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Pseudomonas community structure and antagonistic potential in the rhizosphere: insights gained by combining phylogenetic and functional gene-based analyses

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Summary

The *Pseudomonas* community structure and antagonistic potential in the rhizospheres of strawberry and oilseed rape (host plants of the fungal phytopathogen *Verticillium dahliae*) were assessed. The use of a new PCR-DGGE system, designed to target *Pseudomonas*-specific *gacA* gene fragments in environmental DNA, circumvented common biases of 16S rRNA gene-based DGGE analyses and proved to be a reliable tool to unravel the diversity of uncultured *Pseudomonas* in bulk and rhizosphere soils. *Pseudomonas*-specific *gacA* fingerprints of total-community (TC) rhizosphere DNA were surprisingly diverse, plant-specific and differed markedly from those of the corresponding bulk soils. By combining multiple culture-dependent and independent surveys, a group of *Pseudomonas* isolates antagonistic towards *V. dahliae* was shown to be genotypically conserved, to carry the *phlD* biosynthetic locus (involved in the biosynthesis of 2,4-diacetylphloroglucinol – 2,4-DAPG), and to correspond to a dominant and highly frequent *Pseudomonas* population in the rhizosphere of field-grown strawberries planted at three sites in Germany which have different land use histories. This population belongs to the *Pseudomonas fluorescens* phylogenetic lineage and showed closest relatedness to *P. fluorescens* strain F113 (97% *gacA* gene sequence identity in 492-bp sequences), a biocontrol agent and 2,4-DAPG producer. Partial *gacA* gene sequences derived from isolates, clones of the strawberry rhizosphere and DGGE bands retrieved in this study represent previously undescribed *Pseudomonas gacA* gene clusters as revealed by phylogenetic analysis.

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Introduction

Antagonistic microbe–microbe interactions mediated by *Pseudomonas* species are major drivers in the biological control of phytopathogenic fungi in the rhizosphere and may indirectly benefit plant growth and survival (Cook *et al*., 1995; Walsh *et al*., 2001; Winding *et al*., 2004). The global response regulator gene *gacA* (for global *a*ntibiotic and *c*yanide control; Laville *et al*., 1992) is required for the production of many secondary metabolites and exoenzymes in both plant-beneficial and -pathogenic *Pseudomonas* spp. (Sacherer *et al*., 1994; Heeb and Haas, 2001). The synthesis of molecules involved in antagonistic interactions and disease suppression such as the antibiotics 2,4-diacetylphloroglucinol (2,4-DAPG) and pyoluteorin, biocides such as HCN, the autoinducer *N*-butyryl-homoserine lactone, the blue phenazine pigment pyocyanin and the lytic enzyme lipase is positively controlled by *gacA* (Reimmann *et al*., 1997; Haas and Keel, 2003; Haas and Défago, 2005). Modifications in *gacA* and *gacS* genes induce phenotypic variation in *Pseudomonas* spp. (van den Broek *et al*., 2005a), thereby regulating not only the production of antibiotics and exoenzymes, but also, e.g. biofilm formation, motility and biosurfactant activities (Deziel *et al*., 2001; Drenkard and Ausubel, 2002; van den Broek *et al*., 2003; Achouak *et al*., 2004). All the aforementioned traits are key in the root colonization behaviour (Sanchez-Contreras *et al*., 2002; Martinez-Granero *et al*., 2005), biocontrol capability (Laville *et al*., 1992; van den Broek *et al*., 2003) and therefore ecological success of rhizosphere *Pseudomonas* spp. (Achouak *et al*., 2004; van den Broek *et al*., 2005b). Moreover, the *gacA* gene has been proposed to be a reliable phylogenetic marker within the genus *Pseudomonas* (de Souza *et al*., 2003), suggesting that it might

Table 1. Antimicrobial properties of *Pseudomonas* spp. isolates with antagonistic activity towards *V. dahliae*.

		Microenvironment[b]			Enzymatic activity[c]				Antagonistic[d]		PCR hybridization[e]			
Sites	N[a]	Soil	Str.	Oils.	Chit	Prot	Gluc	Cell	*R. sol.*	*F. oxy.*	*prnD*	PhlD	*phz*	*gacA*
Berlin	45	9	28	8	2	40	0	0	38	32	2	30	0	40
Braunschweig	25	1	8	16	2	23	0	0	20	20	2	18	0	18
Rostock	31	3	18	10	1	28	0	1	24	16	1	27	0	30
Total	101	13	54	34	5	91	0	1	82	68	5	75	0	88

a. Number of *Verticillium* antagonists isolated from each sampling site.
b. Number of *Verticillium* antagonists isolated from each microenvironment (Str., Strawberry rhizosphere; Oils., Oilseed rape rhizosphere).
c. Number of *Verticillium* antagonists responding positively to chitinase (Chit), protease (Prot), glucanase (Gluc) and cellulase (Cell) activity assays.
d. Number of *Verticillium* antagonists showing *in vitro* antagonistic activity towards *R. solani* (*R. sol*.) and *F. oxysporum* (*F. oxy*.).
e. Number of *Verticillium* antagonists hybridizing with specific probes for the biosynthetic loci *prnD* (pyrrolnitrin) of *P. fluorescens* CHA0, *phlD* (2,4-diacetylphloroglucinol) of *P. fluorescens* Q2-87, *phz* (phenazine-1-carboxilic acid) of *P. fluorescens* pf-5 and for the *gacA* gene of *P. fluorescens* CHA0.

be a suitable target for the simultaneous analysis of *Pseudomonas* community structure and function in soil.

Recent studies by Berg and colleagues (2002; 2006) revealed that the largest fraction of culturable rhizobacteria showing antagonistic activity towards the broad host range, phytopathogenic fungus *Verticillium dahliae* Kleb. belonged to the genus *Pseudomonas*. Based on sequence analysis of *Pseudomonas*-specific DGGE ribotypes amplified from rhizosphere DNA of strawberry and oilseed rape, Costa and colleagues (2006a) suggested that dominant *Pseudomonas* DGGE bands might correspond to rhizosphere populations with antagonistic potential. In the present study, we tested the hypothesis that *Pseudomonas* spp. with antifungal activity represent dominant populations in the rhizosphere. A novel PCR-DGGE system was developed and used to characterize *gacA* types within a collection of *Pseudomonas* isolates antagonistic to *V. dahliae* and to assess the diversity of *gacA* gene fragments PCR-amplified from total-community (TC) DNA of bulk and rhizosphere soils. To connect *Pseudomonas* antagonistic potential and community structure, we attempted a link between DGGE fingerprinting (of both 16S rRNA and *gacA* gene fragments) and the phenotypic and genotypic characteristics of antagonists. In addition, we aimed to scrutinize whether a rhizosphere effect on bulk soil *gacA* gene composition is detectable and to what extent such an effect can be regarded as plant-specific. The use of the *gacA* gene as a tool to assess *Pseudomonas* diversity and phylogeny is discussed.

Results

Antimicrobial properties of Pseudomonas *spp. antagonistic towards* V. dahliae

Many *Verticillium* antagonists analysed in this study also displayed *in vitro* antagonistic activity towards *Rhizoctonia solani* and *Fusarium oxysporum* (Table 1). The vast majority of the antagonists showed protease activity (Prot$^+$). No detection of the phenazine biosynthetic locus and no glucanase activity were observed. Only five in 101 isolates were shown to carry the pyrrolnitrin biosynthetic locus *prnD* (*prnD*$^+$) and to display chitinase activity (Table 1). Hybridization with specific *phlD* (*phlD*$^+$) and *gacA* (*gacA*$^+$) gene probes was frequently observed (Table 1). Figure 1 is a principal components analysis (PCA) ordination biplot representing the grouping of *Verticillium* antagonists in respect to their profiles of antimicrobial attributes as surveyed in this study. No association between a given antagonistic property and the origin of the isolates (sampling site, plant species, rhizosphere versus bulk soils) was found (Table 1, Fig. 1). The horizontal PCA axis accounts for more than 50% of the overall variation in the dataset, determining the ordination of *phlD*$^+$ and *phlD*$^-$ genotypes along this axis: the 75 *phlD*$^+$ antagonists detected in this study were plotted on the left side of the diagram, while the 26 *phlD*$^-$ genotypes were ordinated on the right side (Fig. 1). All 75 *phlD*$^+$ antagonists also hybridized with the *gacA* probe of *P. fluorescens* CHA0 and this correspondence can be depicted from the ordination diagram (Fig. 1). Thirteen antagonists represented *gacA*$^-$*phlD*$^-$ genotypes, while other 13 *gacA*$^+$*phlD*$^-$ genotypes, on the basis of hybridization results with specific probes, were observed. The positive correlation between *gacA* and *phlD* detection by hybridization suggests that antagonists whose *gacA* genes share high homology to the *gacA* sequence of *Pseudomonas fluorescens* CHA0 likely contain the *phlD* biosynthetic locus in their genomes. Antagonism towards *F. oxysporum* and *R. solani* as well as protease activity were more frequently detected within the group of *phlD*$^+$ antagonists as compared with *phlD*$^-$ antagonists (Fig. 1). For instance, 62 of the 75 *phlD*$^+$*Verticillium* antagonists also showed *in vitro* activity against *F. oxysporum*, while 19 of the 26 *phlD*$^-$ antagonists lacked this ability. Similarly, 68 *phlD*$^+$ antagonists inhibited *R. solani in vitro*, while 12 *phlD*$^-$ genotypes

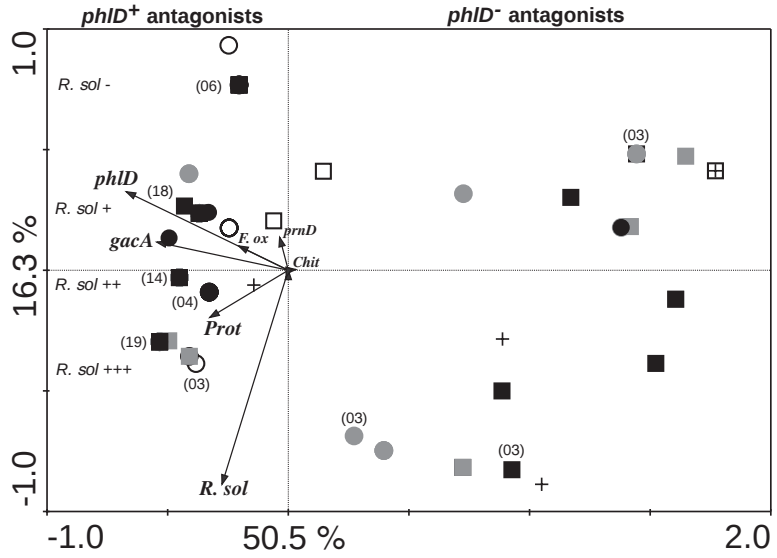

Fig. 1. Principal components analysis (PCA) ordination biplot of antagonistic *Pseudomonas* spp. (symbols, $n = 101$) and their respective antimicrobial properties (arrows). Antagonists were grouped in the ordination diagram according to Euclidean distances calculated on the basis of their profiles of antimicrobial traits. Ranks of intensity (semiquantitative data) were created to register the data on *in vitro* antagonistic activity towards *R. solani* (*R. sol*) and *F. oxysporum* (*F. ox*). Binary data (1 or 0) were used to register results for PCR hybridization with probes specific for the *prnD, phlD*, *phz* and *gacA* genes (see *Experimental procedures*) and for enzymatic activity assays (*Prot*, protease; *Chit*, chitinase). Arrows pointing in the same direction indicate positive correlations between phenotypic/genotypic traits. *R. sol* – means no zone of inhibition while *R. sol* +, ++ and +++ represent until 2 mm, 2–5 mm and > 5 mm wide zone of inhibition of the growth of *R. solani* by *phlD*+ antagonists respectively. Black symbols: antagonists isolated from Braunschweig. Grey and white symbols: antagonists from Berlin and Rostock respectively. Squares and circles: antagonists isolated from the oilseed rape and strawberry rhizospheres respectively. Crosses: bulk soil antagonists. In brackets: number of antagonists (if more than two) with the same profile of antagonistic traits.

did not. In Fig. 1 we highlight four groups of *phlD*$^+$ antagonists: they could be discriminated along the vertical PCA axis of the ordination diagram on the basis of the strength with which the growth of *R. solani* was inhibited as shown by *in vitro* assays (*R. sol* –, *R. sol* +, *R. sol* ++ and *R. sol* +++).

Genotypic diversity and 16S rRNA gene sequencing of antagonistic Pseudomonas *spp.*

We assessed the genotypic diversity within *Verticillium* antagonists by means of whole-genome BOX-PCR (BP) fingerprinting. Six major clusters (Table 2), encompassing a total of 88 antagonists, could be discerned at a level of 85% genotypic similarity from the resulting BP dendrogram (data not shown). The remaining 13 antagonists shared much lower similarity levels (less than 70%) with the defined clusters and within each other and are here collectively referred to as an 'outgroup' (Table 2). While BP clusters 2, 3, 4 and 5 were homogeneous, being formed, each one, by highly similar (> 90% similarity) genotypes, BP cluster 1 could be divided into four subgroups and cluster 6 was highly heterogeneous (data not shown). Representative strains of each BP cluster were submitted to partial sequencing of the 16S rRNA gene (Table 2). The heterogeneity observed within BP profiles of antagonists belonging to cluster 6 and to the 'outgroup' is, to a given extent, reflected by the different closest 16S rRNA gene hits obtained for the members of these groups (Table 2). On the other hand, all closest hits found for the representative members of BP clusters 2 and 3 resembled the 16S rRNA gene sequence of *P. fluorescens* strain Q2-87 (Table 2). With the exception of one isolate, BP clusters 2, 3, 4 and 5 were exclusively composed by *phlD*$^+$ genotypes, whereas BP cluster 6 and the 'outgroup' were both formed by *phlD*$^-$ antagonists (Table 2). This ties in very well with the PCA illustrated in Fig. 1, which was generated independently of BOX-PCR fingerprinting data: antagonists belonging to the BP clusters 2, 3, 4 and 5 represented, in general, *phlD*$^+$ *gacA*$^+$ Prot$^+$ genotypes plotted on the left side of the ordination diagram, separately from the *phlD*$^-$ antagonists.

DGGE fingerprinting of 16S rRNA gene fragments

Figure 2A illustrates 16S rRNA gene DGGE fingerprints of oilseed rape and strawberry rhizosphere samples collected at the sampling site Braunschweig. We observed that the PCR-DGGE mobility of 16S rRNA gene fragments of antagonists (bands A1, A2 and A3 are highlighted) matched the position of a few dominant *Pseudomonas*-specific DGGE ribotypes amplified from rhizosphere DNA. The DGGE double-band 'A3' (Fig. 2A), typically represented the 16S rRNA gene mobility of *phlD*$^+$ antagonists

Table 2. 16S rRNA partial gene sequencing of representative *Pseudomonas* spp. antagonistic to *V. dahliae*.

				Partial 16S rRNA sequence of representative antagonists		
BOX cluster[a]	N[b]	*gacA*[c]	*PhlD*[d]	Antagonist[e]	Closest 16S rRNA gene relative	%
1	20	14	11	**BE2-2-22**	*P. fluorescens* Q2-87 AJ278813	98.6
				RR2-2-43	*P. fluorescens* Q2-87 AJ278813	98.0
				BE1-2-38	*Pseudomonas* sp. S16-2 AF456229	99.9
				BE3-2-21	*Pseudomonas* sp. S16-2 AF456229	99.8
2	13	13	13	**RR4-5-23**	*P. fluorescens* Q2-87 AJ278813	98.8
				BSR2-5-20	*P. fluorescens* Q2-87 AJ278813	99.8
3	32	32	32	**RE4-2-23, RB1-2-39**	*P. fluorescens* Q2-87 AJ278813	100
				RE2-2-23, RE2-2-44		
				BSR4-2-37		
4	3	3	3	**BB3-2-32**	*Pseudomonas* sp. S57-2 AF456220	99.8
5	11	11	10	**BE3-2-35**	*Pseudomonas* sp. S57-2 AF456220	99.9
				BB2-2-35	*Pseudomonas* sp. S57-2 AF456220	99.6
6	9	7	2	RB1-2-40	*P. fluorescens* PC20 AY538264	100
				RR3-2-29	*P. tolaasii* AF348507	100
				BB4-2-39	*P. filiscindens* AY259924	98
				BR3-2-7*	*Pseudomonas* sp. K92.46 AY456702	99.6
Outgroup	13	8	4	BSE2-2-35*	*P. reactans* AF320987, *Pseudomonas* sp. NZ024	100
				BSB1-2-37*	*Pseudomonas* sp. NZ062	100
				BR1-2-40*	*P. putida* ATCC 17494 AF094740	100
				BE3-2-40	*Pseudomonas* sp. S16-2 AF456229	99.5
				BE3-2-28	*Pseudomonas* sp. S16-2 AF456229	100
				BR2-2-11	*P. gessardii* R34 AY972276	99.9
				RR4-5-13	*Pseudomonas* sp. CPE30	99.6
				BSR3-5-15*	*Pseudomonas* sp. DG01 AY546087	99.0
Total	101	88	75			

a. Antagonists sharing more than 85% genotypic similarity as determined by UPGMA clustering of BOX-PCR fingerprints were included in the same BOX-PCR cluster. The 'outgroup' is not a cluster per se.
b. Number of antagonists belonging to each BOX-PCR cluster.
c. Number of antagonists which hybridized with the *gacA* gene probe of *P. fluorescens* CHA0.
d. Number of antagonists which hybridized with the *phlD* gene probe of *P. fluorescens* Q2-87.
e. Strain codes in bold represent *phlD*$^+$ genotypes. *phlD*$^-$ *gacA*$^-$ genotypes are indicated with an asterisk. Sequences of strains BE2-2-22, RR2-2-43, RR4-5-23 and BB4-2-39 were first published by Berg and colleagues (2006).
Isolates' coding indicates: (1) location (B = Berlin, BS = Braunschweig, R = Rostock); (2) microenvironment (B = bulk soil, E = rhizosphere of strawberry, R = rhizosphere of oilseed rape); (3) sampling plot; (4) sampling time (2 = flowering plants 2002, 5 = flowering plants 2003) and (5) consecutive number of the isolate per plant.

on Denaturing Gradient gels (DG-gels): 72 of 75 *phlD*$^+$ *Verticillium* antagonists displayed this melting behaviour. Within the group of *phlD*$^-$ genotypes, on the other hand, many examples of sequence heterogeneities within copies of the 16S rRNA gene were detected. Two to seven DGGE bands could be observed for some of these isolates. These bands sometimes overlapped with DGGE double-band A3, characteristic of *phlD*$^+$ antagonists (Fig. 2A). Thus, a clear differentiation of *phlD*$^+$ and *phlD*$^-$ genotypes by means of 16S rRNA gene DGGE fingerprinting was not possible. The assemblage of PCR-DGGE ribotypes amplified from culturable *Pseudomonas* spp. antagonistic to *V. dahliae* corresponded only to a portion of the whole *Pseudomonas* rhizosphere DGGE profiles (Fig. 2A). This trend was also observed for the sampling sites Berlin and Rostock (data not shown).

DGGE fingerprinting of gacA *gene fragments*

We developed a novel PCR-DGGE system to characterize *gacA* gene types within *Pseudomonas* spp. antagonistic towards *V. dahliae*. Extensive data derived from both *in silico* and *in vitro* (Table S1) surveys of primer specificity and from cloning and sequencing approaches (Table S2, unpubl. data) pointed out the specific status of our newly developed system. The *gacA* DGGE types of 53 antagonists corresponding to different BOX-PCR clusters and antimicrobial profiles (42 *phlD*$^+$ and 11 *phlD*$^-$ genotypes) were recorded. In contrast to 16S rRNA gene-based DGGE analysis (Fig. 2A), the *gacA* mobilities of *phlD*$^+$ and *phlD*$^-$ antagonists could be clearly differentiated on DG-gels (Fig. 2B). One advantage provided by the newly developed PCR-DGGE system was the generation of single *gacA* DGGE bands for each isolate tested. Moreover, all *phlD*$^+$ genotypes characterized by the technique displayed the same *gacA* DGGE type (*gacA* type 'G3'), with the exception of isolate BB3-2-32 (Tables 2 and 3), the single *phlD*$^+$ antagonist found to display the electrophoretic mobility of *gacA* type 'G4' (Fig. 2B). The *gacA* types G1, G2, G6 and G7 were represented exclusively by *phlD*$^-$ antagonists. GacA fragments of antagonists which did not hybridize with the probe generated from

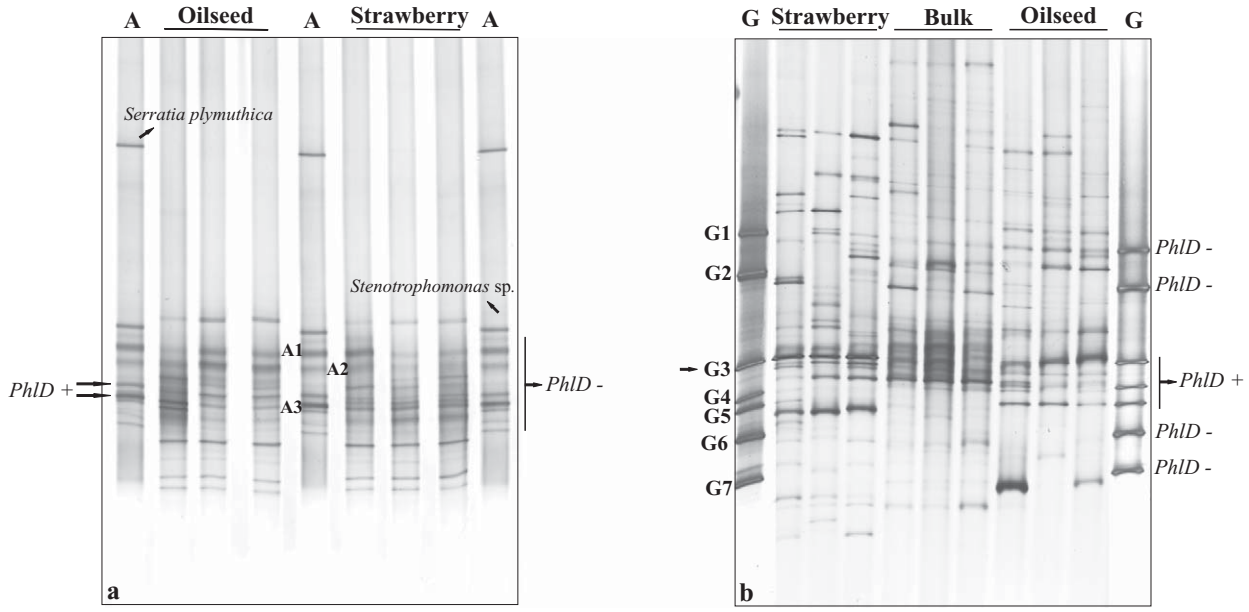

Fig. 2. A. *Pseudomonas*-specific DGGE fingerprints of 16S rRNA gene fragments PCR-amplified from TC-DNA of oilseed rape ('Oilseed') and strawberry ('Strawberry') rhizosphere triplicates collected at the sampling site Braunschweig (year 2003). Fingerprints were compared with a mixture of the 16S rRNA gene fragments amplified from genomic DNA of representative *Verticillium* antagonists isolated from strawberry and oilseed rape rhizospheres ('A' lanes). Amplicons of two *Verticillium* antagonists identified as *Serratia plymuthica* and *Stenotrophomonas* sp. were included in the PCR mixture and their DGGE mobilities are indicated. Dominant *Pseudomonas* ribotypes of the rhizosphere DGGE profiles that matched the 16S rRNA gene DGGE mobilities of *Verticillium* antagonists (bands A1, A2 and A3) are highlighted, as well as the typical DGGE mobilities displayed by *phlD*$^+$ and *phlD*$^-$ antagonists.
B. *Pseudomonas*-specific DGGE fingerprints of *gacA* gene fragments PCR-amplified from TC-DNA of oilseed rape ('Oilseed') and strawberry ('Strawberry') rhizosphere triplicates collected at the sampling site Braunschweig (year 2003) as compared with their corresponding bulk soil profiles ('Bulk'). At the extremities of the gel a lane is shown (lane G) which is composed by bands representing the melting behaviours of *gacA* gene fragments amplified from representative *Verticillium* antagonists (*gacA* DGGE types G1–G4, G6 and G7, Table 3) and from *P. fluorescens* strain CHA0 (*gacA* DGGE type G5). *GacA* DGGE mobilities displayed by *phlD*$^+$ and *phlD*$^-$ antagonists are indicated. The arrow points to *gacA* DGGE type G3, for which a matching band was found in the *gacA* DGGE profiles. The relative abundance of this band is enhanced in the DGGE fingerprints of the strawberry rhizosphere in comparison with those of the bulk soils.

P. fluorescens CHA0, such as strain BSB1-2-37 (Table 2, gacA type G6), could be amplified by the system. Partial *gacA* gene sequences were obtained for antagonists corresponding to each *gacA* DGGE type shown in Fig. 2B. Their closest phylogenetic relatives are listed in Table 3. None of the *gacA* DGGE types registered for our group of *Verticillium* antagonists shared the *gacA* DGGE mobility of *P. fluorescens* CHA0 (*gacA* type G5, Fig. 2B). The new PCR-DGGE system was further employed for the assessment of *Pseudomonas gacA* gene diversity in bulk soils

Table 3. *gacA* gene sequencing of *Pseudomonas* antagonists representing different *gacA* types as determined by DGGE.

			gacA gene partial sequencing			16S rRNA gene partial sequencing[d]			
DGGE[a]	N[b]	Antagonist[c]	bp	Closest hit	%	Closest hit	%	*phlD*[e]	*prnD*[f]
G1	4	BE3-2-40	545	*P. fluorescens* AB054363.1	91.7	*Pseudomonas* sp. S16-2 AF456229	99.5	–	–
G2a	2	RB1-2-40	535	*P. fluorescens* PfO-1 CP000094.1	88.8	*P. fluorescens* PC20 AY538264	100	–	–
G2b	1	RR3-2-29	538	*P. fluorescens* F113 AY254172.1	90.1	*P. tolaasii* AF348507	100	–	–
G3	42	RB1-2-39	541	*P. fluorescens* F113 AY254172.1	97.0	*P. fluorescens* Q2-87 AJ278813	100	+	
G4	1	BB3-2-32	535	*P. fluorescens* F113 AY254172.1	96.3	*Pseudomonas* sp. S57-2 AF456220	99.8	+	–
G6	2	BSB1-2-37	546	*P. fluorescens* AB219365.1	88.6	*Pseudomonas* sp. NZ062	100	–	+
G7	1	RR4-5-13	521	*P. putida* KT2440 AE015451.1	85.8	*Pseudomonas* sp. CPE30	99.6	–	–

a. Electrophoretic mobilities of *gacA* DGGE types as shown in Fig. 2B. The *gacA* DGGE type G5 (not shown in the table) corresponds to the *gacA* electrophoretic mobility of the biocontrol strain *P. fluorescens* CHA0.
b. Number of antagonists tested whose *gacA* gene fragments run at the same DGGE position.
c. One representative antagonist per *gacA* type was selected for sequencing.
d. See alsoTable 2.
e. PCR-hybridization detection of the *phlD* biosynthetic locus.
f. PCR-hybridization detection of the *prnD* biosynthetic locus.

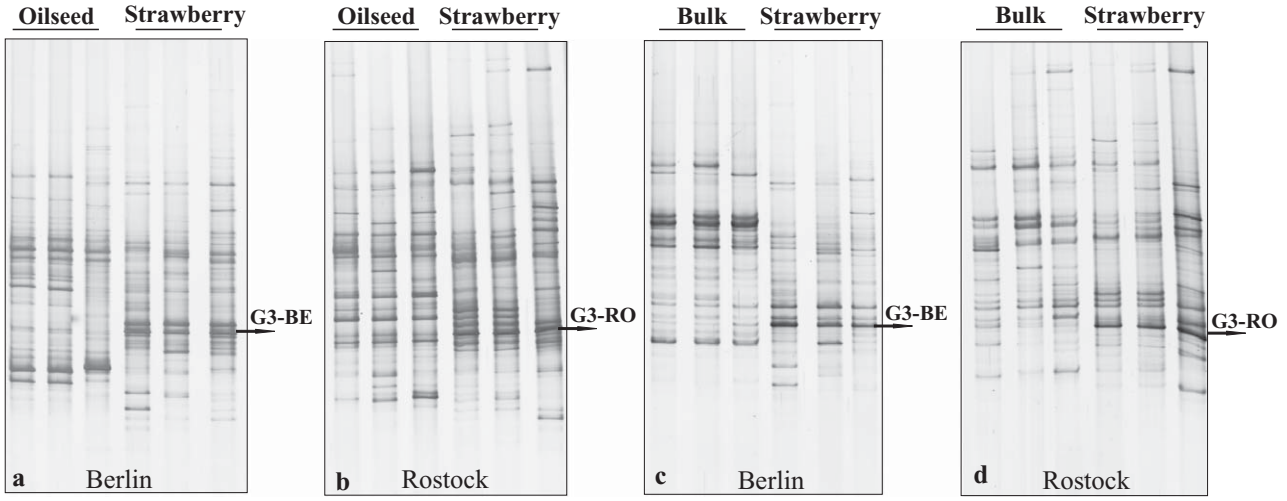

Fig. 3. Plant-dependent composition of *Pseudomonas*-specific *gacA* gene fragments in the rhizospheres of strawberry and oilseed rape (A and B) and the effect of the strawberry rhizosphere on the assemblage of *Pseudomonas gacA* gene fragments of the corresponding bulk soils (C and D) as evidenced by PCR-DGGE analysis of TC DNA templates (sampling of year 2002). DGGE profiles obtained for the sampling sites Berlin (A and C) and Rostock (B and D) are shown in triplicates. Arrows indicate a *gacA* DGGE band which is enriched in the strawberry rhizosphere profiles as compared with those of the bulk soils in all sampling sites studied (see Fig. 2B for the Braunschweig site). This band displays an electrophoretic mobility on DG-gels which corresponds to that of strain RB1-2-39 (*gacA* type G3; Fig. 2B, Table 3). Strawberry: DGGE fingerprints of strawberry rhizosphere samples. Oilseed: DGGE fingerprints of oilseed rape rhizosphere samples. Bulk: DGGE fingerprints of bulk soil samples.

and in the rhizospheres of strawberry and oilseed rape. High diversity of *Pseudomonas gacA* gene fragments was detected (Figs 2B and 3). The richness of *gacA* DGGE bands observed in the rhizosphere profiles (from 22 to 26 fairly visible bands; Fig. 2B) exceeded that of *Pseudomonas*-specific 16S rRNA DGGE bands (approximately 15 bands; Fig. 2A) in all sampling sites (data not shown). As observed for the 16S rRNA gene profiles, *gacA* fragments amplified from antagonists represented only a minor portion of the *gacA* gene assemblage revealed by TC rhizosphere DNA analysis (Figs 2B and 4). The composition of *Pseudomonas gacA* gene fragments in the rhizosphere was plant-specific (Fig. 3A and B): well-defined and separated strawberry and oilseed rape groups were generated by cluster analysis (data not shown). Both the strawberry (Fig. 3C and D) as well as the oilseed rape (Fig. 2B) rhizosphere *gacA* DGGE profiles differed markedly from those of the corresponding bulk soil samples. A *gacA* DGGE band equivalent to the *gacA* type G5 of *P. fluorescens* CHA0 was found to be dominant in, and specific to, the oilseed rape rhizosphere *gacA* fingerprints of the sampling site Braunschweig (Fig. 2B). Moreover, we observed that the relative abundance of a *gacA* DGGE band was enhanced in the profiles of the strawberry rhizosphere as compared with the bulk soil profiles at all sampling sites studied (DGGE bands G3-BE, G3-BS and G3-RO; Figs 3 and 4). This *gacA* band exhibited the same melting behaviour of *gacA* DGGE type G3 (Figs 2B and 4), which represented our collection of genotypically conserved *phlD*$^+$ antagonists (Table 3). As already indicated in Fig. 2A, these antagonists were also shown to correspond to a dominant, rhizosphere-associated 16S rRNA DGGE band (band 'A3'). Partial *gacA* gene sequence of strain RB1-2-39, a representative of this group of antagonists, was closest related to the *gacA* sequence of *P. fluorescens* F113 (Table 3), while its 16S rRNA gene sequence showed the highest similarity to that of *P. fluorescens* Q2-87 (Table 2). Both *P. fluorescens* strain F113 and strain Q2-87 are biocontrol agents (BCAs) and 2,4-DAPG producers (Shanahan *et al*., 1992; Bangera and Thomashow, 1999; Moënne-Loccoz *et al*., 2001).

Sequence analysis and phylogeny of Pseudomonas gacA *gene fragments*

After checking the melting behaviour of 40 *gacA* clones derived from TC strawberry rhizosphere DNA on DG-gels, 16 clones which represented different or slightly different patterns of DGGE migration were selected for sequencing. Nine of such *gacA* DGGE mobilities are highlighted in Fig. 4 ('C' lane). Nucleotide–nucleotide alignment (BLAST algorithm) of all the *gacA* clone sequences obtained in this fashion revealed that the closest matches to our queries were, in all cases, assigned as *gacA* sequences of the genus *Pseudomonas*, but, in general, rather low levels of sequence identity (mostly ranging from 87.5% to 90%) were observed (Table S2). Higher degrees of relatedness could be detected when the *gacA* clone sequences were compared with the partial *gacA*

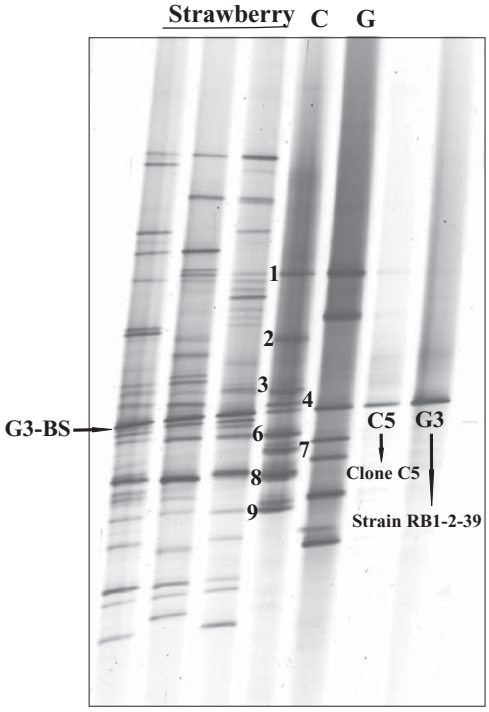

Fig. 4. Comparison of *gacA* DGGE fingerprints of PCR-amplified rhizosphere DNA of field-grown strawberry (sampling site Braunschweig, three independent replicates shown) with the DGGE melting behaviour of *gacA* gene fragments amplified from clones of the strawberry rhizosphere (lane C) and from *Verticillium* antagonists (lane G, see also Fig. 2B and Table 3). The nine DGGE band positions (1–9) indicated on lane 'C' correspond to the melting behaviour of representative *gacA* gene clones used in phylogenetic analysis (Fig. 5). The arrow indicates a dominant *gacA* DGGE band which is enriched in the strawberry rhizosphere as compared with bulk soils (see also Figs 2B and 3) and exhibits the same melting behaviour of the *gacA* gene fragments of clone C5 and strain RB1-2-39 (*gacA* type G3; Fig. 2B, Table 3).

sequences of our own collection of *Verticillium* antagonists (Table S2). Partial *gacA* gene sequences of representative isolates (*gacA* DGGE types G1 to G7; Fig. 2B), clones of the strawberry rhizosphere (*gacA* DGGE types C1 to C9; Fig. 4) and clones derived from the DGGE bands G3-BS, G3-BE and G3-RO (Figs 3 and 4) were used for the construction of a *gacA* gene-based phylogenetic tree (Fig. 5). Clusters of *Pseudomonas gacA* gene sequences so far absent in public databases were found. The cluster formed by *gacA* clones corresponding to band positions C1, C2, C3, C4 and C6 (Fig. 4) likely represents a *P. fluorescens gacA* gene cluster as indicated by 16S rRNA and *gacA* gene sequencing of their closest relative, strain RB1-2-40 (Table 3) and by *gacA* tree topology (Fig. 5). A very robust and consistent *gacA* gene cluster formed by the sequences of isolate RB1-2-39 (*gacA* type G3; Fig. 2B), clone C5 (*gacA* type C5; Fig. 4) and the DGGE band clones from Berlin, Braunschweig and Rostock (Figs 3 and 4) could be visualized (Fig. 5). All these latter sequences, retrieved from different 'sources' but representing the same *gacA* mobility on DG-gels, shared very high similarity as evidenced by nucleotide–nucleotide alignment (data not shown) and phylogenetic analysis (Fig. 5). The *gacA* gene sequence of *P. fluorescens* strain F113 was the closest phylogenetic relative to this cluster (Fig. 5) with about 97% sequence identity (Table 3, Table S2).

Discussion

In this study, a suite of tools was employed to test the hypothesis that antagonistic *Pseudomonas* spp. correspond to dominant populations in the rhizosphere of *V. dahliae* host plants. Culturable *Pseudomonas* spp. antagonistic towards *V. dahliae* were isolated from the rhizosphere of field-grown strawberry and oilseed rape plants (Berg *et al*., 2006). Their functional and genotypic diversity was assessed by multiple means. *In situ* molecular analysis of *Pseudomonas* diversity relied on the use of two phylogenetic marker genes, namely the 16S rRNA and *gacA* genes. A link between culture-dependent and -independent methods was then used to determine the portion of *Pseudomonas* rhizosphere communities represented by culturable *Pseudomonas* spp. with the potential to control *V. dahliae*.

Significance of Pseudomonas*-specific gacA gene-based analysis*

The GacS/GacA two-component regulatory system positively controls the expression of genes that encode the biosynthesis of many secondary metabolites within *Pseudomonas* spp. (Haas and Keel, 2003; Haas and Défago, 2005; Dubuis and Haas, 2007). By recognizing as-yet-unknown environmental stimuli, the membrane-bound sensor kinase protein GacS activates the cytoplasmic response regulator GacA, which triggers the expression of genes controlled by the system (Heeb and Haas, 2001). This system has been shown to be of utmost importance in respect to the fitness of *Pseudomonas* strains in microcosms (Natsch *et al*., 1994; Valverde *et al*., 2003; Zuber *et al*., 2003). For instance, mutations in the *gacA* gene of the biocontrol strain *P. fluorescens* CHA0 were shown to pleiotropically block the production of the secondary metabolites 2,4-DAPG, HCN and pyoluteorin and, moreover, to drastically reduce the ability of this strain in suppressing black root rot of tobacco (Laville *et al*., 1992). However, little is known about the distribution, frequency and diversity of these genes in rhizosphere-associated bacteria. de Souza and colleagues (2003) first suggested that the *gacA* gene could be used as a complementary phylogenetic marker for the study of *Pseudomonas* spp. in the rhizosphere. In this study, we explored this assumption by developing a PCR-DGGE system to target

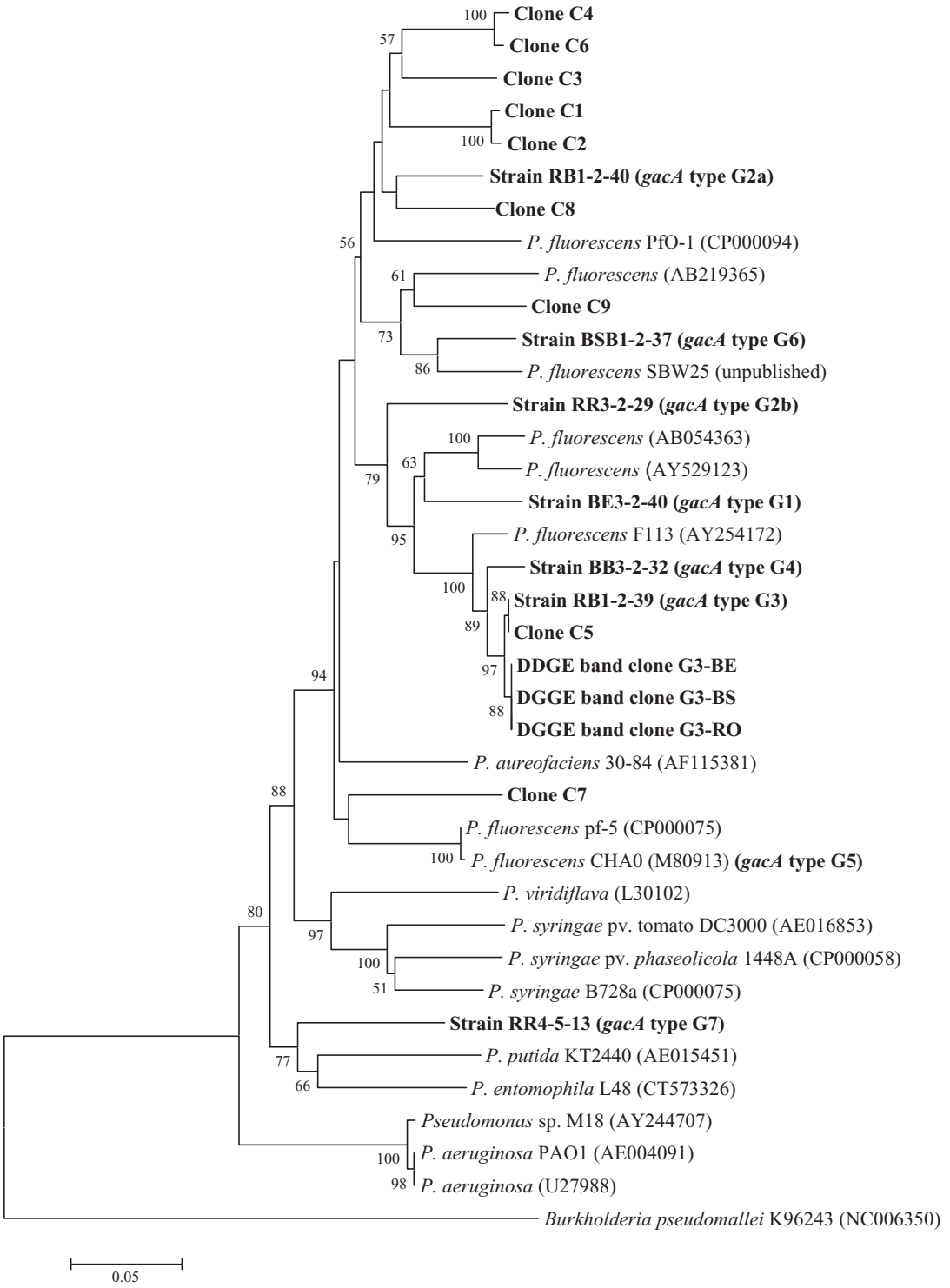

Fig. 5. Phylogeny of 492 bp-long *gacA* gene sequences (positions 46–537 of the *gacA* gene of *P. fluorescens* CHA0, 77% of whole *gacA* coding sequence) as inferred by applying the neighbour-joining method to *p*-nucleotide distances calculated with the MEGA software (version 3.0). Sequences were edited and aligned using the CLUSTALW algorithm prior to bootstrapped (500 repetitions) tests of phylogeny. Bootstrap values above 50 are displayed on tree nodes. Sequences of *Verticillium* antagonists representative of different *gacA* DGGE mobilities ('*gacA* types' G1–G7; Fig. 2B), of *gacA* clones of the strawberry rhizosphere (clones 1–9; Fig. 4) and of a DGGE band enriched in the *gacA* DGGE fingerprints of strawberry rhizospheres collected in Berlin, Braunschweig and Rostock (DGGE band clone G3-BE, G3-BS and G3-RO respectively) were all obtained in this study and are shown in bold. Accession numbers of *gacA* gene sequences of *Pseudomonas* strains present in relevant databases are indicated in brackets. The *gacA* gene homologue of *Burkholderia pseudomallei* strain K96243 was used as outgroup. Bar indicates number of substitutions per site.

Pseudomonas-specific *gacA* gene fragments in TC-DNA. This is the first cultivation-independent assessment of *Pseudomonas gacA* genes in soil. A so-far-unknown richness of rhizosphere-associated *gacA* genes of *Pseudomonas* was revealed. A rhizosphere effect on *Pseudomonas gacA* gene composition and both plant- and site-specific *gacA* gene structure in rhizosphere and bulk soils respectively, could be clearly detected. These findings support and improve previous knowledge of molecular-based community structure and diversity of *Pseudomonas* in the rhizosphere (Costa *et al*., 2006a). The high numbers of *gacA* gene fragments in DGGE profiles of bulk and rhizosphere soils revealed that the genotypic diversity of uncultured *Pseudomonas* in such environments is higher than previously indicated by 16S rRNA gene-based DGGE studies (Gyamfi *et al*., 2002; Garbeva *et al*., 2004; Milling *et al*., 2004; Costa *et al*., 2006a). A better resolving capacity provided by *gacA* gene-based analysis in distinguishing *Pseudomonas* spp. explains this outcome: although both *gacA* and 16S rRNA gene-based phylogenetic trees share a similar topology, a higher power of resolution in distinguishing closely related strains was observed for the former as compared with the latter (de Souza *et al*., 2003). Moreover, as our PCR-DGGE system targets about 83% of the whole *gacA* coding sequence (535 bp), diversity observed in DGGE profiles might even underestimate whole-length *gacA* diversity of soil *Pseudomonas* spp. It is tempting to speculate that, e.g. plant-specific *gacA* gene composition could be an indicator of distinct patterns of secondary metabolite production and antagonistic interactions that take place in the rhizosphere of different plants. The results obtained by characterizing the *gacA* genes of *Verticillium* antagonists support this notion: DGGE *gacA* mobilities of *phlD*$^+$ antagonists differed from those of *phlD*$^-$ and *prnD*$^+$ antagonists. In contrast, these strains could not be differentiated by DGGE of 16S rRNA gene fragments. However, such a differentiation might simply mirror a phylogenetic distinction within *Pseudomonas* spp., because the biosynthetic loci involved in 2,4-DAPG production were so far mainly detected in *P. fluorescens* strains (de la Fuente *et al*., 2005; Validov *et al*., 2005). More comprehensive *gacA* gene-based studies of *Pseudomonas* spp., possibly including representatives of other 'functional groups' within the genus, such as plant-pathogenic strains, are needed to improve our understanding of whether *Pseudomonas gacA* gene phylogeny indeed relates to antagonistic function.

Detection of a 2,4-DAPG-producing Pseudomonas *population enriched in the strawberry rhizosphere*

The assessment of the genotypic diversity within culturable *Pseudomonas* spp. antagonistic towards *V. dahliae* by BP fingerprinting clearly showed that the screening for such organisms ($n = 101$, Table 1) has functioned as a 'genomic bottleneck': basically, antagonists could be split into two categories. The first, composed by isolates ($n = 75$) that hybridized with the *phlD* probe generated from *P. fluorescens* Q2-87 and were found to be highly similar genotypically according to BP fingerprints, 16S rRNA gene sequencing and DGGE of both 16S rRNA and *gacA* genes. Representatives of this group were all identified as *P. fluorescens*. This is in agreement with the findings of previous studies reporting on the occurrence of the 2,4-DAPG biosynthetic loci within diverse strains of this species (McSpadden Gardener *et al*., 2000; Mavrodi *et al*., 2001; de la Fuente *et al*., 2005; Validov *et al*., 2005). The second category was constituted by a smaller group of diverse *phlD*$^-$ antagonists ($n = 26$). The electrophoretic mobilities of *phlD*$^+$ antagonists matched those of dominant rhizosphere DGGE bands of both 16S rRNA and *gacA* gene fingerprints. In a previous study (Costa *et al*., 2006a), we cloned and sequenced dominant *Pseudomonas* 16S rRNA DGGE fragments amplified from the rhizosphere DNA samples analysed in the present report. There we showed that *Pseudomonas* DGGE ribotypes corresponding to band 'A3' (Fig. 2A) shared the highest 16S rRNA gene identity with the BCA and 2,4-DAPG producer *P. fluorescens* strain F113 (Shanahan *et al*., 1992; Moënne-Loccoz *et al*., 2001). Here, we reveal that partial 16S rRNA gene sequences of *phlD*$^+$ antagonists that display this same melting behaviour on DGGE were closest to *P. fluorescens* Q2-87, also known to produce 2,4-DAPG (Bangera and Thomashow, 1999). The 16S rRNA sequences of strains F113 and Q2-87 (Ramette *et al*., 2001) are highly related (1515 matches and no gaps in 1521 bp-long sequences). Thus, the independent strategies of cloning and sequencing *Pseudomonas* DGGE ribotypes (*c*. 400 bp) PCR-amplified from rhizosphere DNA (Costa *et al*., 2006a) and partial 16S rRNA sequencing (*c*. 800 bp) of the corresponding antagonistic isolates (this study) were in agreement. Moreover, partial *gacA* gene sequencing of isolate RB1-2-39, which represented *phlD*$^+$ antagonists that displayed DGGE melting behaviours of bands 'A3' (16S rRNA gene fingerprints) and 'G3' (*gacA* fingerprints) revealed closest identity, again, to strain *P. fluorescens* F113 (97% relatedness). We have shown that the relative abundance of a *gacA* DGGE band corresponding to the *gacA* type 'G3' of *phlD*$^+$ antagonists was clearly enhanced in the DGGE profiles of the strawberry rhizosphere in comparison with that of the corresponding bulk soil profiles in all sampling sites. Phylogenetic analysis confirmed that the *gacA* sequences retrieved from these bands (G3-BE, G3-BS and G3-RO; Fig. 5) resembled the *gacA* gene sequence amplified from strain RB1-2-39 (Fig. 5). Based on this, we concluded that a genetically conserved population of *P. fluorescens* shown to antagonize *V. dahliae in*

vitro and to carry the *phlD*$^+$ biosynthetic locus was a dominant member of the *Pseudomonas* community in the rhizospheres of field-grown *V. dahliae* host plants at three field sites in Germany with different histories of land use and management. The role of 2,4-DAPG production by *Pseudomonas* spp. in the control of plant diseases caused by soil-borne fungal pathogens has been unequivocally demonstrated (Keel *et al*., 1992; Raaijmakers and Weller, 1998; Weller *et al*., 2002). However, no assays have so far been conducted to investigate whether 2,4-DAPG-producing *Pseudomonas* are capable of suppressing *Verticillium* wilt *in situ*. Although our study was not designed to specifically address this question, the major trends derived from our surveys suggest that an involvement of 2,4-DAPG production in controlling *V. dahliae* growth in soil is likely. To test this hypothesis, the performance of *phlD*$^+$ antagonists in suppressing *V. dahliae* growth in soil, as well as the extent to which such a suppression, if existent, is due to the production of 2,4-DAPG, need to be carefully evaluated.

Overall, our results suggest that *gacA* sequence heterogeneities not only reflect the evolutionary relatedness within *Pseudomonas* species, but also allow a more in-depth analysis of the diversity of this highly complex group as compared with 16S rRNA gene phylogeny. Recently, other marker genes (e.g. *rpoB* and *oprF*) or their use in concatenation (e.g. *gyrB-rpoD*) have been proposed to reasonably reflect phylogenetic relationships among *Pseudomonas* spp. (Yamamoto *et al*., 2000; Bodilis *et al*., 2004; Hilario *et al*., 2004; Ait Tayeb *et al*., 2005). In addition, Bodilis and colleagues (2006) have suggested that the *oprF* gene (coding the major outer-membrane protein of *Pseudomonas sensu stricto*) can be used as an ecotype marker within *Pseudomonas* spp. New attempts must be made that combine such emergent phylogenetic, functional and ecological gene markers for the study of *Pseudomonas* species in their environments. Future *gacA* gene-based studies of uncultured *Pseudomonas* spp. hold a great promise in providing a better glimpse of the distribution, abundance and *in situ* activity of these species in nature. The polyphasic approach employed in this study led to new insights on the phylogeny, occurrence, potential role and ecological fitness of rhizosphere-associated *Pseudomonas* spp. capable of suppressing the growth of *V. dahliae*.

Experimental procedures

Field design and sampling

Strawberries [*Fragaria ananassa* (Duchense) Decaisne and Naudin cv. Elsanta] were planted and oilseed rape (*Brassica napus* L. cv. Licosmos) was sown in field plots using a randomized block design consisting of four replicate plots per plant. For each plot, one composite bulk soil sample and one composite rhizosphere sample were taken at three stages of plant development (young, flowering and senescent plants) as described in detail by Berg and colleagues (2005). In the present study, analyses were carried out exclusively for the rhizosphere samples of flowering plants and their corresponding bulk soil samples. Samplings took place in two consecutive years (2002 and 2003) at three locations in Germany: Braunschweig (52°16′N, 10°31′E), Berlin (52°31′N, 13°24′E) and Rostock (54°05′N, 12°07′E). Soil texture was classified as sand in Berlin and weakly loamy sand in Braunschweig and Rostock. Physical-chemical parameters of soils were determined by Berg and colleagues (2005). All sampling sites differ from each other in respect to land use and management (Costa *et al*., 2006a). Samples were immediately transported to the laboratory and processed for further analysis.

Pseudomonas spp. antagonistic to V. dahliae

Berg and colleagues (2006) performed an *in vitro* screening for antagonistic activity towards *V. dahliae* of over 6000 bacterial isolates retrieved from the rhizosphere and bulk soil samples collected as explained above. In total, 590 antagonists were detected, among which *c.* 75% were considered to belong to the genus *Pseudomonas* (Berg *et al*., 2006). From this collection, 101 strains embracing the *Pseudomonas* antagonists isolated from the rhizospheres of flowering strawberries ($n = 54$) and oilseed rape plants ($n = 34$), as well as from the corresponding bulk soils ($n = 13$) were selected to be characterized in the present study. More detailed information on the isolation procedures was provided by Berg and colleagues (2006).

Antimicrobial attributes of Verticillium *antagonists*

Verticillium antagonists were tested for *in vitro* activity towards two other phytopathogenic fungi: *R. solani* AG3 and *F. oxysporum* Foln3 (basidiomycete and ascomycete respectively, with a chitin-glucan-containing cell wall). Tests were performed in dual culture assays on Waksman Agar as described by Berg and colleagues (2000). *Rhizoctonia solani* AG3 was obtained from Plant Research International, Wageningen, the Netherlands. *Fusarium oxysporum* f.sp. lini (isolate Foln3) was received from INRA, Dijon, France. *In vitro* assays to detect the production of cell-wall degrading enzymes (protease, chitinase, β-1,3-glucanase and cellulase activities) by *Verticillium* antagonists were performed as described elsewhere (Chernin *et al*., 1995; Nielsen and Sørensen, 1997; Berg *et al*., 2002).

Genotypic diversity of antagonists and screening for antibiotic-encoding genes

DNA extraction and whole-genome BP fingerprinting of antagonists were carried out following the procedures described by Costa and colleagues (2006b). BOX-PCR profiles were compared using the software package GelCompar version 4.1 (Applied Maths, Kortrijk, Belgium) as explained elsewhere (Costa *et al*., 2006b).

Antagonists were tested for the presence of genes involved in the synthesis of antibiotics using specific PCR amplifications from genomic DNA. PCR detection of the biosynthetic *loci phlD* (2,4-Diacetylphloroglucinol, 2,4-DAPG) and *phz* (phenazin-1-carboxylic acid, PCA) was carried out using the primer pairs and protocols described by Raaijmakers and colleagues (1997). PCR detection of the pyrrolnitrin biosynthetic locus *prnD* was performed using the primers PRND1 and PRND2 (de Souza and Raaijmakers, 2003). PCR detection of the global regulator gene *gacA* was done according to the protocol of de Souza and colleagues (2003). Dot-blot and Southern hybridizations of the PCR amplicons were performed by standard methods (Sambrook *et al*., 1989) under conditions of medium stringency (Fulthorpe *et al*., 1995). Probes were generated by labelling the PCR products amplified from *P. fluorescens* strains CHA0 (*prnD* and *gacA* probes, 786 and 425 bp respectively), Q2-87 (*phlD* probe, 745 bp) and 2–79 (*phz* probe, 1150 bp) using DIG-labelled dUTP as recommended by the manufacturer (Roche Diagnostics). Hybridized probes were detected with a DIG luminescent detection kit and exposure to X-ray film (Roche Diagnostics, Mannheim, Germany) as specified by the manufacturer. In this report, hybridization (rather than PCR) results are used throughout to assign antagonists as positive or negative (e.g. *gacA*$^+$ or *gacA*$^-$) in respect to the detection of a given biosynthetic locus in their genomic DNAs.

Principal components analysis was used to group *Pseudomonas* spp. antagonistic towards *V. dahliae* according to their phenotypic and genotypic characteristics. Data on antagonistic activity assays (towards *R. solani* and *F. oxysporum*), enzymatic activity assays (chitinase, cellulase, 1,3-glucanase and protease) and dot-blot hybridization of PCR-amplified functional genes (*gacA*, *phlD*, *phz* and *prnD*) were used to run PCA with the software Canoco for Windows 4.5 (Microcomputer Power, Ithaca, NY, USA).

TC DNA extraction from rhizosphere and bulk soil samples

Total-community DNA was isolated from environmental samples as described by Costa and colleagues (2006c) using the BIO-101 DNA extraction kit (Q Biogene, Carlsbad, CA, USA) and re-purification with the GENECLEAN Spin kit (Q Biogene) following the supplier's instructions. Genomic DNA yields were checked under UV light after electrophoresis in 0.8% agarose gels and ethidium bromide staining. DNA concentrations were estimated visually by applying the 1 kb gene-ruler™ DNA ladder (Fermentas, St. Leon-Rot, Germany) on the agarose gels as a mass ruler. Genomic DNA samples were diluted differentially to obtain *c*. 1–5 ng DNA to be used as PCR templates for further analysis.

PCR amplification of Pseudomonas *16S rRNA gene fragments for DGGE*

A nested PCR approach was used to amplify *Pseudomonas* spp. 16S rRNA gene fragments from TC rhizosphere DNA. A first, taxon-specific, PCR amplification was carried out with the primer pair F_{311} Ps/R1459 Ps as described by Milling and colleagues (2004) using 1 μl of rhizosphere DNA template (*c*. 5 ng) and 25 thermal cycles. Twenty-fold diluted PCR products were then used as templates (1 μl) for the amplification of 16S rRNA gene fragments using the primers F984GC and R1378 (Heuer *et al*., 1997) as performed by Costa and colleagues (2006b) prior to DGGE analysis. PCR products were checked after electrophoresis on agarose gels under UV transillumination (254 nm).

PCR amplification of Pseudomonas *gacA gene fragments for DGGE*

Primers were designed to target conserved regions within *Pseudomonas gacA* gene sequences longer than 500 bp available at the GenBank database (*Pseudomonas aeruginosa* AE004091 and U27988; *P. aureofaciens* AF115381; *P. entomophila* CT573326; *P. fluorescens* AY254172, AY529123, M80913, CP000076 and CP000094; *P. putida* AE015451 and AJ629219, *P. syringae* CP000075 and AE016853, *P. viridiflava* L30102 and *Pseudomonas* sp. AY244707). The sequences were aligned using the GeneCompar software (version 1.3, Applied Maths, Kortrijk, Belgium). Primers were optimized with the programme Oligo 4.0 (National Biosciences Inc., Plymouth, MN, USA). Primer specificity was tested *in silico* by basic local alignment search tool (BLAST) and *in vitro* by PCR means using a large collection of target and non-target strains (Table S1). The reverse primer gacA2 (de Souza *et al*., 2003), and the newly designed primers gacA-1F (5′-TGA TTA GGG TGY TAG TDG TCG A-3′), gacA-1FGC (5′-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G-GAT TAG GGT GCT AGT GGT CGA-3′) and gacA-2R (5′-GGT TTT CGG TGA CAG GCA-3′) were used in a nested PCR approach. For the first PCR, a reaction mixture was prepared containing 1 μl of template DNA (*c*. 5 ng), 1× PCR buffer II (Applied Biosystems, Foster, CA, USA), 0.2 mM dNTPs, 3.75 mM $MgCl_2$, 2.5 μg of BSA, 5% (w/v) dimethylsulfoxide (DMSO), 0.2 μM primers gacA-1F and gacA2 and 1.25 U Ampli*Taq* Gold™ Polymerase (Applied Biosystems). After 5 min denaturation at 95°C, 30 thermal cycles of 1 min at 94°C, 1.5 min at 57°C and 1 min at 72°C were carried out. A final extension step at 72°C for 10 min was performed to finish the reaction. Twenty-fold diluted amplicons (599-bp) were used as templates for a second PCR using primers gacA-1FGC and gacA-2R. A GC-clamp attached to the 5′ end of the forward primer was used to prevent complete melting of double-stranded DNA during DGGE. Reaction mixtures (25 μl) consisted of 1 μl of diluted template DNA, 1× Stoffel buffer (Applied Biosystems), 0.2 mM dNTPs, 3.75 mM $MgCl_2$, 5% (w/v) DMSO, 0.2 μM primers and 2.5 U *Taq* DNA polymerase (Stoffel fragment, Applied Biosystems). Denaturation for 5 min at 94°C was carried out, after which 25 thermal cycles of 1 min at 95°C, 1.5 min at 52°C and 1.5 min at 72°C were performed. A final extension step of 10 min at 72°C finished the reaction. PCR products (575 bp, GC-clamp included) were checked after electrophoresis on agarose gels under UV transillumination (254 nm).

Denaturing gradient gel electrophoresis of 16S rRNA and gacA *gene fragments*

The Dcode System apparatus (Bio-Rad, Hercules, CA, USA) was used to perform DGGE runs. For both 16S rRNA and

gacA fingerprinting of TC rhizosphere and bulk soil DNA, a double gradient consisting of 26–58% denaturants (100% denaturants defined as 7 M urea and 40% formamide) and 6–9% acrylamide was prepared. Electrophoresis was carried out as performed elsewhere (Costa *et al*., 2006c). Gels were loaded with approximately 3 μl of PCR products. Both DGGE systems were also used to characterize the electrophoretic mobilities of 16S rRNA and *gacA* gene fragments of *Pseudomonas* isolates antagonistic towards *V. dahliae*. Gels were silver-stained according to Heuer and colleagues (2001) and air-dried. DGGE image conversion and statistical analyses were done as described by Costa and colleagues (2006c).

Partial sequencing of 16S rRNA and gacA *genes from antagonistic* Pseudomonas *spp.*

Representative antagonists ($n = 26$) – corresponding to different genotypes as revealed by BOX-PCR, 16S rRNA electrophoretic mobilities on DGGE and profiles of antagonistic attributes – were subjected to partial sequencing of the 16S rRNA gene. In addition, partial *gacA* gene sequences were obtained for antagonists representative of different *gacA* types as determined by DGGE.

DGGE-assisted gacA *clone library of the strawberry rhizosphere*

A *Pseudomonas gacA* clone library of the rhizosphere of strawberries grown at the sampling site Braunschweig was constructed. After mixing TC *gacA* PCR products (gacA-1F/gacA2 PCR) of four strawberry rhizosphere replicates, the resulting PCR mixture was purified using the GENECLEAN Spin kit (Q Biogene, Carlsbad, CA, USA) and cloned into pGEM-T vectors as recommended by the manufacturer (Promega, Madison, USA). The resulting *gacA* clones ($n = 40$) were PCR-amplified (gacA-1FGC/gacA-2R) as described above and loaded on DG-gels. Their melting behaviours were compared with those of *gacA* bands present in rhizosphere *gacA* DGGE fingerprints. Clones representing different electrophoretic mobilities on DG-gels were selected for sequencing. In addition, a dominant *gacA* DGGE band, observed in the strawberry rhizosphere profiles of all sampling sites studied and found to correspond to the *gacA* type 'G3' (Fig. 2B) of *phlD*$^+$ antagonists, was targeted for an extraction, cloning and sequencing procedure as explained by Costa and colleagues (2006a). Sequences retrieved from this band (DGGE bands G3-BE, G3-BS and G3-RO; Figs 3 and 4) were used in *gacA* phylogenetic analysis as explained below.

Sequence analysis

The nucleotide–nucleotide BLAST search tool (BLASTN) of the National Center for Biotechnology Information (NCBI, USA) was applied to all sequences obtained in this study. Sequences selected for phylogenetic analysis were aligned using the CLUSTALW algorithm. The multiple alignments were used for the construction of bootstrapped (500 repetitions) Neighbor-Joining and Maximum Parsimony trees applied to *p*-distances calculated between the sequences using the software MEGA version 3.0 (Kumar *et al*., 2004). All sequences presented in this report were deposited in the GenBank database under the accession numbers DQ464378–DQ464401 and EF417461–417476.

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Supplementary material

The following supplementary material is available for this article online:

Table S1. *In vitro* tests of specificity of the primer pairs used for *Pseudomonas gacA* gene fingerprinting.

Table S2. Closest relatives to non-redundant *gacA* gene clones sequences retrieved from the strawberry rhizosphere.

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