consisted of soil that was autoclaved twice (with 24 h between the two autoclave runs) to eliminate putative indigenous *phlD*$^+$ isolates. Each treatment consisted of three replicates. After 10 to 12 days of cultivation in a growth chamber at 20°C with a 16-h photoperiod, the wheat plants were harvested, and rhizosphere samples were prepared for (i) enumeration of the introduced strain on selective agar plates and (ii) direct DNA extraction, followed by PCR-DGGE analysis. For enumeration of the introduced strain, 0.5 g of roots with associated rhizosphere soil was suspended in 5.0 ml of sterile distilled water and shaken vigorously for 1 min on a Vortex mixer; samples were subsequently sonicated in an ultrasonic cleaner for 1 min and dilution plated onto KMB agar supplemented with delvocid (100 mg/liter), chloramphenicol (13 mg/liter), ampicillin (40 mg/liter), and rifampin (100 mg/liter) (48). The plates were incubated for 3 days at 25°C, and colonies were enumerated. For direct DNA extraction from the rhizosphere, 0.5 g of roots with associated rhizosphere soil was suspended in 1.0 ml of saline phosphate buffer, shaken vigorously for 1 min on a Vortex mixer, and sonicated in an ultrasonic cleaner for 1 min. The roots were discarded, and the suspension was centrifuged for 1 min at 10,000 rpm (19,000 × *g*). An additional 0.5 g of rhizosphere soil that had been subjected to the same treatment was added, and the sample was subsequently processed by bead beating (three times for 90 s each). Cells were lysed by using

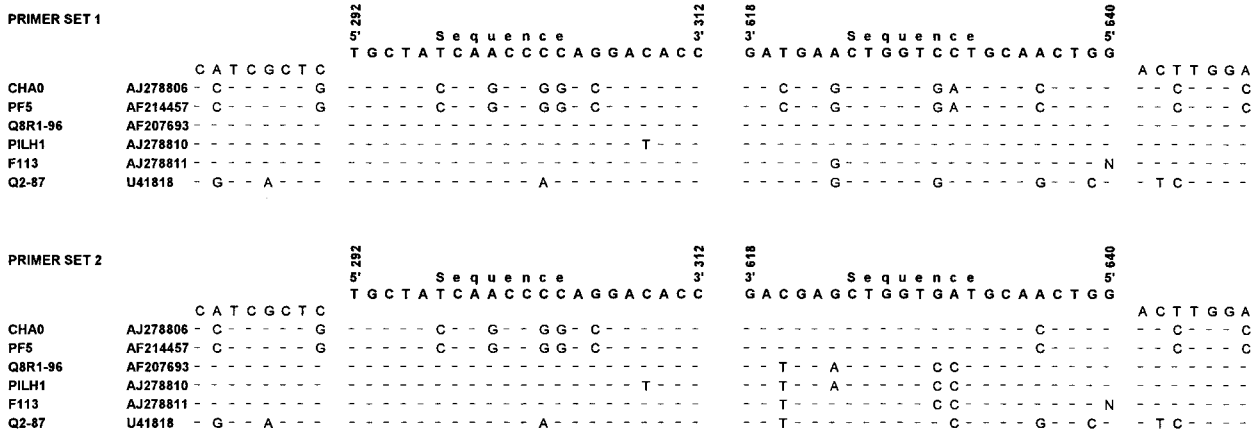

FIG. 1. Comparison of partial *phlD* sequences from several *Pseudomonas* strains. Bases that are identical in all sequences are indicated by dashes. The sequences and positions of the two primer sets used in PCR-DGGE analysis are shown above each of the two alignments. The positions of the 5′ and 3′ ends of each of the primers correspond to the positions in the *phlD* sequence of Pf-5 (accession number AF214457).

the protocol of a FastDNA SPIN kit for soil (Bio 101). The DNA pellet was dissolved in 50 μl of Tris-EDTA (10 mM Tris, 0.1 mM EDTA; pH 8). PCR amplification of extracted DNA was performed in 50-μl reaction mixtures containing approximately 10 to 50 ng of DNA. In most cases, this amount of DNA was acquired after 100-fold dilution of the DNA obtained with the FastDNA SPIN kit. To enhance the specificity of the PCR, a ramping PCR was carried out as follows: the annealing temperature was initially 60°C, and it was increased to 72°C in steps of 0.1°C. The PCR program consisted of an initial denaturation at 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 60°C for 30 s, with an increase at a rate of 0.1°C/s up to 72°C, and 72°C for 1 min. PCR amplification was carried out as described above. The primer concentration used was 20 pmol per reaction mixture instead of 40 pmol per reaction mixture.

In addition to wheat, experiments were performed with sugar beet (*Beta vulgaris* cv. Auris). Twenty-eight sugar beet seeds were sown in small square pots containing 250 g (dry weight) of soil and cultivated in a climate room with a controlled environment at 20°C and a 16-h photoperiod. Genotypically different *phlD*$^+$ isolates were introduced separately into soil at a density of approximately 10^4 CFU/g of soil. The *phlD*$^+$ isolates were spontaneous rifampin-resistant derivatives of isolates PWB233 (DGGE group A), PSC2218 (DGGE group B), PPB2310 (DGGE group C), PSB211 (DGGE group D), PWB532 (DGGE group E), PPB3512 (DGGE group F), PSC415 (DGGE group Z), and Q8R1-96 (DGGE group G). For short-term colonization experiments, the sugar beet plants were harvested after 10 to 12 days of cultivation. For long-term colonization studies, sugar beet plants were grown in the same pots for six successive cycles consisting of 10 to 12 days each. Twice a week, the plants were treated with one-third-strength Hoaglund's solution (macroelements only). After 10 to 12 days of growth, plants were harvested, and their root systems with rhizosphere soil were collected. Excess root material was mixed with the cultivated soil and represented approximately 0.125% (wt/wt) of the soil dry weight. The cultivated soil was subsequently returned to the same pot, and sugar beet seeds were replanted. This process of plant growth and harvesting was repeated for six successive cycles. Four replicates were included per treatment. For both short-term and long-term colonization experiments, rhizosphere samples were plated onto selective media and subjected to direct DNA extraction as described above for the experiments with wheat.

Statistical analysis. Population densities of the introduced *phlD*$^+$ fluorescent *Pseudomonas* strains were $\log_{10}$ transformed prior to statistical analysis. For the colonization assays with sugar beet seedlings, differences in population densities between the introduced strains were analyzed for each successive growth cycle by analysis of variance, followed by Tukey's Studentized range test (SAS Institute Inc., Cary, N.C.).

Nucleotide sequence accession numbers. *phlD* sequences obtained in this study have been deposited in the GenBank database under the accession numbers shown in Table 1.

RESULTS AND DISCUSSION

Primer design and specificity. For detection of DAPG-producing *Pseudomonas* spp., a number of primers directed against sequences in the *phlD* gene have been developed previously (32, 39). The sizes of the amplification products of these primers range from approximately 600 to 750 bp, which is relatively large for further analysis by DGGE. Therefore, two new sets of oligonucleotide primers were developed for conserved sequences in the *phlD* gene of multiple reference strains (Fig. 1). In both primer sets, the forward primer is identical (DGGE292for), whereas there is a four-nucleotide difference between the two different reverse primers (DGGE618rev and 6DGGE618rev). Additionally, a 40-bp GC clamp is attached at the 5′ end of the forward primer (Table 2). The first primer set resulted in amplification of fragments of the predicted size (approximately 350 bp) from DNA of four genotypically different *phlD*$^+$ reference strains (PILH1, F113, Q8R1-96, and Q2-87) and from DNA of each of 184 *phlD*$^+$ isolates obtained previously from the rhizospheres of three

TABLE 2. Properties of the oligonucleotide primers used in the PCR-DGGE analysis

Primer	Sequence (5′-3′)	G+C content (%)	Melting temp (°C)
DGGE618rev	CCAGTTGCAGGACCAGTTCATC	55	67.9
6DGGE618rev	CCAGTTGCATCACCAGCTCGTC	59	67.9
DGGE292for	TGCTATCAACCCCAGGACACC	57	68.1
DGGE292forCG	CGCCGGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGGTGCTATCAACCCCAGGACACC	84	57.9

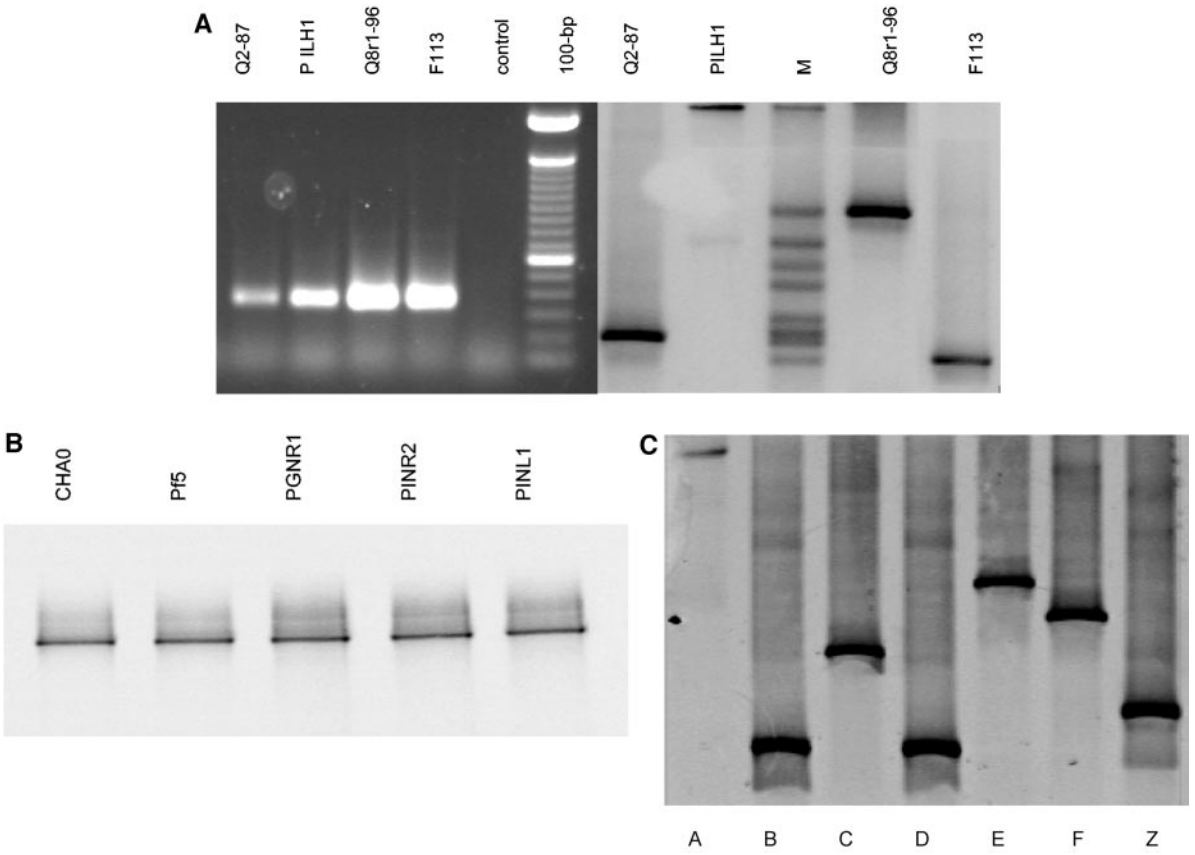

FIG. 2. (A) Agarose gel (left gel) and 32 to 41% denaturant DGGE gel (right gel) of *phlD* fragments amplified from *phlD*$^+$ reference strains Q2-87, PILH1, Q8r1-96, and F113. Lane M contained the DGGE marker, which included independently amplified *phlD* fragments from nine reference and indigenous strains. (B) A 40 to 51% denaturant DGGE gel of *phlD* fragments amplified from *phlD*$^+$ reference strains CHA0, Pf-5, PGNR1, PINR2, and PINL1. (C) A 32 to 41% denaturant DGGE gel of *phlD* fragments amplified from *phlD*$^+$ indigenous isolates obtained from the rhizosphere of multiple plant species. DGGE groups A (PWB152), B (PWB134), C (PSB516), D (PSB211), E (PSB113), F (PPB617), and Z (PWB5516) were included.

different plant species. No amplification product was obtained from DNA of *phlD* mutant R1SS101 or from DNA of *phlD*$^+$ reference strains CHA0 and Pf-5. At this stage, several attempts were made to further optimize the first primer set, including adjustment of the primer concentrations, the annealing temperature (the temperatures tested ranged from 48 to 70°C), and the Mg^{2+} concentration. However, none of these attempts were successful. The second primer set resulted in amplification of the predicted 350-bp fragment from DNA of all six *phlD*$^+$ reference strains, including strains CHA0 and Pf-5, and from DNA of each of the 184 *phlD*$^+$ isolates (Fig. 2). For successful amplification with the second primer set, a two-step PCR approach was required. In the first PCR step, no GC clamp was present at the 5′ end of the forward primer. After the first step, the PCR products were diluted 100- to 1,000-fold, after which the second PCR step was performed with the forward primer containing the GC clamp. The PCR programs for the one-step and two-step PCR amplifications were the same as the program described above.

DGGE analysis. DGGE and temperature gradient gel electrophoresis are widely used to study the microbial diversity in environmental samples and to monitor changes in specific microbial groups or communities (7, 10, 11, 22, 35, 45, 55). DGGE allows analysis of a large number of samples, which is essential for studying spatial and temporal variations in microbial populations (14, 33, 34, 35, 36, 51). To date, most primers used in DGGE analysis target rRNA genes of different microbial genera. In this context, group-specific primers have been developed for a number of bacterial genera, including *Pseudomonas* and *Bacillus* (10, 11), as well as for *Burkholderia* (45), *Actinomycetes* (15), and ammonium oxidizers and methanotrophs (2, 22). Recently, primers targeting specific biosynthetic genes have been developed, and, when combined with DGGE fingerprinting, these primers have led to a better level of discrimination within specific bacterial groups. For example, primers directed against the *fliC* gene allowed specific detection of the bacterial wilt pathogen *Ralstonia solanacearum* in soil and subsequent discrimination between strains obtained from various places (46). Rapid assessment of the diversity of methanogens was performed by DGGE analysis of the *nifH* gene (25, 57). In the same line of research, terminal RFLP analysis of PCR-amplified *nifH* fragments was shown to be a rapid technique for profiling diazotrophic microbial communities (50). Additionally, the community structures of ammonia-oxidizing bacteria (17) and bacteria from the marine environ-

TABLE 3. Genotypic classification of 184 indigenous *phlD*$^+$ *Pseudomonas* isolates originating from the rhizospheres of multiple plant species by DGGE and RAPD analyses

DGGE group	RAPD group	n^a	Frequency (% of all isolates)	
			DGGE	RAPD
A	A1	28	16.85	15.22
	A2	3		1.63
B	B1	19	10.33	10.33
C	C1	11	5.98	5.98
D	D1	2	1.09	1.09
E	E1	68	52.16	36.96
	E2	18		9.78
	E3	3		1.62
	E4	6		3.26
	E5	1		0.54
F	F1	5	2.72	2.72
Z	Z1	20	10.87	10.87
Total		184	100	100

[a] Total number of isolates belonging to each RAPD group.

TABLE 4. Genotypic classification of 12 indigenous *phlD*$^+$ *Pseudomonas* isolates originating from the rhizospheres of multiple plant species and five *phlD*$^+$ reference *Pseudomonas* strains by DGGE and *phlD* RFLP analyses[a]

DGGE genotype	RFLP genotype	Representative strain
A	M	D27B1
A	M	PWB152
A	M	PSB459
B	B	Q2-87
B	NEW1	PWB134
C	NEW1	PSB516
D	NEW2	PSB211
E	D	PSB113
E	D	PPB433
E	D	PWB522
E	D	PPB239
E	D	PWB257
F	NEW3	PPB617
G	D	Q8r1-96
I	K	F113
M	A	CHA0
Z	NEW4	PWB5516

[a] For the *phlD* RFLP analysis, 629-bp fragments of the *phlD* gene were amplified with primers B2BF and BPR4 and this was followed by restriction with HaeIII, MspI, and TaqI (32). NEW indicates groups based on the RFLP analysis which do not correspond with any of the previously described 17 genotypes (23).

ment (3) were explored by DGGE analysis of the *amoA* and *rpoB* genes, respectively.

In the present study, DGGE analysis was performed with the *phlD* fragments amplified with the first and second primer sets described above. A linear 32 to 41% denaturant gradient allowed detection of the amplified fragments and gave optimal discrimination between genotypically different *phlD*$^+$ reference strains and isolates, except for strains CHA0 and Pf-5 (Fig. 2A and C). For CHA0 and Pf-5, a 40 to 51% denaturant gradient was required to detect the PCR products amplified with the second primer set (Fig. 2B). Attempts to design a DGGE gradient (32 to 51% denaturant) for all *phlD*$^+$ strains, including strains CHA0 and Pf-5, resulted in a loss of discrimination between *phlD*$^+$ genotypes other than those of strains CHA0 and Pf-5. The difference in the behavior of strains CHA0 and Pf-5 compared to other *phlD*$^+$ strains in both PCR and DGGE is supported by *phlD* sequence data (see below) and has been described previously for PCR with other *phlD* primers (32). For strains other than CHA0 and Pf-5, the migration positions of PCR fragments obtained with the first and second primer sets were different, but the DGGE groups of the *phlD*$^+$ genotypes were the same for both primer sets (data not shown). DGGE analysis of PGNR1, PINR2, and PINL1, three other strains that are also very closely related to CHA0 and Pf-5 (19), showed that their amplified *phlD* fragments migrated to the same positions as the *phlD* fragments of strains CHA0 and Pf-5 (Fig. 2B).

DGGE analysis of the 184 *phlD*$^+$ isolates obtained from the rhizospheres of wheat, sugar beet, and potato resulted in seven DGGE groups, designated DGGE groups A, B, C, D, E, F, and Z (Fig. 2C and Table 3). Several of the 184 *phlD*$^+$ isolates were assigned to the same DGGE group as reference strains PILH1 (DGGE group A) and Q2-87 (DGGE group B). None of the 184 *phlD*$^+$ isolates were assigned to DGGE groups containing the reference strains Q8R1-96 (DGGE group G), F113 (DGGE group I), and CHA0 (DGGE group M). Therefore, the isolates assigned to DGGE groups C, D, E, F, and Z may represent *phlD*$^+$ genotypes not described previously. DGGE group E was the dominant *phlD*$^+$ genotype found among the collection of 184 indigenous *phlD*$^+$ isolates, representing approximately 52% of the diversity (Table 3).

Comparison of PCR-DGGE, RAPD, and *phlD* RFLP analyses. To determine the resolving capacity of the classifications obtained by PCR-DGGE analysis of the *phlD* gene, RAPD analysis with three 10-mer primers and *phlD* RFLP analysis were performed. The results of RAPD analysis of the 184 indigenous *phlD*$^+$ isolates correlated well with the results obtained by PCR-DGGE, but there was a higher degree of discrimination than there was in the PCR-DGGE analysis (Table 3). RAPD analyses resulted in 12 different RAPD groups, whereas PCR-DGGE resulted in seven different groups. Isolates belonging to DGGE group E were assigned to five different RAPD groups (RAPD groups E1 thru E5), and isolates belonging to DGGE group A were assigned to two RAPD groups (RAPD groups A1 and A2). A subset of five *phlD*$^+$ reference strains and 12 indigenous *phlD*$^+$ isolates, representing the 12 different RAPD groups, were analyzed by RFLP analysis of a 629-bp *phlD* fragment with three restriction enzymes, a technique routinely used to determine the genotypic diversity of DAPG-producing *Pseudomonas* spp. (32). Based on the RFLP analysis, the two isolates (isolates A1 and A2) belonging to DGGE group A were identical to D27B1, a reference strain representing DGGE genotype A (Table 4). Representative isolates belonging to DGGE groups D, F, and Z could be distinguished on the basis of the RFLP analysis. In contrast, however, the RFLP profiles of reference strain Q8r1-96 (DGGE group G) were identical to those of all five isolates belonging to DGGE group E (RAPD groups E1 thru E5). Similarly, the RFLP profiles of isolates belonging to DGGE groups B and C were identical. These results indicated that PCR-DGGE provides a higher level of discrimination between *phlD*$^+$ genotypes than the currently used *phlD* RFLP analysis.

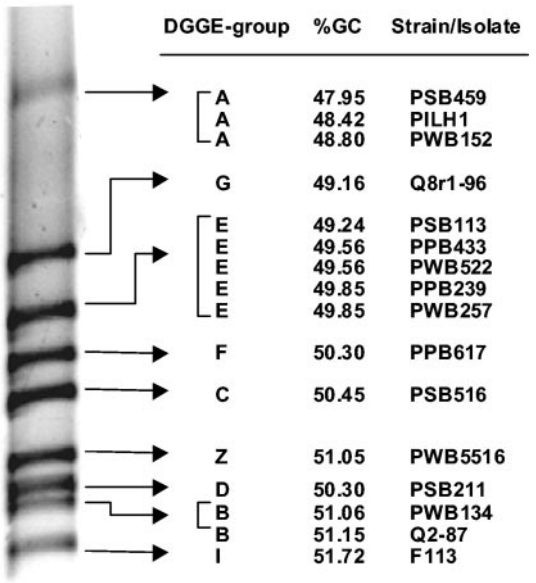

FIG. 3. Relationship between the electrophoretic mobilities of *phlD* fragments and their G+C contents. The electrophoretic mobilities of 350-bp *phlD* fragments in a denaturing gradient gel (32 to 41% denaturant) are shown for various strains and isolates representing nine different DGGE groups. For three DGGE groups (DGGE groups A, B, and E), multiple isolates were included in the analysis.

Sequence and phylogenetic analyses. In general, sequence analyses of the 350-bp *phlD* fragments amplified from DNA of 12 isolates representing the different DGGE genotypes (genotypes A, B, C, D, E, F, and Z) showed a good correlation between the G+C contents of the amplified fragments and the migration patterns in the denaturing gel (Fig. 3). The G+C contents ranged from 47.95% for DGGE genotype A (located in the upper part of the 32 to 41% denaturant gradient) to 51.72% for DGGE genotype I (located at the bottom). The results further showed that not only the G+C content but also the position of the so-called melting domains (34) within the amplified fragment determines the electrophoretic mobility. The relatively high G+C content (59.6%) of the *phlD* fragments of reference strains CHA0 and Pf-5 supported the requirement for a gradient with a higher percentage of denaturant (40 to 51% denaturant), as described previously.

Phylogenetic analyses of the *phlD* sequences obtained in this study and present in the database (Table 1) revealed a total of six distinct clusters based on bootstrap values higher than 75% (Fig. 4). Cluster I contained the reference strains Q8R1-96 (DGGE genotype G), Q65c80, and CM1A2, as well as isolates E1 through E5, which were five representatives of DGGE genotype E. Representative isolates of DGGE genotype B were clustered together (cluster II) with *phlD*$^+$ reference strains F113 (DGGE genotype I) and MI-96. Cluster III contained only representative isolates of DGGE genotypes C, D, F, and Z. Isolates A1 and A2, representing DGGE genotype A, were classified in the same cluster (cluster IV) together with reference strains PILH1 and PITR2, both of which were also classified by DGGE analysis as genotype A. Reference strain Q2-87 (DGGE genotype B) formed a unique cluster (cluster V) that included the recently described strain HR3-A13 (PfY) (29). However, Q2-87 was not grouped close to PWB134, a strain also classified in DGGE genotype B. These results are in accordance with results obtained by the RFLP analysis of strain Q2-87 and isolate PWB134. Cluster VI was the most distant cluster obtained in the phylogenetic analyses and contained reference strains CHA0 and Pf-5 (both DGGE genotype M) and the recently described strain PR3-A52 (PfZ) (29). Phylogenetic analyses of the *phlD* gene sequences by neighbor joining or maximum likelihood yielded similar results and tree topologies.

PCR-DGGE of rhizosphere samples. A notable difficulty of *phlD* RFLP and RAPD analyses is the requirement for isolation and cultivation of *phlD*$^+$ *Pseudomonas* isolates from soil and rhizosphere environments prior to genotypic characterization of the organisms. A PCR-DGGE analysis was performed with DNA extracted from rhizosphere samples obtained from roots of wheat plants grown for 10 to 12 days in soil treated with a spontaneous rifampin-resistant derivative of isolate PWB532 (DGGE group E). Isolate PWB532 was introduced into the soil at initial densities ranging from 10 to 10^6 CFU/g. Rhizosphere samples were subjected to direct DNA extraction, followed by PCR-DGGE, and they were also dilution plated onto KMB supplemented with rifampin for comparison purposes. Based on dilution plating, the rhizosphere population densities of introduced isolate PWB532 ranged from 5×10^2 to 5×10^6 CFU/g of root after 10 to 12 days of cultivation. PCR with DNA directly extracted from the wheat rhizosphere resulted in amplification of the 350-bp *phlD* fragment when the density of PWB532 was equal to or higher than 5×10^3 CFU/g (fresh weight) of root. Subsequent DGGE analysis of the 350-bp fragments amplified from DNA extracted from the rhizosphere of wheat colonized by isolate PWB532 showed a single band corresponding to DGGE group E (Fig. 5A). No other DGGE genotypes were detected. Additionally, no 350-bp amplification products were detected in the control treatments, which included rhizosphere samples from natural CB soil and from CB soil autoclaved twice prior to wheat cultivation (data not shown). The ramping PCR protocol used for DNA extracted from rhizosphere samples was crucial as it considerably increased the sensitivity of the PCR amplification. Similar results were obtained in short-term experiments with sugar beet plants grown in soils treated with isolates PSC415 (DGGE group Z), Q8R1-96 (DGGE group G), and PPB3512 (DGGE group F) (Fig. 5B). Collectively, these results indicate that PCR-DGGE can be used to detect specific *phlD*$^+$ genotypes directly in rhizosphere samples with a detection limit of approximately 5×10^3 CFU/g of root when ethidium bromide staining of the gel after electrophoresis is used. For ethidium bromide-stained gels, a detection limit of 10^5 CFU/g of soil was reported previously for *R. solanacearum* (46). When the results were combined with Southern hybridization, however, cell densities of *R. solanacearum* of approximately 10^3 CFU/g of soil could be detected (46). The results of the latter study suggest that the detection limit for indigenous *phlD*$^+$ isolates may be increased further when the technique is combined with Southern hybridization or when silver staining is used instead of ethidium bromide staining.

A potential problem for PCR-DGGE analysis of DNA extracted from environmental samples may be that in mixed populations of *phlD*$^+$ isolates, certain *phlD* genes are prefer-

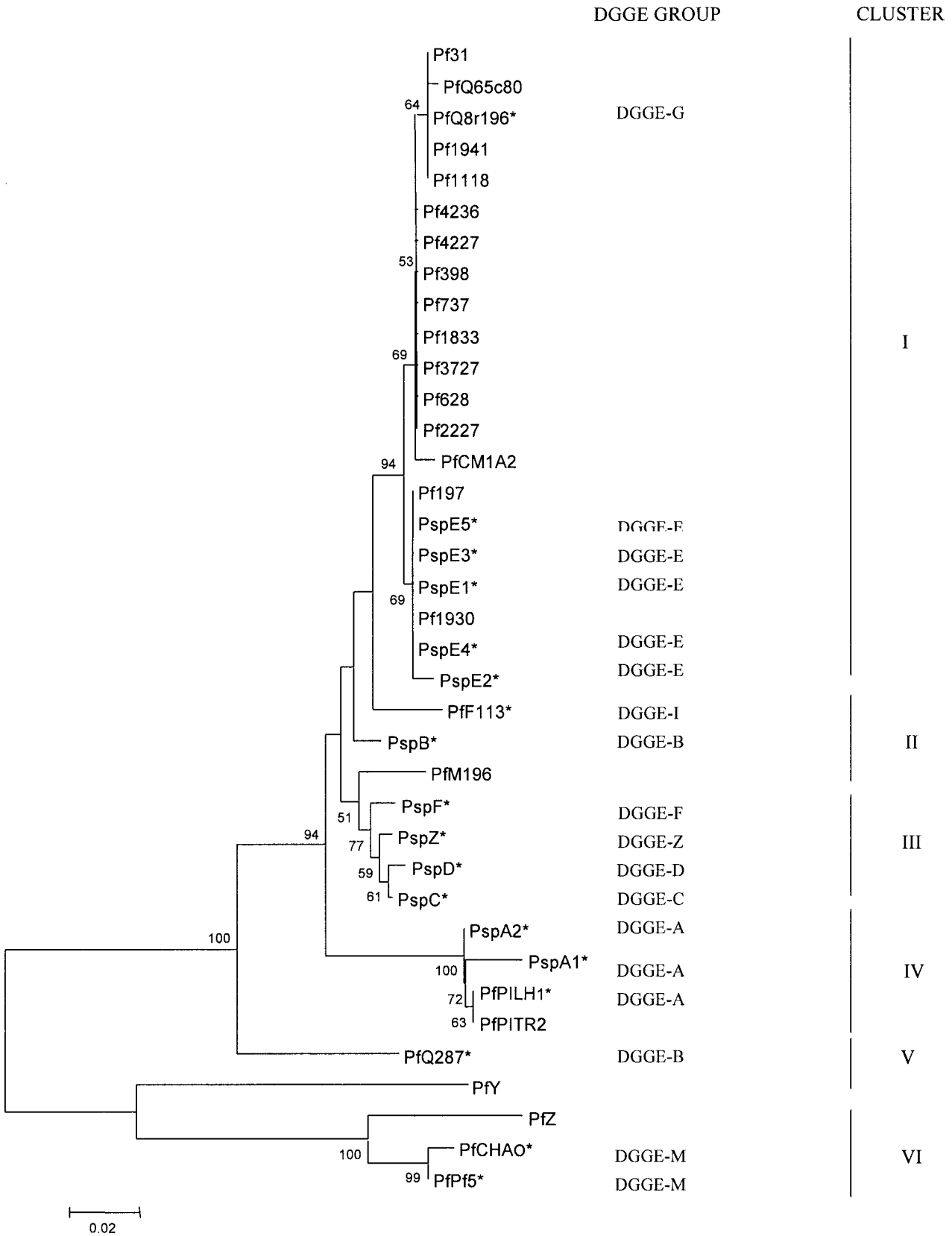

FIG. 4. Phylogenetic tree (not rooted) of 325-bp *phlD* fragments inferred by the neighbor-joining method. Bootstrap values higher than 50% are indicated at the nodes. Asterisks indicate the representative reference *phlD*$^+$ strains or isolates belonging to the different DGGE groups. Clusters I through VI are defined by bootstrap values of >75%.

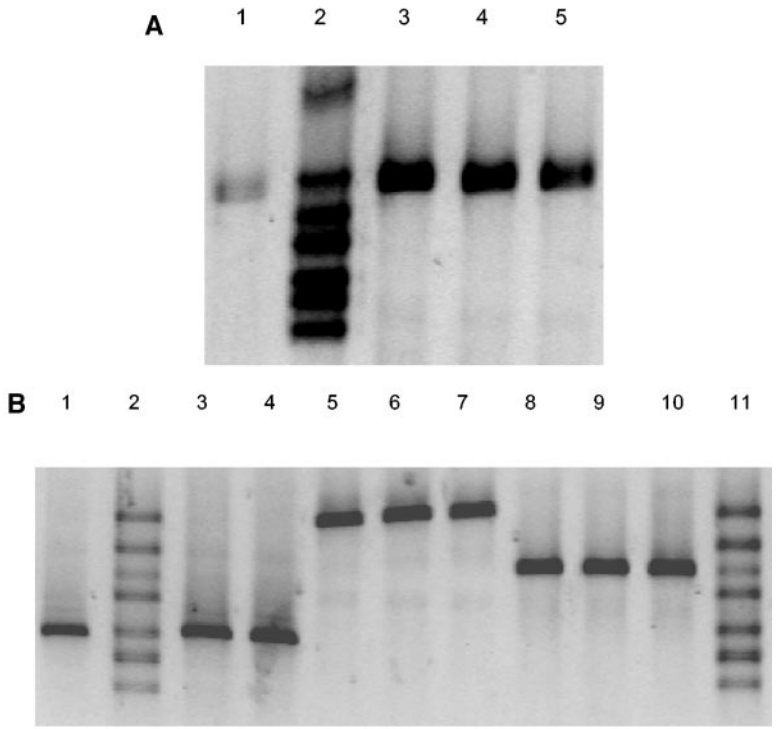

FIG. 5. (A) DGGE patterns (32 to 41% denaturant) of the 350-bp *phlD* fragment amplified from DNA extracted from the rhizosphere of wheat colonized by the rifampin-resistant derivative of *phlD*$^+$ isolate PWB532 representing DGGE group E. Based on dilution plating, isolate PWB532 was present at densities of approximately 5×10^3 CFU/g of root (lane 1), 5×10^4 CFU/g of root (lane 3), 5×10^5 CFU/g of root (lane 4), and 5×10^6 CFU/g of root (lane 5). Lane 2 contained a marker composed of *phlD* fragments amplified from DNA of seven isolates corresponding to DGGE groups G, E, F, C, Z, B, and I (from top to bottom). (B) DGGE patterns (32 to 41% denaturant) of 350-bp *phlD* fragments amplified from isolates PSC415 (DGGE group Z), Q8R1-96 (DGGE group G), and PPB3512 (DGGE group F) introduced into the sugar beet rhizosphere. Lane 1, DGGE group Z at a density of 5.3×10^5 CFU/g of root; lane 2, marker; lane 3, DGGE group Z at a density of 2.7×10^5 CFU/g of root; lane 4, DGGE group Z at a density of 1.5×10^5 CFU/g of root; lane 5, DGGE group G at a density of 2.5×10^6 CFU/g of root; lane 6, DGGE group G at a density of 7.6×10^5 CFU/g of root; lane 7, DGGE group G at a density of 1.9×10^6 CFU/g of root; lane 8, DGGE group F at a density of 8.5×10^3 CFU/g of root; lane 9, DGGE group F at a density of 2.8×10^4 CFU/g of root; lane 10, DGGE group F at a density of 2.4×10^4 CFU/g of root; lane 11, marker. The marker consisted of *phlD* fragments amplified from DNA of seven isolates corresponding to DGGE groups G, E, F, C, Z, B, and I (from top to bottom).

entially amplified, leading to incorrect assessment of all the genotypes present. In the present study, a mixture of six genotypically different *phlD*$^+$ isolates (representatives of DGGE genotypes B, C, E, F, G, and Z) was introduced into wheat rhizosphere samples to a final density of approximately 5×10^5 CFU/g of root for each isolate prior to DNA extraction. PCR-DGGE analysis showed that all six genotypes were detectable in both replicates included. In both mixed samples, one additional band was detected in the DGGE gel; this band may have been a heteroduplex between the different *phlD* sequence variants, as described previously by Kowalchuk et al. (22), or it may have represented another indigenous *phlD*$^+$ isolate present in the wheat rhizosphere sample. The latter possibility was not pursued further. Collectively, these results indicated that PCR-DGGE analysis of the *phlD* gene allows simultaneous detection of multiple genotypes present in a rhizosphere sample.

In the same experiment, the PCR-DGGE methodology was compared with the currently used rapid PCR-based protocol for rhizosphere samples (32). For the latter rapid PCR-based protocol, different dilutions of the rhizosphere samples required incubation in nutrient broth for 48 h prior to PCR and genotypic characterization. PCR and subsequent genotypic characterization showed that the rapid PCR-based protocol resulted in detection of only DGGE group G (strain Q8r1-96), whereas PCR-DGGE analysis resulted in detection of all six genotypes. In conclusion, these results indicated that cultivation of a rhizosphere sample in nutrient broth prior to genotypic characterization introduces a bias toward detecting either the most dominant genotype or the genotypes with higher growth rates or competitive abilities in the nutrient broth relative to the other genotypes present. This bias is circumvented by direct PCR-DGGE analysis of the *phlD* gene.

Biological significance of PCR-DGGE classification of *phlD*$^+$ genotypes. In order to investigate the biological significance of the additional classifications of *phlD*$^+$ genotypes obtained by the PCR-DGGE methodology described in this study, the population dynamics of eight isolates representing different DGGE groups were monitored during six successive growth cycles of sugar beet seedlings in soil obtained from an agricultural field (Fig. 6). Each of the eight isolates was introduced only once (growth cycle 0) at an initial density of approximately 5×10^4 CFU/g of soil. After the first sugar beet growth cycle, the densities of strains Q8r1-96 (DGGE genotype G) and PWB233 (DGGE genotype A) were the highest

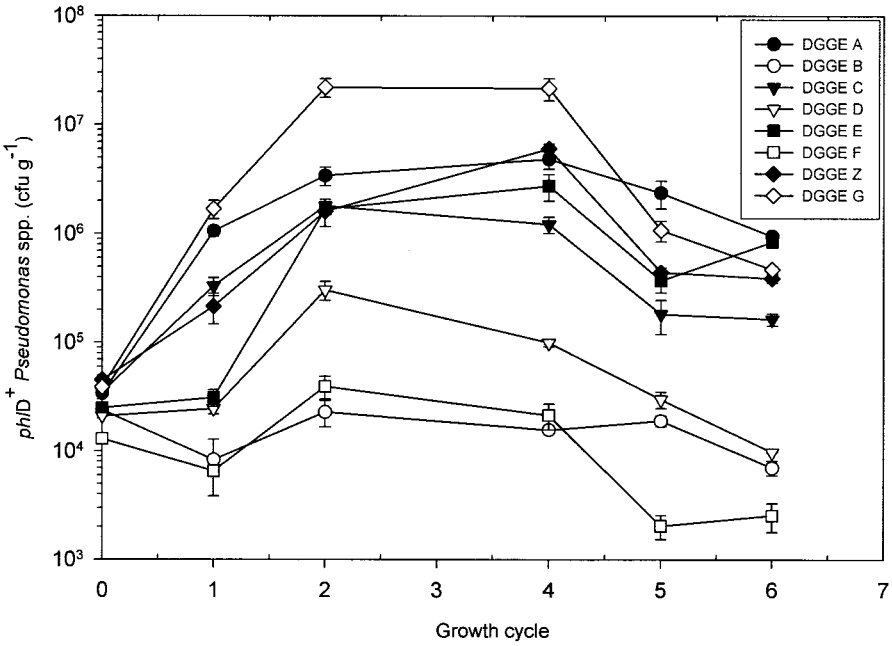

FIG. 6. Population dynamics (expressed in CFU per gram of root) of representative isolates for eight different DGGE genotypes in the sugar beet rhizosphere. Isolates were introduced separately into soil only once (cycle 0) at a density of approximately 5×10^4 CFU/g of soil. The population dynamics of each of the isolates was monitored during six successive sugar beet growth cycles consisting of 10 to 12 days each. Cycle 3 was not included. For each growth cycle, mean values for four replicates are shown. The error bars indicate the standard errors.

densities; the population densities of strain Q8r1-96 (DGGE genotype G) increased further during growth cycles 2 to 4 to values of approximately 3×10^7 CFU/g of root, whereas the population densities of PWB233 (DGGE genotype A) leveled off at values of approximately 5×10^6 CFU/g of root. After the first growth cycle, strains PWB532 (DGGE genotype E), PPB2310 (DGGE genotype C), and PSC415 (DGGE genotype Z) did not exhibit densities as high as those of strains Q8r1-96 (DGGE genotype G) and PWB233 (DGGE genotype A), but they reached densities similar to those of PWB233 (DGGE genotype A) in growth cycles 2 to 4. After six growth cycles of sugar beet seedlings, the population densities of the genotype A, E, and G strains were significantly similar ($P < 0.05$). Strains PSB211 (DGGE genotype D), PSC2218 (DGGE genotype B), and PPB3512 (DGGE genotype F) colonized the rhizosphere of sugar beet seedlings to a significantly lesser extent than the strains representing the other five DGGE genotypes. The population densities of the three strains (DGGE groups D, B, and F) did not increase above 10^5 CFU/g of root but instead declined in the last sugar beet growth cycle to values of 10^4 CFU/g of root (DGGE group D), 8×10^3 CFU/g of root (DGGE group B), and 4×10^3 CFU/g of root (DGGE group F) (Fig. 6).

These results showed that there were considerable differences in the abilities of different *phlD*$^+$ genotypes to colonize the rhizosphere of sugar beet seedlings, confirming and extending results obtained in previous studies (24, 40). Strain Q8r1-96, representing DGGE genotype G, was shown to be superior for colonization of the sugar beet rhizosphere, especially in the first four growth cycles. Similar observations were described previously for wheat (40) and pea (24). These results indicate that the ability of strain Q8r1-96 to rapidly establish and maintain high population densities in the rhizosphere is not linked to a specific plant species but may be due to specific characteristics that enable this strain to be competitive in different rhizosphere environments. In this context, Mavrodi et al. (27) recently identified possible new traits by subtractive hybridization that may contribute to the superior rhizosphere competence of strain Q8R1-96. These traits include bacteriocin production, a trait that may be advantageous in intraspecific competition with other indigenous pseudomonads. Although strain Q8r1-96 (DGGE genotype G) could not be distinguished from strain PWB532 (DGGE genotype E) on the basis of *phlD* RFLP analysis, these organisms differed considerably in the ability to colonize the rhizosphere of sugar beet seedlings. Similarly, strains PPB2310 (DGGE genotype C) and PSC2218 (DGGE genotype B), which could not be distinguished by *phlD* RFLP analysis, differed significantly in the ability to colonize the sugar beet rhizosphere. These results highlight the conclusion that the additional classification of this widely distributed group of antibiotic-producing *Pseudomonas* spp. by PCR-DGGE analysis of the *phlD* gene also provides biologically relevant discrimination. Given the level of polymorphism in specific genes involved in the regulation (*gacA*) (6) or biosynthesis of other antibiotic compounds, including pyrrolnitrin (5, 21) and phenazine antibiotics (26, 39), this technique could easily be used to provide an additional level of discrimination between isolates and strains producing other metabolites involved in rhizosphere competence and biological control of plant pathogens.

Conclusion. Establishing the presence of individual populations of antagonistic microorganisms in soil and rhizosphere environments is an important first step toward fully understanding the functional roles of the organisms in these natural

environments. Additionally, the diversity within such indigenous populations of antagonistic microorganisms with a common biocontrol trait holds promise for further improvement of biological control, especially when this diversity reflects important interactions at the host-antagonist level. The technique described in this paper allows direct detection and assessment of the genotypic diversity of a specific group of bacteria that produce DAPG, a broad-spectrum antibiotic that has been implicated in biological control of multiple plant diseases and in the natural suppressiveness of soils. More specifically, our results indicated that the PCR-DGGE methodology can be used to detect specific *phlD*$^+$ genotypes directly in rhizosphere samples with a detection limit of approximately 5×10^3 CFU/g of root and that it allows simultaneous detection of multiple genotypes present in a rhizosphere sample. Subsequent bioassays clearly showed that there is differential ability of the genotypic groups with respect to colonization of the sugar beet rhizosphere, confirming the biological significance of this methodology.

ACKNOWLEDGMENTS

This research is supported by the Technology Foundation STW (project WBI.4843), the applied science division of NWO, and the technology program of the Ministry of Economic Affairs. The contribution of Jos Raaijmakers was financially supported by the Royal Netherlands Academy of Arts and Science.

We thank Pierre de Wit for critically reading this manuscript and for his valuable suggestions.

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