RESEARCH ARTICLE

A new DGGE protocol targeting 2,4-diacetylphloroglucinol biosynthetic gene *phlD* from phylogenetically contrasted biocontrol pseudomonads for assessment of disease-suppressive soils

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Abstract

In the rhizosphere, biocontrol pseudomonads producing 2,4-diacetylphloroglucinol (Phl) can protect plants from soil-borne pathogens. DGGE of *phlD* has been proposed to monitor these bacteria, but two distinct protocols were needed for analysis of both the '*Pseudomonas fluorescens*' species complex and the strains from *rrs* restriction group ARDRA-1. Here, a single DGGE protocol performed on 668-bp GC-clamp-containing *phlD* amplicons was effective with both types of pseudomonads, and 36 reference biocontrol strains from the '*P. fluorescens*' complex or group ARDRA-1 gave a total of 11 distinct DGGE bands. *phlD* amplicons with at least two to seven nucleotidic differences could be discriminated, and the discrimination level was similar to that of *phlD* restriction analysis with four enzymes. Multiple *phlD*-DGGE bands were obtained when studying rhizosphere soil containing indigenous *phlD*$^+$ pseudomonads, and *phlD* diversity was higher when DGGE was implemented after incubation of tobacco rhizosphere extracts in semi-selective medium (MPN approach) in comparison with approaches based on direct analysis of rhizosphere DNA extracts or assessment of *phlD*$^+$ colonies. *phlD*-DGGE profiles differed for a soil suppressive and a soil conducive to black root rot of tobacco, and each soil yielded new *phlD* sequences. In conclusion, this DGGE protocol was useful for monitoring indigenous rhizosphere consortia of *phlD*$^+$ pseudomonads.

Introduction

Many strains from the fluorescent *Pseudomonas* spp. can protect plant roots from soil-borne pathogens, and this biocontrol ability often relies on the production of inhibitory compounds (Haas & Défago, 2005). Among the latter, 2,4-diacetylphloroglucinol (Phl) has been studied extensively (Fenton *et al.*, 1992; Keel *et al.*, 1992). It has been established that Phl both inhibits several pathogens of plants (Keel *et al.*, 1992; de Souza *et al.*, 2003) and induces resistance pathway(s) in the host plant (Iavicoli *et al.*, 2003). Genetic inactivation of Phl production ability abolished or reduced the biocontrol efficacy of Phl-producing strains (Fenton *et al.*, 1992; Keel *et al.*, 1992; Duffy *et al.*, 2004), and recently the statistical comparison of a world-wide collection of biocontrol pseudomonads indicated that Phl-producing strains displayed a higher plant-protecting activity in two pathosystems in comparison with nonproducing biocontrol pseudomonads (Rezzonico *et al.*, 2007).

The importance of Phl in biocontrol explains why indigenous Phl-producing fluorescent *Pseudomonas* populations have also been studied in the rhizosphere of various crops (Raaijmakers & Weller, 1998; Picard *et al.*, 2000; Weller *et al.*, 2002; Ramette *et al.*, 2003). This is particularly the case when dealing with soils that are suppressive to certain diseases (Raaijmakers & Weller, 1998; Weller *et al.*, 2002; Ramette *et al.*, 2003). Often, Phl-producing pseudomonads have been characterized by restriction or sequencing of the biocontrol-relevant gene *phlD* (Mavrodi *et al.*, 2001; Ramette *et al.*, 2001; Wang *et al.*, 2001; De La Fuente

et al., 2006; Ramette *et al*., 2006). However, all these results were based on the analysis of *phlD*$^+$ pseudomonads isolated on different growth media, which means that only a rather limited number of strains could be assessed.

Recently, a *phlD*-based denaturing gradient gel electrophoresis (DGGE) method was developed for the assessment of consortia of Phl-producing fluorescent pseudomonads (Bergsma-Vlami *et al*., 2005b). The principle of DGGE is based on differential migration of same-length PCR amplicons of different sequences when run on an acrylamide gel with increasing DNA denaturant conditions (Myers *et al*., 1985; Muyzer *et al*., 1993; Muyzer & Smalla, 1998). *phlD*-DGGE proved useful for the characterization of Phl-producing pseudomonads isolated from the rhizosphere of different plant species (Bergsma-Vlami *et al*., 2005a, b). However, the method of Bergsma-Vlami *et al*. (2005b) requires parallel use of two different primer sets and two gels with different denaturant gradients to enable exhaustive analysis of Phl-producing fluorescent pseudomonads, because of the divergence between members of the '*Pseudomonas fluorescens*' species complex and strains from group ARDRA-1, which display a distinct *rrs* restriction profile (Keel *et al*., 1996) and probably correspond to a new species (Frapolli *et al*., 2007). In addition, the *phlD* amplicon is only 350 bp long in this method, which did not enable discrimination within group ARDRA-1. Finally, the method of Bergsma-Vlami *et al*. (2005b) has not been tested directly on rhizosphere DNA extracts.

The objective of the current work was to design and validate a single *phlD*-DGGE protocol enabling simultaneous analysis of phylogenetically contrasted Phl-producing fluorescent pseudomonads in the rhizosphere. To this end, a single set of PCR and gel-denaturant gradient conditions was selected to fit all targeted strains. Method validation was carried out using a collection of Phl-producing biocontrol fluorescent *Pseudomonas* strains of a phylogenetically contrasted status. Experiments were performed using soils suppressive or conducive to *Thielaviopsis basicola*-mediated black root rot of tobacco, because (1) a diverse range of Phl-producing pseudomonads including members of both the '*P. fluorescens*' complex and group ARDRA-1 are known to colonize the tobacco rhizosphere in these soils and (2) a better description of the diversity of Phl-producing pseudomonads in these suppressive soils is needed to improve our understanding of soil suppressiveness.

Materials and methods

phlD-DGGE protocol

For all DGGE analyses, *phlD* was amplified using forward primer B2BF (25-mer, 5′-ACCCACCGCAGCATCGTTTAT GAGC-3′) containing a 40-bp GC clamp (5′-CGCCCGCC GCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG-3′) at the 5′ end, and reverse primer BPR4 (26-mer, 5′-CCGCCG GTATGGAAGATGAAAAAGTC-3′) (McSpadden Gardener *et al*., 2001). PCR was carried out in 10-μL reactions containing 2 μL of DNA lysate, 1 × PCR buffer (Amersham Biosciences, Piscataway, NJ), 5% bovine serum albumin (10 g L^{-1}; Fluka), 5% dimethyl sulfoxide (Fluka), 100 μM of each dATP, dCTP, dGTP, and dTTP (Amersham Biosciences), 0.40 μM of each primer, and 1.4 U of *Taq* DNA polymerase (Amersham Biosciences). The thermocycling program was as follows: initial denaturation at 94 °C (2 min 30 s), followed by 30 cycles (94 °C for 30 s, 60 °C for 30 s, and 72 °C for 60 s) and a final extension at 72 °C for 10 min. PCR products were analyzed on a 1% agarose gel, which confirmed the presence of a single, 670-bp band in each positive amplification.

DGGE analysis was performed using the DCode Universal Mutation Detection System (Bio-Rad, Hercules, CA). DGGE gels were cast using a double gradient ranging from 7% to 12% of acrylamide and from 30% to 60% denaturants (100% denaturant corresponds to 7 M urea and 40% deionized formamide). A marker consisting of individually amplified *phlD* amplicons from up to 10 collection strains of known DGGE migration patterns was loaded next to the samples to be analyzed. The samples were run for 14 h at 140 V in 1 × TAE buffer (40 mM Tris base, 20 mM acetic acid, 1 mM EDTA, pH 8) preheated at 60 °C. The gels were stained with SYBR Gold (Molecular Probes, Eugene, OR) for 1 h and visualized with a UV trans-illuminator.

phlD-DGGE analysis of indigenous pseudomonads grown *in vitro*

phlD$^+$ pseudomonads were grown on King's B agar (King *et al*., 1954) for 2 days, and colonies were heat-lysed (99 °C for 10 min) in water and then used as a PCR template. *phlD*-DGGE was implemented to assess individual strains, as well as mixtures of individual strains (by mixing DNA from 2 to 10 different pseudomonads, each at a final concentration of 10 ng μL^{-1}).

Preparation of soil microcosms

The *T. basicola*-suppressive soil MS8 and the conducive soils MC6 and MC112 are located in the Swiss region of Morens (Stutz *et al*., 1986, 1989; Ramette *et al*., 2003). They were collected in November 2003 and June 2006. Soil samples were taken from 10 to 30 cm depth (sandy loams, 1.9–2.2% organic matter, pH 6.8–7.6) using sterilized shovels and were maintained at 15 °C before use (within the subsequent 14 days). Root residues and stones were removed. Tobacco (*Nicotiana glutinosa* L.) was grown for 4 weeks in coarse quartz sand (1.9–2.2 mm diameter) in a growth chamber (70% relative humidity) with 16 h of light (80 mE m^{-2} s^{-1};

22 °C) and 8 h of darkness (18 °C), with weekly watering with Knop's nutrient solution (Ziegler, 1983), before transplantation into soil.

Estimation of *phlD*-DGGE detection limit

Cells of strains CHA0 and Q2–87 from log-phase cultures in Luria–Bertani medium (Sambrook *et al.*, 1989) were washed and used to inoculate UV-treated (overnight) MS8 soil with either pseudomonad, at final concentrations of 10^3, 10^4, 10^5, 10^6, and 10^7 CFU g^{-1} soil. Total DNA was extracted using the FastDNA SPIN Kit (Bio101 Systems, Q.BIOgene, Carlsbad, CA) from 0.5 g of soil, for each concentration of each strain. PCR amplification with primers B2BFgc and BPR4 was performed (as described above) and amplified products were checked on a DGGE gel (as described below) in order to detect the presence of *phlD* bands for CHA0 or Q2–87. Duplicates of each sample were analyzed.

phlD-DGGE analysis of indigenous pseudomonads from soils MS8 and MC112

The pot experiment was carried out in June 2006 and aimed at studying the indigenous populations of Phl^+ pseudomonads present in suppressive soil MS8 and conducive soil MC112. At the start of the experiment, one 4-week-old tobacco seedling was transplanted into each pot (containing 150 cm^3 of soil), and half the pots were inoculated with *T. basicola* to obtain black root rot disease, as described by Ramette *et al.* (2003). Briefly, *T. basicola* Ferraris strain ETH D127 was cultured on malt agar for 3 weeks at 24 °C. The mycelia and the endoconidia were suspended in distilled water, filtered through sterile glass wool to eliminate the mycelia, and the resulting endoconidia suspension (5 mL, to reach 10^3 endoconidia cm^{-3} soil) was added to soil around the stems of tobacco plants on the day of transplantation in Morens soils. The same volume of distilled water was added to the noninoculated controls. Plants were grown for 3 weeks, during which soil water content was adjusted to 25% w/w (retention capacity for these soils) by watering pots every 1–2 days with distilled water. Eight pots (half of them inoculated with *T. basicola*) were studied per soil and each plant was used as one replicate. Inoculation with *T. basicola* resulted in a disease index (Stutz *et al.*, 1986) of 5–38% for plants in soil MS8 and 5–95% for plants in soil MC112, whereas there was no disease in the absence of inoculation.

Three approaches were followed to study tobacco rhizosphere pseudomonads, based on the analysis of (1) $phlD^+$ *Pseudomonas* colonies grown on a solid semi-selective medium, (2) bacterial cell suspensions (obtained from rhizosphere extracts and serial dilutions) after incubation in a most probable number (MPN) microtiter plate containing liquid semi-selective medium for $phlD^+$ *Pseudomonas* consortia, and (3) total rhizosphere DNA.

For the first two approaches, the root systems were shaken to remove loosely adhering soil, washed with sterile distilled water and placed (usually 0.05–0.10 g roots and tightly adhering soil) in sterile 50 mL falcon tubes filled with 10 mL of 0.9% NaCl solution. The tubes were shaken for 30 min at 350 r.p.m., and they were used as rhizosphere extracts. For the third approach, which uses a kit optimized for soil samples (i.e. the FastDNA SPIN Kit), a distinct sample consisting of 0.5 g of rhizosphere soil and fine roots was used per plant.

For isolation of $phlD^+$ *Pseudomonas* colonies, 200 μL of the rhizosphere extracts were used to perform serial dilutions in 0.9% NaCl solution and then 100 μL of the dilutions were plated onto a *Pseudomonas* semi-selective medium consisting of King's B medium amended with cycloheximide (100 μg mL^{-1}; Fluka, Buchs, Switzerland), chloramphenicol (13 μg mL^{-1}; Fluka), and ampicilline (40 μg mL^{-1}; Sigma Chemicals, St Louis, MO), i.e. medium KB^{+++} (Simon & Ridge, 1974). The plates were incubated at 27 °C for 2 days, and fluorescent colonies were selected (up to 24 colonies for each of the 16 plants). Cell lysis and subsequent *phlD* PCR were performed as described above. Long-term storage of bacteria was performed at − 80 °C in 40% glycerol.

For growth of $phlD^+$ *Pseudomonas* consortia, rhizosphere extracts as well as serial dilutions were mixed with KB^{+++} medium, using the following MPN design. A 96-well microtiter plate in which each well contained 180 μL of KB^{+++} was used to perform decimal dilutions using 20 μL of rhizosphere extract (four wells per dilution). The microtiter plates were covered with a thermal seal film (Axygen scientific, Union City, CA) to avoid steaming and were incubated for 2 days at 27 °C under shaking at 150 r.p.m. Microtiter plates were then checked for the occurrence of (1) bacterial growth and fluorescence and (2) $phlD^+$ pseudomonads by PCR (described above), after cell lysis performed by incubating 20-μL samples (mixed with 50 μL water) at 99 °C for 10 min. The MPN plates were stored at − 80 °C in 40% glycerol.

In each of the three approaches, PCR amplification was performed (as described above) to verify the actual presence of $phlD^+$ pseudomonad(s), i.e. with colonies on KB^{+++}, positive MPN wells (i.e. displaying both bacterial growth and fluorescence), and rhizosphere DNA. *phlD*-DGGE was performed as described above, except that PCR products were treated with 1 U of Mung Bean Nuclease (New England Biolabs, Beverly, MA) per microgram of DNA in order to remove single-stranded DNA and cleave hairpin loops resulting from DNA-heteroduplex formation.

phlD-DGGE analysis of soils inoculated with *Pseudomonas* strains

The second pot experiment aimed at studying mixtures of inoculant strains. Three strains of distinct *phlD* migration

patterns (i.e. CHA0, F113, PITR2) were mixed and inoculated to soil next to 3-week-old tobacco plants growing in soils MS8 and MC6 (both collected from the field in November 2003), to reach a final concentration of 10^8 CFU g^{-1} of soil for each strain. *Thielaviopsis basicola* was not added. After one week of plant growth, extracts from the tobacco rhizosphere were prepared and studied using the MPN method. Cell lysis was performed and *phlD* amplicons were analyzed by DGGE, as described above. Samples were also studied from noninoculated plants (control). Three pots, each containing one plant, were studied per treatment, and each plant was used as one replicate.

Sequencing of *phlD* from DGGE bands and *Pseudomonas* isolates

The *phlD* sequence was available in GenBank for nine of the 36 collection strains. Here, sequencing of *phlD* fragments was performed for (1) the 27 other collection strains (listed in Table 1), (2) Morens isolates from the first pot experiment, and (3) DGGE bands from the *in vitro* experiments and the two pot experiments. In the latter case, the DGGE bands of interest were cut out from the stained DGGE gel with sterile tips. The band discs (*c.* 1 mm in diameter) were placed each in 100 μL of sterile Millipore water and left at room temperature for about 30 min with shaking at 300 r.p.m. Then, water was discarded, discs were blotted dry on towel paper and placed directly in PCR tubes.

The *phlD* amplicons were obtained using primers B2BF/BPR4 for DGGE bands and 20-mer primers Phl2a (5′-GAGGACGTCGAAGACCACCA-3′) and Phl2b (5′-ACCGCAGCATCGTGTATGAG-3′) (Raaijmakers *et al.*, 1997) for isolates (because they yielded longer amplicons). PCR amplification was performed as described above, except that 35-μL reaction mixtures and 7 μL of DNA lysate were used. PCR products were analyzed on a 1% agarose gel, which confirmed the presence of a single band (670 or 745 bp, respectively) in each positive amplification, and the migration pattern of the *phlD* sequences thus obtained was checked by DGGE, to verify that it matched the one of the original amplicon. PCR products were purified by two washes with 40 μL of double-distilled water on a

Table 1. Origin and biocontrol properties of the 36 reference Phl^+ fluorescent *Pseudomonas* strains used in this study

Strain	Biocontrol activity*	Plant host	References
Strain from Ireland			
F113	Sugar beet (Pu)	Sugar beet	Fenton *et al*. (1992)
Strain from Texas (USA)			
Pf-5	Cotton (Pu, Rs), cucumber (Pu)	Cotton	Howell *et al*. (1979)
Strains from Quincy (WA, USA)			
Q2-87	Wheat (Ggt)	Wheat	Vincent *et al*. (1991)
Q37-87	Wheat (Ggt)	Wheat	Keel *et al*. (1996)
Q65c-80	Wheat (Ggt)	Wheat	Harrison *et al*. (1993)
Strains from Morens (Switzerland)			
C6-2, C6-9, C6-11, C6-16, C6-23 (soil MC6)	Tobacco (Tb)	Tobacco	Ramette *et al*. (2003)
C10-181, C10-186, C10-189, C10-190, C10-197, C10-204, C10-205 (soil)	Tobacco (Tb)	Tobacco	Ramette *et al*. (2003)
S7-29, S7-42, S7-46, S7-52 (soil MS7)	Tobacco (Tb)	Tobacco	Ramette *et al*. (2003)
S8-62, S8-110, S8-130, S8-151 (soil MS8)	Tobacco (Tb)	Tobacco	Ramette *et al*. (2003)
CHA0	Tobacco (Tb), wheat (Ggt), cucumber (Pu)	Tobacco	Stutz *et al*. (1986)
CM1′A2	Cucumber (Pu, Ps), cotton (Rs)	Cucumber	Fuchs *et al*. (1991)
K93.2	Cucumber (Pu), tomato (FORL)	Tobacco	Wang *et al*. (2001)
P12	Tobacco (Tb), cucumber (Pu)	Tobacco	Keel *et al*. (1996)
Strains from El Batan (Mexico)			
P97.38	Cucumber (Pu), tomato (FORL)	Cucumber	Wang *et al*. (2001)
Strains from Albenga (Italy)			
PILH1	Cucumber (Pu), tomato (FORL)	Tomato	Keel *et al*. (1996)
PITR2	Cucumber (Pu), tomato (FORL)	Wheat	Keel *et al*. (1996)
Strains from Nitra (Slovakia)			
K94.31, K94.37	Tomato (FORL)	Cucumber	Wang *et al*. (2001)
P97.30	Cucumber (Pu), tomato (FORL)	Wheat	Wang *et al*. (2001)
Strain from Tallinn (Estonia)			
F96.27	Cucumber (Pu), tomato (FORL)	Cucumber	Wang *et al*. (2001)

*Ggt, *Gaeumannomyces graminis* var. *tritici*; FORL, *Fusarium oxysporium*; Ps, *Phomopsis sclerotioides*; Pu, *Pythium ultimum*; Rs, *Rhizoctonia solani*; Tb, *Thielaviopsis basicola*.

MultiScreen PCR plate (Millipore, Molsheim, France), resuspended in 30 μL of double-distilled water, and visually quantified using an agarose gel.

Double-strand sequencing was performed with 3–10 ng of purified PCR product and 0.16 μM of each primer (B2BF/BPR4 or Phl2a/Phl2b), using an ABI PRISM BigDye Terminator v3.0 cycle sequencing kit and an ABI PRISM 3100 genetic analyzer (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions. DNA chromatograms were edited with the Sequencher package (version 4.1, Gene Codes Corporation, Ann Arbor, MI).

Phylogenetic inference

The *phlD* sequences obtained in this study are available at accession numbers EF554331–EF554357 (for collection strains) and EF569137–EF569184 (for PCR clones). The accession numbers of previous *phlD* sequences included in clustering analyses are shown in parentheses in Fig. 2.

The *phlD* sequences were aligned using CLUSTAL W (Thompson *et al.*, 1994). Sites presenting gaps were excluded from the analysis with BIOEDIT (Castresana, 2000). The Molecular Evolutionary Genetics Analysis (MEGA) program version 3.1 (Kumar *et al.*, 2001) was used to infer (1) a phylogenetic tree based on the neighbor-joining method and Kimura's two-parameter and (2) a dendrogram based on the unweighted pair group method with arithmetic mean (UPGMA) using the number of nucleotide differences. The reliability of both trees was evaluated by 1000 bootstrap replicates. In the UPGMA tree, clusters were defined at an arbitrarily chosen similarity level of 99.1%.

Results

phlD-DGGE analysis and sequencing of *phlD* bands for known *Pseudomonas* strains

When 36 known *phlD*$^+$ *Pseudomonas* strains (Table 1) were assessed by *phlD*-DGGE, it appeared that (1) all 36 strains produced a PCR amplicon, (2) only one band was obtained per strain, and (3) 11 different bands were evidenced (Fig. 1a). These 11 bands were termed a–k, from the lowest to the highest denaturant concentration (Table 2).

The 36 *phlD*$^+$ *Pseudomonas* strains yielded 23 different partial *phlD* sequences, i.e. 23 alleles (listed in Table 2). When the relation between the electrophoretic migration of the bands (628-bp *phlD* fragment plus the 40-bp clamp) and alleles (based on the sequenced 620-bp) was assessed, it appeared that a difference of two nucleotides between *phlD* sequences was enough to produce different DGGE bands in some cases (e.g. for Pf-5 vs. C6–23). In other cases, however, *phlD* sequences differing by as many as seven nucleotides could have the same migration pattern (e.g. for Q65c-80 vs. K94.31). Alleles with more than seven nucleotides difference always produced different DGGE bands. GC content, which ranged between 53.39% and 61.94%, was one significant factor determining electrophoretic mobility ($R^2 = 0.93$, $n = 36$). Yet, in a few cases, certain alleles migrated further than sequences of a higher GC content, especially for strain Q2–87 (band g, 55.00% GC) in comparison with strains Q65c-80, CM1′A2, K93.2, S8–130, S8–110, C10-197, P12, C10-204, C10-181, F113, Q37-87, and K94.37 (bands b–f, 55.16–56.61% GC).

Relation between *phlD*-DGGE results and other *phlD* properties for known *Pseudomonas* strains

Clustering analysis of partial *phlD* sequences, based on (1) the current 36 *phlD*$^+$ *Pseudomonas* strains (Table 1), (2) other pseudomonads from the literature, and (3) DGGE bands from rhizosphere analysis in this work, evidenced nine *phlD* clusters at a cut-off level of 99.1% similarity (Fig. 2). When considering only the 36 *phlD*$^+$ pseudomonads, three of the clusters each corresponded to a distinct *phlD*-DGGE band, i.e. clusters 4 (b and d; only one strain tested by DGGE), 6 (a), and 7 (g; only one strain tested by DGGE) (Table 2). In addition, two other clusters corresponded to two bands each, i.e. clusters 1 (bands e and c) and 5 (b and c), but with certain bands that were shared by different clusters, i.e. bands b (clusters 2 and 5), c (1, 2, and 5), and e (1 and 2). Finally, two clusters displayed two cluster-specific bands each, i.e. clusters 8 (band h or i) and 9 (j or k). Therefore, the distinctions made based on *phlD*-DGGE banding pattern were compatible overall with *phlD* phylogenetic data, but the relations derived from the *phlD*-DGGE did not coincide entirely with phylogenetic information.

Ramette *et al.* (2006) had assessed 26 of the 36 *phlD*$^+$ *Pseudomonas* strains by restriction analysis of *phlD* in four separate enzymatic reactions. Strains with different restriction patterns yielded different *phlD*-DGGE bands, except that strains with restriction pattern FBDC or FDBC produced DGGE band b and strains with pattern HBNC or HBDC produced DGGE band c (Table 2). In parallel, ARDRA-1 strains yielding DGGE band j (e.g. CHA0) or k (Pf-5) could not be discriminated by *phlD* restriction analysis. Therefore, the level of discrimination of both methods was rather similar.

Relation between *phlD*-DGGE results and broad genotypic properties for known *Pseudomonas* strains

The relation between *phlD*-DGGE results and broad genotypic properties for known *phlD*$^+$ *Pseudomonas* strains was assessed by considering (1) the distinction between the '*P. fluorescens*' complex and group ARDRA-1, (2)

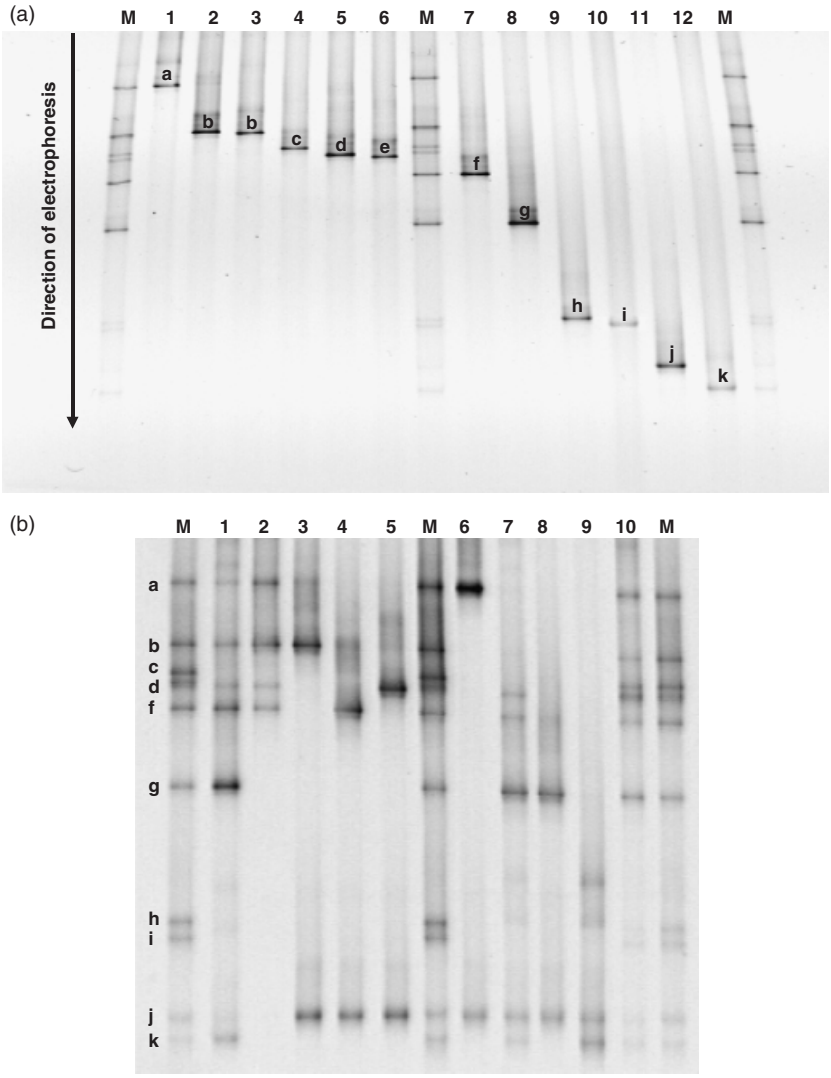

Fig. 1. DGGE of *phlD* fragments amplified from individual *Pseudomonas* strains (a) and mixtures of cell lysates of two to 10 different pseudomonads (b). In (a), the strains were PITR2 (lane 1), Q65c-80 (lane 2), K93.2 (lane 3), CM1′A2 (lane 4), Q37-87 (lane 5), C10-181 (lane 6), F113 (lane 7), Q2-87 (lane 8), P97.38 (lane 9), F96.27 (lane 10), CHA0 (lane 11), and Pf.5 (lane 12). Letters a–k indicate bands at different migration distances. In (b), mixtures were prepared using strains PITR2 (band a), Q65c-80 (b), CM1′A2 (c), Q37-87 (d), F113 (f), Q2-87 (g), P97.38 (h), F96.27 (i), CHA0 (j), and Pf-5 (k), as follows. Lane 1: cell lysates from strains with a, b, d, f, g, j, and k bands were coamplified in the PCR reaction; lane 2: a, b, d and f; lane 3: b and j; lane 4: f and j; lane 5: d and j; lane 6: a and j; lane 7: d, f, g, and j; lane 8: g and j; lane 9: j and k; lane 10: a, b, c, d, f, g, h, i, j, and k. M, mix of independently amplified *phlD* fragments representing each DGGE pattern except that of strain C10-181 (pattern e).

phylogenetic groups based on multiple housekeeping loci, and (3) enterobacterial repetitive intergenic consensus (ERIC) PCR profiling (Table 2). First, among the 11 *phlD*-DGGE bands, nine were obtained for strains from the '*P. fluorescens*' species complex (i.e. bands a–i) and the two others for ARDRA-1 strains (i.e. bands j and k).

Second, five phylogenetic groups (A–E) of *phlD*$^+$ pseudomonads have been defined within the '*P. fluorescens*' complex based on multiple housekeeping loci (Frapolli *et al.*, 2007). At this lower taxonomic level, phylogenetic group D corresponded to *phlD*-DGGE band a, C to band g, and E to bands h and i (Table 2). Group A corresponded mainly to band b (11 strains) and to a lesser extent band c (one strain), whereas group B corresponded to bands c (five strains), d (two strains) and b, e, and f (one strain each).

Third, based on the ERIC-PCR data of Ramette *et al.* (2006), it appeared that the *phlD*-DGGE band a corresponded to ERIC-PCR clusters VI and VII, bands b and e to cluster III, band c to cluster IV, band f to cluster II, band g to cluster V, and bands j and k to cluster I (Table 2). However, the strains with *phlD*-DGGE band a (five strains), b (11 strains), and c (four strains) gave, respectively, 5, 8, and 3 distinct individual ERIC-PCR genotypes. Therefore, the discrimination power of *phlD*-DGGE was inferior to that of ERIC-PCR for the 26 *phlD*$^+$-producing pseudomonads studied.

phlD-DGGE analysis of strain mixtures *in vitro*

Because the advantage of DGGE is the possibility of analyzing samples containing multiple alleles, we checked whether mixed *phlD* fragments of different migration patterns could be detected simultaneously by DGGE. When DNA extracted from up to 10 different pseudomonads was mixed and

Table 2. Relationship between *phlD*-DGGE bands and genotypic characteristics of Phl-producing pseudomonads

		phlD properties				ERIC-PCR properties‖	
DGGE band*	Strains	*phlD* allele†	*phlD* cluster‡	*phlD* restriction analysis§	Multilocus groups¶	ERIC-PCR genotype††	ERIC-PCR cluster
a	PITR2	a1	6	DCCC	D	S	VI
	C10-190, C10-189	a2	6	DCCC	D	Q, T	VII
	C10-205, C10-186	a3	6	DCCC	D	R, U	VII
	PILH1	a4	6	–**	D	–	–
b	Q65c-80	b1	5	FBDC	A	F	III
	P97.30, K94.31, C6-9, C6-16, S7-	b2	5	FDBC	A	–, –, D, D, E,	III
	46, S8-151, C6-11, S7-29 and S7-	b3	5	FBDC	A	G, J, H, I	III
	52						
	S7-42	b4	2	–	B	K	–
	K93.2					–	
c	CM1′A2	c1	5	–	A	–	–
	C10-197	c2	1	HBNC	B	N	IV
	C10-204	c3	1	HBDC	B	M	IV
	S8-110 and S8-130	c4	1	HBNC	B	M, O	IV
	P12	c5	1	–	B	–	–
d	Q37-87	d1	4	–	B	–	–
	K94.37	d2	2	–	B	–	–
e	C10-181	e1	1	JDCC	B	S	III
f	F113	f1	–	GBCC	B	C	II
g	Q2-87	g1	7	BBBB	C	P	V
h	P97.38	h1	8	–	E	–	–
i	F96.27	i1	8	–	E	–	–
j	CHA0	j1	9	AAAA	F	A	I
	C6-2, S8-62 and C6-23	j2	9	AAAA	F	A	I
k	Pf-5	k1	9	AAAA	F	B	I

*Migration of bands consisting of 628-bp *phlD* sequence and the 40-bp GC clamp.

†Differences between *phlD* alleles were determined from the alignment of 620 nucleotides.

‡The *phlD* clusters were those determined in Fig. 2.

§Restriction analysis was done by Ramette *et al*. (2006), one enzyme at a time, using respectively HaeIII, CfoI, MspI, and NdeII. Restriction profiles of strains C6-11, S7-29, S7-52 and S8-130 have been corrected in comparison with data in Ramette *et al*. (2006).

¶Multilocus groups were determined by Frapolli *et al*. (2007) based on phylogenetic analysis of concatenated sequences for 10 housekeeping genes.

‖ERIC-PCR clusters and genotypes were determined in Ramette *et al*. (2006).

**Not determined.

††When more than one strain, the order of letters corresponds to the order in which strains are listed.

coamplified by PCR, all corresponding strains could be recovered by *phlD*-DGGE, i.e. the expected migration patterns were all present on the gel (Fig. 1b). However, the intensity of the bands for strains F96.27, P97.38, CHA0, and Pf-5 (all present in the part of gel with high denaturant concentration) was weaker compared with that of the other strains.

phlD-DGGE analysis of indigenous pseudomonads in two nonsterile soils and impact of sample preparation on *phlD*-DGGE patterns

phlD-DGGE analysis of indigenous rhizosphere pseudomonads was performed in soils MS8 and MC112, using 16

Fig. 2. UPGMA tree inferred from partial *phlD* sequences obtained in this and previous studies. Except for reference strains CHA0, CM1′A2, K93.2, P12, and P97.38, *phlD* sequences for *Pseudomonas* isolates from Morens are named according to the soil of origin (S7-, S8-, C6-, C10-, or C112-), followed by a strain number. *phlD* sequences for PCR clones (from MPN culture or rhizosphere DNA) are named according to the soil of origin (S8-, C6-, or C112-), followed by (1) the abbreviation mc (for MPN culture) or rd (for rhizosphere DNA) and (2) plant (x) and clone (y) numbers (i.e. x.y). In addition to sequence names, soil harvest time is indicated in parenthesis i.e. *A99* (April 1999; isolates described in Ramette *et al*., 2003, 2006), *N03* (November 2003), and *J06* (June 2006). For visual clarity, *phlD* sequences from Morens suppressive soils MS7 and MS8 are depicted by ▲ and ■, respectively, and those from Morens conducive soils C6, C10, and C112 by △, □, and ◇, respectively. The *phlD*-DGGE band and (when available) allele are shown using letters a–k and m–o, followed by numbers. For *phlD* sequences obtained in previous studies, the accession number is given in parenthesis. Clusters were arbitrarily defined at a similarity level of 99.1%. Bootstrap values > 50% are shown.

tobacco plants per soil. Half the plants were inoculated with *T. basicola* to obtain black root rot disease, but this only had negligible effects on DGGE profiles (i.e. when compared with noninoculated plants). Therefore, for simplicity, the results for inoculated and noninoculated plants are shown without distinction (Table 3 and Fig. 3). The DGGE profiles varied when processing *phlD*$^+$ *Pseudomonas* isolates, MPN cultures, or total rhizosphere DNA. *phlD*$^+$ *Pseudomonas*

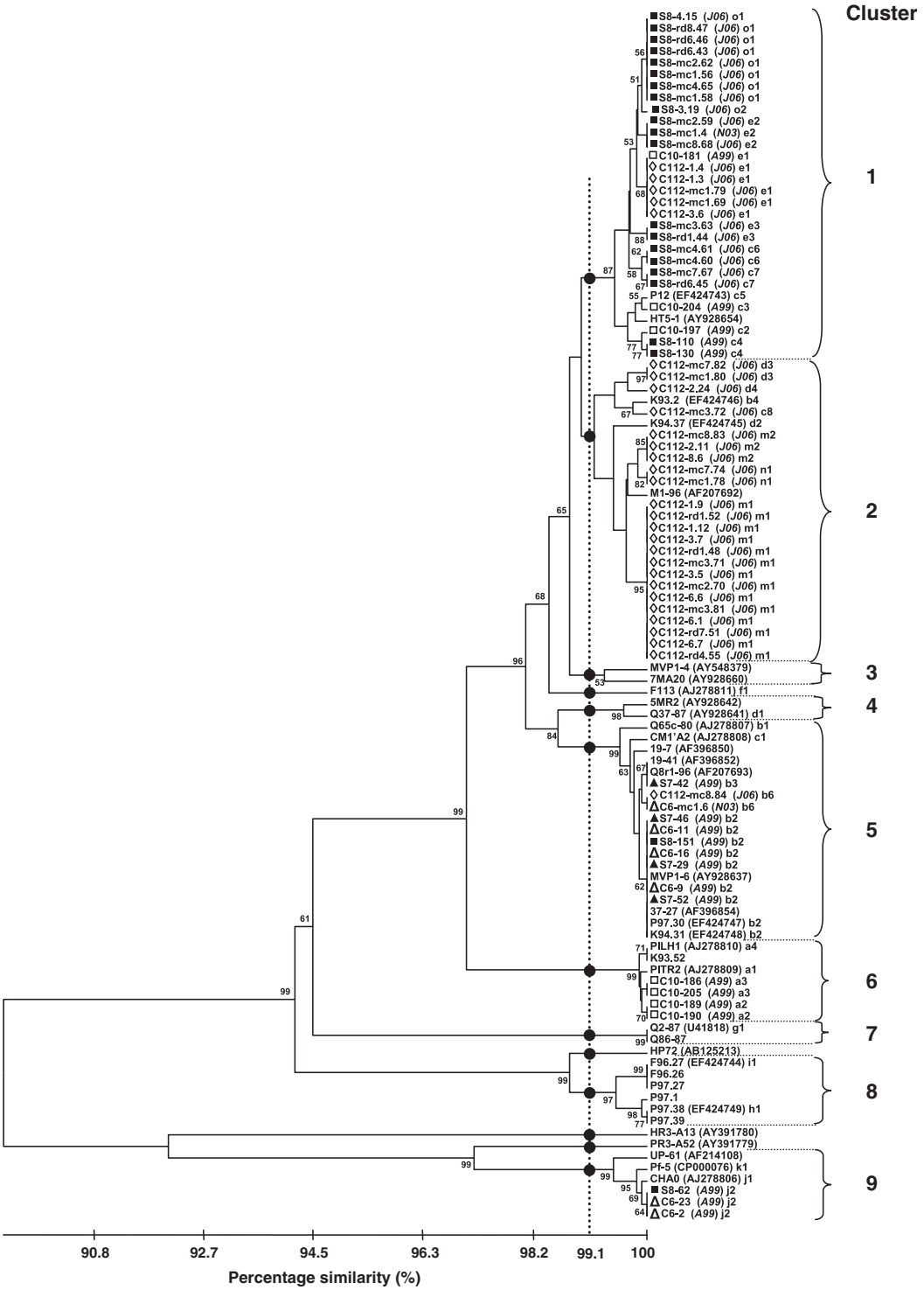

Table 3. DGGE analysis of *phlD* genes for indigenous pseudomonads using MPN cultures, *Pseudomonas* colonies, and rhizosphere DNA

	Number of positive *phlD* amplifications*	Samples analyzed by DGGE	DGGE bands per plant†	Total DGGE bands‡	Sequenced bands§	Number of alleles
Pseudomonas consortia in MPN cultures						
MS8	64/64	64	3	3	11	5
MC112	64/64	64	3	6	13	6
Pseudomonas colonies						
MS8	2/384	2	1	1	2	1
MC112	120/303	107	2	4	13	4
Rhizosphere DNA						
MS8	16/16	16	2	3	5	3
MC112	16/16	16	2	2	4¶	1

*The number of positive *phlD* amplifications with primers B2BF/BPR4 out of the total number of samples when studying (1) MPN wells at the 10^{-1} dilution (four wells for each of the 16 plants; MPN culture approach), (2) randomly-selected colonies (up to 24 colonies for each of the 16 plants; *Pseudomonas* colony approach), and (3) rhizosphere DNA extracts (one DNA extract for each of the 16 plants; rhizosphere DNA approach). In all cases, the 16 plants consisted of eight *Thielaviopsis basicola*-inoculated and eight noninoculated plants.

†Total number of different DGGE bands when considering together all results from one plant.

‡Total number of different DGGE bands when considering together all results from all 16 plants.

§Each band was sequenced one to three times (using PCR products from the same plant or different plants).

¶Sequencing of DGGE band *n* (Fig. 1b) was not successful.

isolates represented two of the 384 *Pseudomonas* colonies from soil MS8 vs. 120 of the 303 colonies from soil MC112 (Table 3). Overall, these 122 *phlD*$^+$ *Pseudomonas* isolates gave a total of five different DGGE bands.

When *phlD*$^+$ *Pseudomonas* consortia enriched in MPN cultures were studied (four wells per plant at the 10^{-1} dilution, 128 wells in total), a total of seven different DGGE bands were obtained, four of them already found with isolates (Table 3). For each treatment, the same *phlD* bands were obtained in all four replicates (data not shown). When more diluted *phlD*-positive wells (i.e. 10^{-2} to 10^{-5} dilutions) were compared with the 10^{-1} dilution (done only for *T. basicola*-inoculated plants), the number of bands was lower (i.e. six bands at 10^{-2}, six at 10^{-3}, four at 10^{-4}, and four at 10^{-5}). However, a new band was obtained at both the 10^{-3} and the 10^{-5} dilutions and so, based on all dilutions, a total of eight different DGGE bands were found. When considering individual plants, up to 3, 2, and 1 bands were identified at, respectively, the 10^{-1} and 10^{-2} dilutions, the 10^{-3} and 10^{-4} dilutions, and the 10^{-5} dilution.

Direct analysis of total rhizosphere DNA for the 32 plants studied gave five different DGGE bands (Table 3). All of them were already found with MPN cultures and sometimes also with isolates, although distinct samples were used to obtain rhizosphere DNA.

From a given soil, up to three different *phlD*-DGGE bands were obtained per plant with the MPN approach vs. up to two when using colonies or rhizosphere DNA (Table 3). When combining all the results for the two soils, it appears that the highest number of DGGE bands was found when analyzing MPN cultures at the lowest dilution (10^{-1}). Sequencing indicated that bands recovered with all three methods did not always display the same sequence, but this was also the case when comparing the same band obtained on different occasions with the same method (Fig. 3). Five different *phlD* alleles were found in MPN cultures from soil MS8 (harvested in June 2006), all of which differed from the five alleles evidenced by Ramette *et al.* (2003) in rhizosphere isolates from the same soil (harvested in April 1999). In conclusion, the most extensive results were found with the MPN method, and the other rhizosphere experiment was performed using this method.

phlD-DGGE analysis of *Pseudomonas* mixtures inoculated in two nonsterile soils

Strains CHA0 (group ARDRA-1), F113, and PITR2 (both belonging to the '*P. fluorescens*' complex) were coinoculated at the same amounts to nonsterile soil where tobacco plants were growing, and rhizosphere extracts were obtained after another week of plant growth. *phlD*-DGGE analysis of rhizosphere extracts after incubation in MPN cultures produced the three bands corresponding to strains PITR2 (i.e. band a), F113 (f), and CHA0 (j) in soil MS8 and the two bands corresponding to strains F113 and CHA0 in soil MC6 (Fig. 4). Indeed, band sequencing evidenced the alleles of strains PITR2 (a1), F113 (f1, found twice), and CHA0 (j1). The bands produced by the three inoculants were not found when studying noninoculated soil.

The inoculation experiment was also a first opportunity to assess the potential of *phlD*-DGGE to evidence indigenous *phlD*$^+$ pseudomonads. In the noninoculated controls, one band (i.e. band e; Fig. 4) with the migration pattern of

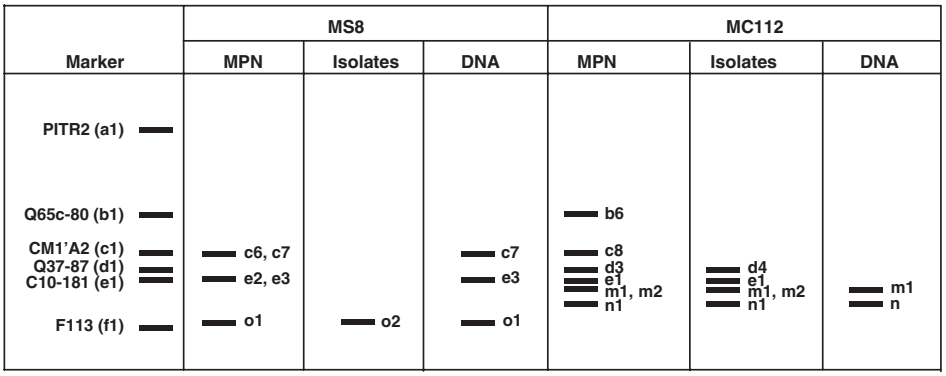

Fig. 3. *phlD*-DGGE banding patterns obtained from the tobacco rhizosphere of soils MS8 and MC112 based on the MPN culture approach (MPN), *Pseudomonas* colonies (isolates), and rhizosphere DNA (DNA). Band positions are indicated with letters a–f (for bands of collection strains) and m–o (for new bands), and different numbers are used for bands of the same position but different *phlD* sequences. The bands displayed summarize all results obtained with both *Thielaviopsis basicola*-inoculated and noninoculated plants.

that produced by strain C10-181 was found in MS8, and another band (band b) was present in MC6. Based on 545 aligned sites, the sequence of the *phlD* band recovered from MS8 did not match the sequence of any of the inoculants. The sequence of the *phlD* band found in MC6 (i.e. b1) matched the sequence from a strain isolated three years later in another conducive soil (MC112).

Indigenous *phlD*$^+$ pseudomonads were also evidenced in *Pseudomonas*-inoculated soil. DGGE analysis and sequencing indicated that *phlD*-DGGE band e1 found in noninoculated MS8 soil was also identified when inoculation was performed, whereas in soil MC6, band b1 was evidenced only in the noninoculated soil (Fig. 4).

Detection limit for *phlD*-DGGE

When *phlD*-DGGE was performed after enrichment in MPN cultures, the theoretical detection limit was 5×10^4 cells g^{-1} soil based on the analysis of the first dilution in the microtiter plate (which can be lowered if the dilution process is changed). When total soil DNA was used directly, the experimental detection limit based on visual observation of the *phlD* amplicon was 10^5 CFU g^{-1} soil with Q2-87, for which *phlD* was readily amplified by the primer set chosen. The detection limit was only 10^7 CFU g^{-1} soil with CHA0, a strain for which there were three mismatches for each of the primers. DGGE analysis of the amplicons confirmed the presence of *phlD* bands corresponding either to CHA0 or to Q2-87 (data not shown). No other bands were observed.

Comparison of the new *phlD* alleles evidenced in this study

The UPGMA tree in Fig. 2 was inferred based on 545-bp *phlD* sequences (1) from collection strains of the present (i.e. 27 of 36 strains listed in Table 1 plus Q86-87, F96.26, P97.39, K93.52, P97.1, P97.27, and K94.38, which were not used in DGGE analysis) and previous studies (23 sequences representing the main alleles documented so far) and (2) obtained here when studying soils MS8, MC6, and MC112 (48 sequences). The latter sequences, which corresponded mostly to new *phlD* alleles (43 of 48), clustered within three of the nine clusters defined in the tree (i.e. clusters 1, 2, and 5) next to previously known sequences. Cluster 1 gathered 20 sequences from MS8 and five sequences from MC112 (as well as two earlier sequences from Morens conducive soil MC10), cluster 2 gathered 23 sequences from MC112, and cluster 5 gathered one sequence from MC112 and one sequence from MC6 (as well as three earlier sequences from MC6, one from MS8, and four from Morens suppressive soil MS7). No *phlD* allele from this study was found in other clusters although clusters 3, 6, and 9 contain earlier sequences from Morens soils MS8, MC6, and MC10. The neighbor-joining tree inferred from the same set of sequences was very similar to the UPGMA tree (data not shown).

Discussion

This work was undertaken to improve the *phlD*-DGGE approach developed by Bergsma-Vlami *et al.* (2005b) for analysis of Phl-producing pseudomonads, which play an important role in biocontrol interactions. More specifically, the aim was (1) to enable DGGE analysis of all *phlD* alleles using a single *phlD*-DGGE protocol rather than having to combine two protocols, (2) to monitor not only *Pseudomonas* inoculants but also to extend the analysis to indigenous pseudomonads, (3) to use a larger *phlD* amplicon, thereby enhancing polymorphism, (4) to identify the most efficient sample preparation method to recover high *Pseudomonas* diversity, and (5) to strengthen DGGE data by sequencing of

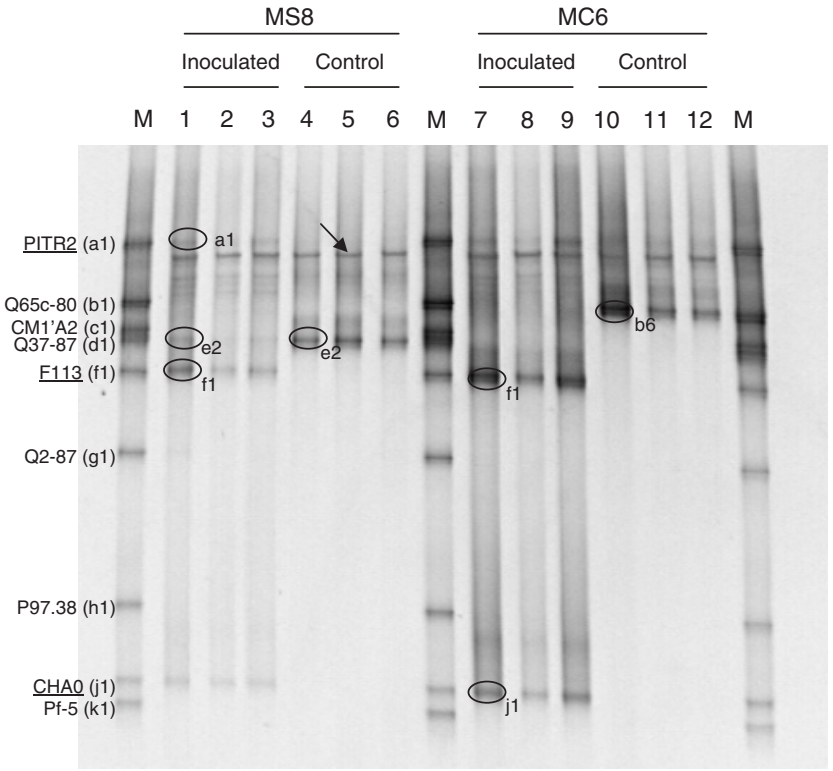

Fig. 4. *phlD*-DGGE analysis of the rhizosphere of tobacco plants grown in non-sterile soils MS8 and MC6 inoculated or not with a mixture of *phlD*$^+$ *Pseudomonas* strains CHA0 (band j), F113 (f), and PITR2 (a). Each lane shows the results from one replicate (i.e. one plant). DNA from circled bands was sequenced. M, mix of independently amplified *phlD* fragments representing DGGE patterns a, b, c, d, f, g, h, j, and k. Inoculated, soils inoculated with CHA0, F113, and PITR2; Control, noninoculated soils. Unlike in the previous soil experiment (Fig. 3), samples were not treated with Mung Bean nuclease, which resulted in the presence of a heteroduplex band (arrow).

phlD-DGGE bands recovered from the gels. Soil experiments were condusted with Morens soils because they harbor a diverse range of indigenous Phl-producing pseudomonads (Ramette *et al.*, 2006), which collectively colonize tobacco roots at levels of 10^5 cells g^{-1} or higher.

First, PCR primers and PCR conditions derived from McSpadden Gardener *et al.* (2001) were chosen to allow simultaneous *phlD* analysis of two phylogenetically contrasted groups. The first one is a group of related *Pseudomonas* species corresponding (or closely related) to *Pseudomonas aurantiaca*, *Pseudomonas brassicacearum*, *Pseudomonas fluorescens*, *Pseudomonas kilonensis*, *Pseudomonas Synxantha,* and *Pseudomonas thivervalensis*, which all fall within the '*P. fluorescens*' species complex. The second is group ARDRA-1 (Keel *et al.*, 1996), which does not belong to any established species complex but rather falls in between the '*Pseudomonas chlororaphis*' and '*Pseudomonas syringae*' complexes. Because the phylogenetic distance between both groups of Phl-producing pseudomonads is extensive, the divergence level can make it difficult to design primers and PCR conditions effective with both types of pseudomonads, as found previously in *phlD* restriction studies (Wang *et al.*, 2001). Here, indeed, *phlD* amplification was less efficient for certain strains than others, as shown when studying strain mixtures (Fig. 1b), which may be explained at least in part by mismatches at PCR priming sites (not shown). Yet, the design of a common DGGE protocol effective with all *phlD* alleles was successful, and it was an important goal because both groups of Phl-producing pseudomonads can coexist in certain rhizospheres (Ramette *et al.*, 2003, 2006). The two previous *phlD*-DGGE protocols enabled simultaneous detection of multiple *phlD* alleles, but only one group of Phl-producing pseudomonads could be studied at a time (Bergsma-Vlami *et al.*, 2005b).

Second, the DGGE approach of Bergsma-Vlami *et al.* (2005b) was successfully used to detect mixtures of Phl-producing *Pseudomonas* inoculants after rhizosphere DNA extraction, and similar findings were obtained in the current work when coinoculating tobacco with ARDRA-1 strain CHA0 and two strains from the '*P. fluorescens*' species complex (Fig. 4). In Bergsma-Vlami *et al.* (2005b) the applicability of *phlD*-DGGE to assess indigenous pseudomonads was not tested extensively, despite their importance in disease-suppressive soils (Weller *et al.*, 2002; Haas & Défago, 2005). Here, several different *phlD*-DGGE bands corresponding to distinct *phlD* alleles were recovered from the rhizosphere of tobacco grown in soil not inoculated with pseudomonads, including alleles not documented in previous studies. The comparison of a suppressive soil and a conducive soil evidenced different *phlD*-DGGE banding patterns and *phlD* alleles (Fig. 3), which strengthened previous results from the study of *Pseudomonas* isolates (Ramette *et al.*, 2006) and substantiated the usefulness of the current DGGE protocol. Pseudomonads comparable to

the model strain CHA0 were not found, in accordance with previous findings on strain endemism at Morens and the absence of strain CHA0 among the dominant strains of soils MS8 and MC112 (Ramette *et al.*, 2006).

Third, the 628-bp *phlD* fragment used in this study displayed more polymorphic sites (210 vs. 107 when considering the alignment of 36 *phlD* sequences of the strains listed in Table 1) than the 349-bp fragment in Bergsma-Vlami *et al.* (2005b). For instance, ARDRA-1 strains CHA0 and Pf-5 could be discriminated using the DGGE protocol developed here, unlike the ones of Bergsma-Vlami *et al.* (2005b). The tree inferred from partial *phlD* sequences (Fig. 2) gave nine UPGMA clusters of DGGE alleles at a similarity level of 99.1%. Most strains with the same or a similar DGGE band clustered together in the *phlD* tree (Fig. 2), and *phlD* clustering of strains was similar to that obtained with the ERIC-PCR method. The current *phlD*-DGGE protocol was less effective than ERIC-PCR but similar to *phlD* restriction analysis in its ability to discriminate different *phlD*$^+$ strains (Table 2). In comparison with *phlD* restriction, however, *phlD*-DGGE had the advantage of allowing (1) discrimination within group ARDRA-1, one of the two taxonomic groups harboring Phl-producing pseudomonads, and (2) analysis of strain mixtures (also in contrast to ERIC-PCR). Therefore, it appears that this *phlD*-DGGE protocol was promising to monitor dominant *phlD* alleles in rhizosphere studies.

Fourth, the method of Bergsma-Vlami *et al.* (2005b) has not been tested directly on rhizosphere DNA extracts of plants not inoculated with pseudomonads, which prompted a comparison of various modes of template preparation. When different sample preparations were tested, it appeared that the highest numbers of *phlD* bands and alleles were found by DGGE analysis of indigenous *phlD*$^+$ *Pseudomonas* consortia after incubation of rhizosphere extracts in semi-selective medium (i.e. the MPN approach) (Fig. 3). It was the case when considering results for individual plants or after combining all the results of the study. This came as a surprise, because the analysis of culturable bacteria is assumed to underestimate the total level of diversityconsiderably because only a minority of species are culturable under a given set of conditions (Amann *et al.*, 1995), and this justifies the use of culture-independent methods based on direct analysis of environmental DNA extracts (Sanguin *et al.*, 2006). Yet, the results obtained with culture-independent methods can be affected by several factors including (1) varying DNA recovery depending on the extraction method used (Martin-Laurent *et al.*, 2001), leading to a bias in the dominance and composition of genotypes, and (2) soil humic acids, which can reduce the performance of PCR (von Wintzingerode *et al.*, 1997). One explanation for the higher diversity of *phlD*$^+$ pseudomonads with MPN cultures could be that the samples used for DNA extraction consisted of more rhizosphere soil than roots compared with the other two approaches. In addition, it is likely that the incubation of indigenous *phlD*$^+$ *Pseudomonas* consortia in MPN cultures provided favorable conditions to recover a diverse range of these bacteria, because (1) although *phlD*$^+$ *Pseudomonas* strains may enter a viable but a nonculturable physiological status (Mascher *et al.*, 2003), as a whole, *Pseudomonas* species correspond to culturable taxa (Troxler *et al.*, 1997), (2) KB^{+++} is suitable for contrasted taxonomic groups of *phlD*$^+$ pseudomonads (Raaijmakers *et al.*, 1997), which probably enabled growth of minority strains above the PCR detection limit, as suggested by Bergsma-Vlami *et al.* (2005b), and (3) PCR is more effective in pseudomonads during growth than when exposed to stress (Rezzonico *et al.*, 2003).

Fifth, while direct sequencing of *phlD*-DGGE bands was not performed in Bergsma-Vlami *et al.* (2005b), it was done in the current work. The results with indigenous pseudomonads showed that a given band could be produced by *phlD* fragments of different sequences (Fig. 3). Together with the results from collection strains, it appears that the electrophoretic mobility was influenced mainly by the GC content, as expected. However, the findings suggest that the position of the melting domains within the amplicon also played a (smaller) role, as proposed by Muyzer & Smalla (1998) for 16S rRNA gene.

In conclusion, this *phlD*-DGGE protocol is an improvement in comparison with the two protocols of Bergsma-Vlami *et al.* (2005b). It is a useful tool (1) to screen for different *phlD* alleles within a collection of cultured *phlD*$^+$ *Pseudomonas* strains, (2) to monitor the fate of *phlD*$^+$ inoculants in the rhizosphere (provided the inocula are dense enough to be detected), and (3) to assess rhizosphere *phlD*$^+$ consortia (when applied in combination with an enrichment strategy such as the MPN approach). Indeed, this protocol proved effective for deepening our understanding of the diversity of *phlD*$^+$ *Pseudomonas* populations present in a disease-suppressive and a conducive soil (proof of principle), and it will be useful in future studies aiming at characterizing suppressive soils and more generally the microbiological basis of soil quality.

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