

RESEARCH PAPER

Influence of *Arabidopsis thaliana* accessions on rhizobacterial communities and natural variation in root exudates

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Abstract

Plant species is considered to be one of the most important factors in shaping rhizobacterial communities, but specific plant–microbe interactions in the rhizosphere are still not fully understood. *Arabidopsis thaliana*, for which a large number of naturally occurring ecotype accessions exist, lacks mycorrhizal associations and is hence an ideal model for rhizobacterial studies. Eight *Arabidopsis* accessions were found to exert a marked selective influence on bacteria associated with their roots, as determined by terminal-restriction fragment length polymorphism (T-RFLP) and ribosomal intergenic spacer analysis (RISA). Community differences in species composition and relative abundance were both significant ($P < 0.001$). The eight distinct and reproducible accession-dependent community profiles also differed from control bulk soil. Root exudates of these variants were analysed by high performance liquid chromatography (HPLC) to try to establish whether the unique rhizobacterial assemblages among accessions could be attributed to plant-regulated chemical changes in the rhizosphere. Natural variation in root exudation patterns was clearly exhibited, suggesting that differences in exudation patterns among accessions could be influencing bacterial assemblages. Other factors such as root system architecture are also probably involved. Finally, to investigate the *Arabidopsis* rhizosphere further, the phylogenetic diversity of rhizobacteria from accession Cvi-0 is described.

Key words: *Arabidopsis* accessions, multi-dimensional scaling, natural variation, rhizobacterial communities, rhizosphere, root exudates.

Introduction

Soil is a complex habitat, characterized by a vast array of carbon sources, spatial heterogeneity, and physicochemical gradients that provide an intricate mosaic of microniches for exploitation by diverse micro-organisms. The colonization of soil by plants adds another level of complexity to the soil environment. As roots penetrate soil to form the rhizosphere, they not only alter soil structure, improve aeration, and provide a surface area for microbial attachment, but also provide nutrients via the secretion of exudates (Rovira, 1969; Keith *et al.*, 1986; Lynch, 1990) and root decomposition and turnover (Swinnen *et al.*, 1994). While microbial activity is enhanced in the rhizosphere (Söderberg and Bååth, 1998; Ramos *et al.*, 2000),

there is evidence that the interaction between plants and microbes is bi-directional. Micro-organisms can stimulate rhizodeposition and increase exudate turnover rates (Kuzyakov *et al.*, 2003). In turn, it has been shown that inoculation of *Arabidopsis* (*Arabidopsis thaliana*) roots with non-pathogenic strains of *Pseudomonas syringae* induces the plants to exude more low-molecular mass compounds (Bais *et al.*, 2005). More specifically, *Arabidopsis NahG* transgenics, which produce catechol as a catabolite of salicylic acid, have been shown to be defective in their resistance to the pathogen *Pseudomonas syringae* pv. *phaseolicola* NPS3121 (van Wees and Glazebrook, 2003) and disrupted biofilm formation on roots in the plant growth-promoting

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rhizobacterium *Bacillus subtilis* FB17 (Rudrappa *et al.*, 2007). Although beneficial plant–microbe interactions have been the focus of rhizosphere research for many years (Barea *et al.*, 2005), more recent ideas that rhizosphere micro-organisms are possibly involved in resource partitioning in soil, leading to differentiation of plant niches and maintenance of plant diversity (Reynolds *et al.*, 2003), as well as affecting plant competition and biomass (Bever, 2003; Bonkowski and Roy, 2005), have elicited new interest in rhizosphere biology.

The selective influence of plant species on bacteria associated with their roots is regarded as being of major importance in shaping microbial communities in the rhizosphere (Grayston *et al.*, 1998; Miethling *et al.*, 2000; Sharma *et al.*, 2005). Mazzola *et al.* (2004) showed that different wheat cultivars supported larger populations of specific *P. fluorescens* strains. This phenomenon has been linked to plant genetic make-up (Neal *et al.*, 1973), possibly through differences in root exudation (Baudoin *et al.*, 2003). Specific exudate-dependent plant–bacterial interactions have been identified in transgenic plants engineered in exudate synthesis, where the growth of *P. putida* was enhanced in a direct response to the stimulated secretion of phenylpropanoid compounds (Narasimhan *et al.*, 2003). There is still little evidence, however, to explain how different plant taxa mediate a differential rhizosphere influence to give rise to distinct rhizobacterial communities.

Significant advances in plant exudate chemistry have been made in the last few years. Secondary metabolites (Walker *et al.*, 2003) and volatile organic compounds (Steeghs *et al.*, 2004) in *Arabidopsis*, mucilage in pea (Knee *et al.*, 2001), and allelochemicals in a number of plants (Weir *et al.*, 2004), have been analysed, but a complete description of the full complement of exudate constituents from any one species remains a challenge, in part, due to limitations in analytical approaches. Exudate production and composition vary with plant taxa (Warembourg *et al.*, 2003), even among closely related species grown under the same conditions (Czarnota *et al.*, 2003). To date, very limited comparative systematic profiling of root exudates has been carried out. Fan *et al.* (2001) provided comprehensive profiles of root exudate components of wheat, barley, and rice plants, revealing distinct profiles for each species, however, a dearth of knowledge regarding differences in exudate activity in diverse plants and among various genotypes of the same plant species persists. Root exudate chemistry remains a neglected area of study in root biology.

To advance our understanding of the rhizosphere further and to discover why rhizobacterial communities are influenced by plant genotype, the role of root exudates in shaping microbial communities was investigated using the genetic plant model *Arabidopsis thaliana*. The vast majority of plants form symbioses with mycorrhizal fungi, which have been shown to have a great impact on resident bacterial assemblages by competing for the same exudate resources, supplying their own exudates, and serving as direct nutrient sources (Boer *et al.*, 2005). *Arabidopsis* roots are not colonized by mycorrhiza, making it an ideal plant

model for studying a true rhizosphere effect on bacterial communities, without the added complexity of fungal co-habitation. Our approach involved the use of various ecotype accessions of *Arabidopsis*. These accessions are natural variants collected from widely separated geographical areas and hence should reflect genetic adaptation to local environmental conditions (Koornneef *et al.*, 2004). Since rhizobacteria can be modulated by exudates, the production of which is, in turn, genetically regulated by plants, it is hypothesized that natural plant genotypic variants of a single species would select for specific microbial consortia as a result of their unique exudate profiles. Our first aim was to ascertain that bacterial selectivity occurred at this level of plant genetic variation. Secondly, there was a need to determine whether phenotypic differences in root exudate activity exist among *Arabidopsis* accessions. Two culture-independent approaches, terminal-restriction fragment length polymorphism (T-RFLP) analysis and ribosomal intergenic spacer analysis (RISA) were used to study microbial communities in soil. Root exudates from the eight accessions were also collected for analysis by high performance liquid chromatography (HPLC). A third objective of this study was to explore the rhizobacterial community of the *Arabidopsis* accession Cvi-0, which, to our knowledge, is a first description of the phylogenetic diversity of the *Arabidopsis* rhizosphere.

Materials and methods

Arabidopsis thaliana accessions and bacterial samples

A loam soil, composed of 44% sand, 49% silt, and 7% clay, was collected from a fallow, experimental, agricultural site, not recently exposed to pesticides or herbicides, at the Center for Agricultural Research, (University of Massachusetts) in Waltham, MA and stored in the dark at 4 °C. The loam had a pH of 5.7, a high (9.8%) organic matter content, and low (4 µg g⁻¹ soil) nitrate content. Eight different *Arabidopsis thaliana* accessions, C24 (C24; Portugal), Columbia (Col-0; Germany), Cape Verde Islands (Cvi-0; Cape Verde), Landsberg *erecta* (Ler; Poland), Nossen (NO-0; Germany), Playa de Aro (Pla-0; Spain), Rld (Rld-1; Russia) and Wassilewskija (Ws; Ukraine), were obtained from the *Arabidopsis* Biological Resource Center (ABRC), Columbus, OH. Seeds were sterilized by imbibing in sterile water with a drop of Tween for 30 min at room temperature, then soaked in 30% bleach with constant shaking for 10 min and thoroughly rinsed with sterile water. Seeds were suspended in sterile 0.1% phytoagar and allowed to imbibe at 4 °C in the dark for 48 h prior to plating on to sterile, half-strength Murashige and Skoog (MS) medium containing 0.9% phytoagar and supplemented with 1.5% sucrose. Plants were grown in square Petri dishes positioned vertically, allowing seedlings to grow on the surface of the medium. After 2 weeks of growth at 22 °C under short-day conditions (12 h of light), in an incubator (Percival

Scientific, Inc., Perry, Iowa), five seedlings from each accession were transplanted into soil. To minimize injury and physiological changes, seedlings were gently lifted off with sterile forceps from under the cotyledons and transferred to individual pots containing a mixture of 10 g of loam and 35 g of sterilized, commercial potting compost. This blend had improved water drainage, more suited for the cultivation of *Arabidopsis*. The soil mixture was prepared all at once for all pots and was thoroughly mixed prior to use to ensure homogeneity. Following transfer, the seedlings were kept covered for 2 d to keep the environment humid. Transplanted seedlings and pots containing control bulk soil were incubated at 22 °C for 12 h of light in a plant growth room kept under controlled conditions, and watered with sterile, deionized water until plants reached maturity. The position of the pots was switched around regularly, to ensure that all pots were subject to the same conditions. Rhizosphere soil was collected just prior to flowering, approximately 4 weeks after transplantation. For the purpose of this study, rhizosphere soil was operationally defined as soil that remained firmly adhered to plant roots, after the plant was uprooted and shaken. Soil was scraped off roots using sterile scalpel blades, collected into sterile Petri dishes and stored in sterile Eppendorf tubes. Samples of control bulk soil were also collected. All soil samples were stored at -20 °C until DNA was extracted using an UltraClean Soil DNA Kit Mega Prep (Mo Bio Laboratories, Inc., Carlsbad, CA) as recommended by the supplier. Genomic DNA was eluted in 10 mM TRIS and stored at -20 °C until further analysis.

T-RFLP with capillary electrophoresis

Genomic DNA isolated from soil samples (four replicates per accession, three replicates for control bulk soil) was subjected to PCR amplification with primers specific to the coding region of the Bacterial 16S rRNA gene. The forward primer (5'-AGA GTT TGA TCC TGG CTC AG-3') corresponded to the *Escherichia coli* region 8–27 (Edwards *et al.*, 1989) and was labelled with 6-carboxyfluorescein (6-FAM) at the 5'-end. The reverse primer (5'-TGA CGG GCG GTG TGT ACA AG-3') corresponded to the *E. coli* region 1389–1408 (this study). For each sample, ~10 ng of DNA were mixed in a final volume of 50 µl for PCR in a reaction mixture containing 1× PCR buffer, 1.5 mM MgCl₂, 0.8 mM dNTPs, 0.4 µM each of the forward and reverse primers, and 0.75 units of Platinum *Taq* DNA polymerase (Invitrogen Corp, Carlsbad, CA). Amplification involved an initial denaturing step of 95 °C for 3 min, followed by 30 cycles of denaturing at 94 °C for 1 min, annealing at 57 °C for 1 min, and extension at 72 °C for 1 min 45 s, with a final extension of 10 min. The entire volume of the PCR product was gel purified from 1% agarose gels using the QIAquick Gel Extraction Kit (Qiagen Inc., Valencia, CA) as recommended by the supplier and eluted in 50 µl of 10 mM TRIS-HCl (pH 8.5). Aliquots of 44 µl of DNA were digested with 20 units of the 4-base cutter *Msp* I in a reaction mixture containing 1× NE buffer

2 (New England BioLabs Inc., Beverly, MA) by incubating at 37 °C for 15 h. The enzyme was inactivated by incubating the mixture at 65 °C for 20 min and the products subsequently purified, desalted, and concentrated to ~10 µl in sterile deionized H₂O using Microcon centrifugal filter units YM-30 (Millipore Corp., Billerica, MA), as per the manufacturer's instructions. Subsequently, 3 µl of the concentrated DNA was added to 20 µl of hi-di formamide (Applied Biosystems, Foster City, CA) and 0.25 µl of internal standard MapMarker 1000 (Bioventures Inc., Murfreesboro, TN). This standard consists of single-stranded DNA fragments that produce peaks ranging from 50 to 1000 base pairs (bp) and was therefore suitable for the denaturing conditions of fragment analysis. The 6-FAM-labelled terminal-restriction fragments (T-RFs) were resolved on an ABI PRISM 3100-*Avant* Genetic Analyser (Applied Biosystems) with an injection time and voltage set at 60 s and 15 kV, respectively. All samples were analysed twice. Data were analysed by GeneMapper software v.3.5 (Applied Biosystems).

RISA with gel electrophoresis

Five replicates per accession and three replicates for control bulk soil were subjected to RISA. Primers used were specific to the Domain Bacteria, G1 (5'-GAA GTC GTA ACA AGG-3') and L1 (5'-CAA GGC ATC CAC CGT-3') (Jensen *et al.*, 1993), which amplify the intergenic spacer region (ISR) between the 16S and 23S subunits of the rRNA genes. PCR conditions were modified from Thompson *et al.* (2002), and designed to try and minimize heteroduplex and chimera formation. Approximately 10 ng of DNA were added to a mix of 1× PCR buffer, 2 mM MgCl₂, 0.8 mM dNTPs, 0.4 µM each of the forward and reverse primers, and 0.02 units of Ampli*Taq* Gold (Applied Biosystems, Roche Molecular Systems, Inc.) The reaction mixture was incubated in a T3 Thermocycler (Biometra) at 95 °C for 10 min, followed by 15 cycles of 94 °C for 15 s, 55 °C for 4 min, and 72 °C for 4 min, with a final extension step of 72 °C for 7 min. PCR was subsequently performed with 5 µl of this PCR product, in fresh reaction mixture and re-amplified under the same conditions for another 15 cycles. To ensure the elimination of any potential heteroduplexes that could still have formed during amplification, the final PCR products were treated with a T7 Endonuclease I (New England BioLabs) that digests single-stranded, but not double-stranded DNA, as described in Lowell and Klein (2000). Samples were incubated at 37 °C for 2 h with 1× NE buffer and 0.2 units of enzyme. The products were resolved on 5% polyacrylamide gels run for 2 h at 105 V in 1× TBE and stained with SYBR Gold Nucleic Acid Stain (Molecular Probes, Inc., Eugene, OR) diluted to 1/10,000 in 1× TBE, by constant shaking at room temperature for 30 min, as recommended by the supplier.

Preparation of exudate samples

Exudates from the eight different *Arabidopsis* accessions were analysed by HPLC. Seeds were sterilized as previously

described and plated on half-strength MS supplemented with 1.5% sucrose. After 7 d, germinated seedlings were transferred to 6-well plates containing liquid 0.5× MS+1.5% sucrose. Two seedlings were grown in each well, in 8 ml of MS, in three replicate plates per accession. The plates were incubated at 22 °C under long-day conditions (16 h light) and continuously shaken at 100 rpm. After 21 d of growth, the liquid media containing root exudates were collected. Exudates from 12 plants were pooled together to obtain three exudate samples for each accession. The exudates were filtered with 25 mm diameter, 0.22 µm HPLC grade nylon syringe filters to remove debris (Corning Inc., Germany) and frozen at –80 °C. The frozen samples were lyophilized (Edwards Pirani 501 Super Modulyo) and reconstituted in 100 µl of MilliQ water prior to analysis by HPLC.

Exudate analysis by HPLC

A volume of 20 µl of each exudate sample was injected into an Agilent 1100 Series HPLC system (Agilent Technologies, Inc., Santa Clara, CA). Exudate compounds were resolved on a 4.6 mm×150 mm reverse phase Zorbax Eclipse XBD C₁₈ column (Agilent Technologies, Inc., Santa Clara, CA) by gradient elution, using water (solvent A) and acetonitrile+0.1% formic acid (solvent B), set up as follows: 5 min with 5% solvent B, 60 min with 60% solvent B, 65 min with 5% solvent B. The flow rate was set at 1 ml min⁻¹ and the temperature at 40 °C. Compounds were detected at 280 nm and 310 nm with a UV-Vis detector (Agilent Technologies, Inc., Santa Clara, CA).

Data transformation and statistical analysis

Data generated by GeneMapper v.3.5 included peaks measured in base pairs and the area of each peak. Only T-RF peaks falling between 50–750 bp were considered, as peak detection beyond this range appeared to have an accuracy of less than 0.5 of a base pair. Area data from each profile were standardized by calculating the percentage area of each peak relative to the total area of all peaks within that sample. A cut-off of 0.05% relative area was adopted, to eliminate background fluorescence from the data set. Relative area data from replicates for each sample were manually aligned according to their corresponding peak size. Only peaks that were detected in both runs were included in the analysis, for which averages were calculated. Data from all samples were then manually aligned to ensure accurate peak binning. T-RFs that were missing from a sample were assigned a value of zero. The resultant aligned relative area data matrix was subjected to statistical analysis.

Bacterial community profiles obtained by RISA were visually analysed on 5% polyacrylamide gels using a 50 bp DNA step ladder (Promega Corp., Madison, WI) and a 100 bp ladder (New England BioLabs) for the calibration of band sizes. For each sample, a string of binaries, where '1' denotes the presence and '0' the absence of a band on a scale

of fragment size graduated at 25 bp intervals, was generated. These data were directly amenable to statistical analysis.

Data from HPLC analysis of *Arabidopsis* exudates were captured by Chemstation for LC Systems Rev. B.01.03 [204]. Retention times in minutes and peak height in mAU were tabulated and relative peak heights were calculated for each sample. The 30 most prominent peaks were considered for further analysis. Data detected at wavelengths of 280 nm and 310 nm were analysed, but only data from 280 nm are shown here, since the results were similar. Relative peak heights were aligned according to their retention times, and this data matrix was subjected to multivariate statistical analysis.

Data from T-RFLP, RISA, and HPLC were imported into PRIMER 5 (Plymouth Routines in Multivariate Ecological Research - version 5.2.9) from PRIMER-E Ltd., Plymouth, UK, a statistical software package for the analysis of ecological, multivariate data. By taking individual T-RF sizes from T-RFLP and ISR sizes from RISA to be separate species or OTUs, similarity matrices for the bacterial community profiles were constructed by calculating similarities between each pair of samples using the Bray–Curtis coefficient S :

$$S_{jk} = 100 \left\{ 1 - \frac{\sum_{i=1}^p |y_{ij} - y_{ik}|}{\sum_{i=1}^p (y_{ij} + y_{ik})} \right\}$$

where y_{ij} is the abundance or presence/absence value for the i th T-RF size in the j th sample. The Bray–Curtis coefficient is particularly suitable for biological community data as explained by Clarke and Warwick (2001) and has been used for T-RFLP data of bacterial communities (Rees *et al.*, 2004). Two separate similarity matrices were calculated from T-RFLP data, one from presence–absence data and the other from square root ($\sqrt{}$) transformed relative area values. Root transformation down-weights the dominance of the most abundant T-RFs. Data from RISA generated only one similarity matrix from the presence–absence data. A $\sqrt{}$ -transformed matrix was constructed with relative peak heights from HPLC data.

To visualize the relationship among samples, the similarity matrices were analysed by Hierarchical Cluster Analysis (HCA), a classification method that aims to group samples into discrete clusters based on similarity. HCA was performed by a weighted, group-average linkage agglomerative method and dendrograms were constructed from the ranked similarities. The similarity matrices were also used in non-metric Multi-Dimensional Scaling (MDS), an ordination method that seeks to reveal possible relationships on a continuous scale in reduced space. Non-metric MDS is particularly suitable for ecological, multivariate data as, unlike other ordination methods such as principal component analysis, makes no assumptions about the distribution of the data. The MDS plots in two- and three dimensions were plotted to represent the relationship among samples. In an MDS plot, the actual location of each data point in space is arbitrary, and the axes can be rotated freely. It is the relationship of the data points to each other that is of

importance, with two near points representing more similarity to each other than to another point located at a distance. The stress of the plot is a measure of how much distortion was introduced to allow the representation of the data in the specified dimensions. A stress of 0 gives a perfect representation, while a measure of stress ≤ 0.2 indicates that the plot is a good representation of the data set and can be used for interpretation. A measure >0.3 indicates that the level of distortion that was required to display the data on the map is too high for any reliable inferences to be made from the configuration. In our analyses, MDS plots were generated from the best possible ordination following 100 random restarts.

For significance testing of sample data, the non-parametric permutation procedure ANOSIM (analysis of similarity), available in PRIMER, was employed. This test applies ranks to similarity matrices used for HCA and MDS and combines this ranking similarity with Monte Carlo randomization to generate significance levels (P values). ANOSIM tests the null hypothesis, for which a test statistic R will have a value of 0, that all samples are the same. As R approaches 1, the null hypothesis is rejected and this describes a case where replicates from one group are more similar to each other than to replicates from other groups.

16S rRNA gene clone library

A clone library was constructed from a pool of DNA taken from Cvi rhizosphere DNA samples used for T-RFLP and RISA. DNA was amplified using the same primers and conditions employed in T-RFLP, excluding the fluorescent probe. Amplicons were ligated into the pCR4-TOPO vector (Invitrogen, Carlsbad, CA) and products transformed into One Shot TOP10 chemically competent *E. coli* cells (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. Transformed cells were grown overnight at 37 °C and 82 single colonies were picked at random for restreaking. Isolated colonies were grown overnight in 1.5 ml liquid Terrific Broth (TB) with 50 $\mu\text{g ml}^{-1}$ kanamycin at 37 °C and 250 rpm. Plasmids were isolated using UltraClean Standard Mini Plasmid Prep Kit (Mo Bio Laboratories, Inc., Carlsbad, CA) as per the manufacturer's instructions. Plasmids were sequenced at the Dana-Farber/Harvard Cancer Center DNA Resource Core (Cambridge, MA). Sequencing was done in one direction only using the sequencing primer M13F (5'-GTA AAA CGA CGG CCA G-3') (Invitrogen, Carlsbad, CA), and when the amplicon in the vector was found to be in a reverse orientation, M13R (5'-CAG GAA ACA GCT ATG AC-3') (Invitrogen, Carlsbad, CA). Sequencing in one direction yielded 750 bp of high quality data that could be used for alignment with ClustalW (<http://www.ebi.ac.uk/Tools/clustalw/>). A sequence similarity value of 97% was used to define an OTU. The presence of chimeras in the dataset was checked by Mallard version 1.02 (<http://www.cardiff.ac.uk/biosi/research/biosoft/>) and any anomalous queries verified against identical sequences in Genbank using the Pintail program (<http://www.bioinformatics-toolkit.org/Web-Pintail/>). Con-

firmed chimeras were omitted from further analysis. Phylogenetic analysis of the aligned data was conducted with MEGA version 4 using the Jukes–Cantor method for calculating evolutionary distances and a phylogenetic tree was subsequently constructed using the Neighbor–Joining method with bootstrap analysis of 1000 replicates. The 16S rDNA sequences obtained were compared to sequences in GenBank and the Ribosomal Database Project II and sequences with the highest homology were incorporated into the phylogenetic tree.

Nucleotide sequence accession numbers

All clone sequences from the Cvi-0 rhizosphere sample have been deposited in the GenBank database under accession numbers FJ712828–FJ712886.

Results

T-RFLP analysis

T-RFLP analysis of triplicate control bulk soil, and eight rhizosphere soil samples of the different *Arabidopsis* accessions, carried out in replicates of four, generated bacterial community profiles of fluorescence from amplified DNA plotted against a T-RF size measured in base pairs. Examples of bacterial community profiles generated by the restriction enzyme *Msp* I appear in Fig. 1. In all, 215 distinct T-RFs were detected, ranging from 88–131 per sample. Peaks occurring at 72, 89, 146, 432, 461, 479, and 540 bp were among those detected in all samples with strong fluorescence signals. Table 1 shows the number of T-RFs detected from each community. T-RF diversity was lowest for the accessions *Ler* and C24 and for control bulk soil, and the highest for Rld. The number of T-RFs was significantly different for several accessions and control bulk soil, as revealed by ANOVA and Tukey's post-hoc comparisons (Table 1). Community structure was examined by plotting dominance curves, displaying the relative abundance of the detected T-RFs plotted against the T-RFs ranked in order of decreasing abundance on a log scale (see Supplementary Fig. S1 at *JXB* online).

The relationships among rhizobacterial communities of different accessions and bacterial communities from control bulk soil were assessed by HCA and MDS ordination. Both these statistical methods revealed distinct accession-specific groups of bacterial communities when relative abundance and presence–absence data obtained by T-RFLP analysis were used (Fig. 2, 3). The relative abundance data (Fig. 2A) clustered the community types more closely than the presence–absence data (Fig. 2B), with six of the nine bacterial community types forming type-specific branches. The MDS map configurations of relative abundance and presence–absence data, on the other hand, were comparable (Fig. 3). The stress of both MDS plots in Fig. 3 is at the limits of acceptable distortion to allow the community data to be reduced to two dimensions. While having a lower stress value of 0.13, the three-dimensional plots (data not

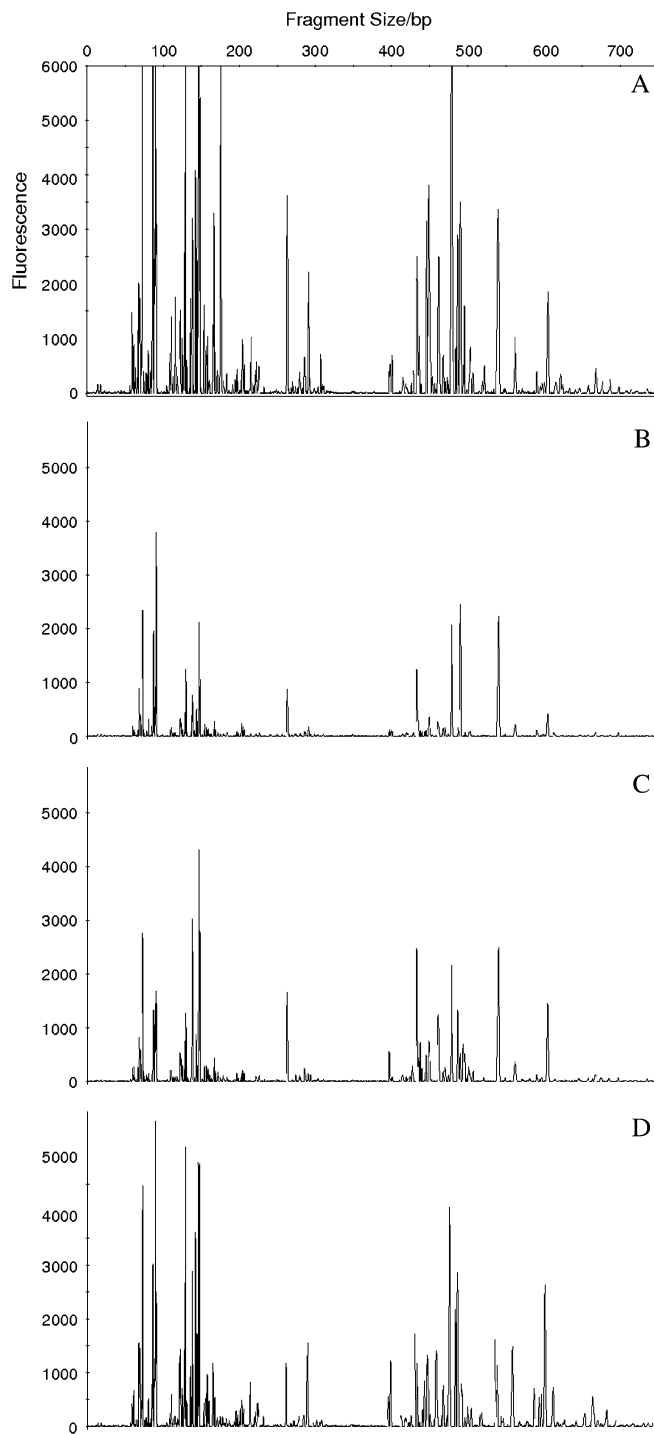


Fig. 1. Examples of *Msp* I T-RFLP electropherograms of bacterial community profiles from (A) control bulk soil and *Arabidopsis* accessions (B) C24, (C) Cvi-0 and (D) Rld-1.

shown), showed similar clustering to the two-dimensional maps. Furthermore, multiple restarts for two-dimensional MDS ordination always generated the same configuration, exhibiting confidence in the output. For ease of visualization therefore, two-dimensional maps are presented (Fig. 3).

Differences among the eight rhizosphere soils and control bulk soil bacterial communities were statistically analysed

Table 1. Average T-RF number for each community type sampled

Bacterial community type	Average T-RF no. ^a
Bulk (b)	98 ^{nw}
Rhizosphere	
C24 (c)	97 ^{nw}
Col (o)	108
Cvi (v)	110
Ler (l)	96 ^{nw}
No (n)	118 ^{bcd}
Pla (p)	109
Rld (r)	123 ^{bcd}
Ws (w)	117 ^{bcd}

^a Superscript letters, denoting community types in the first column, indicate significant differences between those types ($P < 0.05$), using Tukey's *post-hoc* comparisons after ANOVA revealed significant differences between some types.

using the non-parametric ANOSIM test of the null hypothesis that there are no differences among communities. The resultant global R statistics of 0.82 ($P = 0.001$) and 0.70 ($P = 0.001$) for relative abundance and presence–absence similarity matrices, respectively, strongly support the alternative hypothesis that bacterial communities from the same accession or from control bulk soil are more similar to each other than to communities of other types. The global R value indicates that there is a statistically significant difference between at least some community types. Pairwise comparisons revealed that all pairs, except for the Pla/Ws communities, were significantly different. For relative abundance data, 31 out of 36 possible paired comparisons gave an R value between 0.7–1.0 ($P < 0.03$), while for presence–absence data, 29 gave an R value between 0.6–1.0 ($P < 0.03$), indicating that the rhizobacterial communities from those accessions are significantly distinct.

RISA analysis

The RISA performed on control bulk and rhizosphere soil collected from the different accessions yielded community profiles of presence–absence data for 40 possible ISR sizes. While band intensities varied considerably within and among samples (data not shown), possibly indicating abundance differences among bacterial taxa, this was not taken into account. The dendrogram from HCA of presence–absence data displays a clear accession-specific grouping of rhizobacterial communities, with control bulk soil communities forming a separate branch to most of these (Fig. 4). Ordination of presence–absence RISA data produced MDS maps with obvious accession-dependent grouping as seen for T-RFLP data (data not shown). ANOSIM testing of the RISA profiles gave a global R statistic of 0.78 ($P = 0.001$). ANOSIM of pairs of groups gave statistically significant R values for all pairs, except for the Col/Pla rhizobacterial communities. The R value was greater than 0.5 ($P < 0.02$) for 34 of the 36 paired groups.

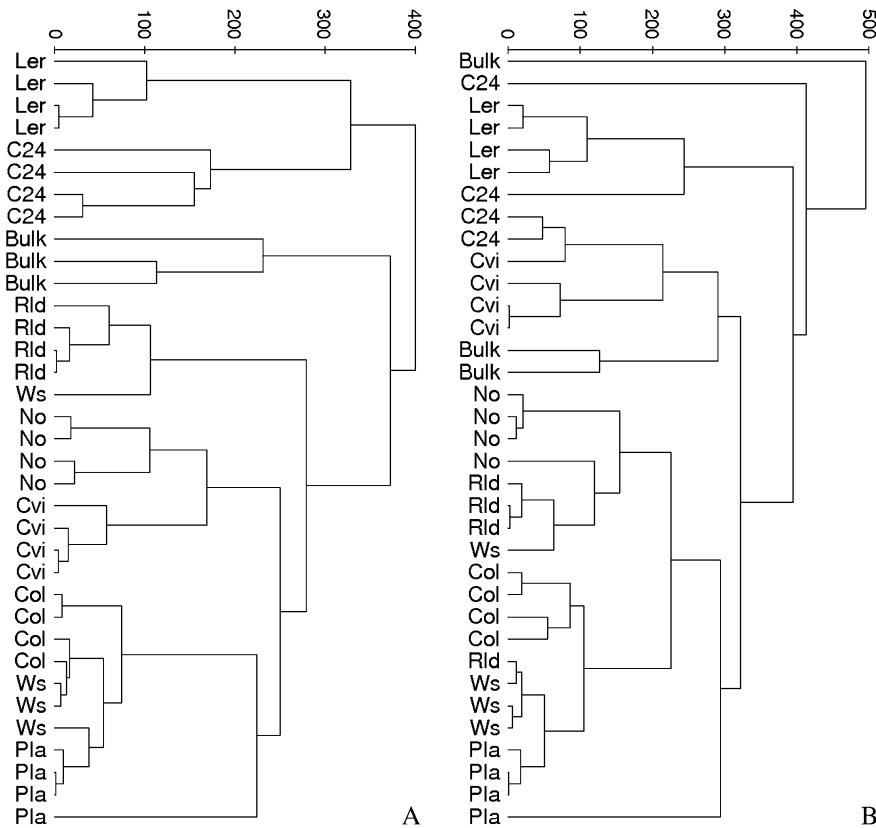


Fig. 2. Dendrograms generated by HCA of ranked similarities of (A) relative abundance data and (B) presence–absence data from T-RFLP profiles of rhizobacterial communities from eight *Arabidopsis* accessions and control bulk soil.

Hierarchical cluster analysis of rhizobacterial community profiles obtained by T-RFLP and RISA gave dendrograms with comparable topologies (Figs 2, 4). The ISR dendrogram is most similar to the T-RF dendrogram constructed from relative abundance data, although common features with the presence–absence T-RF dendrogram are also shared. Most of the Ws-Pla-Col rhizobacterial community replicates form a tight cluster in both T-RFLP dendrograms (Fig. 2A, B) and RISA (Fig. 4), although the relationship of the other rhizobacterial communities with this group vary. The Ler–C24 communities constitute a separate cluster in both T-RFLP relative abundance and RISA dendrograms. There was one Pla sample that fell as an outlier in all analyses, suggesting that this community was different from other Pla sample replicates. The outlying Col sample in RISA was the excluded replicate from the T-RFLP analysis. Control bulk soil communities also composed a separate group in Figs 2A, 3, 4, although the variation between this and other communities was not more pronounced.

Exudate analysis by HPLC

The HPLC analysis carried out on replicate samples of exudates collected from each *Arabidopsis* accession yielded highly reproducible chromatograms. Very distinct differences in exudate profiles were observed among accessions (Fig. 5). Using retention times and relative peak heights in the ANOSIM test gave a global *R* statistic of 0.96

($P=0.001$), providing very robust evidence that different *Arabidopsis* genotypes deposit a unique suite of exudates into the rhizosphere. The ANOSIM test of pairs of groups gave statistically significant *R* values for all pairs, with *R* values of 1 for all pairs except for C24/Col. For the latter, an *R* value of 0.85 was obtained. This difference in exudate profiles among the eight accessions was very clearly revealed by HCA (Fig. 6A). All accessions displayed tight clustering of replicate samples, indicating a strong genetic control of root exudation among *Arabidopsis* variants. C24 and Col appeared to be the most closely related in terms of exudate composition and relative amounts of compounds present. Pla was also most similar to these two accessions. Rld, Ws, and Cvi formed a second group, while Ler and No formed a third cluster. The MDS plot provided further support for accession-specific exudate profiles, as well as a means of graphically representing the relationship among accessions (Fig. 6B).

Phylogenetic diversity of the Cvi rhizosphere

A total of 82 clones from the Cvi rhizosphere were sequenced. Of these, nine were discarded due to a low quality read and eight were omitted from the analysis because of apparent anomalies when checked with the pintail program. Of the 65 remaining sequences, a total of 59 operational taxonomic units (OTUs) were assigned, using 97% identity as a definition of an OTU. Phylogenetic

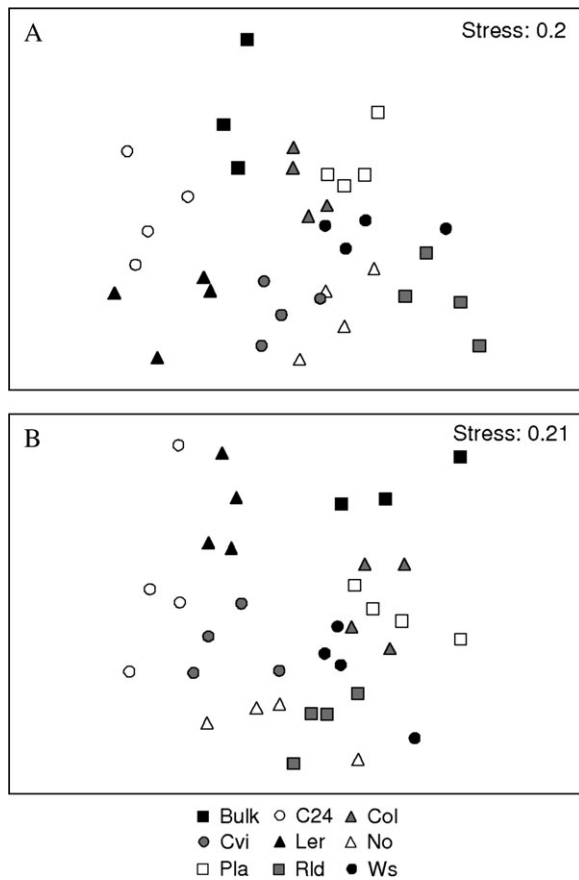


Fig. 3. Non-metric MDS plots of (A) relative abundance and (B) presence-absence data from T-RFLP profiles of bacterial communities from rhizosphere and control bulk soil.

analysis revealed that the clones represented nine different phyla (Table 2). The largest group was the α -Proteobacteria, comprising 27% of the library, of which 25% (almost 7% of the entire library) were from the order Rhizobiales. The second major phylum was Acidobacteria, making up 17% of all clones sequenced, followed by Bacteroidetes at 14%. A phylogenetic tree (Fig. 7) shows the evolutionary relationship among the partial 16S rDNA ribotypes detected, as calculated by the Jukes-Cantor method of estimating evolutionary distance and a Neighbor-Joining algorithm for tree assembly.

Discussion

As demonstrated by two independent methods of bacterial community fingerprinting, RISA and T-RFLP analysis, the *Arabidopsis* rhizosphere mediates a significant change in resident soil bacterial assemblages, relative to control bulk soil. Furthermore, each *Arabidopsis* accession tended to support a distinct and reproducible rhizobacterial community. The effect of different accessions is detectable both in bacterial community composition and the relative abundance of ribotypes present and implies that the development of a rhizobacterial community is a regulated event influenced by plant-related changes in soil parameters. This phenome-

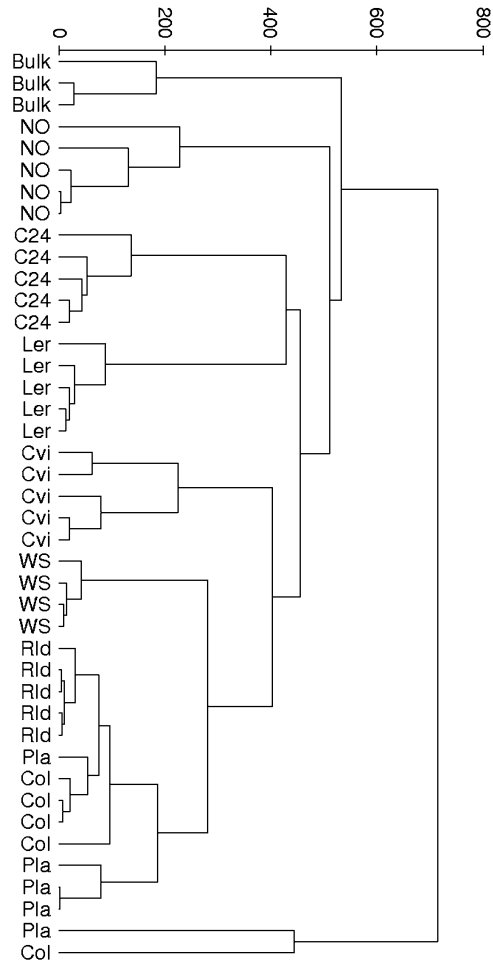


Fig. 4. Dendrogram generated by HCA for presence-absence data of rhizobacterial and soil bacterial community profiles obtained by RISA.

non is attributable to differences in plant genetic make-up among accessions. Although the impact of plant species on rhizobacterial communities has been well documented, bacterial selection among closely related variants of a single species has only previously been demonstrated for five wheat (Mazzola *et al.*, 2004) and two maize (Dalmastri, 1999) cultivars on populations of specific bacteria, *P. fluorescens* and *Burkholderia cepacia*, respectively. In the present study, an *Arabidopsis* accession-dependent influence on the entire rhizobacterial community has been revealed, providing further evidence of the importance of plant genotype in shaping rhizobacterial assemblages. This finding is significant, since, unlike wheat and maize, *Arabidopsis* lacks mycorrhizal associations, and thus exhibits a true genotype-specific impact on bacterial communities. Moreover, *Arabidopsis* is a powerful plant genetic tool and these accession-specific differences in rhizobacteria could provide the means to genetically characterize how plants and microbes communicate at the molecular level in the rhizosphere.

Profiling of root exudates of eight different *Arabidopsis* accessions showed that each accession releases a unique suite of compounds into its rhizosphere. This is the first report of natural variation in root exudates of *Arabidopsis*

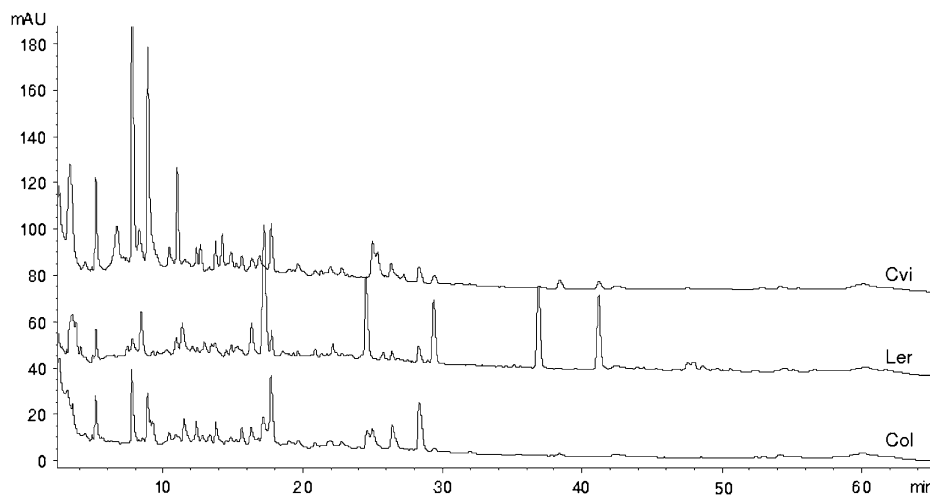


Fig. 5. Examples of chromatograms obtained by HPLC analysis of the *Arabidopsis* accessions Cvi, Ler and Col.

accessions. Exudate compounds varied both qualitatively and quantitatively among the different accessions studied (Fig. 5). Although accurate quantification of each exudate component was beyond the scope of this study, quantitative differences among accessions were obvious, and compounds that were predominantly detected in one accession, could be present in negligible amounts, or absent altogether, in others. Moreover, HPLC chromatograms were highly reproducible for each accession, implying that root exudation is tightly genetically regulated.

The natural variation in root exudation among such closely related plant variants could explain, at least in part, the genotypic influence on bacterial communities in the rhizosphere. Although a direct causal link between rhizobacteria and root-exuded phytochemicals could not be ascertained in the present study, different microbial groups have been shown to exploit specific exudate-derived carbon sources for growth (Paterson *et al.*, 2007). A specific example of this is the release of L-malic acid by *Arabidopsis* roots to select for the beneficial bacterium *Bacillus subtilis* FB17 (Rudrappa *et al.*, 2008). The unique set and varying amounts of exudate compounds derived from each *Arabidopsis* genotype studied could be influencing the resident microbes in a distinct manner, and future work will focus on establishing this connection.

Accession-specific differences in both rhizobacterial communities and root exudates were revealed, but no real correlation was evident between the relationships of these two processes. While this could simply be a product of the inherent limitations of the techniques employed, another plausible explanation could be the lack of a temporal component in the sampling method employed. Nonetheless, there are, undoubtedly, several plant-derived factors contributing to bacterial selection in the rhizosphere. Natural variation in other root traits, such as protein profiles from root extracts (Chevalier *et al.*, 2004) and regulation of root growth and root system architecture, have been reported (Loudet *et al.*, 2005). Furthermore, among the accessions used in this study, differences in root system architecture, including characteristics such as primary root length, degree

of lateral root formation and root hair density and length, were strongly evident (data not shown). An attempt at measuring these differences was not made because of the plasticity of root system architectural traits. However, a strong genetic element does exist in regulating these parameters (Osmont *et al.*, 2007). The number of growing root tips is of particular significance, since root exudation is enhanced in this region (Cardon and Gage, 2006). In addition, root border cells appear to play a crucial role in defining the rhizosphere ecology of several plant species (Hawes *et al.*, 2000, 2003). Although root border cells have not been identified for *Arabidopsis*, recent studies have shown that cell layers are released from root tips of *Arabidopsis* in an organized manner (Vicre *et al.*, 2005), rather than as isolated cells as described for other plant species (Hawes and Lin, 1990). These cells have been termed root border-like cells, since, like border cells, they have been implicated in plant–microbe interactions (Vicre *et al.*, 2005). Root system architecture, therefore, could have multiple effects on rhizobacterial growth, not only in terms of surface area availability for bacterial attachment, but also by determining patterns of rhizodeposition, encompassing both root exudation and sloughing of cells.

The bacterial communities of the *Arabidopsis* rhizosphere were further investigated by evaluating 65 16S rDNA sequences. Of these, 59 were considered to be unique ribotypes, representing a high diversity with members belonging to nine different phyla. The major group was the vastly diverse α -Proteobacterial group. This group, together with the β - and γ -Proteobacteria, have been found to be more abundant in the rhizosphere than in bulk soil (Fierer *et al.*, 2007). The Rhizobiales comprised 25% of these, despite the fact that *Arabidopsis* does not form symbioses with nitrogen-fixers. The Acidobacteria are a very diverse lineage of mostly uncultivated acidophilic bacteria, considered to be oligotrophic and more predominant in nutrient-deficient bulk soils (Fierer *et al.*, 2007), but have been shown to have some affinity to rape seed roots and rhizosphere (Ludwig *et al.*, 1997). A high proportion of Acidobacteria were also identified in the Cvi rhizosphere,

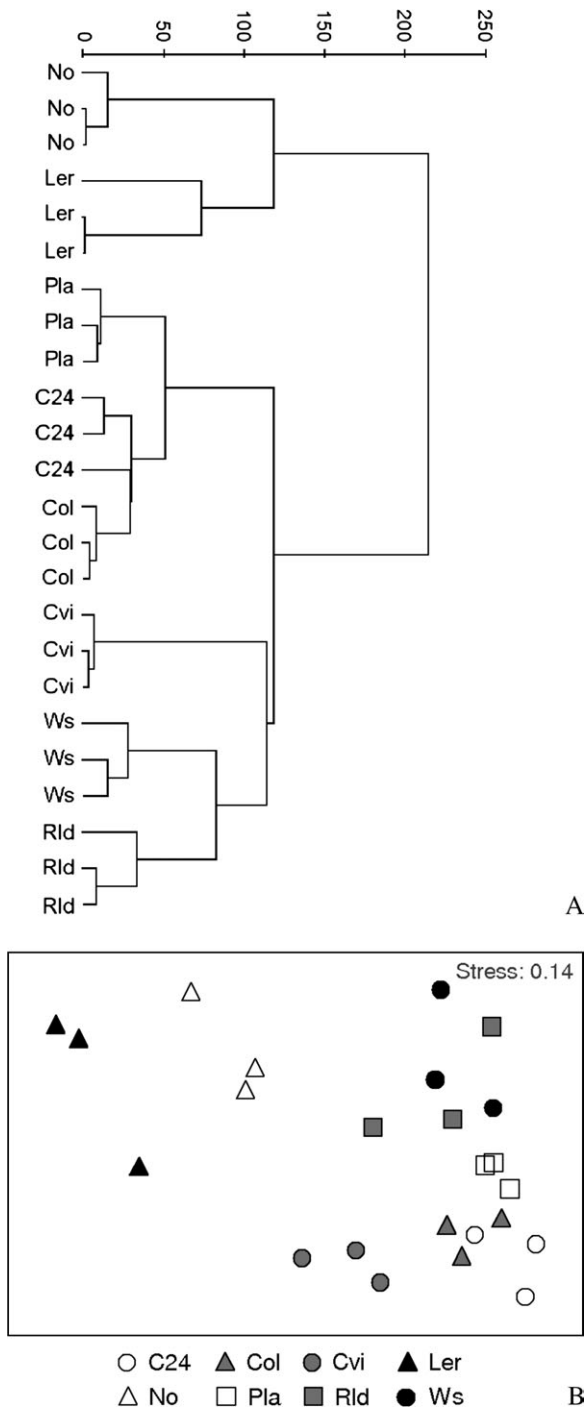


Fig. 6. (A) HCA dendrogram and (B) non-metric MDS plot constructed from HPLC data obtained for *Arabidopsis* exudates.

and could be reflecting a large representation of this group in the acidic bulk soil used, a reminder that, although often considered as distinct habitats, bulk and rhizosphere soils actually exist as a continuum, with the bulk soil being the source of all rhizosphere flora. The Pseudomonads were unexpectedly underrepresented, however, the closely related Burkholderiaceae comprised 10% of the ribotypes identified. Like *Pseudomonas*, *Burkholderia* encompasses a wide range of ecologically important organisms, including plant pathogens and plant-growth promoting bacteria. Xanthomonads,

Table 2. Phylogenetic groups detected in the Cvi rhizosphere

Phylogenetic group	Number of OTUs	% Representation
α -Proteobacteria	16	27%
(Rhizobiales)	(4)	
Acidobacteria	10	17%
Bacteroidetes	8	14%
γ -Proteobacteria	7	12%
(Xanthomonadaceae)	(5)	
(Pseudomonads)	(3)	
β -Proteobacteria	6	10%
(Burkholderiales)		
Verrucomicrobia	4	7%
Actinobacteria	3	5%
Gemmatimonadetes	3	5%
δ -Proteobacteria	2	3%

an important group of plant pathogens, were also present in the Cvi rhizosphere.

Bacterial community diversity and structure were estimated using individual ribotypes (number of T-RFs) as a measure of detectable species richness. Ribotype abundance, as determined by the relative amount of PCR product, was used as an approximate estimator of that diversity. This is a useful approach that has been used previously to assess microbial diversity (Buchan *et al.*, 2003; Conn and Franco, 2004; Rees *et al.*, 2004), but data should be interpreted with caution. The number of 16S rDNA T-RFs generated does not directly correspond to the number of species present in the community. One T-RF peak size may represent several distinct T-RF sequences originating from different taxa. Alternatively, one species can account for more than one T-RF peak, if it possesses multiple rRNA operons, although sequence redundancy and low levels of divergence between rRNA operons within a single genome appear to be more commonly found in Bacteria (Acinas *et al.*, 2004). Furthermore, genome size (Farrelly *et al.*, 1995) and PCR bias preclude the direct extrapolation from the data to the actual situation *in situ*. These limitations should be borne in mind when inferences regarding community structure and diversity are made. Despite these limitations, T-RFLP proves to be an invaluable tool in revealing microbial diversity and in comparative studies (Hartmann and Widmer, 2006; Singh *et al.*, 2006).

Data were transformed to presence-absence form so that abundant and rare OTUs have equal weight when computing the similarity matrix for subsequent hierarchical agglomeration and ordination. Relative abundance data, on the other hand, considers the relative contribution of each OTU in that community, such that the resultant similarity matrix is not based solely on community composition, but also on community structure. This is of relevance in PCR-based techniques, since DNA from taxa that are not actively growing may be amplified and appear present in a community, even if they are not abundant *in situ*. Although relative abundance values cannot be directly related to the abundance of bacterial species in the samples, these data can be used for sample-to-sample comparison, since PCR bias is

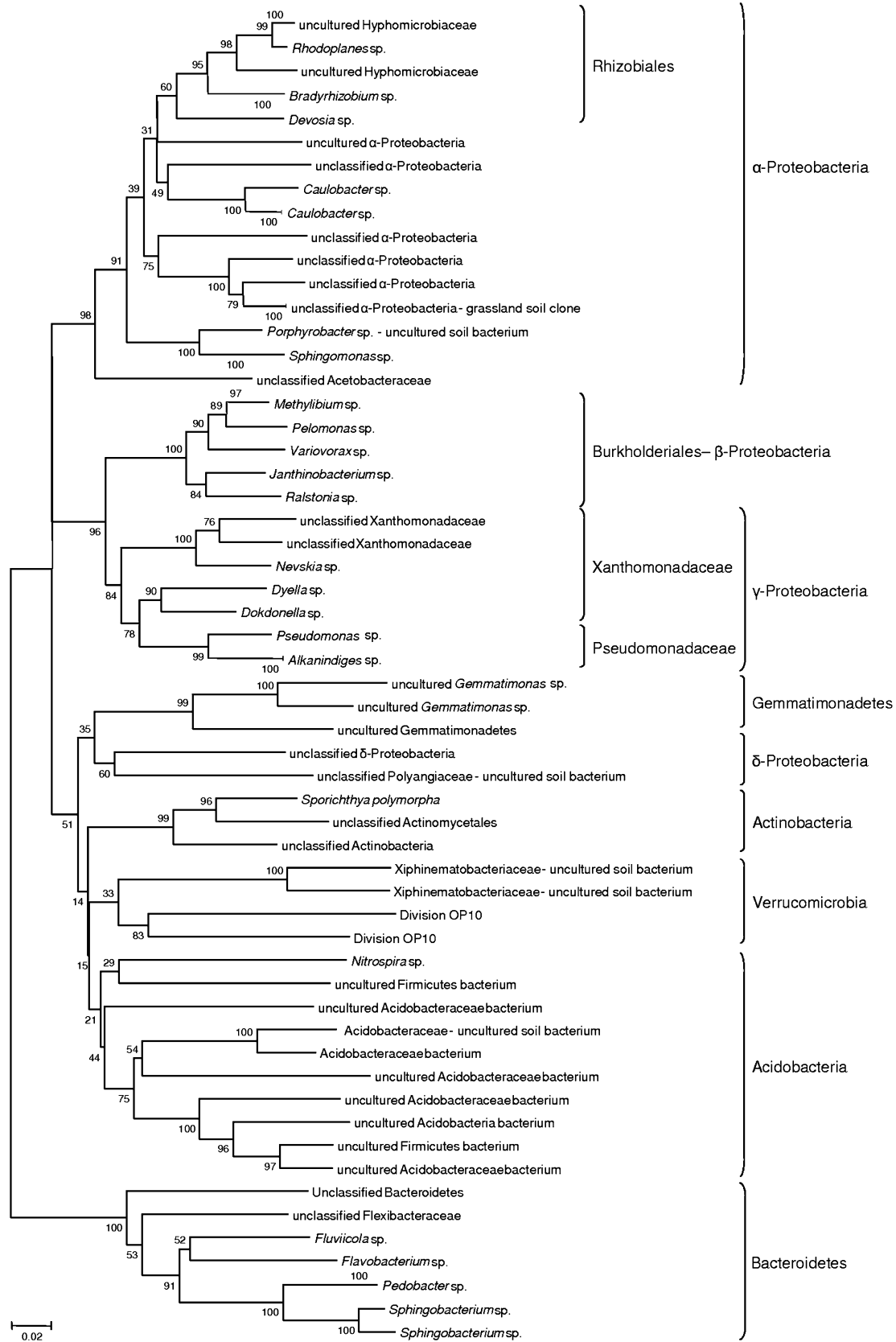


Fig. 7. Phylogenetic tree constructed with bacterial 16S rDNA genes sequenced from the Cvi rhizosphere.

similar for all samples. Relative abundance data resulted in more distinct clustering, suggesting that the accession-dependent grouping of bacterial communities was due not only to differences in species present but also to relative differences in individual bacterial species abundance.

As expected, control bulk soil communities almost always formed a separate group in HCA dendrograms and MDS plots, consistent with the idea that plants play a major role in shaping microbial communities in soil (Mavingui *et al.*, 1992; Grayston *et al.*, 1998; Miethling *et al.*, 2000; Smalla *et al.*, 2001). The degree of dissimilarity exhibited by this cluster, however, was equivalent to the dissimilarity among clusters formed by the different accessions, implying that the *Arabidopsis* rhizosphere effect does not cause a radical shift in community species or strain composition in the New England soil used. This could be attributable to the high organic matter content in the soil used, edaphic conditions known to attenuate, if not completely eliminate, a rhizosphere effect (Semenov *et al.*, 1999). Moreover, the possibility of a substantial shift in microbial activity between control bulk and rhizosphere soil as roots start to develop cannot be excluded. The data were generated from 16S rDNA and not rRNA, which would have yielded a more representative picture of active community members. Moreover, the diversity estimates and similarity coefficient used to generate the similarity matrix were based on relative and not absolute abundances. Thus any differences in activity and growth dynamics prevalent among these communities, reflected in actual bacterial numbers, could not be assessed.

The genotype-specific selectivity of plants on their rhizobacterial community structure could have valuable applications and provide ecological insight into plant-microbe interactions. Interest in manipulating the rhizosphere to create a nutritional bias favouring the growth of a desired suite of micro-organisms has previously been proposed (O'Connell *et al.*, 1996). Its application in plant-pathogen control, phytoremediation, and biofertilization has both economical and environmental benefit. However, rhizoengineering strategies have primarily been based on the use of plants that have been genetically modified in exudate production (Oger *et al.*, 1997, 2004; Savka and Farrand, 1997; Narasimhan *et al.*, 2003). Since bacterial communities in the rhizosphere are influenced by plants at the accession level of genetic variation in *Arabidopsis*, rhizoengineering could also encompass the alternative approach of exploiting naturally-occurring variants of wild-type genotypes for the management and manipulation of associated microbes. Moreover, since recombinant inbred lines (RILs) are readily available for *Arabidopsis*, the rhizosphere model system and exudate profiling technique developed in this study could be used to dissect the genetic basis of exudate synthesis and release into the rhizosphere.

Supplementary data

Supplementary data containing Figure S1 are available at *JXB* online.

Fig. S1. Dominance curves of average relative abundance of T-RFs for all 8 accessions and control bulk soil plotted against the log of T-RF ranks, ranked in order of decreasing abundance.

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