ELSEVIER

Soil Biology & Biochemistry 38 (2006) 2434–2447

Soil Biology & Biochemistry

www.elsevier.com/locate/soilbio

Diversity and antagonistic potential of *Pseudomonas* spp. associated to the rhizosphere of maize grown in a subtropical organic farm

Rodrigo Costa[a], Newton C.M. Gomes[a], Raquel S. Peixoto[b], Norma Rumjanek[c], Gabriele Berg[d], Leda C.S. Mendonça-Hagler[b], Kornelia Smalla[a,*]

[a]*Federal Biological Research Centre for Agriculture and Forestry (BBA), Messeweg 11/12, D-38104 Braunschweig, Germany*
[b]*Instituto de Microbiologia Prof. Paulo de Góes, Universidade Federal do Rio de Janeiro, CCS, Bloco I, Ilha do Fundão, CEP 21941-590, Rio de Janeiro, RJ, Brazil*
[c]*Institute of Environmental Biotechnology, Graz University of Technology, Petersgasse 12, A-8010 Graz, Austria*
[d]*Empresa Brasileira de Pesquisa Agropecuária (Embrapa)-Agrobiologia, Seropédica, RJ, Brazil*

Received 26 January 2006; received in revised form 28 February 2006; accepted 3 March 2006
Available online 18 April 2006

Abstract

Pseudomonas spp. are one of the most important bacteria inhabiting the rhizosphere of diverse crop plants and have been frequently reported as biological control agents (BCAs). In this work, the diversity and antagonistic potential of *Pseudomonas* spp. in the rhizosphere of maize cultivars Nitroflint and Nitrodent grown at an organic farm in Brazil was studied by means of culture-dependent and -independent methods, respectively. Sampling of rhizosphere soil took place at three different stages of plant development: 20, 40 and 106 days after sowing. A PCR-DGGE strategy was used to generate specific *Pseudomonas* spp. fingerprints of 16S rRNA genes amplified from total community rhizosphere DNA. Shifts in the relative abundance of dominant populations (i.e. PCR-DGGE ribotypes) along plant development were detected. A few PCR-DGGE ribotypes were shown to display cultivar-dependent relative abundance. No significant differences in diversity measures of DGGE fingerprints were observed for different maize cultivars and sampling times. The characterisation and assessment of the antagonistic potential of a group of 142 fluorescent *Pseudomonas* isolated from the rhizosphere of both maize cultivars were carried out. Isolates were phenotypically and genotypically characterised and screened for in vitro antagonism towards three phytopathogenic fungi and the phytopathogenic bacterium *Ralstonia solanacearum*. Anti-fungal activity was displayed by 13 fluorescent isolates while 40 isolates were antagonistic towards *R. solanacearum*. High genotypic and phenotypic diversity was estimated for antagonistic fluorescent *Pseudomonas* spp. PCR-DGGE ribotypes displayed by antagonists matched dominant ribotypes of *Pseudomonas* DGGE fingerprints, suggesting that antagonists may belong to major *Pseudomonas* populations in the maize rhizosphere. Antagonists differing in their genotypic and phenotypic characteristics shared the same DGGE electrophoretic mobility, indicating that an enormous genotypic and functional diversity might be hidden behind one single DGGE band. Cloning and sequencing was performed for a DGGE double-band which had no corresponding PCR-DGGE ribotypes among the antagonists. Sequences derived from this band were affiliated to *Pseudomonas* stutzeri and *P. alcaligenes* 16S rRNA gene sequences. As used in this study, the combination of culture-dependent and -independent methods has proven to be a powerful tool to relate functional and structural diversity of *Pseudomonas* spp. in the rhizosphere.

Keywords: *Pseudomonas*; Maize; Rhizosphere; Biocontrol; DGGE

1. Introduction

Soil-borne bacteria antagonistic to plant pathogens might contribute substantially to prevent plant diseases and represent an alternative to the use of chemical pesticides in agriculture (Walsh et al., 2001). Due to its crucial role to plant health and soil fertility, the rhizosphere has been frequently used as a model environment to screen for putative biological control agents (BCAs) of soil-borne plant pathogens (Weller, 1988; Raaijmakers et al., 1997; Picard et al., 2000; Bergsma-Vlami et al., 2005). *Pseudo-*

*Corresponding author. Tel.: +49 531 2993814; fax: +49 531 2993013.
E-mail address: k.smalla@bba.de (K. Smalla).

0038-0717/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.soilbio.2006.03.003

monas spp. are excellent root colonisers (Lugtenberg et al., 2001, Raaijmakers & Weller, 2001), have been frequently reported as successful BCAs (Keel et al., 1992; Leeman et al., 1995) and might contribute to soil suppressiveness (Raaijmakers et al., 1997, 1999; Haas and Keel, 2003). *Pseudomonas* spp. and, more specifically, fluorescent *Pseudomonas* strains, can suppress soil-borne plant pathogens by a variety of mechanisms, such as competition for resources (e.g. iron, nutrients provided by the root), synthesis of antibiotics (e.g. 2,4- diacetylphloroglucinol, -DAPG-, phenazine-1-carboxilic acid -PCA-, pyrrolnitrin) and biosurfactants, production of cell wall-degrading enzymes (e.g. cellulases, proteases, glucanases, chitinases) and elicitation of induced systemic resistance (ISR) in the host plant against the pathogen (Walsh et al., 2001; Winding et al., 2004). Among others, species such as *Pseudomonas aeruginosa*, the type species of the genus, *P. chlororaphis*, *P. fluorescens*, *P. putida* and the phytopathogenic species *P. syringae* are capable of producing fluorescent pigments (Dwivedi and Johri, 2003). Fluorescent *Pseudomonas* BCAs have received particular attention also because they are easy to grow in vitro and readily amenable to genetic manipulation (Haas and Keel, 2003).

Different studies have evaluated the influence of plant species (Bergsma-Vlami et al., 2005), natural or transgenic cultivars (Fromin et al., 2001), stages of plant development (Picard et al., 2000) and geographic origin (Keel et al., 1996; Wang et al., 2001) on the structural and functional diversity of *Pseudomonas* spp. in bulk or rhizosphere soils. Such studies relied almost exclusively on culture-dependent investigations and have improved substantially our understanding of the distribution and abundance of functional genes in the environment. Recently, studies on the diversity of non-cultured *Pseudomonas* spp., based on specific PCR-amplification of *Pseudomonas* 16S rRNA gene fragments from total community soil DNA, have been reported (Gyamfi et al., 2002; Milling et al., 2004; Garbeva et al., 2004; Pesaro and Widmer, 2006). The use of such techniques is fundamental to better comprehend soil microbial community structure (Heuer and Smalla, 1997) and, to our knowledge, no culture-independent studies specifically targeting *Pseudomonas* spp. in the rhizosphere of crop plants grown in subtropical regions have been conducted so far. In this work we used a suite of techniques to assess the structural and functional diversity of *Pseudomonas* spp. in the rhizosphere of maize grown in an organic farm in Southeast Brazil. Culture-independent analysis comprised DNA extraction of rhizosphere samples followed by a PCR-DGGE strategy that allows fingerprinting of *Pseudomonas* 16S rRNA gene fragments amplified from total community rhizosphere DNA. Our objective was to determine whether *Pseudomonas* spp. structural diversity shifts along plant development and whether populations specific to a given maize cultivar could be detected by DGGE. To assess the putative functional and whole-genome diversity behind DGGE bands, a culture-dependent approach was used in combination with DGGE profiles. Antagonistic activity of fluorescent *Pseudomonas* spp. isolates towards soil-borne plant pathogens with broad host-ranges was surveyed. Antagonists were characterised genotypically by BOX-PCR and phenotypically according to their antagonistic properties. Both approaches were linked by determining whether culturable, antagonistic fluorescent *Pseudomonas* spp. corresponded to dominant *Pseudomonas* spp. populations in the rhizosphere of maize using culture-independent analysis.

2. Materials and methods

2.1. Field design and sampling

Maize seeds were cultivated at the "Integrated System for Agroecological Production" (SIPA) coordinated by Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA), located in the city of Seropédica (22°44′38″S/43°42′27″ W), Rio de Janeiro State, Brazil. The climate of the region is characterised by wet summers and dry winters, with annual average temperature and rainfall of 24.6°C and 1500 mm, respectively. The soil is classified as Planosol with low fertility, characterised by a sandy superficial layer sustained by a compact subsurface layer of clay. Soils contained 0.88% of C and 1.55% of organic matter, with pH ranging from 4.7 to 5.1. A completely organic management of the soil at SIPA is in progress since 1993, with emphasis on the use of local resources, maximisation of nutrient cycling and nitrogen self-sustainability through intense crop rotation and diversification. More information about the SIPA can be obtained at the web site http://www.cnpab.embrapa.br/.

Sampling procedures were performed as described by Gomes et al. (2001). Briefly, six composite rhizosphere samples of each cultivar (Nitroflint and Nitrodent) were taken at 20, 40 and 106 days after sowing (DAS) (late autumn, 1999). One composite rhizosphere sample comprised total roots from five maize plants. Rhizosphere samples were shaken vigorously to eliminate soil not adherent to the roots, transported to the laboratory in sterile plastic bags and processed for the isolation of fluorescent *Pseudomonas* spp. and DNA extraction.

2.2. Isolation and phenotypic identification of fluorescent *Pseudomonas spp.*

Fresh rhizosphere samples were processed according to Gomes et al. (2001). Serial dilutions were plated onto King's B agar (KB) and incubated at 29 °C for 48h for the isolation of fluorescent *Pseudomonas* spp. Fluorescent colonies were picked randomly under UV light. Isolates were purified and kept frozen at −80 °C in KB broth with 40% glycerol. Isolates were identified by fatty acid methyl esther analysis (FAME) of total cellular fatty acids by gas chromatography using the MIDI (Newark, Del, USA) microbial identification system (MIS). The FAME Similarity Index expresses how closely the fatty acid composi-

tion of an unknown strain compares with the mean fatty acid composition of its most similar match in the MIS databank. In addition, the API 20 NE System (API) (BioMérieux, Marcy-L'Etoile, France), consisting of nine enzymatic and twelve carbohydrate assimilation tests, was used for a rapid identification of the isolates. The numerical profiles obtained were compared to the profiles stored in the Analytical Profile Index software database (BioMérieux).

2.3. DNA extraction from isolates

Pure cultures were grown in King's B broth at 28 °C for 48 h and subsequently centrifuged at 3000 g for 20 min. The pellets were re-suspended in 1ml saline, centrifuged at 14000 g for 15 min and washed with 750 µl $MgSO_4$ (10 mM). The suspensions were centrifuged at 14000 g for 10 min. To obtain crude cell lysates, the Qiagen genomic DNA extraction kit (Qiagen, Hilden, Germany) was used. DNA extraction was performed using the Ultra Clean TM15 DNA Purification Kit (MoBio Laboratories, Carlsbad, CA, USA). DNA yields were checked on UV light (254 nm) after agarose gel electrophoresis and ethidium bromide staining.

2.4. Genotypic fingerprinting of fluorescent Pseudomonas spp.

Whole-genome BOX-PCR fingerprints were generated for all isolates. A 50 µl Master Mix was prepared containing 2 µl template DNA (ca. 10 ng), 1 × Stoffel Buffer (Applied Biosystems, Foster, USA), 3.75 mM $MgCl_2$, 0.2 mM each dNTP, 5% DMSO, 0.2 µM primer BOXA1R (5′-CTA CGG CAA GGC GAC GCT GAC TGA CG-3′) (MWG Biotech, Ebersberg, Germany) and 5 units (0.1 U/µl) Taq Polymerase (Stoffel fragment, Applied Biosystems). After 7 min of denaturation at 94 °C, 35 cycles of 1 min at 94 °C, 1 min at 53 °C and 8 min at 65 °C were carried out. A final extension step of 16 min at 65 °C was used to finish the reaction. Products (10 µl) were loaded on 1.2% SEAKEM (BMA, Rockland, ME, USA) agarose gels, electrophoresed at 100 V for 4 h and checked under UV transillumination (254 nm). Cluster analysis of BOX-PCR profiles (GelCompar version 4.1. Applied Maths, Kortrijk, Belgium) was performed using Pearson correlation indices of similarity with the unweighted pair-group method using arithmetic averages (UPGMA).

2.5. In vitro screening for antagonistic activity

Isolates were tested for in vitro antifungal activity in dual culture assays on Waksman Agar with the plant pathogens *Verticillium dahliae* Kleb., *Rhizoctonia solani* Kühn. and *Sclerotinia sclerotiorum* Lib. as described by Berg et al. (2000). All isolates were surveyed in three independent replicates. Zones of inhibition were measured after 5 days of incubation at 20 °C as described by Berg et al. (2000). *Ralstonia solanacearum* strains B3B and 1609 (Race 3, Biovar 2) were grown in 1:10 Trypticase Soy Broth (TSB) (Becton, Dickinson and Co, Sparks, MD, USA) at 28 °C for 72 h. *Ralstonia solanacearum* suspensions (1.5 ml) were added to 1L sterile TSB containing 4% glucose and 1.5% agar prior to pouring the medium onto Petri dishes. Freshly grown fluorescent *Pseudomonas* isolates were then streaked on the medium and haloes indicating inhibition of *Ralstonia* growth were measured after incubation at 28 °C for 72 h.

Strains *V. dahliae* V25 and *S. sclerotiorum* were obtained from the strain collection of the University of Rostock, Department of Microbiology. *Ralstonia solanacearum* 1609 and *Rhizoctonia solani* (AG3) were obtained from Plant Research International, Wageningen, The Netherlands. *Ralstonia solanacearum* B3B was obtained from the Federal Biological Research Centre for Agriculture and Forestry (BBA), Kleinmachnow, Germany. Isolates that showed antagonistic activity towards phytopathogenic fungi and / or *Ralstonia solanacearum* strains were selected for further assays.

2.6. Production of extra-cellular metabolites with antagonistic properties

Antagonists were tested for the production of extracellular secondary metabolites. Growth inhibition of *Rhizoctonia solani* Kühn was determined in sterile filtrate assays as described in detail by Berg et al. (2000). The tests were carried out in duplicates for each isolate. Siderophore production was determined on agar plates as described by Schwyn and Neilands (1987).

2.7. Production of cell-wall degrading enzymes

Clear zones in skimmed milk agar were measured and used as indicators of protease activity/casein degradation (Nielsen and Sørensen, 1997) displayed by antagonists. Chitinolytic activity was tested by streaking antagonistic isolates on chitin-agar (CA) and measuring clearance halos after 5 days of incubation at 30 °C (Berg et al., 2000). β-1,3-glucanase activity was determined by measuring the production of reducing sugars from laminarin as described by Berg et al. (2000).

2.8. PCR detection of antibiotic-encoding genes

Antagonists displaying anti-fungal activity were submitted to PCR detection of the antibiotic-encoding loci *phlD* (2,4-diacetylphloroglucinol — DAPG) and *phz* (phenazin-1-carboxylic acid — PCA) using the primer pairs and protocols described by Raaijmakers et al. (1997). PCR detection of the pyrrolnitrin biosynthetic locus (*prnD*) was carried out using the specific primers PRND1 and PRND2 as described by De Souza and Raaijmakers (2003). To confirm specificity of amplicons, Southern-blot hybridisation using digoxigenin-labelled probes with high

stringency conditions were carried out as described by Top et al. (1995).

2.9. Whole-community DNA extraction from the rhizosphere and PCR amplification of Pseudomonas 16S rRNA gene fragments

Total community DNA extraction from 1 g rhizosphere (maize roots with tightly adhering soil particles) was performed as described by Gomes et al. (2001). Crude DNA samples were purified using the Geneclean spin Kit (Q Biogene, Carlsbad, CA, USA). To amplify *Pseudomonas* spp. 16S rRNA gene fragments from total community rhizosphere DNA, a nested-PCR approach was used. A first, taxon-specific, PCR amplification was carried out with the primer pair F311 Ps / R1459 Ps as described by Milling et al. (2004) using 1 μl rhizosphere DNA template (c. 5 ng) and 25 thermal cycles. One to 20 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 and Smalla, 1997) as performed by Costa et al. (2006) prior to DGGE analysis. PCR products were checked after electrophoresis on agarose gels under UV transillumination (254 nm).

2.10. Denaturing gradient gel electrophoresis of 16S rRNA gene fragments

DGGE analysis was performed with the Dcode System apparatus (Bio-Rad Inc., Hercules, CA, USA). Gel casting was done as recommended by Heuer et al. (2001). A double gradient consisting of 26–58% denaturants (100% denaturants defined as 7 M urea and 40% formamide) and 6–9% acrylamide was prepared (Costa et al., 2004). Aliquots containing equal amounts of PCR products (c. 3 μl) were loaded on the gel and electrophoresis was carried out with 1 × Tris-acetate-EDTA buffer at 58 °C and constant voltage of 220 V for 6 h. Gels were silver-stained according to Heuer et al. (2001) and air-dried. Computer-assisted analysis of DGGE fingerprints was done using GelCompar version 4.1. The DGGE mobility of all fluorescent *Pseudomonas* spp. isolates with antagonistic activity was checked. A marker based on each PCR-DGGE ribotype observed was loaded on DGGE to detect whether these ribotypes match dominant bands of the *Pseudomonas* DGGE profiles obtained from PCR-amplified rhizosphere DNA.

2.11. Cloning and sequencing of DGGE ribotypes

Band 1 (Fig. 3), present in the rhizosphere of both cultivars at all sampling times and represented by at least two slightly different PCR-DGGE ribotypes, was extracted from DGGE lanes representing each sampling time (20, 40 and 106 DAS), re-amplified and cloned as described by Costa et al. (2006). Clones containing inserts which shared the electrophoretic mobility of the original DGGE ribotypes were selected and evaluated by Amplified Ribosomal DNA Restriction Analysis (ARDRA) with the enzymes Alu I and Msp I (Costa et al., 2006). Clones representing different ARDRA profiles were submitted to sequencing analysis.

2.12. Measurements of diversity

The Shannon measure of diversity (H'), determined as $H' = -\sum pi \log pi$, where pi represents the relative abundance of the ith category within the sample, was applied to estimate genotypic and phenotypic diversity within fluorescent *Pseudomonas* isolates. Indices were used to estimate how diverse are the groups of (1) isolates representing dominant populations in the rhizosphere of maize and of (2) isolates which showed either antifungal or antibacterial properties. Physiological profiles obtained with the characterisation of the isolates by API were employed as the categories to assess phenotypic diversity. Genotypic diversity was determined at two different levels of resolution, i.e., by means of (1) whole-genome BOX-PCR fingerprinting and (2) PCR-DGGE of 16S rRNA gene fragments. Evenness ($J' = H'/H'\max$) and dominance ($1–J'$) were calculated based on the diversity indices obtained (Zar, 1996). Diversity indices were compared by performing a t test adapted for the Shannon's measure of diversity as described by Brower et al. (1997) and applied by Salles et al. (2006). Diversity indices of *Pseudomonas* spp. DGGE fingerprints were also calculated. Band positions and intensities were used as parameters to indicate categories (PCR-DGGE ribotypes) and relative abundance (peak area) of each category within the sample, respectively. The relative abundance of a given band was obtained by dividing the band's peak surface by the sum of the surfaces of all bands within the same DGGE lane, thus eliminating variation in band intensity caused by possible differences in the amount of PCR products loaded on the gels. Average diversity measures were calculated for each microenvironment and tested for significant differences by applying the two-sample t test of Student.

3. Results

3.1. Phenotypic identification of fluorescent Pseudomonas spp.

A total of 142 fluorescent *Pseudomonas* spp. isolated from the rhizosphere of maize grown in Brazil was identified by FAME and API. Fifty-two, 42 and 48 fluorescent strains were isolated from the first (20 DAS), second (40 DAS) and third (106 DAS) sampling times, respectively. By using these methods of identification a rather narrow species richness within the isolates was observed, with identities being almost exclusively restricted to the species *P. putida*, *P. fluorescens* and *P. chlororaphis*. Among all strains characterised, 84 were identified as *P. putida* by both methods: 38 at 20 DAS ($n = 52$), 30 at 40

DAS ($n = 42$) and 16 at 106 DAS ($n = 48$). Higher frequencies of *P. fluorescens*/*P. chlororaphis* isolates were observed at 106 DAS. Here, isolates identified as *P. fluorescens* by API were frequently assigned to *P. chlororaphis* according to FAME. *P. fluorescens*/*P. chlororaphis* phenotypes were rarely observed at 20 and 40 DAS. Although species richness within the isolates was limited as indicated by both identification procedures, API physiological profiling revealed 8 different phenotypes among *P. putida* isolates ($n = 94$) and, as well, eight different *P. fluorescens* ($n = 29$) physiological profiles, suggesting a certain degree of intra-specific diversity within the isolates.

3.2. Antagonistic activity and phenotypic characterisation of fluorescent Pseudomonas spp.

Tables 1 and 2 show phenotypic characterisation of isolates with antagonistic activity towards phytopathogenic fungi and *Ralstonia solanacearum*, respectively. Thirteen isolates displayed antagonistic activity at least to one of the phytopathogenic fungi used in the bioassays (Table 1). A much higher proportion of antagonists with antibacterial properties was revealed: 40 of 142 isolates tested exhibited antagonistic activity towards *Ralstonia solanacearum* strain B3B, while 26 were active against *R. solanacearum* strain 1609 (Table 2). Only three isolates (PRD 16, PRD 18 and PRD 38) have shown both antifungal and antibacterial activity and isolate PFC 14 was the only one active against more than one fungal plant pathogen. Nearly all antagonists active against fungal phytopathogens were isolated from the first sampling time (Table 1). The majority of fungal antagonists (9 of 13 — Table 1) were isolated from the maize cultivar Nitrodent (strain codes PRD, PDC and PDP), while 28 out of 40 strains active against *Ralstonia solanacearum* were retrieved from the rhizosphere of the cultivar Nitroflint (strain codes PRF, PFC and PFP — Table 2). The majority of the antagonists were able to produce siderophores, while protease activity was found to be a common feature among fungal antagonists (Table 1). PCR-detection of antibiotic-encoding genes resulted in only three positive signals (2 *phlD*+ and 1 *prnD*+) obtained among antagonists showing anti-fungal activity (Table 1).

3.3. Assessment of diversity among fluorescent Pseudomonas spp.

It is important to emphasise that, concerning the characterisation of isolates, diversity measures were exclusively used as criteria to determine which of the methods employed here was better suited to differentiate strains belonging to the same sample unit, since our main goal was to assess the degree of genotypic or phenotypic variability that can be potentially hidden behind one DGGE band. As presented here, diversity indices cannot be considered as an actual, representative measure of diversity of culturable fluorescent *Pseudomonas* in the maize rhizosphere.

3.3.1. Phenotypic and genotypic diversity of Pseudomonas putida populations

As the vast majority of the isolates was identified as *P. putida* by both API and FAME ($n = 84$), we assessed the diversity within this group of isolates and referred to it as to "intra-specific diversity of *P. putida* populations". Table 3 shows measures of richness, diversity, evenness and dominance among *P. putida* strains isolated at 20, 40 and 106 DAS as assessed by API physiological profiling, PCR-DGGE of 16S rRNA gene fragments and whole-genome BOX-PCR. A cut-off level of 83% similarity was used to determine whether isolates shared the same BOX-PCR profile. This value was calculated after comparing the profiles of the same strain (PRF 01) generated by independent BOX-PCR amplifications loaded on the agarose gels needed for the analysis of BOX profiles with GelCompar. Overall, 53 genotypes within 84 *P. putida* strains were detected by BOX-PCR fingerprinting. Four of these genotypes were found to be represented by 5 or more (up to 8) isolates and 3 of these small clusters contained exclusively isolates of the cultivar Nitroflint. No genotype was found to be represented in all stages of plant development, but the analysis of the BOX-PCR dendrogram at lesser levels of similarity revealed that groups of similar, although not equal, genotypes could be retrieved at all sampling times and also from the rhizosphere of both cultivars (data not shown). Such an impressive genotypic richness exceeded by far the numbers of different API physiological profiles (8) and of 16S rRNA DGGE types (6) observed among these strains. Whole-genome BOX-PCR diversity indices were significantly higher than the values obtained for the other two parameters used to assess diversity of *P. putida* populations at all sampling times (Table 3). We found no significant differences between diversity indices (when estimated using the same method of characterisation) calculated for *P. putida* strains isolated at different stages of plant development.

3.3.2. Phenotypic and genotypic diversity of antagonistic Pseudomonas spp.

To assess phenotypic diversity among antagonists, we extended the number of physiological attributes used to characterise the isolates by adding the results of in vitro antagonistic activity tests (towards *V. dahliae*, *Rhizoctonia solani*, *S. sclerotiorum* and *Ralstonia solanacearum*), sterile filtrate assays (*Rhizoctonia solani*), production of lytic enzymes (chitinase, protease and β-1,3 glucanase) and synthesis of siderophores to the physiological profiles generated by API. This strategy resulted in a diverse number of different phenotypic profiles: out of 50 isolates with antagonistic activity, 28 different phenotypes were recognised in comparison to 25 BOX-PCR profiles and the 6 PCR-DGGE ribotypes shown in Fig. 1. Phenotypic diversity ($H' = 1{,}362$) and whole-genome BOX-PCR diversity ($H' = 1{,}270$) did not differ significantly and, as performed here, phenotypic characterisation was also a sensitive parameter to typing the group of antagonistic

Table 1
Characterisation of fluorescent *Pseudomonas* spp isolated from the rhizosphere of maize with antagonistic activity towards fungal plant pathogens

Strain	DGGE[a]	DAS[b]	Antagonistic activity[c]			Filtrate[d]	Lytic enzyme production[e]			Sider.[f]	PCR[g]		Tentative phenotypic identification			
			V. d.	*R. s.*	*S. s.*	*R. s*	Chit	Prot	Gluc		*prnD*	*PhlD*	API	SI[h]	FAME	SI
PRF 01	A6	20	–	+	–	++	–	+	–	+	–	+	*Pseudomonas putida*	98.4	*Pseudomonas putida*	91.8
PRF 19	A4		–	–	++	++	–	+	–	+	–	+	*P. fluorescens*	62.4	*P. putida*	19.7
PRD 01	A6		++	–	–-	++	–	+	–	+	–	–	*P. putida*	99.8	*P. putida*	70.1
PRD 02	A6		+	–	–	++	–	+	–	+	–	–	*P. putida*	98.4	*P. putida*	65.6
PRD 14	A5		–	–	+	++	–	+	–	+	–	–	*P. putida*	98.4	*P. chlororaphis*	71.1
PRD 16	A6		+	–	–	++	–	+	–	+	–	–	*P. putida*	99.7	*P. putida*	58.0
PRD 18	A5		–	+	-	++	–	-	–	-	+	–	*P. fluorescens*	84.0	*P. putida*	65.2
PRD 21	A2		–	–	++	++	–	+	–	+	–	–	*P. putida*	98.4	*P. putida*	24.0
PRD 23	A2		–	–	+	+	–	+	–	+	–	–	*P. putida*	98.4	*P. putida*	81.5
PRD 37	A3	40	–	–	+	++	–	+	–	+	–	–	*P. putida*	99.7	*P. putida*	16.9
PRD 38	A5		+	–	–	++	–	+	–	++	–	–	*P. putida*	98.4	*P. putida*	87.3
PDC 23	A3	106	+	–	–	+	–	+	–	–	–	–	*P. fluorescens*	96.0	*P. chlororaphis*	89.0
PFC 14	A4		–	+	+	++	–	+	–	–	–	–	*P. fluorescens*	79.3	*P. chlororaphis*	74.4

[a]PCR-DGGE ribotype of the respective strain according to the electrophoretic mobilities shown in Fig. 1.

[b]Sampling time (days after sowing).

[c]Antagonism towards *Verticillium dahliae (V.d.), Rhizoctonia solani AG3* (*R.s.*) and *Sclerotinia sclerotiorum* (*S.s.*). - indicates no zone of inhibition, + represents until 5mm wide zone of inhibition, ++ represents 5-10mm wide zone of inhibition and +++ represents > 10mm wide zone of inhibition.

[d]Antibiosis assay. The growth of *Rhizoctonia solani* on agar plates was monitored by using a sterile filtrate test. ++ indicates more than 70% inhibition of fungi growth compared to the control, + indicates a range of 35-70% growth inhibition.

[e]Chitinase, protease and β-1,3 glucanase activities were determined by plate assay. (+) hydrolysis; (-) no hydrolysis.

[f]Production of siderophores.

[g]PCR-detection of the *prnD* (pyrrontrin) and phlD (2,4 DAPG) loci.

[h]Similarity Index.

Table 2
Characterisation of fluorescent *Pseudomonas* spp. isolated from the rhizosphere of maize with antagonistic activity towards *Ralstonia solanacearum* biovar 2, race 3

Strain	DGGE[a]	DAS[a]	Antagonism *R. solanacearum*[b]		Filtrate[a]	Lytic enzyme production[a]			Sider.[a]	Tentative phenotypic identification			
			Strain B3B	Strain 1609	*R. solani*	Chit	Prot	Gluc		API	SI	FAME	SI
PRF 02	A5	20	1.5–2 mm	1.5–2 mm	+	–	–	–	+	*Pseudomonas putida*	98.4	*Pseudomonas putida*	72.0
PRF 04	A5		1.5–2 mm	1.5–2 mm	–	–	–	–	+	*P. putida*	99.8	*P. putida*	70.0
PRF 09	A5		1 mm	–	+	–	–	–	+	*P. putida*	99.8	*P. putida*	86.4
PRF 14	A6		4–5 mm	4–5–5 mm	-	–	+	+	+	*P. putida*	99.8	*P. putida*	82.6
PRF 17	A5		1 cm	–	+	–	–	–	+	*P. putida*	99.8	*P. putida*	89.5
PRF 18	A5		4 mm	4 mm	+	–	–	–	+	*P. putida*	96.0	*P. putida*	84.5
PRF 20	A6		3 mm–1cm	1 mm	+	–	+	+ + +	+	*P. putida*	99.2	*P. putida*	89.2
PRF 22	A5		1 mm	–	–	–	–	–	+	*P. putida*	99.8	*P. putida*	78.1
PRF 24	A5		2–3 mm	1 mm	+	–	–	–	+	*P. putida*	98.4	*P. putida*	38.7
PRF 25	A5		1 mm	–	+	–	–	–	+ + +	*P. putida*	99.8	*P. putida*	70.4
PRD 03	A5		1.5–2 mm	–	+	–	–	–	+	*P. fluorescens*	79.3	*P. fluorescens*	38.7
PRD 13	A6		1 mm	1–2 mm	+	–	–	–	+	*P. putida*	98.4	*P. putida*	79.4
PRD 16	A6		6–7 mm	1 cm	+	–	+ +	–	+	*P. putida*	99.7	*P. putida*	58.0
PRD 18	A5		4 mm	6 mm	+	–	–	–	–	*P. fluorescens*	84.0	*P. putida*	65.2
PRD 26	A5		3–4 mm	5 mm	+	–	–	–	+	*P. putida*	98.4	*P. chlororaphis*	66.2
PRD 27	A5		3–4 mm	5–6 mm	+	–	+	–	–	*P. putida*	99.8	*P. putida*	70.7
PRD 30	A6		5 mm	5 mm	+	+	+	–	+	*P. putida*	99.7	*P. putida*	57.6
PRF 50	A4	40	1 mm	1 mm	-	–	–	–	–	P. putida	98.4	*P. putida*	35.5
PRF 56	A4		1 mm	1 mm	+	–	–	–	+	*P. putida*	98.4	*P. putida*	84.7
PRF 59	A4		1 mm	1 mm	+	–	–	–	+	NI		*P. putida*	82.2
PRF 60	A4		1 mm	–	+	–	–	–	+	NI		*P. putida*	67.3
PRF 65	A6		1 mm	–	–	–	–	–	+	*P. putida*	98.4	*P. putida*	86.2
PRF 68	A5		1.5–2mm	2–3 mm	+	–	–	–	+	NI		*P. putida*	81.7
PRF 81	A5		1.5 mm	1.5 mm	+	–	–	–	+	*P. putida*	99.2	*P. putida*	62.0
PRF 94	A5		3–4 mm	4–5 mm	+	–	–	–	+	*P. putida*	98.4	*P. putida*	78.9
PRD 36	A3		1.5 mm	2 mm	–	–	–	–	-	*P. putida*	99.8	*P. chlororaphis*	84.1
PRD 38	A5		1 cm	4–6 mm	–	–	+ +	–	+	*P. putida*	98.4	*P. putida*	87.3
PRF 120	A1	106	1 mm	1 mm	+	–	+	–	+	*P. fluorescens*	96.4	*P. fluorescens*	70.1
PRF 121	A3		1 mm	–	+	–	+	–	+	*P. fluorescens*	79.3	*P. fluorescens*	95.6
PFC 12	A1		1 mm	–	+	–	+ +	–	+	*P. fluorescens*	79.3	*P. chlororaphis*	89.0
PFC 15	A5		4 mm	–	+	–	+	–	+	*P. fluorescens*	96.9	*P. chlororaphis*	97.0
PFC 16	A1		1.5 mm	–	+	–	–	–	+	*P. fluorescens*	79.3	*P. chlororaphis*	91.0
PFP 02	A5		3 mm–1 cm	–	+	–	–	–	+	*P. putida*	99.2	*P. putida*	91.2
PFP 06	A5		1.5 mm	–	–	–	–	–	+ + +	*P. putida*	98.4	*P. putida*	89.4
PFP 13	A6		5 mm–1 cm	1 mm	–		-	–	+	*P. putida*	91.4	*P. putida*	88.6
PFP 21	A3		1 mm	1 mm	+	–	+	–	+	*P. fluorescens*	71.3	*P. chlororaphis*	87.8
PFP 25	A4		1 mm	1 mm	+	–	-	–	+ + +	*P. fluorescens*	79.3	NI	
PDC 12	A1		5 mm	1 mm	+	–	-	–	+	*P. fluorescens*	97.3	*P. chlororaphis*	85.8
PDP 15	A1		1.5 mm	1.5 mm	+	–	-	–	–	P. fluorescens	79.3	*P. fluorescens*	88.8
PDP 17	A1		1 mm	1 mm	–	–	+ +	–	+ + +	*P. fluorescens*	96.4	*P. chlororaphis*	90.0

[a]See legend provided in Table 1.
[b]In vitro assays for antagonistic activity towards *Ralstonia solanacearum*. Values are measures of clearance haloes indicating inhibition of *R. solanacearum* growth.

fluorescent *Pseudomonas* analysed in this study. Fig. 2 shows BOX-PCR profiles of 9 fungal antagonists in comparison to a BOX-PCR profile from a non-antagonistic *P. putida* strain, exemplifying the high genotypic diversity observed among the fluorescent *Pseudomonas* spp. cultured onto King's B agar obtained in this study.

3.4. Cultivation-independent diversity of Pseudomonas spp. in the maize rhizosphere

Rhizosphere PCR-DGGE fingerprints of *Pseudomonas* 16S rRNA gene fragments revealed a rather high level of variability among replicates of the same treatment (sampling time or maize cultivar). This impaired a clear differentiation between treatments based on DGGE profiles by cluster analysis (data not shown). Figs. 3 and 4 show fingerprints obtained for both cultivars at different sampling times. Despite the variability among replicates, cultivar-dependent shifts in the relative abundance of specific PCR-DGGE ribotypes could be detected at all sampling times, as shown by arrows in Figs. 3 and 4 (ribotype 3 at 20 DAS, ribotypes 1 and 2 at 40 DAS, ribotype 1 at 106 DAS). The relative abundance of dominant PCR-DGGE ribotypes also changed along plant

Table 3
Intra-specific Richness (S), Diversity (H′), Evenness (J′) and Dominance (1 – J′) of *Pseudomonas putida* populations isolated from the maize rhizosphere

	20 DAS (n = 38)[a]		BOX[d]	40 DAS (n = 30)		BOX	106 DAS (n = 16)		BOX	Total (n = 84)		BOX
	Phen[b]	16S rRNA[c]		Phen	16S rRNA		Phen	16S rRNA		Phen	16S rRNA	
S	6	5	30	7	5	18	5	3	12	8	6	53
H'	0,666	0,4893	1,378**	0,684	0,586	1,139**	0,635	0,2615	1,016**	0,727	0,529	1,54**
J'	0,856	0,7	0,933	0,809	0,838	0,91	0,908	0,548	0,942	0,805	0,68	0,893
1 - J'	0,144	0,3	0,067	0,191	0,162	0,09	0,092	0,452	0,058	0,195	0,32	0,107

Tests of significance were conducted only for diversity indices (H'). $P<0.01$**
[a]DAS, days after sowing; n, number of isolates identified as P. putida by FAME and API at the corresponding sampling time.
[b]Phenotypic characterisation.
[c]DGGE mobilities of 16S rRNA gene fragments.
[d]BOX-PCR profiles.

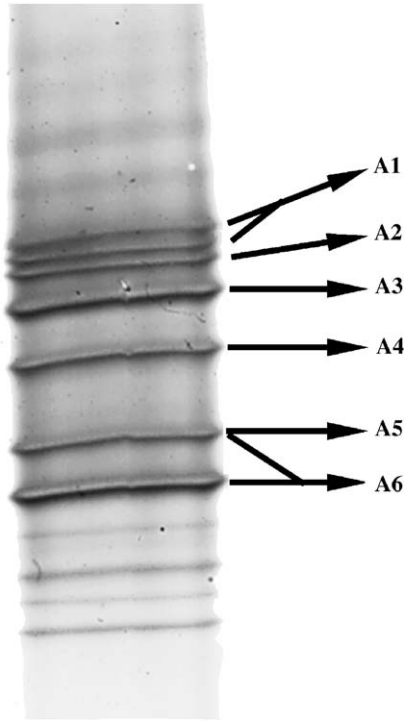

Fig. 1. Types of 16S rRNA gene fragments (ribotypes) amplified from genomic DNA of fluorescent *Pseudomonas* spp. isolated from the maize rhizosphere as revealed by DGGE. Ribotypes A1 and A6 are represented by double bands. The fainter bands below ribotype A6 are 16S rRNA gene copies belonging to isolates running either in the A5 or A6 position.

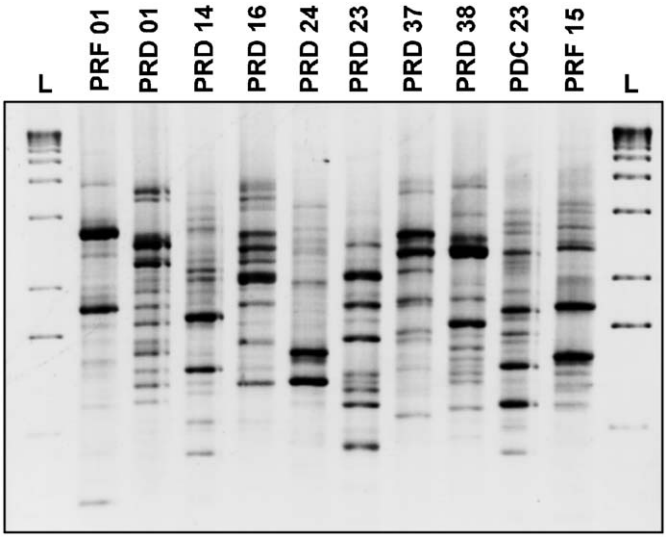

Fig. 2. BOX-PCR fingerprints of fluorescent *Pseudomonas* spp. isolated from the maize rhizosphere. Profiles obtained for nine strains showing antagonistic activity towards fungal pathogens (Table 2) and one strain (PRF 15, *P. putida*) without antagonistic activity *in vitro* are shown.

development as arrows in Fig. 5 indicate. However, no ribotype was found to be exclusively associated to a given maize cultivar or sampling time. Diversity indices were calculated for each track in the gels and are indicated at the bottom of the gel images in Figs. 3 and 4. Significant differences in diversity indices were observed neither for different maize cultivars nor for different sampling times.

3.5. Combining culture-dependent and -independent methods to link functional and structural diversity

At the extremities of the gels in Figs. 3–5, PCR-DGGE ribotypes derived from the antagonists isolated from the maize rhizosphere are shown (ribotypes A1—A6 — Fig. 1). Band 1, shown by arrows in Figs. 3–5, was found to be a dominant double-band (in most cases) amplified from rhizosphere DNA samples of both cultivars at all sampling times and had no corresponding PCR-DGGE ribotypes among the antagonistic isolates. The relative abundance of band 1 shifted along plant development: it decreased substantially at 106 DAS in the Nitroflint rhizosphere (Fig. 5) and was negligible at 40 DAS in three replicates of the Nitrodent rhizosphere (Fig. 4), reappearing at 106 DAS (Fig. 3). The bands 1a, 1b and 1c (Fig. 5) were cut from this gel, re-amplified, cloned and sequenced in order to reveal the identity or identities behind the overlapping PCR-DGGE ribotypes composing this double-band. Clones that displayed the same mobility as the original band on DGGE gels were submitted to sequencing analysis. Sequences affiliated to *P. stutzeri*, a non-fluorescent pseudomonad, were retrieved from all bands 1a, 1b and 1c (20, 40 and 106 DAS, respectively — Table 4), while sequences related to

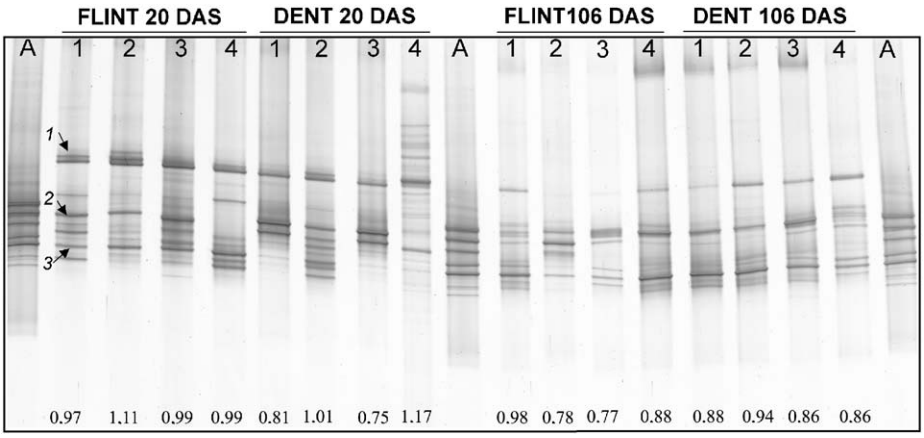

Fig. 3. DGGE-fingerprinting of *Pseudomonas* 16S rRNA gene fragments amplified from community DNA extracted from the rhizosphere of the maize cultivars Nitroflint (FLINT) and Nitrodent (DENT). Profiles of rhizospheres collected 20 and 106 days after sowing (DAS) are shown. Four independent replicates per treatment were applied to the gel. Arrows indicate dominant bands whose abundance differed in respect to at least one of the factors studied (maize cultivar/sampling time). Values at the bottom of the gel indicate diversity indices estimated for each DGGE lane based on band positions and intensities. The "A" lanes show electrophoretic mobilities displayed by fluorescent *Pseudomonas* isolates with antagonistic properties (ribotypes A1–A6 – Fig. 1).

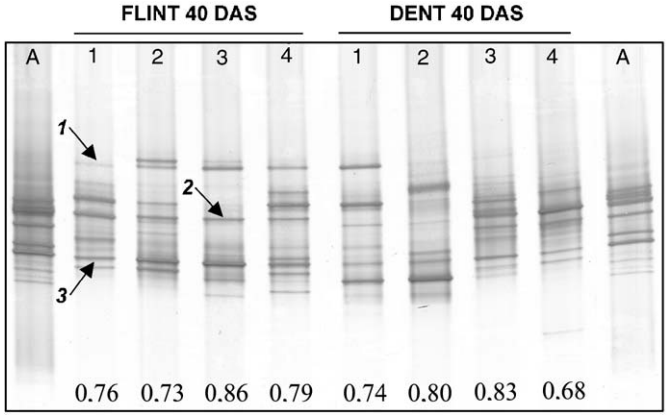

Fig. 4. Comparison of *Pseudomonas* 16Sr RNA gene DGGE fingerprints of the rhizosphere of maize cultivars Nitroflint (FLINT) and Nitrodent (DENT) collected 40 days after sowing (DAS). Four independent replicates per treatment are shown. Arrows indicate dominant bands whose abundance differed in respect to cultivar type. Diversity indices obtained for the DGGE profiles are shown at the bottom of each lane. A, ribotypes A1–A6 (Fig. 1).

P. alcaligenes, also non-fluorescent, were found for bands 1a and 1c (Table 4). With the exception of this double-band, PCR-DGGE ribotypes generated from isolates with antagonistic activity (Fig. 1) matched most dominant bands of the *Pseudomonas* DGGE fingerprints (Figs. 3–5). The number of isolates represented by each PCR-DGGE ribotype, their origin and their main antagonistic features are listed in Table 5. Many strains diverging in their antagonistic attributes and in their genotypic profiles shared the same DGGE electrophoretic mobility. For instance, ribotype 3 from the DGGE profiles (Figs. 3 and 4), found to be very stable at the Nitroflint rhizosphere at all sampling times (Fig. 5), matched ribotype A6 from the isolates, characteristic of *P. putida* strains that displayed many antagonistic properties in a variable manner. Antagonists towards *Ralstonia solanacearum* that produced siderophores and also secondary metabolites that inhibited the growth of *Rhizoctonia solani* in sterile filtrate assays represented the most common type of antagonist: 20 out of 50 antagonists were characterised with these attributes. Almost all of them were identified as *P. putida* and corresponded to PCR-DGGE ribotypes A5 and A6 (Table 2; Fig. 1). While DGGE ribotypes A3 and A4 comprised also a variable group, containing isolates from different sampling times and cultivars, belonging to different species and having divergent antagonistic properties, ribotypes A1 and A2 were represented by isolates with very similar characteristics as summarised in Table 5.

4. Discussion

In this study, we aimed to assess the diversity and structure of *Pseudomonas* communities in the rhizosphere of maize grown in Brazil by means of DGGE and, moreover, evaluate the potential functional and genotypic diversity behind one single DGGE band by combining cultivation-dependent and -independent methods. As applied here, this combination of techniques may allow a better understanding of population dynamics, community structure and antagonistic activity of *Pseudomonas* in the rhizosphere. For instance, BOX-PCR fingerprinting of *P. putida* populations (as identified by the methods employed here) provided the first indication that an enormous genotypic diversity and variability may be hidden behind the electrophoretic mobility of 16S rRNA gene fragments. An extremely high number of BOX-PCR profiles was observed, which could be detected neither by physiological characterisation nor by electrophoretic mobilities of the isolates in DGGE. Such an extreme diversity resulted in a dendrogram which nearly did not reveal typical clusters

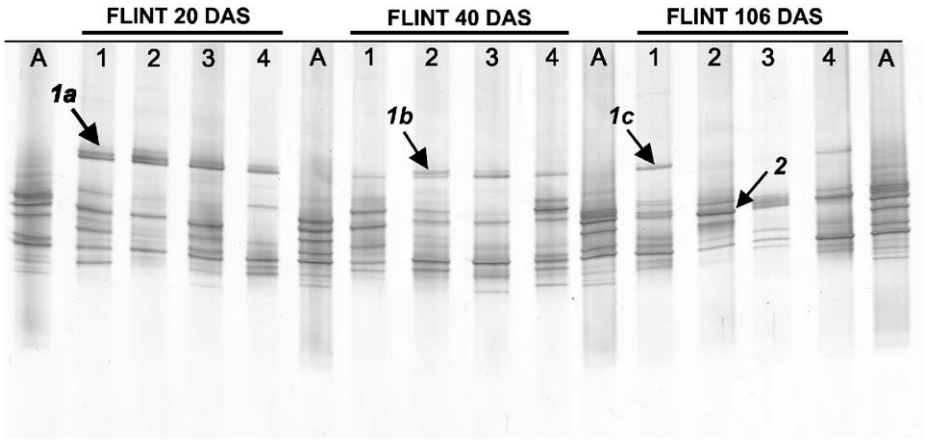

Fig. 5. DGGE-fingerprinting of *Pseudomonas* 16SrDNA fragments amplified from community DNA extracted from the rhizosphere of the maize cultivar Nitroflint collected 20, 40 and 106 days after sowing (DAS). Four independent replicates per treatment were applied to the gel. Bands 1a, 1b, 1c and 2 were extracted from the gel, cloned and sequenced. A, ribotypes A1–A6 (Fig. 1).

Table 4
Tentative phylogenetic affiliation of 16S rRNA gene partial sequences (regions V6–V8) derived from dominant DGGE bands

Band[a]	DAS[b]	Clones[c]	ARDRA[d]	Clone code[e]	Accession nr.[f]	Length(bp)	Most closely related sequence(s)		
							Identity	%	Accession nr.[g]
1a	20	6	2	RF1 2 (5)	DQ137125	392	*Pseudomonas stutzeri*	99.0	AF237677
							Pseudomonas stutzeri strain ST27MN3	99.0	U26419
				RF1 4 (1)	DQ137126	394	*Pseudomonas* sp. PAI-A	100.0	AY288072
							Pseudomonas alcaligenes strain S3	100.0	AY651923
1b	40	9	4	RF5 1 (5)	DQ137127	393	*Pseudomonas straminea* strain IAM1587	100.0	AB06135
				RF5 4 (1)	DQ137128	394	Uncultured *Pseudomonas* sp. Isolate ON3	100.0	AJ306834
							Pseudomonas sp. P400Y-1	100.0	AB076857
				RF5 13 (2)	DQ137129	392	*Pseudomonas stutzeri*	99.0	U65012
							Pseudomonas stutzeri strain ZoBell	99.0	U26420
				RF5 16 (1)	DQ137130	394	*Pseudomonas stutzeri*	99.0	AF237677
							Pseudomonas stutzeri strain ST27MN3	99.0	U26419
1c	106	7	2	BR10 27 (1)	DQ137131	386	*Pseudomonas stutzeri*	99.0	AF237677
							Pseudomonas stutzeri strain ST27MN3	99.0	U26419
				BR10 30 (6)	DQ137132	394	*Pseudomonas* sp. PAI-A	99.0	AY288072
							Pseudomonas alcaligenes strain S3	99.0	AY65193
2	106	6	1	BR10b 1 (6)	DQ137133	394	*Pseudomonas* sp. 3C_12	100.0	AY689037
							Uncultured bacterium clone L7	100.0	AY444988

[a]Codes refer to bands shown by arrows in Fig. 5.
[b]Sampling time (days after sowing).
[c]Number of clones matching the original DGGE band's electrophoretic mobility after extraction, re-amplification and cloning.
[d]Number of different ARDRA profiles observed among clones with the correct insert obtained from the same DGGE band.
[e]Number of clones sharing the same ARDRA profile in brackets.
[f]GenBank sequence accession numbers of the respective clone.
[g]GenBank sequence accession number of most closely related bacterial sequence(s).

(data not shown). High genotypic diversity of rhizobacteria in the maize rhizosphere was also observed by Di Cello et al. (1997), Dalmastri et al. (1999) and Tabacchioni et al. (2000) while studying *Burkholderia cepacia* populations. These authors claimed that such a pattern can be a result of a non-clonal model of population dynamics of certain bacterial groups in soil. In particular, the genomic complexity and plasticity among fluorescent *Pseudomonas* spp. (Hayward, 1994; Bennasar et al., 1996; Moore et al., 1996) favour intra-specific diversity, which, in turn, confers high persistence ability of these populations in changing environments such as the rhizosphere.

It has been shown that, in comparison to other plant microenvironments, the rhizosphere is one of the main reservoirs of antagonistic bacteria (Berg et al., 2005). Fluorescent *Pseudomonas* frequently represented the ma-

Table 5
Absolute number (*n*), origin (sampling time, cultivar) and typical antagonistic attributes of fluorescent *Pseudomonas* isolates sharing the same 16S rRNA gene mobility on DGGE gels (ribotypes A1–A6)

Ribotype[a]	*n*	DAS (*n*)[b]	Cultivar (*n*)[c]	Characteristics (*n*)[d]
A1	6	106	Dent (3) / Flint (3)	*R. solanacearum antagonists*, antibio +(5), siderophore +(5), *Pseudomonas fluorescens* strains
A2	2	20	Dent	*S. sclerotiorum antagonists*, antibio +, protease +, siderophore +, *Pseudomonas putida* strains
A3	5	40 (2) / 106 (3)	Dent (3) / Flint (2)	*R. solanacearum* (4) antagonists, antibio + (4), Protease + (4), siderophore + (4). *V. dahliae* (1) and *S. sclerotiorum* (1) antagonists
A4	7	20 (1), 40 (4), 106 (2)	Flint	Variable. *R. solanacearum* (4), *S. sclerotiorum* (2) and Rhizoctonia solani (1) antagonists. Siderophore + (5), antibio + (5). *P. putida* (4) and *P. fluorescens* (3) strains
A5	20	20 (13), 40(4), 106 (3)	Flint (5) / Dent (5)	*R. solanacearum antagonists* (19), Siderophore + (18), antibio + (16), *P. putida* strains (17)
A6	10	20 (8), 40 (1), 106 (1)	Flint (14) / Dent (6)	Variable. *R. solanacearum* (7) and *V. dahliae* (3) antagonists, antibio + (7), protease + (7), all siderophore +, all *P. putida* strains. Glucanase (2) and chitinase (1) activity detected only for strains belonging to this rybotype group

[a]Rybotypes A1–A6 are shown in Fig. 1 and can be seen at the extremities of DGGE gels in Figs. 3, 4 and 5.
[b]Sampling time (days after sowing). Number of isolates from each sampling time are given in brackets.
[c]Number of isolates from each cultivar are given in brackets.
[d]Number of antagonists displaying a given attribute are given in brackets. No brackets are present in cases where all antagonists belonging to the same PCR-DGGE ribotype were positive to a given attribute.

jority of bacterial strains displaying antagonistic properties when screening strategies were applied to recover antagonistic bacteria from the rhizosphere (Berg et al., 2002, 2006). They are among the most effective plant growth-promoting rhizobacteria and have been shown to be responsible for the reduction of soil-borne disease in natural suppressive soils (Raaijmakers and Weller, 1998). Our results revealed a high antagonistic potential of fluorescent *Pseudomonas* ssp. isolated from the rhizosphere of maize, with 50 out of 142 isolates displaying antagonism towards one or more of the tested plant pathogens. Antagonists displayed a wide spectrum of attributes that might confer these strains the capability of antagonising phytopathogens in situ, highlighting their potential use as BCAs. Indeed, many reports have been attempting to investigate the use of *Pseudomonas* spp. as BCAs either due to their antagonistic properties (De Souza et al., 2003; Bergsma-Vlami et al., 2005) or due to induction of systemic resistance (Van Loon et al., 1998; Pozo et al., 2005). The majority of the antagonists retrieved in this study was able to antagonise *R. solanacearum* in dual culture assays and synthesise siderophores. *Ralstonia solanacearum* is the causative agent of bacterial wilt in potatoes (Hayward and Hartmann, 1994). *Ralstonia solanacearum* Race 3 has a broad host range and causes important yield losses worldwide. Strategies for the biological control of this pathogen are important especially for tropical and subtropical areas (Götz et al., 2006) and the use of antagonistic bacteria for this purpose can be considered a realistic alternative. Siderophores are molecules that bind traces of insoluble Fe^{+3} in the rhizosphere and form stable complexes. The synthesis of these molecules is supposed to be involved in the suppression of pathogens by competition for Fe^{+3}, since availability of these ions in soil is normally low. Species such as *P. putida* and *P. aeruginosa* are known to display siderophore activity (Gray and Smith, 2005).

Gomes et al. (2001) reported that bacterial, alpha- and betaproteobacterial 16S rRNA gene fingerprints of the rhizosphere of maize grown in subtropical soils shifted dramatically along plant development: a higher number of bands was observed in the TGGE rhizosphere profiles of mature plants in comparison to those of young and of flowering plants. In addition, no clear indications for cultivar-dependent microbial community structure could be depicted from the TGGE profiles obtained for these bacterial groups. We have applied, to the same set of DNA samples, a PCR-DGGE system that specifically amplified *Pseudomonas* spp. 16S rRNA gene fragments from whole-community rhizosphere DNA. Shifts in the relative abundance of some dominant PCR-DGGE ribotypes along plant development and in the rhizosphere of different cultivars could be detected, although significant differences in community diversity of DGGE fingerprints were found neither for different maize cultivars nor for different sampling times. High degree of heterogeneity among replicates of the same microenvironment may have contributed to impair, to a given extent, a comprehensive differentiation of microenvironments as distinct units concerning community structure and diversity. In contrast, we have detected a major temporal shift in the frequency of fluorescent *Pseudomonas* isolated from the maize rhizosphere: isolates identified as *P. fluorescens* shared dominance with *P. putida* isolates at 106 DAS. As some *P.*

fluorescens and *P. putida* isolates were found to display, in some cases, the same DGGE mobility (Table 5), such kind of shift may escape detection by DGGE fingerprinting of 16S rRNA gene fragments. Our main outcomes contrasted some of the findings made by Gomes et al. (2001) for other bacterial groups and highlighted the importance of using taxon-specific primers to investigate structure and diversity of different microbial groups in the rhizosphere (Costa et al., 2006). As soil-borne *Pseudomonas* ssp. play major roles as plant pathogens, biodegraders, root-colonisers and biocontrol agents, evaluating the impact of environmental triggers, pollutants, land management and other factors on *Pseudomonas* community structure in the rhizosphere using nucleic acid-based techniques is of utmost importance.

Partial 16S rRNA gene sequencing of clones derived from band 1 (Fig. 5), composed by two similar PCR-DGGE ribotypes, resulted, in most cases, in phylogenetic affiliation to *P. stutzeri* and *P. alcaligenes*. Alignments of "*P. alcaligenes*" and "*P. stutzeri*" sequences obtained from DGGE band 1 showed that they differ from each other in 2–3 base identities within 16S rRNA gene fragments of around 400 bp. This might elucidate why this band is in fact composed by at least two PCR-DGGE ribotypes with slightly different melting behaviours. The fact that *P. stutzeri* and *P. alcaligenes* do not produce fluorescent pigments explains why no PCR-DGGE ribotypes matching band 1 were observed among the antagonists, which are all fluorescent *Pseudomonas*. On the other hand, ribotypes A5 and A6 (Fig. 1), for instance, harboured the DGGE mobility of various "*P. putida*" isolates with a wide range of antagonistic properties and matched dominant (ribotype 3) and rare PCR-DGGE ribotypes amplified from rhizosphere DNA (Figs. 3–5). This observation suggests that these antagonists may represent dominant *Pseudomonas* populations in the maize rhizosphere, an important attribute for biocontrol efficiency, since only metabolically active populations are capable of expressing their antagonistic properties in situ. The rhizosphere competence of one *Pseudomonas putida* isolate (PRD16, Table 2) retrieved in this study has recently been evaluated after seed and root inoculation of tomato plants, a major host of *Ralstonia solanacearum*. The localisation of this strain in the tomato rhizosphere was observed by means of confocal laser scanning microscopy, indicating a rhizosphere-competent status (Götz et al., 2006). Moreover, these authors assessed potential impacts of the inoculant strain PRD16 on non-target bacteria in the rhizosphere of tomato using DGGE fingerprints of 16S rRNA gene fragments amplified from total community DNA. For the first time, it was shown that the inoculation resulted in shifts of the *Pseudomonas* patterns in comparison to the control even 3 weeks after root inoculation (Götz et al., 2006). Further studies on biocontrol efficacy and rhizosphere competence are needed to better evaluate the potential use of this strain and others shown in this report as suitable BCAs.

The PCR-DGGE system employed in this study was an efficient strategy to assess major shifts in the structure of *Pseudomonas* communities in rhizosphere samples. Supplementing the body of knowledge gathered with this system with additional information on the genotypic diversity and antagonistic activity of fluorescent *Pseudomonas* isolates and their respective mobility on DGGE gels has proven to be a suitable approach to better understand the abundance, distribution and antimicrobial function of *Pseudomonas* spp. in the rhizosphere of maize grown in agricultural soils in southeast Brazil. Data provided here can be used as baseline for the selection of putative BCAs for further studies on rhizosphere competence and in situ suppression of phytopathogens.

Acknowledgements

This study was financed by a bilateral (German/Brazilian) cooperation in science and technology (WTZ98/005). The authors thank EMBRAPA (Empresa Brasileira de Pesquisa Agropecuária), Seropédica, Rio de Janeiro, for providing the experimental field and technical support. R. Costa was supported by a Deutscher Akademischer Austauschdienst (DAAD) scholarship during the development of this work.

References

Bennasar, A., Rosselló-Mora, R., Lalucat, J., Moore, E.R.B., 1996. 16S rRNA gene sequence analysis relative to genomovars of *Pseudomonas* stutzeri and proposal of *Pseudomonas* balearica sp. nov. International Journal of Systematic Bacteriology 46, 200–205.

Berg, G., Kurze, S., Buchner, A., Wellington, E.M., Smalla, K., 2000. Successful strategy for the selection of new strawberry-associated rhizobacteria antagonistic to *Verticillium* wilt. Canadian Journal of Microbiology 46, 1–10.

Berg, G., Roskot, N., Steidle, A., Eber, L., Zock, A., Smalla, K., 2002. Plant-dependent genotypic and phenotypic diversity of antagonistic rhizobacteria isolated from different *Verticillium* host plants. Applied and Environmental Microbiology 68, 3328–3338.

Berg, G., Krechel, A., Ditz, M., Sikora, R.A., Ulrich, A., Hallmann, J., 2005. Endophytic and ectophytic potato-associated bacterial communities differ in structure and antagonistic function against plant pathogenic fungi. FEMS Microbiology Ecology 51, 215–229.

Berg, G., Opelt, K., Zachow, C., Lottmann, J., Götz, M., Costa, R., Smalla, K., 2006. The rhizosphere effect on bacteria antagonistic towards the pathogenic fungus *Verticillium* differs depending on plant species and site. FEMS Microbiology Ecology 56, 250–261.

Bergsma-Vlami, M., Prins, M.E., Raaijmakers, J.M., 2005. Influence of plant species on population dynamics, genotypic diversity and antibiotic production in the rhizosphere by indigenous *Pseudomonas* spp. FEMS Microbiology Ecology 52, 59–69.

Brower, J.E., Zar, J.H., von Ende, C.N., 1997. Field and laboratory methods for general ecology, fourth ed. WCB/McGraw-Hill, USA, 273 pp.

Costa, R., Gomes, N.C.M., Milling, A., Smalla, K., 2004. An optmized protocol for simultaneous extraction of DNA and RNA from soils. Brazilian Journal of Microbiology 35, 230–234.

Costa, R., Götz, M., Mrotzek, N., Lottmann, J., Berg, G., Smalla, K., 2006. Effects of site and plant species on rhizosphere community structure as revealed by molecular analysis of microbial guilds. FEMS Microbiology Ecology 56, 236–249.

Dalmastri, C., Chiarini, L., Cantale, C., Bevivino, A., Tabaccioni, S., 1999. Soil type and maize cultivar affect the genetic diversity of maize

root-associated *Burkholderia cepacia* populations. Microbial Ecology 38, 273–284.

De Souza, J.T., Raaijmakers, J.M., 2003. Polymorphisms whithin the prnD and pltC genes from pyrrolnitrin and pyoluteorin-producing *Pseudomonas* and *Burkholderia* spp. FEMS Microbiology Ecology 43, 21–34.

Di Cello, F., Bevivino, A., Chiarini, L., Fani, R., Paffetti, D., Tabacchioni, S., Dalmastri, C., 1997. Biodiversity of a *Burkholderia cepacia* population isolated from the maize rhizosphere at different plant growth stages. Applied and Environmental Microbiology 63, 4485–4493.

Dwivedi, D., Johri, B.N., 2003. Antifungals from fluorescent *pseudomonads*: biosynthesis and regulation. Current Science 85, 1693–1703.

Fromin, N., Achouak, W., Thiéry, J.M., Heulin, T., 2001. The genotypic diversity of *Pseudomonas* brassicacearum populations isolated from roots of Arabidopsis thaliana: influence of plant genotype. FEMS Microbiology Ecology 37, 21–29.

Garbeva, P., van Veen, J.A., van Elsas, J.D., 2004. Assessment of the diversity, and antagonism towards Rhizoctonia solani AG3, of *Pseudomonas* species in soil from different agricultural regimes. FEMS Microbiology Ecology 47, 51–64.

Gray, E.J., Smith, D.L., 2005. Intracellular and extracellular PGPR: commonalities and distinctions in the plant-bacterium signaling processes. Soil Biology & Biochemistry 37, 395–412.

Gomes, N.C.M., Heuer, H., Schönfeld, J., Costa, R., Mendonça-Hagler, L.C., Smalla, K., 2001. Bacterial diversity of maize (Zea mays) grown in tropical soil studied by temperature gradient gel electrophoresis. Plant & Soil 232, 167–180.

Götz, M., Gomes, N.C.M., Dratwinski, A., Costa, R., Berg, G., Peixoto, R., Mendonça-Hagler, L.C.S., Smalla, K., 2006. Survival of gfp-tagged antagonistic bacteria in the rhizosphere of tomato plants and their effects on the indigenous bacterial community. FEMS Microbiology Ecology 56, 207–218.

Gyamfi, S., Pfeifer, U., Stierschneider, M., Sessitsch, A., 2002. Effects of transgenic glucosinate-tolerant oilseed rape (Brassica napus) and the associated herbicide application on eubacterial and *Pseudomonas* communities in the rhizosphere. FEMS Microbiology Ecology 41, 181–190.

Haas, D., Keel, C., 2003. Regulation of antibiotic production in root-colonizing *Pseudomonas* spp. and relevance for biological control of plant disease. Annual Review of Phytopathology 41, 117–153.

Hayward, A.C., 1994. Systematics and phylogeny of *Pseudomonas* solanacearum and related bacteria. In: Hayward, A.C., Hartmann, G.L. (Eds.), Bacterial Wilt. The Disease and its Causative Agent, *Pseudomonas* solanacearum. Cab International, Wallingford, UK, pp. 123–135.

Hayward, A.C., Hartmann, G.L., 1994. Bacterial wilt: The disease and its causative agent, *Pseudomonas* solanacearum. CAB International, Wallingford, UK, 259 pp.

Heuer, H., Smalla, K., 1997. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) for studying soil microbial communities. In: Van Elsas, J.D., Trevors, J.T., Wellington, E.M.H. (Eds.), Modern Soil Microbiology. Marcel Dekker, New York, pp. 353–373.

Heuer, H., Wieland, J., Schönfeld, J., Schönwälder, A., Gomes, N.C.M., Smalla, K., 2001. Bacterial community profiling using DGGE or TGGE analysis. In: Rouchelle, P. (Ed.), Environmental Molecular Microbiology: Protocols and Applications. Horizon Scientific Press, Wymondham, UK, pp. 177–190.

Keel, C., Schnider, U., Maurhofer, M., Voisard, C., Laville, J., Burger, U., Wirthner, P., Haas, D., Défago, G., 1992. Suppression of root diseases by *Pseudomonas* fluorescens CHA0: importance of the bacterial secondary metabolite 2,4-diacetylphloroglucinol. Molecular Plant–Microbe Interactions 5, 4–13.

Keel, C., Weller, D.M., Natsch, A., Défago, G., Cook, R.J., Thomashow, L.S., 1996. Conservation of the 2,4-diacetylphloroglucinol biosynthes is locus among fluorescent *Pseudomonas* strains from diverse geographic locations. Applied and Environmental Microbiology 62, 552–563.

Leeman, M., van Pelt, J.A., Hendrickx, M.J., Scheffer, R.J., Bakker, P.A.H.M, Schippers, B., 1995. Biocontrol of Fusarium wilt of radish in commercial greenhouse trials by seed treatment with *Pseudomonas* fluorescens WCS374. Phytopathology 85, 1301–1305.

Lugtenberg, B.J.J., Dekkers, L., Bloemberg, G.V., 2001. Molecular determinants of rhizosphere colonization by *Pseudomonas*. Annual Review of Phytopathology 34, 461–490.

Milling, A., Smalla, K., Maidl, F.X., Schloter, M., Munch, J.C., 2004. Effects of transgenic potatoes with an altered starch composition on the diversity of soil and rhizosphere bacteria and fungi. Plant & Soil 266, 23–39.

Moore, E.R.B., Mau, M., Arnscheidt, A., Böttger, E.C., Hutson, R.A., Collins, M.D., Van de Peer, Y., Wachter, R., Timmis, K., 1996. The determination and comparison of the 16S rRNA gene sequences of species of the genus *Pseudomonas* (sensu stricto) and estimation of the natural intrageneric relationship. Systematic and Applied Microbiology 19, 478–492.

Nielsen, P., Sørensen, J., 1997. Multi-target and medium-independent fungal antagonism by hydrolytic enzymes in *Paenibacillus polymyxa* and *Bacillus pumilus* strains from barley rhizosphere. FEMS Microbiology Ecology 22, 183–192.

Pesaro, M., Widmer, F., 2006. Identification and specific detection of a novel *Pseudomonadaceae* cluster associated with soils from winter wheat plots of a long-term agricultural field experiment. Applied Environmental Microbiology 72, 37–43.

Picard, C., Di Cello, F., Ventura, M., Fani, R., Guckert, A., 2000. Frequency and biodiversity of 2,4-Diacetylphloroglucinol-producing bacteria isolated from the maize rhizosphere at different stages of plant growth. Applied and Environmental Microbiology 66, 948–955.

Pozo, M.J., Van Loon, L.C., Pieterse, C.M.J., 2005. Jasmonates-signals in plant-microbe interactions. Journal of Plant Growth Regulation 23, 211–222.

Raaijmakers, J.M., Weller, D.M., Thomashow, L.S., 1997. Frequency of antibiotic-producing *Pseudomonas* spp. in natural environments. Applied and Environmental Microbiology 63, 881–887.

Raaijmakers, J.M., Weller, D.M., 1998. Natural plant protection by 2,4-diacetylphloroglucinol-producing *Pseudomonas* spp. in take-all decline soils. Molecular Plant-Microbe Interactions 11, 144–152.

Raaijmakers, J.M., Bonsall, R.F., Weller, D.M., 1999. Effect of population density of *Pseudomonas* fluorescens on production of 2,4-diacetylphloroglucinol in the rhizosphere of wheat. Phytopathology 89, 470–475.

Raaijmakers, J.M., Weller, D.M., 2001. Exploiting genotypic diversity of 2,4-diacetylphloroglucinol-producing *Pseudomonas* spp.: characterization of superior root-colonizing P. fluorescens strain Q8r1-96. Applied and Environmental Microbiology 67, 2545–2554.

Salles, J.F., Samyn, E., Vandamme, P., van Veen, J.A., van Elsas, J.D., 2006. Changes in agricultural management drive the diversity of *Burkholderia* species isolated from soil on PCAT medium. Soil Biology & Biochemistry, in press. doi:10.1016/j.soilbio.2005.06.018.

Schwyn, B., Neilands, J.B., 1987. Universal chemical assay for the detection and determination of siderophores. Analytical Biochemistry 160, 47–56.

Tabacchioni, S., Chiarini, L., Bevivino, A., Cantale, C., Dalmastri, C., 2000. Bias caused by using different isolation media for assessing the genetic diversity of a natural microbial population. Microbial Ecology 40, 169–176.

Top, E.M., Holben, W.E., Forney, L.J., 1995. Characterization of diverse 2,4-dichlorophenoxyacetic acid-degradative plasmids isolated from soil by complementation. Applied and Environmental Microbiology 61, 1691–1698.

Van Loon, L.C., Bakker, P.A.H.M, Pieterse, C.M.J., 1998. Systemic resistance induced by rhizosphere bacteria. Annual Review of Phytopathology 36, 453–483.

Walsh, U.F., Morrissey, J.P., O'Gara, F., 2001. *Pseudomonas* for biocontrol of phytopathogens: from functional genomics to commercial exploitation. Current Opinion in Biotechnology 12, 289–295.

Wang, C., Ramette, A., Punjasamarnwong, P., Zala, M., Natsch, A., Möenne-Loccoz, Y., Défago, G., 2001. Cosmopolitan distribution of phlD-containing dicotyledonous crop-associated biocontrol *Pseudomonas* of worldwide origin. FEMS Microbiology Ecology 37, 105–116.

Weller, D.M., 1988. Biological control of soilborne plant pathogens in the rhizosphere with bacteria. Annual Review of Phytopathology 26, 379–407.

Winding, A., Binnerup, S.J., Pritchard, H., 2004. Non-target effects of bacterial biological control agents suppressing root pathogenic fungi. FEMS Microbiology Ecology 47, 129–141.

Zar, J.H., 1996. Biostatistical Analysis. Prentice-Hall, Nova Jersey, EUA, 662p.