Effects of site and plant species on rhizosphere community structure as revealed by molecular analysis of microbial guilds

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Abstract

The bacterial and fungal rhizosphere communities of strawberry (*Fragaria ananassa* Duch.) and oilseed rape (*Brassica napus* L.) were analysed using molecular fingerprints. We aimed to determine to what extent the structure of different microbial groups in the rhizosphere is influenced by plant species and sampling site. Total community DNA was extracted from bulk and rhizosphere soil taken from three sites in Germany in two consecutive years. Bacterial, fungal and group-specific (*Alphaproteobacteria, Betaproteobacteria* and *Actinobacteria*) primers were used to PCR-amplify 16S rRNA and 18S rRNA gene fragments from community DNA prior to denaturing gradient gel electrophoresis (DGGE) analysis. Bacterial fingerprints of soil DNA revealed a high number of equally abundant faint bands, while rhizosphere fingerprints displayed a higher proportion of dominant bands and reduced richness, suggesting selection of bacterial populations in this environment. Plant specificity was detected in the rhizosphere by bacterial and group-specific DGGE profiles. Different bulk soil community fingerprints were revealed for each sampling site. The plant species was a determinant factor in shaping similar actinobacterial communities in the strawberry rhizosphere from different sites in both years. Higher heterogeneity of DGGE profiles within soil and rhizosphere replicates was observed for the fungi. Plant-specific composition of fungal communities in the rhizosphere could also be detected, but not in all cases. Cloning and sequencing of 16S rRNA gene fragments obtained from dominant DGGE bands detected in the bacterial profiles of the Rostock site revealed that *Streptomyces* sp. and *Rhizobium* sp. were among the dominant ribotypes in the strawberry rhizosphere, while sequences from *Arthrobacter* sp. corresponded to dominant bands from oilseed rape bacterial fingerprints.

Introduction

Strawberry and oilseed rape are host plants of the soil-borne fungal phytopathogen *Verticillium dahliae* Kleb. The wilt disease caused by this fungus can be responsible for important yield losses worldwide (Tjamos *et al.*, 2000). It has been argued that the chemical control of *Verticillium dahliae* in the field has become virtually impossible since the phasing-out of methylbromide and related substances, because microsclerotia can persist for several years in soil in the absence of a susceptible host (Maas, 1998). This problem has increased the interest in antagonists that could be applied for the biological control of this pathogen in the field (Berg *et al.*, 2000, 2001, 2002, 2005a, b). The rhizosphere, which is defined as the portion of soil adjacent to and influenced by the plant root (Sørensen, 1997), has been frequently used as a model environment for the isolation of potential biocontrol strains (Weller, 1988; Raaijmakers *et al.*, 1997; Lottmann *et al.*, 2000; Picard *et al.*, 2000; Walsh *et al.*, 2001; Maurhofer *et al.*, 2004).

An understanding of the microbial community structure in the rhizosphere is, however, critical to the successful application of biological control strains. Previous studies have shown that the structure of rhizosphere microbial communities is influenced by the plant species, because of differences in root exudation and rhizodeposition in different root zones (Jaeger *et al.*, 1999; Brimecombe *et al.*, 2001). Several studies on the bacterial community structure of

rhizospheres that indicated plant-dependent diversity of such communities were performed using cultivation-based techniques (Liljeroth *et al.*, 1991; Lemanceau *et al.*, 1995; Mahaffee & Kloepper, 1997; Germida *et al.*, 1998; Grayston *et al.*, 1998). These techniques allow the analysis of only a minor fraction of the microbial community (Amann *et al.*, 1995). Analysing DNA extracted directly from rhizosphere and soil samples is an alternative that overcomes these limitations. The diversity of target genes, such as the 16 S rRNA or 18S rRNA genes, can be assessed by means of molecular fingerprinting techniques such as denaturing gradient gel electrophoresis (DGGE) (Heuer & Smalla, 1997). These methods are useful for the analysis of large numbers of samples, an essential requirement for ecological studies.

In a previous study, the bacterial diversity in the rhizosphere of potato, strawberry and oilseed rape was assessed in a cultivation-independent fashion by Smalla *et al.* (2001), and a plant-specific selection of bacterial DGGE ribotypes was observed in the rhizosphere of plants grown in a randomized block design at one sampling site. In addition, a plant-dependent selection of bacteria antagonistic towards *Verticillium dahliae* in the rhizosphere of these plants was shown to exist by Berg *et al.* (2002) by means of culture-dependent techniques, and the highest proportion of antagonists was isolated from the strawberry rhizosphere. In order to evaluate whether this phenomenon occurs inexorably for different microbial groups, and how much the location affects the bacterial and fungal community structure in the rhizosphere, this follow-up study was performed. The culture-independent analysis of microbial communities in the rhizosphere of strawberry and oilseed rape grown at three sites over two consecutive years was carried out. We aimed to determine to what extent the so-called 'rhizosphere effect' is detectable among different microbial taxa (Bacteria, Fungi, Alphaproteobacteria, Betaproteobacteria, and Actinobacteria), whether this phenomenon occurs at sampling sites harbouring different soil types, climate conditions and crop histories, and to identify dominant members of these communities. We hypothesised that (1) plant roots are the determinant factors in structuring microbial community composition in the rhizosphere at a given site, (2) the selective force exerted by the rhizosphere in shaping microbial community structure is plant-specific, and (3) plant roots influence microbial community structure in the rhizosphere to a higher extent than soil type/sampling site.

Materials and methods

Field design and sampling

Sampling took place 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. Physicochemical parameters were determined by Berg *et al.* (2005a). Two different crop plants, strawberry (*Fragaria ananassa* [Duchense] Decaisne & Naudin cv. Elsanta) and oilseed rape (*Brassica napus* L. cv. Licosmos) were grown in a randomized block design consisting of four replicate plots per crop plant. Strawberries were planted and oilseed rape was sown in the same field plots in two consecutive years (2002 and 2003). For each plot, one composite bulk soil sample and one composite rhizosphere sample were taken at the flowering stage of the plants. Each composite soil sample consisted of ten cores (15 cm of top soil) taken in areas free from roots and mixed by sieving. Each composite rhizosphere sample taken per plot consisted of the roots of five or more randomly selected strawberry and oilseed rape plants, respectively. The roots were shaken vigorously to separate soil not tightly adhering to the roots. Four composite samples were collected per treatment (strawberry rhizosphere, oilseed rape rhizosphere, soil from strawberry field, and soil from oilseed rape field), sampling site, and sampling time. Samples were immediately transported to the laboratory and processed for further analysis.

Extraction of microbial cells from soil matrices

Microbial cells were dislodged from soil matrices, and pellets were obtained prior to total community DNA extraction by applying the method described by Bakken and Lindahl (1995) as follows. For each sample, 5 g of soil or plant roots with firmly adhering soil was re-suspended in 15 mL of Milli-Q water and treated in a stomacher blender (Stomacher 400, Seward, England) for 1 min at high speed. After centrifugation at low speed (2 min, 500 ***g***), the supernatant was collected into 50 mL falcon tubes. This step was repeated twice, and the supernatants of the three stomacher-centrifugation steps were combined prior to centrifugation at high speed (10 000 ***g***) for 30 min to produce a microbial pellet. The resulting pellets were kept at $-70\,^{\circ}$C.

Total community DNA extraction

The BIO-101 DNA extraction kit (Q Biogene, Carlsbad, CA) was used to extract total community DNA. Cell pellets were added to lysis tubes containing a mixture of ceramic and silica particles, and DNA extraction was carried out according to the manufacturer's recommendations. The procedure combines highly energetic mechanical means (FastPrep Instrument, Q Biogene) with the use of detergents and salts in the very first step to allow disruption of hard-to-lyse cells, minimize shearing of DNA and contribute to inactivate nucleases. After DNA elution, a silica matrix is used to bind DNA, and samples are washed with a salt/ethanol solution.

The GENECLEAN Spin kit (Q Biogene) was applied as described by the manufacturer to re-purify DNA. Genomic DNA yields were checked after electrophoresis in 0.8% agarose gels stained with ethidium bromide under UV light. DNA concentration was estimated visually by applying the 1-kb gene-ruler™ DNA ladder (Fermentas, St Leon-Rot, Germany) on the agarose gels. Genomic DNA samples were diluted differentially to obtain *c.* 1 to 5 ng DNA to be used as PCR-templates for the bacterial taxa, while *c.* 20 ng DNA was used as a template for the fungi.

PCR amplification of universal 16S rRNA gene fragments for DGGE analysis

PCR amplifications were performed with a Tgradient thermal cycler (Biometra, Göttingen, Germany). Prior to DGGE analysis of the bacterial profiles, 16S rRNA gene fragments were amplified by PCR from rhizosphere and soil DNA extracts with the primer pair F984GC/R1378 (Table 1). The reaction mixture (25 μL) was composed of 1 μL template DNA (1–5 ng), 1× Stoffel buffer (Applied Biosystems, Foster, CA), 0.2 mM dNTPs, 3.75 mM $MgCl_2$, 4% (w/v) acetamide, 0.2 μM each primer, and 2.5 U *Taq* DNA polymerase (Stoffel fragment, Applied Biosystems). After 5 min of denaturation at 94 °C, 30 cycles of 1 min at 95 °C, 1 min at 53 °C and 2 min at 72 °C were carried out. A final extension step of 10 min at 72 °C was used to finish the reaction. Products were checked by electrophoresis in 1% agarose gels and ethidium bromide staining.

PCR amplification of group-specific 16S rRNA gene fragments

For the amplification of actinobacterial, alpha- and betaproteobacterial 16S rRNA gene fragments, a nested-PCR approach was applied. The nested-PCR consisted of a first, group-specific PCR-amplification of 16S rRNA gene fragments followed by a F984GC/R1378 PCR for the amplification of the same 16S rRNA gene region (V6–V8 variable regions of the 16S rRNA gene) as used for the DGGE bacterial profiles. Specific alphaproteobacterial 16S rRNA gene fragments were amplified as follows: a reaction mixture (25 μL) was prepared containing 1 μL template DNA (*c.* 1–5 ng), 1× Stoffel buffer (Applied Biosystems), 0.2 mM dNTPs, 3.75 mM $MgCl_2$, 5% (v/v) DMSO, 0.2 μM primers F203α and R1494 (Table 1), and 1 U *Taq* DNA polymerase (Stoffel fragment, Applied Biosystems). After an initial denaturation step of 5 min at 94 °C, DNA templates were amplified with 25 thermal cycles of 30 s at 94 °C, 2 min at 64 °C and 1 min at 72 °C. A final extension step of 10 min at 72 °C finished the reaction. The reaction mixture (25 μL) for the amplification of betaproteobacterial 16S rRNA gene fragments was composed of 1 μL template DNA (*c.* 1–5 ng), 1× Stoffel buffer (Applied Biosystems), 0.2 mM dNTPs, 3.75 $MgCl_2$, 4% (w/v) acetamide, 0.2 μM primers F948β and R1494 (Table 1), and 1 U *Taq* DNA polymerase (Stoffel fragment, Applied Biosystems). The PCR programme applied was the same as for the *Alphaproteobacteria*. For the amplification of actinobacterial 16S rRNA gene fragments, a reaction mixture (25 μL) was prepared containing 1× PCR buffer II (Applied Biosystems), 0.2 mM dNTPs, 2.5 mM $MgCl_2$, 5% (v/v) DMSO, 0.2 μM primers F243 and R1494 (Table 1), and 1.25 U AmpliTaq Gold (Applied Biosystems). After an initial denaturation step of 5 min at 94 °C, DNA templates were amplified with 25 thermal cycles of 1 min at 94 °C, 1 min at 63 °C and 2 min at 72 °C. A final extension step of 10 min at 72 °C finished the reaction. Diluted (1 : 25) group-specific PCR products served as templates for a F984GC/R1378 PCR as described above with 20 thermal cycles. Products were checked after electrophoresis in 1% agarose gels and ethidium bromide staining under UV light.

PCR amplification of fungal-specific 18S rRNA gene fragments

Amplification of 18S rRNA gene fragments prior to fungal community fingerprinting was done using the primer pair

Table 1. Primers used in this study targeting the 16S and 18S rRNA genes

Primer	Sequence 5′–3′	Specificity	Reference
F984	AACGCGAAGAACCTTAC	*Bacteria*	(Heuer & Smalla, 1997)
GC-Clamp	CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCA CGG GGG G	–	(Nübel *et al.*, 1996)
R1378	CGG TGT GTA CAA GGCCCGGGAACG	*Bacteria*	(Heuer & Smalla, 1997)
F203α	CCGCATACGCCCTACGGGGGAAAGATTTAT	*Alphaproteobacteria*	(Gomes *et al.*, 2001)
F948β	CGCACAAGCGGTGGATGA	*Betaproteobacteria*	(Gomes *et al.*, 2001)
F243	GGATGAGCCCGCGGCCTA	*Actinobacteria*	(Heuer *et al.*, 1997)
R1494	CTACGG(A/G)TACCTTGTTACGAC	*Bacteria*	(Gomes *et al.*, 2005)
NS0	TACCTGGTTGATCCTGCC	*Fungi*	(Messner & Prillinger, 1995)
EF3	TCCTCTAAATGACCAAGTTTG	*Fungi*	(Smit *et al.*, 1999)
NS1	GTAGTCATATGCTTGTCTC	*Fungi*	(White *et al.*, 1990)
FR1	AICCATTCAATCGGTAIT	*Fungi*	(Vainio & Hantula, 2000)
GC-Clamp	CCC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GCC G	–	(Vainio & Hantula, 2000)

NS0/EF3 (Table 1) in a PCR assay followed by a second PCR step with the primer pair NS1/FR1GC (1.650 bp–Table 1). For the first amplification step, the reaction mixture (25 μL) consisted of *c.* 25 ng template DNA, Stoffel buffer (Applied Biosystems), 0.2 mM dNTPs, 3.75 mM $MgCl_2$, 2% (v/v) DMSO, 0.2 μM each primer, and 5 U *Taq* DNA polymerase (Stoffel fragment, Applied Biosystems). After 8 min of denaturation at 94 °C, 25 thermal cycles of 30 s at 94 °C, 45 s at 53 °C and 3 min at 72 °C were performed, followed by an extension step at 72 °C for 10 min. PCR products were used as templates for a second PCR with the primer pair NS1/FR1GC prior to DGGE analysis. The reaction mixture was prepared as described above for the first PCR. The amplification took place using the same settings as for the previous PCR, except for the annealing temperature (48 °C) and the number of thermal cycles (20 cycles). Products were checked after electrophoresis in 1% agarose gels and ethidium bromide staining under UV light.

DGGE of 16S rRNA gene fragments

Denaturing gradient gel electrophoresis analysis was performed with the Dcode System apparatus (Bio-Rad Inc., Hercules, CA). Gel casting was performed as described 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 (Gomes *et al.*, 2004). Aliquots of PCR products (*c.* 2 μL) were loaded on the gel and electrophoresis was carried out with 1 × Tris-acetate-EDTA buffer at 58 °C and at a constant voltage of 220 V for 6 h. PCR products amplified from four replicates per treatment (each representing one composite sample) were loaded side by side on the gel. Gels were silver-stained according to Heuer *et al.* (2001) and air-dried. A mixture of the DGGE-PCR products from 11 bacterial species was applied at the extremities of the gels as a marker to check the electrophoresis run and to compare fragment migration between gels, as described by Smalla *et al.* (2001).

DGGE of 18S rRNA gene fragments

Materials used, instructions for gel casting and loading of samples followed the descriptions listed for the bacterial fingerprinting. Aliquots of PCR samples (2 to 4 μL) were applied to DGGE gels containing a denaturing gradient of 18 to 38% denaturants and 6% acrylamide. Electrophoresis was performed in 1× Tris-acetate-EDTA buffer at 58 °C at a constant voltage of 180 V for 18 h. Gels were air-dried after silver-staining according to Heuer *et al.* (2001). Selected PCR-amplified 18S rRNA gene fragments from fungal isolates of the strawberry and oilseed rape rhizospheres were mixed and applied to the gels to be used as a marker and to allow the comparison of fragment migration between gels.

Computer-assisted analysis of DGGE fingerprints

The DGGE loading schemes allowed the evaluation of the following aspects: (1) the rhizosphere effect, i.e. shifts of relative abundances of ribotypes in the rhizosphere compared with in bulk soil (Fig. 1); (2) plant-dependent community structure, i.e. the extent to which the microbial community structures of the rhizosphere soils of strawberry and oilseed rape grown at the same sampling site differ from each other (Fig. 1); and (3) site-dependent community structure, which reveals the similarity of DGGE fingerprints obtained for samples belonging to the same microenvironment but coming from different sampling sites (Figs 2 and 3).

Denaturing gradient gels were scanned transmissively (Epson 1680 Pro, Seiko-Epson Corp. Suwa, Nagano, Japan) with high-resolution settings. The GelCompar 4.0 programme (Applied Maths, Ghent, Belgium) was used to analyse the community fingerprints of each denaturing gradient gel as recommended by Rademaker *et al.* (1999), with the modifications of settings described by Smalla *et al.* (2001). The Pearson correlation index (r) for each pair of lanes within a gel was calculated as a measure of similarity between the community fingerprints. Cluster analysis was performed by applying the unweighted pair group method using average linkages (UPGMA) to the matrix of similarities obtained. In parallel, significance tests to compare the community fingerprints of different microenvironments using pairwise similarity measures were carried out (Kropf *et al.*, 2004). The test of significance is based on permutations of the similarity values of a given matrix in order to determine whether similarity measures calculated within groups (among replicates of the same microenvironment) are significantly higher than those obtained between groups (replicates from different microenvironments). Furthermore, the test allows comparisons of different matrices (gels). This approach was used to verify whether soil samples from different sampling sites, loaded on one DGGE gel (Fig. 3a), differed more from each other than their corresponding rhizosphere samples, loaded on another gel (Fig. 3c). The gels are considered as two different blocks in a statistical sense, and comparisons between similarity values obtained for both gels can be carried out (Kropf *et al.*, 2004).

Extraction and cloning of dominant bacterial DGGE bands

Dominant bands were excised with a scalpel from silver-stained DGGE gels and de-stained as described by Gomes *et al.* (2005) prior to elution and re-suspension according to the protocol described by Schwieger and Tebbe (1998). Two microlitres of the resulting suspension were used in a DGGE-PCR to re-amplify the excised 16S rRNA gene

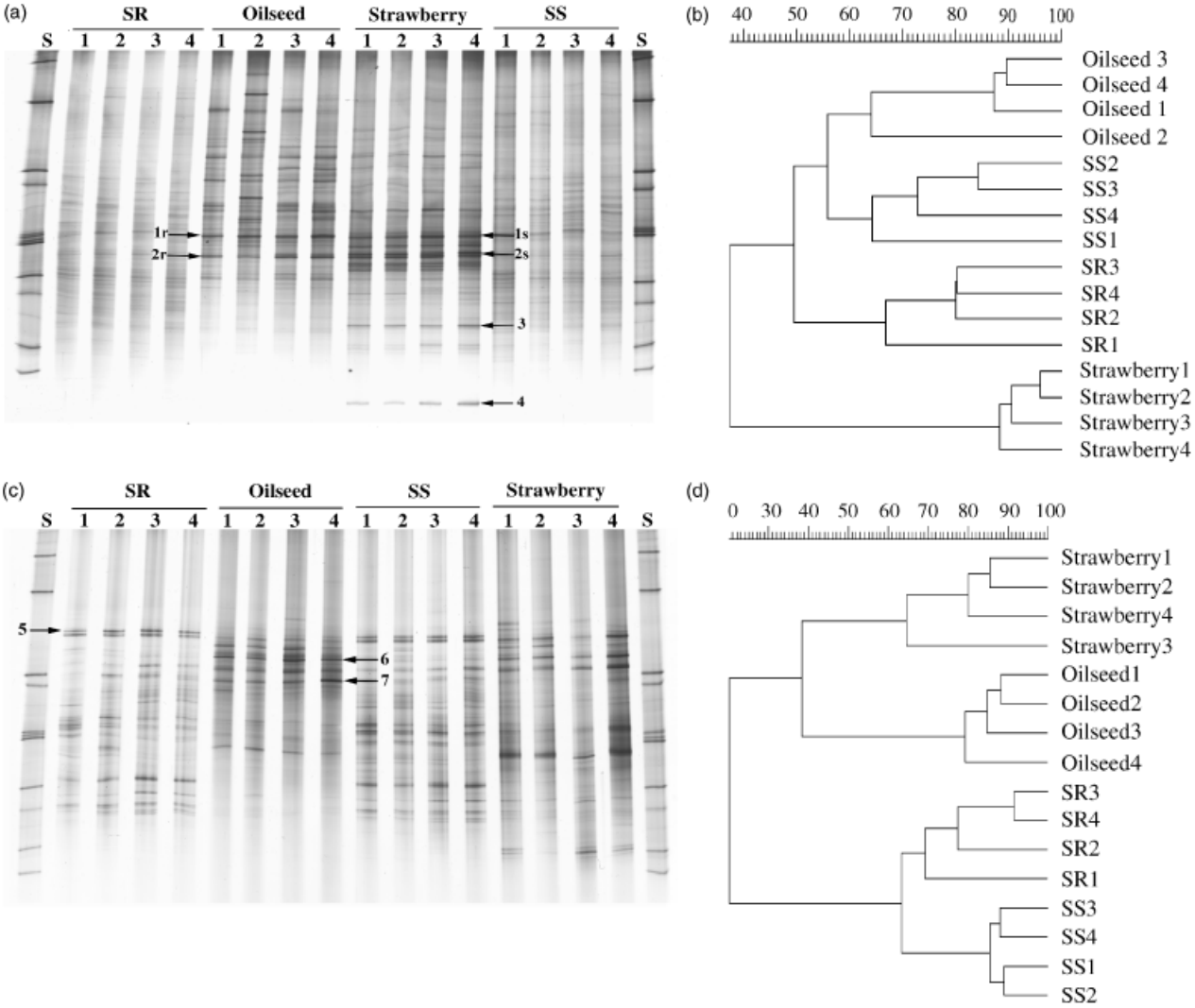

Fig. 1. Within-site comparisons among denaturing gradient gel electrophoresis fingerprints of 16S rRNA gene fragments amplified from bulk and rhizosphere (strawberry and oilseed rape) soil DNA templates. Gels obtained for the sampling site Rostock with their respective dendrograms generated by cluster analysis (UPGMA) are shown. (a) bacterial profiles, 2003; (b) respective dendrogram; (c) betaproteobacterial profiles, 2002; (d) respective dendrogram. Arrows indicate dominant bands, which were extracted from the gels for sequence analysis. SS, bulk soil samples from strawberry field; SR, bulk soil samples from oilseed rape field; Strawberry, strawberry rhizosphere samples; Oilseed, oilseed rape rhizosphere samples.

fragments. After confirming the correct electrophoretic mobility of the excised band by DGGE, the PCR product (without GC-clamp) was ligated into a pGEM-T vector (Promega, Madison, WI) and transformed into competent cells (*Escherichia coli* JM109; Promega) as recommended by the manufacturers. The 16S rRNA gene fragments amplified from clones and from the original community DNA samples were loaded on the same DGGE gel in order to check carefully whether the cloned 16S rRNA gene fragments co-migrated with the band of interest of the corresponding community pattern. Clones containing inserts that shared the electrophoretic mobility of the original band were selected for further analysis.

ARDRA and sequencing of 16S rRNA gene fragments extracted from DGGE gels

Amplified ribosomal DNA restriction analysis (ARDRA) was performed to compare restriction profiles among inserts originating from the same DGGE band. Inserts were amplified with the primers SP6 and T7 (Promega) according to the manufacturer's instructions, and a 10 μL aliquot of each PCR product containing approximately 3 μg of DNA was digested with the restriction enzymes *Alu* I and *Msp* I (0.1 U/μL) in a total volume of 50 μL at 37 °C for 2.5 h. The digested PCR products were precipitated by addition of 125 μL of ethanol and 5 μL of sodium acetate 3 M (pH 5.2)

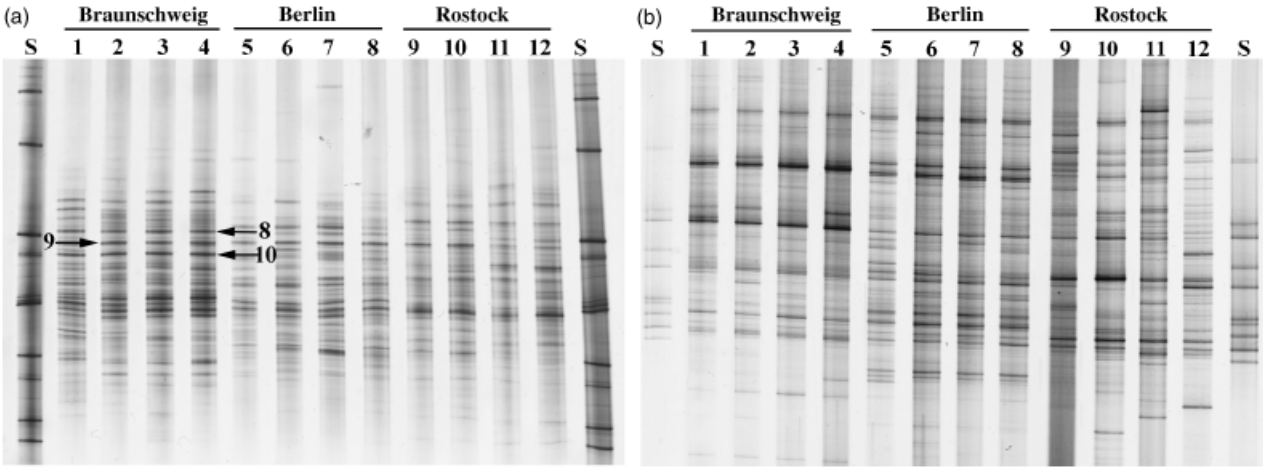

Fig. 2. Denaturing gradient gel electrophoresis fingerprinting of bulk soil samples collected at three sampling sites. (a) Alphaproteobacterial 16S rRNA gene fragments, 2003; (b) fungal 18S rRNA gene fragments, 2003. Arrows indicate alphaproteobacterial bands that were dominant and characteristic of the sampling site Braunschweig. Bands were extracted from the gel prior to cloning and sequencing analysis.

followed by overnight storage at −20 °C. After centrifugation at 12 000 ***g*** for 20 min, samples were washed with 70% ethanol. The centrifugation step was repeated, and pellets were dried and re-suspended in 20 μL TE buffer. A 10 μL aliquot was applied onto a 4% agarose gel (Nu Sieve 3 : 1, Cambrex Bio Science, Rockland, ME) for the separation of the digested PCR fragments. Inserts showing different ARDRA profiles were submitted to sequencing of the V6 to V8 region of the 16S rRNA gene (approximately 400 bp).

Nucleotide sequence accession numbers

Tentative phylogenetic affiliation of partial 16S rRNA gene sequences obtained from 30 clones corresponding to dominant DGGE bands was carried out by comparing the sequences with those available in the database using BLAST-N search. Nucleotide sequence accession numbers of the partial 16S rRNA gene sequences are given in Table 3.

Results

Rhizosphere effect

DNA extraction procedures allowed the recovery of high-molecular-weight DNA from all rhizosphere and bulk soil samples. Community fingerprints of five different microbial groups (*Bacteria, Fungi, Alphaproteobacteria, Betaproteobacteria* and *Actinobacteria*) were generated for each sampling site (Braunschweig, Berlin and Rostock) in two seasons (2002 and 2003). DGGE profiles of bacterial taxa shared in general similar characteristics: at all sampling sites, the bulk soil patterns consisted of a few stronger bands and a large number of fainter bands representing less dominant ribotypes, whereas the relative abundance of several ribotypes was enhanced in the rhizosphere (Figs 1a and c). Furthermore, for all bacterial groups evaluated, similar DGGE patterns were observed among replicates belonging to the same microenvironment. Significant differences between the DGGE patterns of the rhizosphere and bulk soil samples could be detected at all sampling sites for all bacterial groups in both years. The only exceptions to this rule were the actinobacterial DGGE fingerprints of the oilseed rape rhizosphere in Braunschweig and Berlin in the first season, which could not be distinguished from the bulk soil fingerprints by cluster analysis and permutation tests (Table 2). However, significant differences among oilseed rape rhizosphere and bulk soil profiles were obtained in the subsequent year (Table 2). On the other hand, selection and enhancement in abundance of actinobacterial ribotypes in the strawberry rhizosphere could be easily detected at all sampling sites and in both years (Table 2).

Fungal DGGE fingerprints displayed some features that were different from the patterns typically observed for bacteria. Cluster analysis did not allow a clear distinction of rhizosphere from bulk soil fungal fingerprints except for fingerprints generated for the Braunschweig and Berlin sites in the first year. In contrast, pairwise group comparisons revealed significant differences between the microenvironments (rhizosphere and bulk soil) in both years at these sites (Table 2). No significant differences were found among rhizosphere and bulk soil profiles in Rostock in the first season (Table 2), where internal variability within both bulk and rhizosphere soil replicates was strikingly high. No differences encountered between the microenvironments were due to the absence of clear, characteristic patterns. The picture was nevertheless different in the subsequent year, with rhizosphere and bulk soil profiles of samples collected in Rostock differing from each other significantly

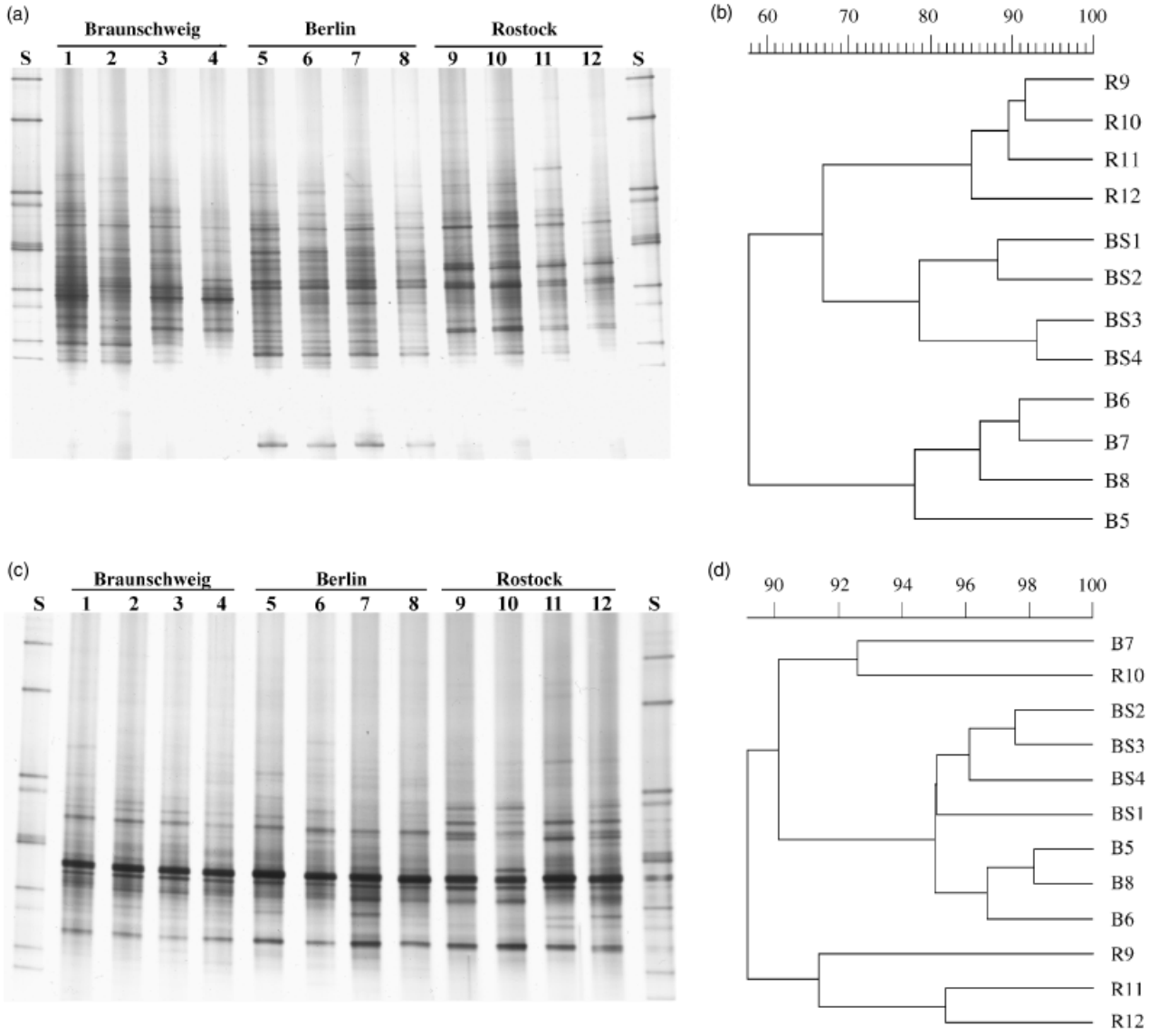

Fig. 3. Actinobacterial denaturing gradient gel electrophoresis community fingerprints of 16S rRNA gene fragments obtained for bulk soil (a) and strawberry rhizosphere (c) samples collected at three sampling sites (2002). The respective dendrograms generated by cluster analysis (UPGMA) of Pearson's similarity indices are shown (b, c). BS, Braunschweig; B, Berlin; R, Rostock.

Table 2. Significant values (*P* values) of pairwise comparisons among rhizosphere and soil samples performed for actinobacterial and fungal denaturing gradient gel electrophoresis community fingerprints

	Braunschweig			Berlin			Rostock		
Season	Str. vs. Soil	Oils. vs. Soil	Str. vs. Oils.	Str. vs. Soil	Oils. vs. Soil	Str. vs. Oils.	Str. vs. Soil	Oils. vs. Soil	Str. vs. Oils.
Actinobacteria									
2002	0.002	0.517	0.029	0.002	0.060	0.030	0.002	0.002	0.029
2003	0.001	0.006	0.029	0.001	0.012	0.030	0.002	0.004	0.029
Fungi									
2002	0.009	0.005	0.003	0.002	0.040	0.029	1.000	0.514	1.000
2003	0.002	0.018	0.030	0.001	0.002	0.080	0.002	0.006	0.029

P values indicate whether measures of dissimilarity among samples from different microenvironments are significant ($P < 0.05$).
Str., Strawberry rhizosphere; Oils., oilseed rape rhizosphere; Soil, bulk soil from the corresponding field.

(Table 2). In general terms, higher variability within replicates was observed in the fungal fingerprints mainly in the bulk soil during the first growing season (2002), impeding a clear detection of specific ribotypes that could possibly be enriched in the rhizosphere. Fungal patterns were clearer and more stable in the second growing season (2003), where the abundance of few ribotypes was obviously enhanced in the rhizosphere. Taken together, typical characteristics of the bacterial profiles, such as homogeneity among replicates, higher evenness of ribotypes in bulk soil than in the rhizosphere, and increasing abundance of specific ribotypes in the rhizosphere, could also be detected, although to a lower extent, in the fungal community fingerprints of the second growing season.

Plant-dependent community structure

The rhizospheres of oilseed rape and strawberry grown at the same sampling site clearly selected different soil bacterial ribotypes (Figs 1a and c). This aspect was observed not only in the universal bacterial fingerprints but also in the actinobacterial, alphaproteobacterial and betaproteobacterial profiles at all sampling sites and in both years. Thus, each plant species was found to display its particular microbial community DGGE profiles, independent of the bacterial group investigated, with replicates of each rhizosphere forming well delineated clusters and differing significantly from each other according to pairwise comparisons (data not shown). As mentioned above, the actinobacterial community profiles in the oilseed rape rhizosphere were similar to those displayed by the bulk soil (Braunschweig and Berlin, first growing season). However, both rhizospheres differed from each other even in these cases, since microbial community composition in the strawberry rhizosphere differed markedly from those of the oilseed rape rhizosphere and bulk soil samples (Table 2). Pairwise comparisons revealed a similar trend for the fungal fingerprints in four of six cases (Table 2), indicating plant-dependent composition of fungal communities in the rhizosphere. No differences were found between oilseed rape and strawberry fungal profiles at the Rostock site in 2002 and at the Berlin site in 2003 (Table 2).

Site-dependent community structure

Soils from Braunschweig, Berlin and Rostock harbour different microfloras (Figs 2a and b). Considering all taxa investigated in this work, unique bulk soil DGGE fingerprints were revealed for each sampling site. With the exception of one gel obtained for the fungi in the first year, where internal variability was high, the evidence that each location displays its particular microbial community composition was remarkable. On the other hand, rhizosphere samples collected from different sampling sites were quite frequently found to belong to the same group after cluster analysis. Nevertheless, this observation did not always indicate that these rhizosphere samples were more similar to each other than their corresponding bulk soil samples, according to the similarity values obtained by cluster analysis. In contrast, it probably reflects the absence of a clear trend. The only case in which cluster analysis clearly revealed a higher similarity among rhizosphere samples from different locations in comparison with the corresponding soil samples was observed for the actinobacterial fingerprints of the strawberry rhizosphere (Fig. 3) in both years. The same trend was found in the universal bacterial profiles of the strawberry rhizosphere, although not as pronounced (data not shown). Since cluster analysis led to unclear results, we performed pairwise comparisons combining results obtained for two matrices using a pair of gels to evaluate whether rhizosphere fingerprints from Braunschweig, Berlin and Rostock were more similar to each other than their corresponding bulk soil fingerprints. This strategy revealed that similarities among rhizosphere samples of a given plant species from different sampling sites were in general significantly higher than those observed for the corresponding bulk soils (data not shown). However, results obtained for the fungi were, again, different: strawberry rhizosphere profiles were more similar to each other than their corresponding soil samples ($P = 0.005$ in 2002 and $P = 0.0005$ in 2003), but this was not the case for the oilseed rape rhizosphere ($P = 0.22$ in 2002 and $P = 0.973$ in 2003).

Sequence analysis of dominant DGGE bands

The arrows in Figs 1 and 2 show the dominant bands that were extracted from DGGE gels and submitted to cloning and sequencing. Their tentative phylogenetic affiliations are shown in Table 3. Interestingly, bands 1 and 2 in Fig. 1a, which were found to be dominant in the bacterial profiles in both rhizosphere soils, were represented by more than one 16S rRNA gene sequence affiliation. Furthermore, some 16S rRNA gene affiliations found for the same band (1 or 2) in different rhizosphere profiles (r – oilseed rape and s – strawberry) were exclusive to each plant species (Table 3). For instance, two of the sequences obtained for the bands 1r and 2r (oilseed rape) were affiliated with *Arthrobacter* sp., but we did not obtain any similar sequence for their corresponding strawberry bands 1s and 2s. Similarly, phylogenetic affiliations related to *Streptomyces* sp. were found for sequences re-amplified from bands 1s and 2s, but not for their corresponding oilseed rape bands 1r and 2r. Although bands 1 and 2 were extracted from DGGE bacterial profiles obtained for the sampling site Rostock, ribotypes with the same electrophoretic mobilities were detected in the strawberry and oilseed rape rhizosphere bacterial profiles of the

Table 3. Tentative phylogenetic affiliation of partial 16S rRNA gene sequences (regions V6 to V8) derived from dominant denaturing gradient gel electrophoresis bands

					Closest phylogenetic relatives		
Band	Origin*	ARDRA†	Clone‡	Length (bp)	Identity/strain	Accession no.§	%
1r	Rostock	4	1r 1 [AY920471]	394	*Arthrobacter* sp. SB	[AY327445]	100
	Oilseed		1r 6 [AY920472]	396	*Arthrobacter* sp. SN16A	[AB024412]	99
	Bacteria		1r 17 [AY920473]	392	*Rhizobium* sp. PRF241	[AY117665]	100
			1r 24 [AY920474]	395	Uncultured bacterium	[AY212714]	99
1s	Rostock	5	1s 1 [AY921580]	392	*Rhizobium mongolense* S110	[AY509212]	100
	Strawberry		1s 2 [AY921581]	392	*Rhizobium* sp. ORS1407	[AY500263]	97
	Bacteria		1s 8 [AY921582]	400	*Streptomyces bicolor* ISP 5140	[AJ276569]	97
			1s 9 [AY921583]	386	Uncultured Verrucomicrobia bacterium	[AY622244]	100
			1s 15 [AY921584]	404	*Streptomyces scabiei* PK-A41	[AY438566]	99
2r	Rostock	4	2r 7 [AY921585]	396	Alphaproteobacterium AP-16	[AY14553]	98
	Oilseed		2r 9 [AY921586]	394	Uncultured gamma-proteobacterium	[AJ532711]	93
	Bacteria		2r 10 [AY921587]	394	*Arthrobacter* sp. An5	[AJ560624]	99
			2r 20 [AY921588]	393	Uncultured Verrucomicrobium DEV005	[AJ401105]	99
2s	Rostock	2	2s 11 [AY921589]	393	Uncultured *Sphingomonas* sp. M8	[AF312671]	99
	Strawberry		2s 24 [AY921590]	400	*Streptomyces* sp.Y70013	[AY623798]	100
	Bacteria						
3	Rostock	1	3-1 [AY921591]	393	*Nocardia carnea* ATCC 6847T	[X80602]	100
	Strawberry						
	Bacteria						
4	Rostock	1	4-1 [AY921592]	399	Bacterium Ellin5012 (actinobacterium)	[AY234429]	94
	Strawberry						
	Bacteria						
5	Rostock	3	5-24 [AY921593]	389	Uncultured soil bacterium clone S1133	[AY622261]	100
	Soil		5-27 [AY921594]	389	Uncultured betaproteobacterium	[AB047127]	96
	Beta		5-34 [AY921595]	389	Uncultured eubacterium ONG1	[AF507756]	99
6	Rostock	2	6-3 [AY921596]	393	Uncultured *Variovorax* sp. clone 83-5	[AF526937]	99
	Oilseed Beta		6-20 [AY921597]	393	Uncultured eubacterium WD2115	[AJ292627]	99
7	Rostock	2	7-3 [AY921598]	393	Uncultured *Variovorax* sp. clone 9-13	[AY755406]	99
	Oilseed		7-9 [AY921599]	389	Uncultured betaproteobacterium clone S-G30 16S	[AY622269]	98
	Beta						
8	Braunsch.	1	8-1 [AY921600]	393	Uncultured alphaproteobacterium	[AJ318111]	97
	Soil						
	Alpha						
9	Braunsch.	2	9-1 [AY921601]	391	Uncultured alphaproteobacterium Kmlps6-15	[AF289910]	97
	Soil		9-18 [AY921603]	396	*Mesorhizobium loti* LMG 6125	[X67229]	99
	Alpha						
10	Braunsch.	3	10-5 [AY921604]	370	*Asticcacaulis* sp.T3-B7	[AY500141]	95
	Soil		10-11 [AY921605]	370	*Asticcacaulis* sp. T3-B7	[AY500141]	95
	Alpha		10-23 [AY921606]	370	*Asticcacaulis* sp. T3-B7	[AY500141]	95

*Indicates sampling site, environment (oilseed rape rhizosphere, strawberry rhizosphere or bulk soil) and group-specific denaturing gradient gel electrophoresis fingerprint (*Bacteria, Alphaproteobacteria* or *Betaproteobacteria*).

†Number of different amplified ribosomal DNA restriction analysis profiles observed among clones with the correct insert obtained from the same denaturing gradient gel electrophoresis band.

‡GenBank sequence accession numbers of the respective clones are given in brackets.

§GenBank sequence accession number of most closely related bacterial sequence.

other two sites, with the exception of band type 2r (oilseed rape profiles), which was not detected in the bacterial profiles of the Berlin site. Phylogenetic affiliations obtained for bands 3 and 4 (Fig. 1a), which were enriched in the strawberry rhizosphere and were also detected in the strawberry bacterial profiles of the Braunschweig and Berlin sites, were assigned to the Actinobacteria (Table 3). Tentative affiliation of the clones obtained for bands 6 and 7 (Fig. 1c), which appear exclusively in the betaproteobacterial profiles of the oilseed rape rhizosphere, revealed two different sequences (one for each band) phylogenetically related to *Variovorax* sp. (Table 3). *Mesorhizobium loti* and *Asticcacaulis* sp. were among the closest sequence affiliations obtained from dominant bands that are characteristic of

the soil alphaproteobacterial DGGE profiles from Braunschweig. Clones 10-5, 10-11 and 10-23 were all affiliated with *Asticcacaulis* sp. T3-B7. Alignment of the sequences revealed that they differed from each other due to only one base identity.

Discussion

The rhizosphere is a dynamic environment whose distribution of resources varies in space and time (Yang & Crowley, 2000). The composition of root exudates was shown to vary depending on the plant species and the stage of plant development (Jaeger *et al.*, 1999). Plants provide a variety of specific carbon and energy sources, and different compositions of root exudates are supposed to influence microbial populations in a specific manner. The plant-dependent enrichment of 16S rRNA gene ribotypes (Smalla *et al.*, 2001) and the selection of bacteria antagonistic to *Verticillium dahliae* (Berg *et al.*, 2002) in the rhizosphere of strawberry, oilseed rape and potato plants have been previously shown in one sampling site (Braunschweig). In the present study, we substantially extended the current body of knowledge by assessing the structure of five different microbial guilds in bulk soil and in the rhizospheres of strawberry and oilseed rape at three locations. DGGE fingerprints of 16S and 18S rRNA genes were generated to investigate to what extent microbial community structure in the rhizosphere is influenced by plant type and location.

Rhizosphere effect and plant-dependent community structure

Denaturing gradient gel electrophoresis fingerprints obtained for the bacterial groups analysed showed that, regardless of the sampling site, rhizospheres and bulk soils harboured microbial communities differing in the relative abundance of ribotypes (rhizosphere effect) and that the increased abundance of certain microbial populations in the root vicinities is plant-species-dependent. The rhizosphere effect of strawberry on the actinobacterial community structure was the most striking one observed among the various microbial groups assessed. On the other hand, no rhizosphere effect was detected in the actinobacterial profiles of oilseed rape in the first season at the Braunschweig and Berlin sites. Data presented here support the idea that the extent to which the plant influences community composition and structure in the rhizosphere may be different depending not only on the plant species, as previously shown by other reports (Germida *et al.*, 1998; Smalla *et al.*, 2001), but also on the microbial group being investigated.

In contrast to bacteria, the plant-dependent enrichment of fungal populations in rhizosphere soils has not yet been extensively studied, despite the importance of fungi to soil fertility and functioning. Recently, cultivation-independent fingerprinting methods have been developed and applied to characterize fungal communities in soil matrices (Kowalchuk *et al.*, 1997; Smit *et al.*, 1999; van Elsas *et al.*, 2000; Ranjard *et al.*, 2001; Klamer *et al.*, 2002; Gomes *et al.*, 2003; Edel-Hermann *et al.*, 2004; Oros-Sichler *et al.*, in press). DGGE fingerprints of PCR-amplified 18S rRNA gene fragments were applied in this study to determine the effect of plant species and site on the structure of fungal communities in the rhizosphere and bulk soil. Owing to the high variability observed among replicates of fungal fingerprints mainly in the first season, it was in some cases difficult to identify ribotypes with increased abundance in the rhizosphere fingerprints. Nevertheless, overall our results indicated that the rhizosphere effect and plant-dependent diversity were also detected for the fungi, although they were less pronounced than observed for the bacterial groups. Significant differences between strawberry and oilseed rape rhizosphere fungal profiles from the same sampling site were detected in four of the six cases analysed (Table 2). Dominant plant-specific ribotypes in the rhizosphere profiles were more frequently detected in the second season. Less variability of the 18S rRNA gene fragment fingerprints and a stronger rhizosphere effect was observed for fungal communities in the rhizosphere of maize grown in Brazil (Gomes *et al.*, 2003). However, several other studies reported on a high variability between replicates of fungal fingerprints (Klamer *et al.*, 2002; Girvan *et al.*, 2004; Oros-Sichler *et al.*, in press). The reason for this variability might be that fungi were more heterogeneously distributed than bacteria. Furthermore, we suspect that low fungal DNA template amounts in PCR mix might contribute to this variability. Based on the analysis of morphotypes isolated from the same set of samples, Berg *et al.* (2005b) also detected plant- and soil-dependent composition and genotypic diversity of fungi antagonistic to *Verticillium dahliae*. The diversity of fungal antagonists in the rhizosphere was lower than in bulk soil for all three sites, suggesting that the relative abundance of some antagonists was increased in the rhizosphere (Berg *et al.*, 2005b).

Site-dependent community structure

Considering the limited geographical scale embraced in our study, plant type could possibly influence the microbial community structure of the rhizosphere to a larger extent than sampling site. If so, rhizosphere samples collected from different sites would display higher levels of similarity to each other than their soil counterparts. Such a 'convergence of DGGE profiles' induced by the plant root was only evident in the bacterial and, more strongly, in the actinobacterial community fingerprints of the strawberry rhizosphere, indicating that, in these cases, plant roots played a

more important role than sampling site in microbial community structure in the rhizosphere. The actinobacteria may thus be the principal microbial group responsible for the high similarity observed in the universal bacterial profiles of the strawberry rhizospheres sampled in Braunschweig, Berlin and Rostock. Although tests of significance indicated significant differences for the other bacterial groups as well, with rhizosphere fingerprints being considered more similar to each other than soil profiles, we did not clearly identify specific ribotypes that were selected in all three locations by the same plant. It seems, here, that both factors, i.e. plant type and sampling site, act together in determining microbial composition in the rhizosphere. It is important to emphasize that what is referred to as 'sampling site' comprises a range of environmental and biotic factors, such as soil structure and physicochemical parameters, nutrient availability, organic matter content, local climatic conditions, crop and land-use history and management. All these factors have been shown to play a role in soil community dynamics (Latour *et al.*, 1996; Horwath *et al.*, 1998; Lupwayi *et al.*, 1998; Marschner *et al.*, 2001; Sessitsch *et al.*, 2001; Schönfeld *et al.*, 2002; Garbeva *et al.*, 2004a; Salles *et al.*, 2004) and may act simultaneously in determining the composition of the indigenous soil microflora, which is, in its turn, the source of organisms that will take part in the process of root colonization, persistence and survival. Rhizodeposition is affected by multiple factors such as light intensity, temperature, nutritional status, activity of retrieval mechanisms and stress factors (Neumann & Römheld, 2001), suggesting that a given plant genotype does not necessarily display the same exudation patterns under different environmental conditions. In addition, microbial activity leads to quantitative and qualitative alterations of root exudate composition as a result of degradation of exudates and the release of microbial metabolites (Neumann & Römheld, 2001). The presence of microbial metabolites influences root exudation (Brimecombe *et al.*, 2001), suggesting that different indigenous soil microbial communities, as observed for the three sampling sites, could possibly lead to differentiated patterns of exudation release or at least influence this process to a certain extent. Previous studies indicated that the soil type, instead of the plant species or cultivar, had the greatest impact on the rhizosphere microflora (Groffman *et al.*, 1996; Horwath *et al.*, 1998; da Silva *et al.*, 2003). However, the plant species was found to be the determinant factor in other reports (Germida *et al.*, 1998; Wieland *et al.*, 2001). Berg *et al.* (2005b) retrieved a higher proportion of fungi antagonistic to *Verticillium dahliae* from the strawberry rhizosphere in comparison to the oilseed rape rhizosphere, and a clear influence of the sampling site was found, with dominant antagonists varying from one place to another. Marschner *et al.* (2001) proposed that a complex interaction between soil type, plant species and root-zone location affects the bacterial community composition, the strength of each factor varying from case to case. Garbeva *et al.* (2004b) suggested that the microbial group under investigation would also interfere in the relative strength of the various forces shaping microbial communities in soil and in the rhizosphere, as observed in the present study.

Sequence analysis of dominant DGGE bands

Cloning and sequencing of 16S rRNA gene fragments re-amplified from bands of bacterial profiles revealed that phylogenetically non-related organisms can share the same electrophoretic mobility in DGGE gels, indicating that various different ribotypes can be hidden behind one DGGE band. Similar observations were made by Smalla *et al.* (2001) and by Schmalenberger *et al.* (2003). In this work, the melting behaviour of all clones obtained from a given band was carefully checked on DGGE gels. Only clones that matched the electrophoretic mobility of the original DGGE band were analysed further. The fact that different and in some cases exclusive taxonomic affiliations were found for bands sharing the same positions, but originating from the rhizosphere of different plant species, suggests that plant-dependent composition of microbial communities might be stronger than indicated by DGGE fingerprints. We revealed that, although the same band types 1 and 2 (a double band) were enriched in the rhizospheres of strawberry and oilseed rape, they did not represent similar taxonomic assemblages. Although ribotypes with the electrophoretic mobilities of bands 1 and 2 could be detected in the profiles of all sites, only bands from the profiles of the Rostock site were excised, re-amplified, cloned and sequenced. Despite the pitfalls of PCR-based rRNA analysis (von Wintzingerode *et al.*, 1997), DGGE profiling of rhizosphere and bulk soil microbial communities proved to be a powerful method for the cultivation-independent analysis of large numbers of samples. We employed group-specific PCR-DGGE systems for the analysis of bacterial groups such as Alphaproteobacteria, Betaproteobacteria and Actinobacteria. This procedure allowed the detection of less abundant ribotypes that were not evident in the universal profiles, enhancing the level of resolution of the PCR-DGGE technique. Evaluating universal and group-specific fingerprints simultaneously resulted in a more comprehensive approach to studying microbial community dynamics in the rhizosphere by investigating how the structure of different microbial guilds is influenced by plant type and sampling site. Recently, new primer systems have been developed for the fingerprinting of other important, and even narrower, bacterial groups, such as *Burkholderia* spp. (Salles *et al.*, 2004), *Pseudomonas* spp. (Garbeva *et al.*, 2004a), *Paenibacillus* spp. (da Silva *et al.*, 2003) and *Bacillus* spp. (Garbeva *et al.*, 2003). The targetting

of such bacterial groups, often involved in mechanisms of antagonistic activity in the rhizosphere, by molecular tools is a promising approach to establishing a proper link between microbial community structure and function in the rhizosphere.

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