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Cosmopolitan distribution of *phlD*-containing dicotyledonous crop-associated biocontrol pseudomonads of worldwide origin

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Abstract

In biocontrol fluorescent pseudomonads, *phlD* encodes a polyketide synthase required for the synthesis of the antifungal compound 2,4-diacetylphloroglucinol (Phl). Here, PCR-restriction fragment length polymorphism analysis was used to compare *phlD* alleles in 77 dicot-associated pseudomonads originating from various countries worldwide and 10 counterparts from a monocotyledonous host (wheat). The 16 restriction patterns obtained were mostly unrelated to geographic location or dicot host. Cluster analysis distinguished eight *phlD* clusters at a similarity level of 0.63. One cluster grouped 18 pseudomonads that produced also the antifungal polyketide pyoluteorin but could not assimilate D-galactose, D-galactonate lactone, D-sorbitol, L-arabinose, D-saccharate or D-xylose. These 18 pseudomonads, along with the eight pseudomonads from a second *phlD* cluster, were the only isolates that failed to deaminase 1-aminocyclopropane-1-carboxylate (ACC), a rare root growth promotion trait. Overall, assessment of *phlD* polymorphism, ACC deaminase activity and catabolic profiles pointed to a cosmopolitan distribution of Phl-producing biocontrol fluorescent pseudomonads of worldwide origin associated with dicotyledonous crop plants.

Keywords: *phlD*; 2,4-Diacetylphloroglucinol; Biocontrol; 1-Aminocyclopropane-1-carboxylate deaminase; Carbon source utilization; *Pseudomonas*

1. Introduction

Many strains of fluorescent *Pseudomonas* spp. can suppress plant root diseases caused by soil-borne fungal pathogens [1,2]. Different mechanisms are involved in disease suppression, among which competition, induction of plant resistance and antagonism mediated by extracellular lytic enzymes and/or antifungal secondary metabolites [2–6]. The production of antifungal secondary metabolites, e.g. 2,4-diacetylphloroglucinol (Phl), pyoluteorin (Plt), hydrogen cyanide, phenazines or pyrrolnitrin, is a prominent feature of many biocontrol fluorescent pseudomonads [7,8]. Indeed, several of the latter can produce more than one type of antifungal secondary metabolites, whose relative importance in biocontrol probably differs depending on soil and climatic parameters, the pathosystem considered, and disease pressure [8,9].

Interestingly, several well-studied biocontrol fluorescent pseudomonads originating from different geographic locations worldwide were all found capable of producing Phl [10]. Phl is a polyketide displaying antifungal as well as antibacterial, antiviral, antihelminthic, antinematode and phytotoxic properties in vitro [11–14]. The use of Phl-minus derivatives of *Pseudomonas fluorescens* CHA0, F113 and Q2-87 in soil microcosms has shown the importance of Phl in disease suppression by the corresponding wild-type strains [3,13,15,16]. Moreover, Phl has been detected in the rhizosphere of plants colonized by Phl$^+$ strains CHA0 (under gnotobiotic conditions; [13]) and Q2-87 (in non-sterile soil; [17]).

In the Phl$^+$ strain Q2-87, isolated from wheat grown in take-all-suppressive soil, a total of six genes involved in Phl production and clustered on a 6.5-kb DNA fragment have been identified [18], and a very similar *phl* locus exists in the sugarbeet isolate F113 [19]. Indeed, a diverse array

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of Phl$^+$ fluorescent pseudomonads hybridized to a same 4.8-kb DNA probe from Q2-87 [10], which included the biosynthetic gene *phlD* (encoding a polyketide synthase), and/or displayed the expected *phlD* PCR amplicon [20,21] when the primers Phl2a and Phl2b [22] were used. However, *phlD* was recently shown to display significant polymorphism [23,24]. This polymorphism correlated with broad genotypic features of the strains, as indicated by amplified 16S ribosomal DNA restriction analysis (ARDRA) and cluster analysis of randomly amplified polymorphic DNA (RAPD) markers and/or BOX-PCR fingerprints [23,24].

In the work of Mavrodi et al. [23], it appeared that a single *phlD*-based genotypic group (i.e. a *phlD* cluster defined at 0.70 similarity level from restriction data) predominated in the rhizosphere at each geographic location studied. However, as many as 114 of the 123 pseudomonads studied were obtained from wheat, which was mainly grown in soils subjected to wheat monoculture. Whether this finding can be extended to other plants remains to be established, especially in the case of dicots, which are often grown in crop rotation systems and whose roots seem to harbor a particular type of Phl$^+$ pseudomonads (i.e. Phl$^+$ Plt$^+$ strains; [10,25]) not found in the rhizosphere of monocots [20,21]. In [24], only certain *phlD* restriction patterns seemed associated with a particular geographic location, but sampling biases prevented from drawing a clear conclusion. Therefore, the first objective of the current work was to determine whether there was a link between *phlD* restriction profile and geographic origin of dicot-associated Phl$^+$ biocontrol fluorescent pseudomonads. A worldwide collection of 87 Phl$^+$ strains was used, which consisted of 77 pseudomonads isolated from various dicotyledonous crop plants grown in soils originating from Europe (Switzerland, Italy, Ireland, Czech Republic, Estonia, West Russia), America (Texas, Washington, Oklahoma, Alaska, Mexico), Africa (Ghana) and Asia (Bhutan), as well as 10 reference isolates from wheat.

The second objective was to strengthen the analysis by including other bacterial properties of relevance to plant–microbe interactions. We chose a number of biocontrol traits, which included the ability to display 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity (implicated in root growth promotion; [26]), as well as carbon source utilization profiles. Indeed, catabolic properties are important for root colonization [27] and production of Phl and other biocontrol secondary metabolites [28,29], which in turn can influence the capacity of pseudomonads to protect plants from fungal soil-borne diseases [2]. The relationship between *phlD* restriction profiles, ACC deaminase activity and catabolic profiles was determined and those traits were used to assess the cosmopolitan or endemic nature of the distribution of Phl$^+$ biocontrol fluorescent pseudomonads associated with the roots of dicotyledonous crop plants.

2. Materials and methods

2.1. Bacterial strains and growth media

The 87 fluorescent pseudomonads used in this study are listed in Table 1. Among them, 10 and 24 were already included in the works by Mavrodi et al. [23] and Ramette et al. [24], respectively. Most of the pseudomonads were isolated from soils suppressive to *Thielaviopsis basicola*-mediated black root rot of tobacco (Morens, Switzerland; [30]), *Fusarium* wilt of tomato (Albenga, Italy; [31] and Ghana; [10]), or take-all of wheat (Quincy, WA, USA; [32]). The others were isolated from soil taken in fields where disease symptoms were not found but for which disease-suppressive ability has not been established yet. All pseudomonads were isolated from rhizosphere soil, root macerates (including all isolates obtained from 1994 on in this study) or roots previously washed of soil, with the exception of strain PF isolated from wheat leaf [33]. In the current work, seeds were surface-sterilized [13,34] prior to use in the soil microcosms. Isolates were obtained after plating samples on King's B agar (KBA; [35]) or Gould's S1 medium [36]. Fluorescence on KBA was verified under UV light (366 nm). The biocontrol ability of the 62 fluorescent pseudomonads isolated in this study was demonstrated in non-sterile soil microcosms (Table 1) prepared using a clay loam soil from St. Aubin (County Fribourg, Switzerland), as described by Sharifi-Tehrani et al. [25].

All strains were routinely cultured at 27°C on KBA or in Luria–Bertani broth [37] with shaking (160 rpm). Bacteria were stored at −80°C in 40% glycerol.

2.2. Restriction analysis of phlD

The gene *phlD* was characterized by restriction fragment length polymorphism (RFLP) analysis after PCR amplification. PCR conditions [22] were optimized by modifying DNA preparation, PCR mix adjuvants and annealing temperature, as follows. Two bacterial colonies obtained on KBA after a 48-h incubation at 27°C were transferred into 100 μl of lysis solution (50 mM KCl, 0.1% Tween 20, 10 mM Tris–HCl (pH 8.3); [10]) and the suspension was centrifuged at 5000 rpm for 1 min (Sigma 4-15C, Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) and incubated for 10 min at 99°C. The heat-lysed bacterial suspension was frozen (−20°C) during 30 min. After thawing, 4-μl volumes of supernatant were carefully taken and used for PCR.

PCR amplification was performed using forward primer Phl2a (20-mer 5′-GAGGACGTCGAAGACCACCA-3′) and reverse primer Phl2b (20-mer 5′-ACCGCAGCATCGTGTATGAG-3′), which were developed from the *phlD* sequence of *P. fluorescens* Q2-87 [22]. Primers were synthesized by MWG Biotech (Basel, Switzerland). PCR amplification was carried out in 20-μl reaction mixtures containing 4 μl of lysed bacterial suspension, 1× PCR

Table 1
Origin and biocontrol ability of the 87 Phl$^+$ fluorescent pseudomonads used in the study

Strain	Geographic origin	Year	Plant host	Biocontrol ability[a]	Source[b]
Switzerland					
CHA0	Morens, Fribourg	1985	Tobacco	Cucumber (Pu), tobacco (Tb), wheat (Ggt), tomato (FORL)	[10,25,30]
CΔ1'B2, CM1'A2, C*1A1	Morens, Fribourg	1992	Cucumber	Cucumber (Pu, Ps), cotton (Rs)	[10,25,58]
K92.14, K92.59	Morens, Fribourg	1992	Cucumber	Cucumber (Pu, Ps), cotton (Rs)	*
TM1A3, TM1'A4, TM1'A5, TM1A5, TM1B2	Morens, Fribourg	1992	Tomato	Cucumber (Pu, Ps), cotton (Rs)	[10,25,58]
K92.48, K92.53	Morens, Fribourg	1992	Cotton	Cucumber (Pu, Ps), cotton (Rs)	*
K93.7, K93.9, K93.13, K93.17, K93.18	Morens, Fribourg	1993	Cucumber	Cucumber (Pu), tomato (FORL)	*
K93.1, K93.2, K93.3, K93.4, K93.5	Morens, Fribourg	1993	Tobacco	Cucumber (Pu), tomato (FORL)	*
K93.6, K93.8, K93.10, K93.11, K93.12, K93.14, K93.15, K93.16, K93.19, K93.20, K93.21, K93.22, K93.23, K93.24	Morens, Fribourg	1993	Tomato	Cucumber (Pu), tomato (FORL)	*
K93.39	Morens, Fribourg	1993	Wheat	Cucumber (Pu), tomato (FORL)	*
K94.4, K94.5, K94.18	Morens, Fribourg	1994	Tomato	Cucumber (Pu), tomato (FORL)	*
Pf1	Morens, Fribourg	1995	Tobacco	Tobacco (Tb), cucumber (Pu)	[10,25]
K95.43, K95.44	Morens, Fribourg	1995	Cucumber	Cucumber (Pu), tomato (FORL)	*
K95.45	Morens, Fribourg	1995	Cucumber	Tomato (FORL)	*
P97.38, P97.39	Morens, Fribourg	1997	Cucumber	Cucumber (Pu), tomato (FORL)	*
K95.34	Riddes, Valais	1995	Cucumber	Cucumber (Pu), tomato (FORL)	*
K94.6	Brusio, Graubünden	1994	Cucumber	Cucumber (Pu), tomato (FORL)	*
K94.30	Brusio, Graubünden	1994	Cucumber	Tomato (FORL)	*
Czech Republic					
K94.31, K94.37, K94.38	Nitra, North Moravia	1994	Cucumber	Tomato (FORL)	*
K94.40, K94.41	Nitra, North Moravia	1994	Cucumber	Cucumber (Pu), tomato (FORL)	*
P97.30, P97.31	Nitra, North Moravia	1997	Wheat	Cucumber (Pu), tomato (FORL)	*
P97.32	Nitra, North Moravia	1997	Cucumber	Cucumber (Pu), tomato (FORL)	*
P97.33	Nitra, North Moravia	1997	Bean	Cucumber (Pu), tomato (FORL)	*
P97.34	Nitra, North Moravia	1997	Tomato	Cucumber (Pu), tomato (FORL)	*
Italy					
PINR2, PINR3	Albenga	1993	Tobacco	Cucumber (Pu), tomato (FORL)	[10,25]
K93.25	Albenga	1993	Tobacco	Cucumber (Pu), tomato (FORL)	*
PILH1	Albenga	1993	Tomato	Cucumber (Pu), tomato (FORL)	[10,25]
K93.52	Albenga	1993	Tomato	Cucumber (Pu), tomato (FORL)	*
PITR2, PITR3	Albenga	1993	Wheat	Cucumber (Pu), tomato (FORL)	[10,25]
Ireland					
F113	County Cork	1992	Sugarbeet	Sugarbeet (Pu)	[3,10,14]
Russia					
P96.20	West Russia	1996	Cucumber	Cucumber (Pu), tomato (FORL)	*
P96.25	West Russia	1996	Wheat	Cucumber (Pu), tomato (FORL)	*
Estonia					
F96.27	Tallinn	1996	Cucumber	Cucumber (Pu), tomato (FORL)	*
USA					
Pf-5	Texas	1979	Cotton	Cucumber (Pu), cotton (Pu, Rs)	[10,25,59]
Q2-87	Quincy, WA	1987	Wheat	Wheat (Ggt)	[10,15,25]
Q6-87	Quincy, WA	1995	Wheat	Wheat (Ggt)	[10,25]
PF	Oklahoma	1992	Wheat	Wheat (St)	[10,25,33]
P97.20	Little Sue, AK	1997	Wheat	Cucumber (Pu), tomato (FORL)	*
Mexico					
F96.26	El Batan	1996	Cucumber	Tomato (FORL)	*
Ghana					
PGNR1, PGNR2, PGNR3, PGNR4, PGNL1	IITA soil	1993	Tobacco	Cucumber (Pu), tomato (FORL)	[10,25]
Bhutan					
P97.1, P97.2, P97.27	Punakha Wangdi	1997	Cucumber	Cucumber (Pu), tomato (FORL)	*
P97.6, P97.26	Punakha Wangdi	1997	Tomato	Cucumber (Pu), tomato (FORL)	*

[a]Pu, *Pythium ultimum*; Tb, *T. basicola*; Ggt, *Gaeumannomyces graminis* var. *tritici*; FORL, *Fusarium oxysporum* f. sp. *Radicis lycopersici*; Ps, *Phomopsis sclerotioides*; Rs, *Rhizoctonia solani*; St, *Septoria tritici*.
[b]Isolates obtained in this study are indicated with an asterisk.

buffer (Amersham Pharmacia, Uppsala, Sweden), bovine serum albumin (0.5 g l^{-1}; Fluka Chemie, Buchs, Switzerland), 5% dimethyl sulfoxide (Fluka Chemie), 100 μM each of dATP, dCTP, dGTP and dTTP (Amersham Pharmacia), 0.40 μM of each primer and 1.4 U of *Taq* DNA polymerase (Amersham Pharmacia). Amplifications were performed with a PTC-100™ cycler (MJ Research Inc., Watertown, MA, USA). The initial denaturation (2 min

at 94°C) was followed by 30 PCR 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.

Restriction analysis was performed in 20-μl volumes prepared with 5 μl of amplified product and 15 μl of restriction buffer, and containing 1.5 U of either *Hae*III, *Sau*3A, *Nde*II, *Cfo*I or *Msp*I (Boehringer, Mannheim, Germany). After a 1.5-h digestion at 37°C, restriction fragments were separated by gel electrophoresis (2.5% agarose), using standard protocols [37]. A 100-bp ladder (Gibco-BRL Life Technologies Inc., Gaithersburg, MD, USA) was used as molecular size marker. For each pseudomonad, restriction analysis was done at least three times for each enzyme.

2.3. Characterization of biocontrol traits

The ability to produce Phl and Plt was determined by Keel et al. [10] for some of the strains. For the others, this was done by reverse-phase high-performance liquid chromatography, as described by Keel et al. [10]. These determinations were carried out routinely as part of an extensive screening program designed to identify potential biocontrol agents, and they were done using King's B medium (solid or liquid) and either malt agar [34], yeast extract malt sucrose asparagine medium (per l: yeast extract (Difco; Detroit, MI, USA) 3 g, malt extract (Oxoid, Hampshire, UK) 3 g, Bacto peptone (Difco) 5 g, sucrose 10 g, L-asparagine 1 g, pH adjusted to 7.0 before autoclaving using NaOH; Keel and Maurhofer, unpublished) or Keel's glycerol-casamino acid medium [38]. Certain pseudomonads can produce in King's B broth an as yet unidentified red/orange pigment [10]. Production of this pigment is associated with the biosynthetic gene *phlE* in strain Q2-87 [39], but its contribution to biocontrol remains to be established. In this study, the ability to produce the red/orange pigment was checked after a 5-day incubation of liquid cultures at 20°C. Hydrogen cyanide production was detected on KBA using HCN indicator paper, as described by Castric and Castric [40]. Extracellular protease activity was assessed using skim milk agar [41] and scored after a 24-h incubation at 27°C using a 0–3 scale (0, none; 1, little; 2; strong; 3, very strong; [25]). ACC deaminase activity was inferred from the ability of the pseudomonads to use ACC as sole nitrogen source [26]. KBA-grown cells were used to inoculate DF salts minimal medium [42] containing ACC (303 mg l^{-1}; i.e. 3 mM) and solidified with agar. The plates were incubated for 2–5 days at 27°C before assessing growth. Determinations were done twice.

2.4. Carbon source utilization profiles

The ability to assimilate 128 carbon sources was assessed using Biolog GN and Biolog GP microplates (Biolog Inc., Hayward, CA, USA). Bacteria were grown for 24 h at 27°C on 1/10 strength tryptic soy agar (Difco) containing a total of 12 g agar l^{-1}. The cells were collected from the plates, washed in 0.85% NaCl solution, and each bacterial suspension was adjusted to approximately 10^8 colony forming units ml^{-1} based on an optical density (OD) reading of 0.125 at 600 nm [25]. The Biolog GN and GP microplates were inoculated with 150 μl of bacterial suspension per well and incubated for 24 h at 27°C with shaking (100 rpm). OD readings (570 nm) were taken using an enzyme-linked immunosorbent assay plate reader and wells were scored positive for growth when readings

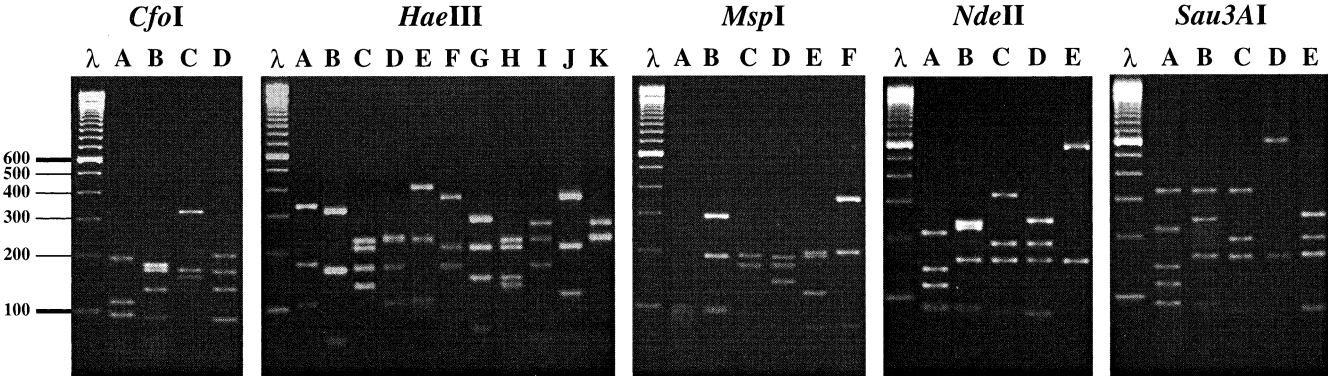

Fig. 1. Banding patterns produced by restriction of PCR-amplified *phlD* for 87 Phl^+ biocontrol fluorescent pseudomonads using *Hae*III, *Sau*3A, *Nde*II, *Cfo*I or *Msp*I. A 100-bp ladder (i.e. from the bottom of the gel 100 bp, 200 bp, etc.; Gibco-BRL) is also included. The 11 banding patterns found using *Hae*III were obtained with e.g. CHA0 (lane A), Q2-87 (B), K92.48 and K93.2 (C), PITR2 (D), PILH1 (E), K94.31, C*1A1, P97.30 and K93.3 (F), F113 (G), K94.37 (H), P97.1 (I), P97.26 (J) and P97.6 (K). The five banding patterns found using *Sau*3AI were obtained with e.g. CHA0 (A), Q2-87 (B), K94.31, C*1A1, K92.48, K94.37, P97.30, K93.3, P97.26, F113, PITR2 and PILH1 (C), P97.38 and F96.26 (D) and K93.2 (E). The five banding patterns found using *Nde*II were obtained with e.g. CHA0 and K94.31 (A), Q2-87 (B), C*1A1, K92.48, K94.37, P97.30, K93.3, P97.26, F113, PITR2 and PILH1 (C), K93.2 (D) and P97.38 and F96.26 (E). The four banding patterns found using *Cfo*I were obtained with e.g. CHA0 and K94.31 (A), Q2-87, C*1A1, K92.48, K94.37, K93.2, P97.30, K93.3, P97.26 and F113 (B), PITR2 and PILH1 (C) and P97.38 and F96.26 (D). The six banding patterns found using *Msp*I were obtained with e.g. CHA0, K94.31 and K93.3 (A), Q2-87 (B), F113, PITR2 and PILH1 (C), C*1A1, K92.48, K94.37 and K93.2 (D), P97.30, P97.38 and F96.26 (E) and P97.26 (F). Restriction patterns are in agreement with those predicted by virtual restriction of seven *phlD* sequences available in GenBank or EMBL databases, i.e. for strains CHA0 (accession number AJ278806), Q2-87 (U41818), F113 (AJ278811), Q65C-80 (AJ278807), PITR2 (AJ278810), PILH1 (AJ278810), CM1'A2 (AJ278808).

exceeded 0.20. Growth was not detected in the wells that did not contain a carbon source.

2.5. Similarity coefficients and cluster analyses

Data of *phlD* restriction analysis and of carbon source utilization were analyzed each using Jaccard's pairwise coefficient of similarity [43], based on presence/absence of restriction bands and growth/no growth, respectively. Cluster analysis was performed using the unweighted pair group method with arithmetic means (UPGMA), and algorithms were computed using the NTSYS-pc numerical taxonomy and multivariate analysis system [44]. To facilitate presentation of results, clusters were arbitra-

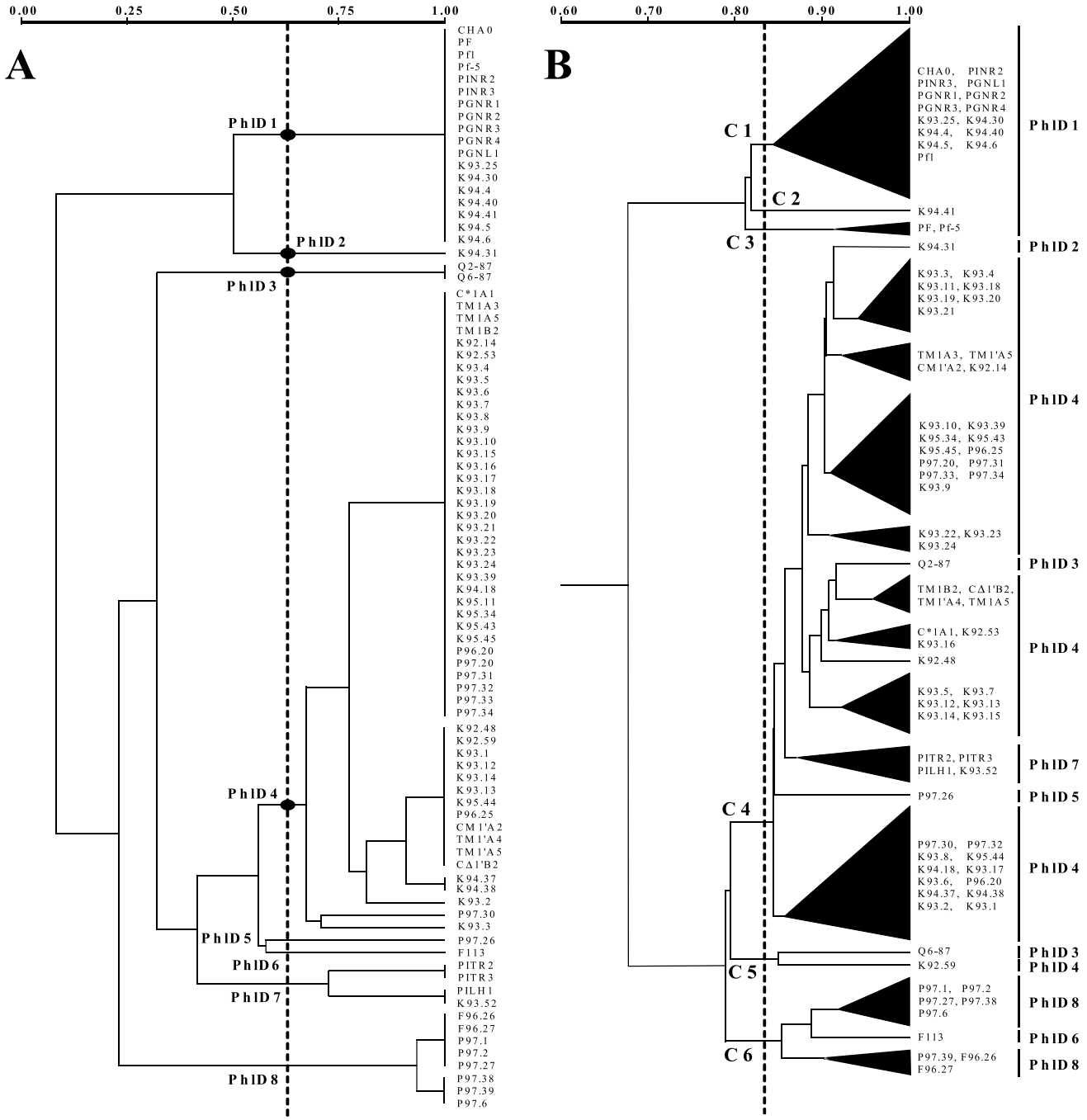

Fig. 2. Cluster analysis of 87 Phl$^+$ biocontrol fluorescent pseudomonads based on PCR-RFLP analysis of *phlD* (A) and carbon source utilization profiles (B). Clusters were arbitrarily defined at similarity levels of 0.63 (A) and 0.83 (B), yielding clusters PhlD1–PhlD8 and C1–C6, respectively. In B, black triangles are used within catabolic clusters C1, C3, C4 and C6 when pseudomonads belonged to a same *phlD* cluster. Among the 128 carbon sources tested, the number of carbon sources assimilated by the pseudomonads was 27.8 ± S.E.M. 0.8 (in cluster C1), 30 (for K94.41; cluster C2), 27 ± 1 (in cluster C3), 37.2 ± 0.8 (in cluster C4), 26 ± 5 (in cluster C5) and 28.6 ± 1.2 (in cluster C6).

rily considered at similarity levels of 0.63 (*phlD* restriction analysis) and 0.83 (carbon source utilization). Comparisons between catabolic clusters based on percentage utilization of particular carbon sources were carried out with the help of χ^2 tests ($P < 0.05$).

3. Results

3.1. PCR-RFLP analysis of phlD in 87 Phl$^+$ biocontrol fluorescent pseudomonads

In our hands, the PCR procedure of Raaijmakers et al. [22] worked well to amplify *phlD* in a majority of pseudomonads (including strain Q2-87), but performed poorly with others, e.g. strain CHA0 (and all other pseudomonads subsequently shown to produce Plt). In contrast, the PCR procedure described here proved satisfactory to amplify *phlD* efficiently from all pseudomonads. A DNA fragment about 745 bp in size was obtained in all cases, as predicted from known *phlD* sequences [18,24], whereas no PCR product was obtained from *Pseudomonas* sp. P3, which does not produce Phl. These results were identical to the ones obtained when PCR was carried out using purified genomic DNA instead of heat-lysed cell suspensions.

Among the 87 pseudomonads, restriction analysis revealed 11 (with *Hae*III), six (with *Msp*I), five (with *Nde*II or *Sau*3A) or four (with *Cfo*I) different banding patterns (Fig. 1). When the information obtained using the five restriction enzymes was combined, as many as 16 different restriction patterns were distinguished (only seven of them already obtained by Ramette et al. [24]). Cluster analysis revealed that each restriction pattern was very different from most of the others (Fig. 2A). The most prevalent restriction pattern was shared by 35 (among which strain C*1A1) of the 87 pseudomonads, whereas six restriction patterns were found only once (i.e. in the case of K94.31, K93.2, P97.30, K93.3, P97.26 and F113).

3.2. Relationship between phlD polymorphism and Pseudomonas origin

When considering restriction patterns obtained for at least three pseudomonads, it appeared that the latter were never all isolated from soil samples taken from a same location (Fig. 2A, Table 1). For instance, the most prevalent restriction pattern comprised 29 pseudomonads from Switzerland (28 of them from Morens), four from the Czech Republic, one from Russia and one from Alaska. Likewise, the restriction pattern displayed by CHA0 was found also with another Swiss isolate from Morens (Pf1), with three of the five isolates from Italy, with the isolates from Texas (Pf-5) and Oklahoma (PF), and with the five isolates from Ghana. As many as 47 of the 87 pseudomonads originated from Morens, and they displayed seven of the 16 restriction profiles found in this study.

Table 2
Number of Phl$^+$ biocontrol fluorescent pseudomonads in each PCR-RFLP *phlD* cluster and each catabolic cluster according to geographic origin of soil or host plant from which isolates ($n = 87$) were obtained

Clusters[a]	Geographic origin[b]										Host plant						
	CH	CzR	It	Irl	NE	Ala	WUS	Mex	Gha	Bhu	Tobacco	Tomato	Cucumber	Cotton	Bean	Beet	Wheat
***phlD* clusters**																	
PhlD1 ($n = 18$)	6	2	3				2		5		10	2	4	1			1
PhlD2 (K94.31)		1											1				
PhlD3 (Q2-87, Q6-87)							2										2
PhlD4 ($n = 52$)	42	7			2	1					5	21	18	2	1		5
PhlD5 (P97.26)										1		1					
PhlD6 (F113)				1												1	
PhlD7 ($n = 4$)			4									2					2
PhlD8 ($n = 8$)	2				1			1		4		1	7				
Catabolic clusters																	
C1 ($n = 15$)	6	1	3						5		10	2	3				
C2 (K94.41)		1											1				
C3 (PF, Pf-5)							2							1			1
C4 ($n = 58$)	41	8	4		2	1	1			1	5	24	18	2	1		8
C5 (Q6-87, K92.59)	1						1						1				1
C6 ($n = 9$)	2			1	1			1		4		1	7			1	
Total	50	10	7	1	3	1	4	1	5	5	15	27	30	3	1	1	10

[a] *phlD* clusters were defined based on the results of PCR-RFLP analysis and an arbitrary 0.63 similarity level in cluster analysis (Fig. 2A), whereas catabolic clusters were defined using the ability of pseudomonads to assimilate 128 carbon sources and an arbitrary 0.83 similarity level in cluster analysis (Fig. 2B).

[b] CH, Switzerland; CzR, Czech Republic; It, Italy; Irl, Ireland; NE, northern Europe (Estonia, Russia); Ala, Alaska; WUS, western USA (Oklahoma, Texas, Washington); Mex, Mexico; Gha, Ghana; Bhu, Bhutan.

Table 3
Relationship between PCR-RFLP *phlD* cluster and biocontrol traits for 87 Phl$^+$ fluorescent pseudomonads

phlD clusters[a]	HCN[b]	Plt	Red/orange pigment	Extracellular protease[c]	ACC deaminase
PhlD1 ($n=18$)	+	+	−	3.17 ± 0.38	−
PhlD2 (K94.31)	+	−	+	1	+
PhlD3 (Q2-87, Q6-87)	+	−	+	0.53 ± 0.67	+
PhlD4 ($n=52$)	±	−	+	1.13 ± 0.50	+
PhlD5 (P97.26)	+	−	+	2	+
PhlD6 (F113)	+	−	+	1	+
PhlD7 ($n=4$)	+	−	+	0.51 ± 0.39	+
PhlD8 ($n=8$)	+	−	+	1.25 ± 0.46	−

[a] *phlD* clusters were defined based on the results of PCR-RFLP analysis and an arbitrary 0.63 similarity level in cluster analysis (Fig. 2A).
[b] All were HCN-positive, with the exception of strain K92.53 (from cluster C4).
[c] Means ± S.E.M. All pseudomonads displayed extracellular proteolytic activity and the latter was scored using a 0–3 scale.

Similarly, pseudomonads sharing a same restriction pattern were never isolated from a single plant species when the number of pseudomonads considered exceeded five (Fig. 2A, Table 1). For instance, the most prevalent restriction pattern was found with two isolates from tobacco, 17 from tomato, 11 from cucumber, one from cotton, one from bean and three from wheat. A majority of pseudomonads were isolated from cucumber (30 isolates) or tomato (27 isolates), and they displayed seven (cucumber isolates) and six (tomato isolates) different restriction profiles.

3.3. Relationship between phlD polymorphism, ACC deaminase activity and other biocontrol traits

The relationship between *phlD* polymorphism and biocontrol traits was assessed using clusters derived from the analysis of *phlD* restriction profiles. A total of eight clusters were identified at a similarity level of 0.63 (Fig. 2A). Three of the eight *phlD* clusters (i.e. clusters PhlD1, PhlD4 and PhlD8), which represented 78 of the 87 pseudomonads studied, were comprised of bacteria originating (i) from soil taken in several different countries and (ii) from different plant hosts (Table 2), as could be anticipated from the analysis of *phlD* restriction profiles.

Certain bacteria can benefit plants by promoting root growth, which in turn may help the plant to resist to fungal pathogens. In several bacterial taxa (including a few fluorescent pseudomonads), this can be achieved by ACC deaminase activity [45], a trait identified in all pseudomonads studied here, with the exception of those belonging to clusters PhlD1 ($n=18$) and PhlD8 ($n=8$). Therefore, ACC deaminase activity was found in 42 of the 50 pseudomonads from Switzerland, eight of the 10 pseudomonads from the Czech Republic, four of the seven pseudomonads from Italy, the Irish strain (F113), the two Russian pseudomonads, the strain from Alaska (P97.20), the two pseudomonads from Washington State and one of the five pseudomonads from Bhutan (P97.26). In addition, all seven plants studied in this work (including wheat) yielded strain(s) with ACC deaminase activity.

Certain Phl$^+$ biocontrol pseudomonads can also produce the antifungal compound Plt, whereas others do not [10]. Here, one of the clusters (PhlD1) corresponded to the 18 pseudomonads (including *P. fluorescens* CHA0 and Pf-5) that could produce both Phl and Plt (Table 3). All 87 bacteria produced a red/orange pigment in King's B broth, with the exception of the Phl$^+$ Plt$^+$ biocontrol pseudomonads (i.e. cluster PhlD1), which did not display significant pigmentation (a weak pigmentation could be found with a longer incubation time [10]). All pseudomonads could produce HCN, a compound implicated in the suppression of black root rot of tobacco by CHA0 [46], with the exception of K92.53 (isolated from cotton grown in Morens soil) from cluster PhlD4. Extracellular proteases can contribute to the ability of bacteria to suppress fungal diseases [47], and here all 87 pseudomonads displayed proteolytic activity (Table 3). However, this capacity seemed more pronounced for Phl$^+$ Plt$^+$ pseudomonads (i.e. cluster PhlD1).

3.4. Relationship between phlD polymorphism and carbon source utilization profiles

Among the 128 carbon sources tested, 29 were assimilated by all pseudomonads, 33 by none, but the 66 remaining carbon sources were useful to discriminate among the 87 Phl$^+$ pseudomonads. Indeed, substantial diversity was found when comparing the pseudomonads based on their carbon source utilization profile (Fig. 2B). No relationship was found between carbon source utilization profile and soil geographic origin or host plant from which the pseudomonads were isolated. When cluster analysis of data was considered at a similarity level of 0.83, a total of six catabolic clusters (i.e. C1–C6) were identified. The average number of carbon sources utilized by the pseudomonads ranged from 26 to 30 in each cluster, except in cluster C4 where it reached about 37 (see legend of Fig. 2). A major distinction could be made between clusters C1–C3 ($n=18$), whose pseudomonads could not assimilate D-galactose, D-galactonate lactone, D-sorbitol, L-arabinose, D-saccharate or D-xylose, and clusters C4–C6 ($n=69$), whose pseudomonads could assimilate all six carbon sources (Table 4).

The catabolic clusters C1–C3 corresponded to the 18

Table 4
Percentage utilization of 128 carbon sources by Phl$^+$ biocontrol fluorescent pseudomonads in each catabolic cluster[a]

Carbon sources[b]	C1 ($n=15$)	C2 (K94.41)	C3 (PF, Pf-5)	C4 ($n=58$)	C5 (Q6-87, K92.59)	C6 ($n=9$)
Useful to discriminate C1–C3 from C4–C6						
D-Galactose, D-galactonic acid lactone, D-sorbitol, L-arabinose, D-saccharic acid, D-xylose	–	–	–	100	100	100
2-Amino ethanol	73	100	100	28	–	–
D-Galacturonic acid	7	–	–	91	50	–
Putrescine	100	100	100	22	–	89
Alaninamide	7	–	–	70	50	44
L-Ornithine	100	100	100	55	–	33
D-Glucuronic acid	–	–	–	91	50	–
L-Threonine	7	–	–	48	–	–
Mono-methyl succinate	–	100	–	57	–	11
Itaconic acid	100	100	100	3	–	–
Glycogen	7	–	–	38	–	11
Sucrose	–	–	100	100	100	11
N-Acetyl-D-glucosamine	100	100	100	91	–	–
D-Glucosaminic acid	7	–	–	98	50	89
Maltose	–	–	–	28	–	–
γ-Hydroxybutyric acid	100	100	100	88	100	–
Glucose-6-phosphate	87	100	100	5	–	–
D-Malic acid	–	–	–	33	–	–
D-Ribose	100	100	100	78	–	100
Fructose-6-phosphate	93	100	100	–	–	–
Other carbon sources						
D-Psicose	27	100	–	47	50	22
Uridine	67	100	–	93	–	100
Formic acid	40	100	–	62	50	44
Succinamic acid	100	–	100	88	–	33
Hydroxy-L-proline	100	–	50	93	100	67
α-Keto-valeric acid	–	100	–	–	–	–
m-Inositol	93	–	50	100	100	100
L-Serine	100	100	100	98	–	89
DL-α-Glycerol phosphate	40	–	100	26	50	67
D-Arabitol	67	100	–	100	100	100
N-Acetyl-L-glutamic acid	13	100	100	52	–	56
Phenyl ethylamine	20	–	–	2	–	–
Tween 80	100	100	50	98	100	100
Malonic acid	100	100	100	85	100	56
D-Trehalose	100	100	100	98	50	78
Propionic acid	67	100	100	76	50	100
2,3-Butanediol	–	–	50	17	–	11
Acetic acid	87	100	100	93	50	100
L-Histidine	100	100	100	98	100	44
Sebacic acid	47	–	50	3	–	–

[a]The sign – was used when none of the pseudomonads could assimilate the carbon source.

[b]All could assimilate D-alanine, L-alanyl-glycine, glycyl-L-glutamic acid, Tween 40, L-pyroglutamic acid and succinic acid, except for 47% (D-alanine) and 13% (L-alanyl-glycine, glycyl-L-glutamic acid) of the pseudomonads in cluster C1, for strain PF (Tween 40) in cluster C3, and for 2% (L-pyroglutamic acid) and 3% (succinic acid) of the pseudomonads in cluster C4. For 14 of the 128 carbon sources, the ability to assimilate them was absent in the 87 pseudomonads, except in 2% (α-cyclodextrin, D-raffinose, gentibiose, α-D-lactose, glucose-1-phosphate, cellobiose, uridine-5′-monophosphate, 3-methyl-glucose), 3% (2′-deoxy adenosine), 5% (D-serine), 7% (α-hydroxybutyric acid), 12% (dextrin, glucuronamide) and 22% (L-phenylalanine) of the pseudomonads in cluster C4. All 87 pseudomonads could use the following 29 carbon sources: *p*-hydroxyphenylacetic acid, bromosuccinic acid, urocanic acid, D-fructose, *cis*-aconitic acid, inosine, citric acid, L-leucine, α-ketoglutaric acid, α-D-glucose, DL-lactic acid, L-alanine, L-proline, D-gluconic acid, L-asparagine, quinic acid, L-aspartic acid, glycerol, L-glutamic acid, D-mannitol, methylpyruvate, β-hydroxybutyric acid, DL-carnitine, γ-aminobutyric acid, adenosine, D-lactic acid methyl ester, L-lactic acid, L-malic acid and pyruvic acid. None of the 87 pseudomonads could assimilate the following 33 carbon sources: *i*-erythritol, D-melibiose, β-methyl-D-glucoside, L-fucose, α-keto-butyric acid, thymidine, L-rhamnose, *N*-acetyl-D-galactosamine, adonitol, lactulose, turanose, xylitol, glycyl-L-aspartic acid, D-tagatose, lactamide, α-methyl D-mannoside, β-cyclodextrin, arbutin, palatinose, maltotriose, inulin, adenosine-5′-monophosphate, mannan, D-melezitose, thymidine-5′-monophosphate, salicin, α-methyl D-galactoside, sedoheptulosan, β-methyl-D-galactoside, *N*-acetyl-D-mannosamine, stachyose, amygdalin and α-methyl D-glucoside.

Phl$^+$ Plt$^+$ pseudomonads (i.e. cluster PhlD1; Fig. 2B and Table 3). Strain K94.31 (i.e. cluster PhlD2) was found in cluster C4 ($n = 58$), whereas the two pseudomonads from cluster PhlD3 (i.e. Q2-87 and Q6-87) were split in clusters C4 ($n = 58$) and C5 ($n = 2$), respectively. Among the 52 pseudomonads from cluster PhlD4, 51 were found in cluster C4 and one (K92.59) in cluster C5. Strain P97.26 (i.e. cluster PhlD5) and the four pseudomonads from cluster PhlD7 were in cluster C4, whereas F113 (i.e. cluster PhlD6) and the pseudomonads from cluster PhlD8 were found in cluster C6.

4. Discussion

Soil populations of fluorescent *Pseudomonas* spp. from pristine ecosystems worldwide appeared to be cosmopolitan when studied at a coarse level of resolution (i.e. by ARDRA; [48]), and ARDRA comparison of root-associated Phl$^+$ pseudomonads gave the same results [10]. For the latter, three ARDRA groups were obtained, each of them likely to be comprised of several different species [24]. However, soil pseudomonads displayed strong endemicity when considered at intra-species levels using BOX-PCR fingerprints [48]. Similar findings were obtained with soil bacteria degrading 3-chlorobenzoate [49]. The results from BOX-PCR, RAPD profiling and PCR-RFLP analysis of *phlD* (i.e. *phlD* clusters defined at 0.70 similarity level) in Phl$^+$ fluorescent pseudomonads isolated from wheat roots [23] suggested also a significant level of endemism. Since *phlD* polymorphism correlates well with genotypic diversity in Phl$^+$ pseudomonads, *phlD* restriction analysis has been advocated as a useful tool in ecological studies of Phl$^+$ populations of root-associated fluorescent pseudomonads [23,24].

In this work, amplification of *phlD* was made easier by modifying several parameters (i.e. DNA preparation, PCR mix adjuvants, annealing temperature) of the PCR procedure of Raaijmakers et al. [22], which worked poorly with PhlD1 (i.e. Phl$^+$ Plt$^+$) strains, such as CHA0 and Pf-5. Recently, improved amplification of *phlD* sequences has also been achieved by designing a new primer set [50], but the procedure amplified only 629 of 1047 bp (i.e. 60%), versus 745 bp (i.e. 71%) with the method proposed here. One implication is that restriction analysis of *phlD* yielded larger bands compared with those obtained by Mavrodi et al. [23], thereby facilitating strain comparisons. The PCR-RFLP results of the current work confirm and expand the findings of Ramette et al. [24] and Mavrodi et al. [23] regarding the high level of polymorphism of *phlD* in Phl$^+$ biocontrol fluorescent pseudomonads. For wheat-associated strains, 11 of the 14 *phlD* clusters were comprised of isolates from a single geographic location only [23]. In contrast, there was little relationship between *phlD* polymorphism and the geographic origin worldwide (Table 1) of dicot-associated pseudomonads in the current work, regardless of whether banding patterns (Fig. 1) or *phlD* clusters (Fig. 2A) were considered. Rather, results in Table 2 suggest that biocontrol pseudomonads belonging to different *phlD* clusters may coexist in the rhizosphere of dicots at a given geographic location. Preliminary results obtained in the case of Morens soils suppressive to *T. basicola*-mediated black root rot of tobacco are in agreement with this hypothesis (data not shown).

Furthermore, when taking plant host into consideration, it appears that the most frequent *phlD* clusters were identified in pseudomonads associated with different dicotyledonous crops (Table 2). Therefore, the results from the current work raise the possibility that functional communities of Phl$^+$ biocontrol fluorescent pseudomonads displaying a given array of *phlD* alleles could perhaps be found in the rhizosphere of several different dicots grown at a same geographic location. For instance, clusters PhlD1 and PhlD4 were found with isolates from both tobacco (CHA0 and K93.3, respectively) and tomato (K94.4 and TM1B2, respectively) grown in Morens soil. This could perhaps take place also when considering functional communities of Phl$^+$ biocontrol fluorescent pseudomonads in the rhizosphere of a same dicotyledonous crop grown in different geographic locations, as suggested by the observation that PhlD1 and PhlD4 were found with cucumber isolates from both southern Switzerland (K94.30 and K95.34, respectively) and Nitra, Czech Republic (K94.40 and P97.32, respectively).

The comparison of catabolic traits, using the six catabolic clusters defined at a similarity level of 0.83 (Fig. 2B), pointed to a rather cosmopolitan distribution of Phl$^+$ pseudomonads associated with roots of dicotyledonous crop plants (Table 2), thereby strengthening the findings derived from *phlD* polymorphism. Indeed, substantial diversity was found when considering carbon source utilization profiles, and at similarity levels higher than 0.83, e.g. 0.9 and 0.95, as many as 27 and 59 different phenotypes, respectively, were identified. In the rhizosphere, seed and root exudates are likely to represent the main supply of carbon sources available to pseudomonads [7,29,51]. Whether the carbon compounds studied in the current work are actually present in the rhizosphere of the dicots investigated is unknown. However, some of the carbon sources useful to discriminate between catabolic clusters, e.g. D-galactose and D-xylose (clusters C1–C3 vs. C4–C6), can be present in plant exudates [52–54]. Each *phlD* cluster corresponded to only one or a few catabolic clusters (Fig. 2B), and overall a good relationship was found between *phlD* polymorphism and catabolic traits. This is not surprising since broad genotypic properties (i.e. results of ARDRA, whole-cell BOX-PCR and ERIC-PCR) correlate with catabolic traits in *phlD*$^+$ pseudomonads [20]. Indeed, the agreement between broad genotypic status and membership to particular *phlD* and catabolic clusters can be illustrated for 25 of the 87 bacteria (Table 5), which have been characterized by ARDRA and RAPD analysis

Table 5
Summary of the relationship between broad genotypic characteristics (ARDRA and RAPD analysis; [10]), *phlD* clusters and catabolic clusters for 25 Phl$^+$ biocontrol pseudomonads

Genotypic group	Strains	*phlD* cluster	Catabolic cluster
ARDRA group[a] 1			
RAPD group[b] 1	CHA0, PINR2, PINR3, PGNR1, PGNR2, PGNR3, PGNR4, PGNL1, Pf1	PhlD1	C1
RAPD group 2	Pf-5, PF	PhlD1	C3
ARDRA group 2			
RAPD group 3	CΔ1'B2, TM1'A4, TM1B2, TM1A5, TM1A3, TM1'A5, CM1'A2, C*1A1	PhlD4	C4
RAPD group 4	Q2-87, Q6-87	PhlD3	C4 (Q2-87), C5 (Q6-87)
RAPD group 5	PITR2, PITR3, PILH1	PhlD7	C4
ARDRA group 3			
RAPD group 7	F113	PhlD6	C6

[a]ARDRA groups of same restriction patterns obtained using eight enzymes [10].
[b]RAPD groups were defined based on the comparison (Jaccard's similarity coefficient of 50% or higher and UPGMA clustering) of RAPD profiles obtained using four primers [10].

[10]. However, certain catabolic clusters gathered pseudomonads belonging to different *phlD* clusters, including *phlD* clusters spread over several catabolic clusters (Fig. 2B). This is the case for clusters C4 and C5, which both contained PhlD3 and PhlD4 bacteria. Perhaps such a situation is the result of horizontal gene transfer events among pseudomonads.

Confirmation of these findings was also sought for by characterizing biocontrol properties, but antagonism-related biocontrol traits did not prove very useful to assess endemicity of the bacteria. The fact that cluster PhlD1 was associated with Plt production (found only in pseudomonads from ARDRA group 1; [10]) is a confirmation, with a larger number of pseudomonads, of the findings of Ramette et al. [24]. In contrast, the analysis of ACC deaminase activity proved valuable. The enzyme catalyzes the degradation of ACC (the precursor of the phytohormone ethylene) into α-ketobutyrate and ammonium [55,56]. Ethylene can inhibit root growth [55], and the degradation of ACC enables certain rhizobacteria to have a positive effect on root growth [45], presumably by lowering ethylene levels [55]. Indeed, strain CHA0, which cannot display ACC deaminase activity, acquired root growth-promoting ability following the expression of heterologous ACC deaminase genes [26]. ACC deaminase activity is a rare trait in pseudomonads [26,56], and thus it came as a surprise that as many as 61 of the 87 Phl$^+$ pseudomonads studied here displayed this property. ACC deaminase activity correlated with *phlD* clusters and was found at most geographic locations (Table 2). Therefore, the distribution of ACC deaminase activity confirmed the cosmopolitan nature of Phl$^+$ pseudomonads associated with dicot roots.

The limited endemicity of Phl$^+$ pseudomonads observed in the current work may result from geographic dissemination of seeds. Since the dicots studied were crops, trading of seeds to be used as food or to propagate the crop arguably provides an opportunity for plant-associated bacteria to be disseminated to a large extent worldwide. This contrasts with the endemic distribution of pseudomonads in bulk soil sampled from pristine environments [48]. Phl$^+$ pseudomonads from wheat also displayed substantial endemicity [23], but in the latter work most isolates were obtained from wheat monoculture sites, which could have provided the opportunity for enrichment of a particular *phlD* genotype at each site. In addition, the main *phlD*-based isolation procedure used to obtain the wheat isolates studied by Mavrodi et al. [23] detected indigenous *phlD*$^+$ pseudomonads less frequently compared with their improved PCR-based assay [50], which raises the possibility that certain *phlD* genotypes were preferentially obtained, thereby amplifying the trend. This is compatible with the observation that a PhlD1 strain (the leaf isolate PF; [33]) could be recovered from wheat, whereas no PhlD1 (i.e. Phl$^+$ Plt$^+$) bacterium was found by Mavrodi et al. [23]. Indeed, the Phl$^+$ Plt$^+$ strain CHA0 could colonize the rhizosphere of field-grown wheat and suppress take-all of wheat at Eschikon, Switzerland [57]. In conclusion, it appears that Phl$^+$ populations of fluorescent pseudomonads in the rhizosphere of dicotyledonous crops are largely cosmopolitan at a worldwide scale.

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