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Host signature effect on plant root-associated microbiomes revealed through analyses of resident *vs.* active communities

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Summary

Plant roots create specific microbial habitat in the soil – the rhizosphere. In this study, we characterized the rhizosphere microbiome of four host plant species to get insight into the impact of the host (host signature effect) on resident *vs.* active communities. Results show a distinct plant host specific signature found among wheat, maize, tomato and cucumber, based on the following three parameters: (i) each plant promoted the activity of a unique suite of soil bacterial populations; (ii) significant variations were observed in the number and the degree of dominance of active populations; and (iii) the level of contribution of active (rRNA-based) populations to the resident (DNA-based) community profiles. In the rhizoplane of all four plants, a significant reduction of diversity was observed, relative to the bulk soil. Moreover, an increase in DNA–RNA correspondence indicated higher representation of active bacterial populations in the residing rhizoplane community. This study demonstrates that the host plant determines the bacterial community composition in its immediate vicinity, especially with respect to the active populations.

Introduction

Soils are among the most complex niches on earth, displaying high spatial and temporal heterogeneity of physical, chemical and biological properties (Hinsinger *et al.*, 2009). Thousands of unique microorganisms may coexist even in a very small volume of soil (Torsvik *et al.*, 2002; Gans *et al.*, 2005), and are highly exposed to external perturbations. One common perturbation is growth of plant roots. Plant roots are considered to exert high selective pressure on the soil microbiome, a force which radiates from the root interior towards the bulk soil. The 'rhizosphere effect' describes the phenomenon in which the activity of microorganisms is enhanced at root proximity in comparison with the bulk soil (Sørensen, 1997; Raaijmakers *et al.*, 2009). Root exudates, collectively termed rhizodeposits, are the driving force in this process. The composition and amount of rhizodeposits varies from plant to plant and depends on plant age and the root section tested (Jaeger *et al.*, 1999; Kravchenko *et al.*, 2003; Jones *et al.*, 2004). The rhizosphere can be subdivided into compartments that include the root interior, which hosts endophytes, the root surface, termed the rhizoplane and the rhizosphere soil. Functions of all rhizosphere compartments are of central importance to plant nutrition and health (Berg and Smalla, 2009; Compant *et al.*, 2010).

The role of the plant host in determining which specific bacteria assemble in its rhizosphere is not clear. On the one hand, a clear plant signature was recently demonstrated in *Arabidopsis thaliana* root endophytes (Bulgarelli *et al.*, 2012; Lundberg *et al.*, 2012), and plant species identity has been found to be the main factor determining the rhizosphere soil microbial community composition (Grayston *et al.*, 1998; Miethling *et al.*, 2000; Kuske *et al.*, 2002). In other cases, however, no such effect has been found (Brodie *et al.*, 2002; Hartmann *et al.*, 2009; Teixeira *et al.*, 2010). Moreover, it has been suggested that the effect of soil type on the microbial community may overpower the effect of the plant species (Buyer *et al.*, 1999). The least explored rhizosphere compartment with respect to host signature is the rhizoplane. It is the first contact point between plant roots and soil. There, the concentration of rhizodeposits is the highest (Gao *et al.*, 2011), and it is the point through which endophytic colonization initiates (Compant *et al.*, 2010). The rhizoplane is therefore a critical point of plant–microbe interactions. Even so, studies of bacterial communities associated with the

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rhizoplane are rather scarce compared to the numerous studies of rhizosphere soil or endophytic bacterial communities (Ofek *et al*., 2011; Oh *et al*., 2012). Considering plant species specificity with respect to the level and composition of root exudates (Gransee and Wittenmayer, 2000) as well as root surface properties, e.g. cell wall composition (Zeier and Schreiber, 1998) or ion exchange properties (Meychik and Yermakov, 2001), rhizoplane properties may be highly species dependent. Such niche divergence may be manifested in the assembled and active rhizoplane communities.

One approach to address this complexity is to target and identify organisms whose activity is stimulated. Combined profiling of community DNA and RNA can provide ample data regarding stimulated populations, particularly when assessed in detail using high-throughput sequencing technology. Such sequencing technologies have been adopted to the study of rhizosphere microbial communities in recent years (Manter *et al*., 2010; Navarro-Noya *et al*., 2010; Uroz *et al*., 2010; Kolton *et al*., 2011; Ofek *et al*., 2011; Lundberg *et al*., 2012). These technologies enable comprehensive sampling, thereby providing high-resolution data and strong statistical power. In this study, these advantages were used for elucidation of the plant host effect on its associated microbiome. Specifically, the effect of the plant host on assembled *vs*. metabolically active (DNA level and RNA level respectively) bacterial communities was evaluated. The objectives of the present study were: (i) to provide a better understanding of the signature effect of plant host species on bacterial community composition and structure within the soil and at the rhizoplane; (ii) to assess the degree of root influence at the rhizoplane compared to the soil; and (iii) to analyze the correspondence between resident (DNA) and active (RNA) communities in different soil fractions. To fulfil these tasks, the soil and rhizoplane bacterial communities of four important crop plants (cucumber, tomato, wheat and maize) were described at both DNA and RNA levels, using high-throughput sequencing technology.

Results and discussion

The bacterial communities within the bulk soil and rhizoplane of 12 days old cucumber, tomato, wheat and maize seedlings were examined. Following normalization and subsampling of the data, from a total of 296 905 high quality bacterial 16S rRNA gene/transcript sequences, a table of operational taxonomic unit (OTU) frequencies containing 1102 OTUs was obtained. Normalized pair-wise similarities between all samples (32 from roots rhizoplane and 30 from soil rhizosphere), were used to examine the effects of root proximity (soil *vs*. rhizoplane), molecule type (DNA *vs*. RNA) and plant species, as individual factors using analysis of similarities (ANOSIM) and analysis of molecular variance (AMOVA), with three different similarity indices (Bray-Curtis index, Jaccard coefficient and Yuan & Clayton theta). These analyses revealed significant effects ($P < 0.01$) for all three factors (Table S1).

Significant plant host signature effect was measured in both bulk soil and rhizoplane communities

The effect of plant species on the community of bulk soil was best resolved at the 16S rRNA level, while DNA-based 16S rRNA gene profiles of soil from the different plant species did not uniquely cluster (Figs 1 and 2). A similar trend was observed in the structural parameters of these communities (Table 1). Bacterial community richness and evenness could not be distinguished between plant species in bulk soil DNA samples but differed significantly among the active soil communities (RNA-based analyses). This result demonstrates that although similar bacteria resided in the bulk soil of all four plants, each

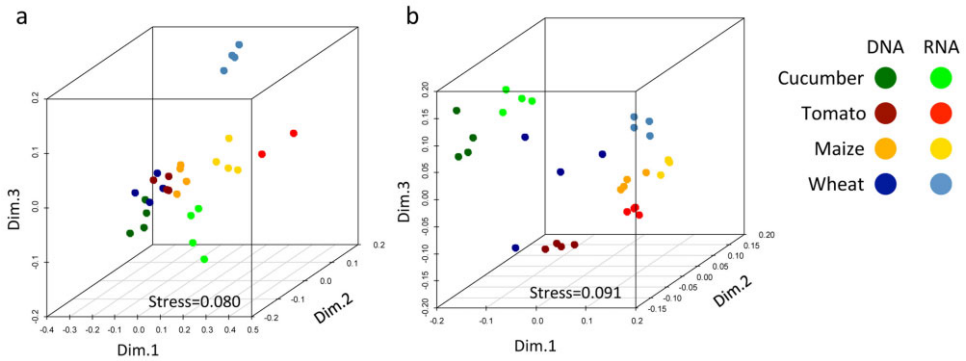

Fig. 1. Composition of bulk soil (A) and rhizoplane (B) bacterial communities of four plant species. Compositions were compared based on a rarefied OTU table, with OTUs defined at 97% sequence similarity threshold. Non-metric multidimensional scaling analyses was performed based on pair-wise, normalized Bray–Curtis distances between samples. The stress values indicate the degree of fit between the original distances in the matrix and the reproduced distances within the ordination plot.

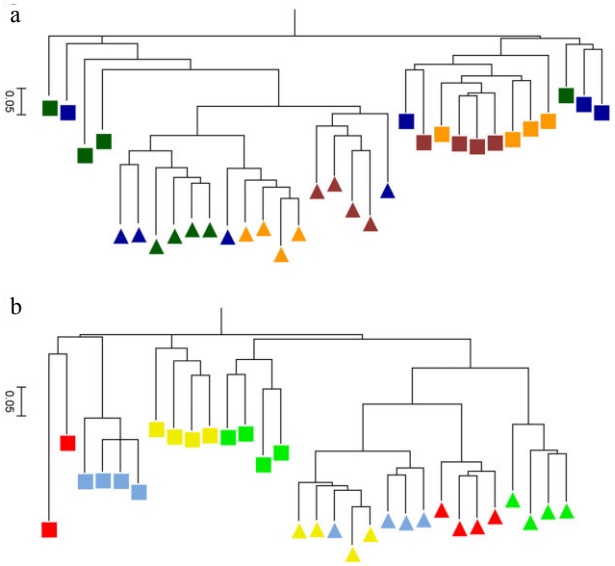

Fig. 2. Neighbour-joining hierarchical clustering of UniFrac metrics between bulk soil (squares) and rhizoplane (triangles) DNA (A) or RNA (B) samples. The UniFrac distances were calculated pair-wise based on OTUs table (defined at a 97% sequence similarity threshold) after subsampling. Color coding is the same as in Fig. 1. The scale bar represents the UniFrac distance metric.

plant species stimulated the activity of a different subset of populations. To examine the role of plant species in stimulation of activity in the soil, we compared bacterial community profiles generated from DNA and RNA samples. Using a stringent false-discovery-rate (FDR) value < 0.001 (Benjamini adjusted), OTUs were verified as enriched in RNA compared to DNA profiles of the different plant species' bulk soil samples (Fig. 3A). The vast majority of those (30 of 35 OTUs) showed a plant species-specific abundance pattern and were substantial components of the respective active communities (Fig. 3A). The difference in the taxonomic composition of plant species-enriched OTUs was notable, with Betaproteobacteria abundant in the maize-enriched OTUs, Bacteroidetes and Actinobacteria abundant in the cucumber soil, and Planctomyces and Cyanobacteria abundant in the wheat and tomato soil respectively (Table S2). Furthermore, for each plant species, the most significantly stimulated soil OTUs belonged to a different phylum (Fig. 3B–E). Recent studies utilizing DNA profiling concluded that only a minor fraction of the rhizosphere soil community is indeed affected by root activity (DeAngelis *et al.*, 2008; Uroz *et al.*, 2010; Bulgarelli *et al.*, 2012; Lundberg *et al.*, 2012; Peiffer *et al.*, 2013). Our results demonstrate a clear plant effect and a unique plant species signature on the active community within the bulk soil. The dominance of unidentified cyanobacterial rRNA sequences in tomato bulk soil library is surprising. Cyanobacteria have been previously reported in rhizosphere DNA libraries at low relative abundance (RA), as in the data presented here (Manter *et al.*, 2010; Zhang *et al.*, 2011). However, due to the scarcity of RNA-based surveys in this niche, the finding of high abundance of cyanobacterial rRNA sequences is rather remarkable.

Rhizoplane communities. The plant species effect described above in the soil was even more salient in the rhizoplane fraction (Figs 1B and 2). At the DNA level, the community profiles of cucumber, maize and tomato each clustered separately, while those of wheat showed high variance and were not tightly clustered (Figs 1B and 2A). At the RNA level, three main clusters could be defined: wheat and maize, cucumber, and tomato (Figs 1B and

Table 1. Rhizoplane and bulk soil bacterial community structural parameters.

		DNA			cDNA		
Sample	Plant species	OTUs	Chao1	Evenness	OTUs	Chao1	Evenness
Rhizoplane	Cucumber	137	224	0.31	186	299	0.41
	Tomato	158	223	0.40	79	100	0.23
	Maize	105	165	0.12	57	74	0.09
	Wheat	124	183	0.29	91	141	0.12
Bulk soil	Cucumber	223	328	0.53	234	405	0.48
	Tomato	222	300	0.51	118	120	0.31
	Maize	226	302	0.52	139	186	0.41
	Wheat	211	283	0.53	163	259	0.24
	Critical range		51	0.09		54	0.12
	Plant		*	***		***	***
	Sample		***	***		***	***
	Plant*sample		ns	***		ns	*

Richness (Chao1) and evenness were calculated based on Hellinger-transformed OTU counts data, and compared by factorial ANOVA ($P < 0.05$) and Post-hoc Tukey HSD test. Critical range ($P < 0.05$) was determined by the Newman-Keuls multiple step test. Means of four replicates are presented; except for the tomato rhizosphere cDNA treatment where the mean of two replicates is shown. ns: not significant. *: $P < 0.05$; ***: $P < 0.001$.

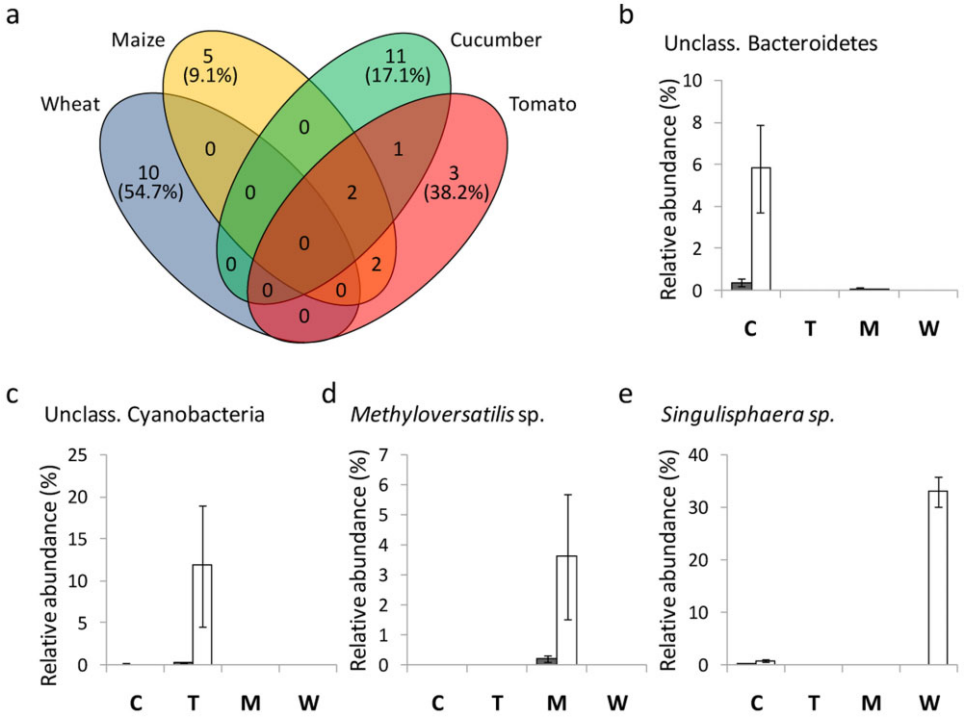

Fig. 3. Bacterial populations (OTUs) with stimulated activity in the bulk soil of four plants. Stimulated OTUs were determined as ones with a mean relative abundance in the active community (16S rRNA based) > 2 fold higher than mean relative abundance in DNA-based community, with a Benjamini adjusted false discovery rate (FDR) of < 0.001. (A) Venn diagram with numbers and the cumulative relative abundance (in parentheses) within the total active community of stimulated OTUs. (B–E) Most stimulated OTUs in the bulk soil of each plant species. Grey and white bars represent relative abundance in DNA and RNA samples respectively. C: Cucumber; T: Tomato; M: Maize; W: Wheat.

2B). The unique plant host signature effect on rhizoplane communities was highly notable when examining community structural parameters (Table 1). Based on DNA data, a significant reduction in measured species richness occurred at the root–soil interface relative to the bulk soil, the degree of which was plant species specific (25–45% reduction in Chao1 richness index). Furthermore, the reduction in evenness index, which serves as an indicator for altered environmental constraints (Hillebrand *et al*., 2008), also significantly varied in magnitude between the plant species. Similar high variance in dominance level was recently described among four tree species (Oh *et al*., 2012). At both DNA and RNA levels, reduction in evenness between soil and rhizoplane compartments was much more robust for maize and wheat compared with cucumber and tomato (Table 1). This apparent loss of diversity between the soil and rhizoplane communities may reflect an actual significant reduction in species richness. However, the strong structural change towards high dominance in rhizoplane communities can mask the actual richness. Furthermore, technical bias (for example in DNA/RNA extraction from root *vs*. soil samples) may also interfere with the accuracy of diversity measurements. Nevertheless, the strong root influence on community structure was unequivocal. A dramatic loss of diversity was recently reported in endophytic bacterial community of *Arabidopsis thaliana* compared to respective bulk soil and rhizosphere soil (Lundberg *et al*., 2012). This significant reduction was associated with a strong selective influence of the plant and supported the elucidation of a robust plant-specific root microbiome (Bulgarelli *et al*., 2012; Lundberg *et al*., 2012). Taking into account the findings of Lundberg and colleagues (2012), the findings here suggest that the degree of selective influence of the rhizoplane on bacterial community structure parameters is comparable with that of the root interior. It can therefore be hypothesized that the rhizoplane poses a strong selective barrier for internal root colonization, reflecting a step-wise enrichment/selection process of colonization from the soil to the root interior.

A non-parametric test was used to compare relative abundances of bacterial classes in active rhizoplane community profiles of the four plant species. The results showed that the rhizoplane of each plant species was characterized by the stimulation of specific bacterial classes, or 'signature dominance' (Fig. 4). In detail, activity of *Gammaproteobacteria* was stimulated in the rhizoplane of maize and wheat; *Alphaproteobacteria*, *Sphingobacteria* and *Flavobacteria* were stimulated

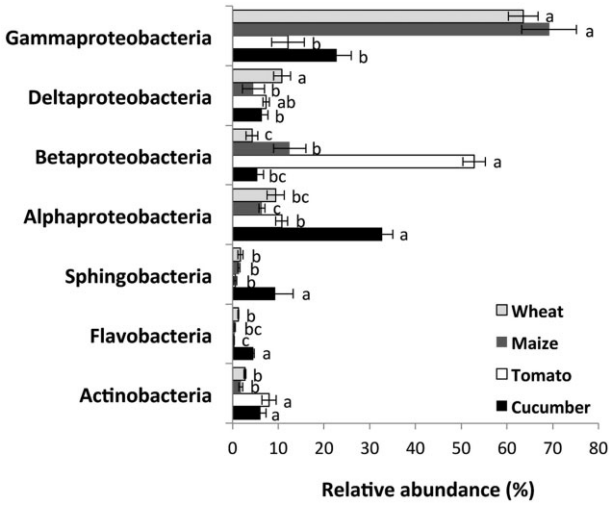

Fig. 4. Relative abundance of dominant classes (> 2% of total reads) in the active bacterial communities of the rhizoplane of the four plant species. Means and standard deviations of four replicates are presented. For each presented class, different letters indicate significant difference based on non-parametric Kruskal–Wallis ANOVA ($P < 0.05$), followed by multiple comparisons of mean ranks.

in the cucumber rhizoplane, members of *Betaproteobacteria* class were highly stimulated in the tomato rhizoplane (Fig. 4). Other classes, including *Actinobacteria* and *Deltaproteobacteria*, although displaying significant variation among plant species rhizoplane communities, contributed less to the plant species-specific signature (i.e. the variance was not linked to a single-plant species). These findings support previous reports that compared active bacterial communities in the rhizosphere of three different legumes plants grown in one soil (Sharma *et al*., 2005). There, high proportions of Firmicutes (*Bacillus*-Clostridia) in community 16S rRNA were found in pea rhizosphere clone libraries, while high proportions of *Betaproteobacteria* and *Deltaproteobacteria* were found in lupin and faba beans 16S rRNA transcript libraries respectively. Many of the stimulated groups found in the current study are generally classified as copiotrophs (e.g. *Gammaproteobacteria* and *Betaproteobacteria*, *Flavobacteria* and *Sphingobacteria*). Bulgarelli and colleagues (2012) demonstrated that growth of root-associated copiotrophs is related to the presence of lignocellulosic material. However, cell wall structure can differ dramatically among plant species (Cosgrove, 1997; Lee *et al*., 2011). For example, Hu and colleagues (1996) measured twice higher content of pectin in root material of cucumber and tomato compared to wheat and maize. Such differences in the quality of the colonized matrix may explain the differential stimulation of copiotrophic microorganisms found between the plant species examined here.

Specific dominant populations

The rhizoplane of the four plant species could clearly be characterized by high dominance of very few active populations defined as OTUs of 97% sequence similarity (Fig. 5). The cumulative RA of the four most abundant OTUs in the rhizoplane communities accounted for 78%, 69%, 55% and 25% of the active community in (respectively) maize, wheat, tomato and cucumber rhizoplane. This level of dominance demonstrates the highly selective, specific impact of roots. Nevertheless, as DNA-based studies demonstrated that community complexity increase with plant age (Gomes *et al*., 2001; Ibekwe and Grieve, 2004), it may represent an early, transient stage in root bacterial community succession, governed by high root growth and rhizodeposition rates.

The dominance of *Pseudomonas* in both maize and wheat rhizoplane communities was most striking. *Pseudomonas* formed 67% and 48% of the active bacterial community of maize and wheat rhizoplane respectively (Fig. 5). Bacteria of this genus are well known for their plant growth-promoting merits (Mercado-Blanco and Bakker, 2007; Lugtenberg and Kamilova, 2009; Santoyo *et al*., 2012) and are often enriched within the rhizosphere of many plant species (Thirup *et al*., 2001; van Elsas *et al*., 2002; Garbeva *et al*., 2008). Few reports, however, provide an assessment of the full taxonomic composition of wheat or maize rhizoplane bacterial communities and the richness of indigenous *Pseudomonas* in particular. Using fluorescence *in situ* hybridization technique, Watt and colleagues (2006) estimated the RA of *Pseudomonas* spp. on the surface of field grown wheat to be around 10% of the total bacterial community. Germida and Siciliano (2001) found *Pseudomonas* spp. to be the dominant cultivable group in the rhizosphere and root interior of

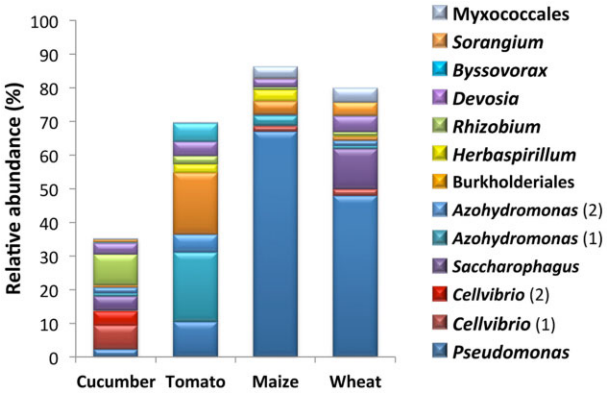

Fig. 5. Average relative abundance of dominant OTUs in active rhizoplane bacterial communities of four plant species (including the five most dominant OTUs for each plant species). Each bar represents a single OTU defined at a 97% sequence similarity threshold.

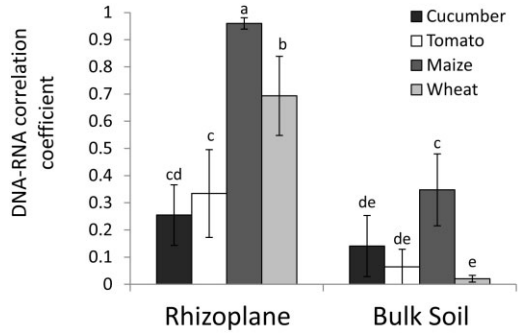

Fig. 6. Correspondence between composition of resident (DNA) and active (RNA) bulk soil and rhizoplane bacterial communities. Correlation coefficients were calculated between all possible pairs of DNA and RNA samples for each plant species within each compartment. Means and standard deviations are presented. Different letters indicate significant difference based on non-parametric Kruskal–Wallis ANOVA ($P < 0.05$), followed by multiple comparisons of mean ranks.

different wheat cultivars. A recent cultivation-based study in Brazil also reported high RA of *Pseudomonas* in the rhizosphere and roots of maize (Arruda *et al*., 2013). A cultivation-independent study in Spain detected *Pseudomonas* in maize rhizosphere at levels of 45% of the total bacterial community (García-Salamanca *et al*., 2012).

Another interesting observation was that of *Cellvibrio* spp. (*Pseudomonadaceae*) dominance on cucumber roots (Fig. 5). While accounting for 11.5% of the active populations, the relative abundance of these bacteria DNA sequence libraries reached 20% in the cucumber rhizoplane. Members of this genus are known for their ability to degrade plant cell wall components and other complex polysaccharides (DeBoy *et al*., 2008), and were detected on root surface and in the rhizosphere of multiple plant species (Schmalenberger and Tebbe, 2002; Kim *et al*., 2006). Interestingly, the RA of *Cellvibrio* spp. was previously shown to be positively affected by plant-beneficial soil treatments. We have previously reported high RA of *Cellvibrio* spp. colonizing root surfaces of cucumber seedling after 48 h of germination in disease suppressive compost-amended potting medium (Ofek *et al*., 2011). Elsewhere, a significant (> 10 fold) increase in the RA of root-colonizing *Cellvibrio* was measured on roots of cucumber seedlings grown in *Fusarium oxysporum*-suppressive soil, compared to conducive soil (Klein *et al*., 2013). Furthermore, the RA of *Cellvibrio* spp. associated with sweet pepper roots also increased following soil amendment with biochar (Kolton *et al*., 2011), a soil amendment associated with disease control. Recently, a strong positive correlation between the RA of *Cellvibrio* spp. and wheat productivity (measured as shoot biomass) was reported (Anderson and Habiger, 2012). Similarly, a positive correlation between *Cellvibrio* RA and root and shoot biomass was reported in potatoes (Manter *et al*., 2010). These reports suggest a general plant-beneficial effect of *Cellvibrio* members and further stress the importance of specific enrichment in the cucumber rhizoplane.

The most dominant active bacterial population in the tomato rhizoplane, with an RA of 37%, was classified to the order *Burkholderiales*. This finding is consistent with previous reports for tomato, based on 16S rRNA gene clone libraries (Shiomi *et al*., 1999; Caballero-Mellado *et al*., 2007).

DNA–RNA correspondence

To the best of our knowledge, this is the first study to employ high-throughput sequencing technology for a coupled DNA and RNA analysis of soil and rhizoplane bacterial community composition. This allows for the differential analysis of the contribution of resident bacterial populations (DNA-based analysis) to the active community (RNA-based analysis). This task has been addressed in the past mainly by employing comparative methodologies, such as PCR-DGGE (Sharma *et al*., 2005; Jossi *et al*., 2006), with relatively low resolution (Muyzer and Smalla, 1998; Petersen and Dahllöf, 2005). In order to examine DNA–RNA correspondence, correlation coefficients between DNA and RNA profiles were calculated for each plant species in each compartment (Fig. 6). Means were then compared by the non-parametric Kruskal–Walis test, which verified significant differences ($P < 0.001$). For all plant species (although to a lesser degree in cucumber), the DNA–RNA mean correlation coefficients were higher in rhizoplane samples compared to soil samples (Fig. 6). This indicates higher proportion of resident bacterial populations in the active community at the rhizoplane, i.e. the rhizoplane bacterial community may be composed mainly of active cells. However, the rhizoplane correlation coefficient values varied greatly among the tested plant species, with maize presenting the highest mean, followed by wheat and lowest were those of tomato and cucumber samples (Fig. 6). This, again, emphasizes the different nature of the rhizoplane fraction of the different plant species.

By simultaneous data collection from RNA and DNA, some clues can be found for the different physiological states of dominant bacterial populations in association with the host plant roots. In order to improve the ability to compare trends among different OTUs, rarefied reads counts were normalized to the average number of rRNA genes of known affiliated species, according to the ribosomal RNA database (rrnDB, Michigen State University). For most of these dominant active populations, the RNA–DNA ratio was > 1, indicating that most cells in the population were highly active. In contrast, one of the two

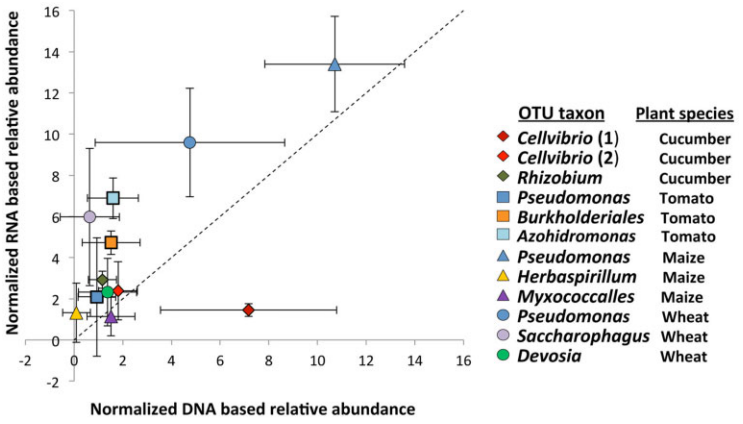

Fig. 7. Correspondence between the relative abundance of dominant active OTUs in DNA and RNA samples. Values for each OTU were normalized by the number of rRNA operons of the closest known related taxa. Each symbol represents the mean of a single OTU defined at a 97% sequence similarity threshold. For each plant species, the three most dominant OTUs are presented. Horizontal and vertical bars represent the range of values for each OTU on each axis.

populations of *Cellvibrio* that dominated the cucumber rhizoplane had RNA-DNA ratio < 1 (Fig. 7). This situation could reflect technical limitations (e.g. variability in cell lysis during nucleic acids extraction) but could indicate different population dynamics scenarios for this group: (i) the physiological state of individual cells in the population is highly variable; (ii) following a peak in activity and abundance, the population RA declines due to shift in niche conditions or due to competitive exclusion. The latter could be related to a natural age-related process of succession, characterizing rhizosphere communities (Green *et al.*, 2006).

In conclusion, recent developments in high-throughput sequencing have allowed us to explore complex bacterial communities in high detail in terms of resident *vs.* active components. The variation in composition or structure of bulk soil resident communities could not be linked to the plant host identity. However, a clear plant effect on the active soil community was demonstrated. Indeed, the use of high-throughput technology enabled the detection and taxonomic identification of distinct bacterial classes, stimulated by different plant hosts, thus pointing towards a host-linked variation in soil microbiome functions. Unlike the bulk soil, though under strong soil influence to which it is exposed, the rhizoplane presents a high degree of selectivity towards both its resident and active colonizers with a significant plant host signature. This selectivity was indicated by a dramatic loss of diversity, a strong shift towards a more structured community, high correspondence between prevalence and activity of populations and major deviations in composition among hosts. Thus, the plant has a major effect on its microbiome that is manifested in the immediate vicinity of its roots. Although DNA evidence points to a short range influence on soil communities, the root effect on the active fraction prevails even to the more distant zone of the surrounding soil. The major influence of the plant host on the composition of active populations suggests a corresponding difference in the functional services rendered by host associated microbiome that remains to be addressed.

Experimental procedure

Plants growth and samples collection

Cucumber (*Cucumis sativus*, cv. Kfir-413), tomato (*Solanum lycopersicum*, cv. Fantasia 1125), wheat (*Triticum turgidum*, cv. Negev) and maize (*Zea mays*, cv. Basso) were cultivated in sandy loam soil (81% sand, 6% silt and 13% clay) obtained from Maon region, Israel. Dry soil was mixed with Hoagland nutrient solution (6 mM KNO_3; 4 mM $Ca(NO_3)_2{\cdot}4H_2O$; 2 mM $MgSO_4{\cdot}7H_2O$; 1 mM $(NH_4)_2PO_4$; 50 μM KCl, 25 μM H_3BO_3; 2 μM $MnSO_4{\cdot}H_2O$; 2 μM $ZnSO_4{\cdot}7H_2O$; 0.3 μM $CuSO_4{\cdot}5H_2O$; 0.5 μM H_2MoO_4, 20 μM Fe-EDTA) at 1 L per 3 kg of soil. Seeds were surface sterilized by soaking in 3% sodium hypochlorite for 1.5 min followed by 70% ethanol for 1.5 min, and washed three times with sterile water. The wet soil was distributed into 200 mL plastic pots, with four seeds in each. The plants were placed in a growth chamber maintained at 30°C, with 18h of daylight. After 12 days of growth, plants were harvested from eight pots for each plant species. Plants were carefully removed with intact roots, and the soil adhering to the roots was removed by shaking. The roots were then immersed in 30 ml of sterile saline (0.85% NaCl) and vortexed at maximum speed for 30 s. This step was repeated with fresh sterile saline, and then roots were removed, blotted on sterile filter paper and stored at –80°C until further use. Bulk soil samples, i.e. soil particles not strongly adhering to roots, were taken from each pot and stored at –80°C until further use.

DNA and RNA isolation and sequencing

For each plant species, four replicates of soil and root samples were used for genomic DNA extraction and four for total RNA extraction. Genomic DNA was extracted according to the protocol described by Angel and colleagues (2011). Total RNA was extracted using a modification of the RNA PowerSoil™ Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA). Briefly, 1 g of roots or 4 g of bulk soil were thawed on

ice and applied to the MoBio bead tube. Total RNA was then extracted according to the manufacturer's protocol with slight modifications. Ten 10 microlitre/millilitre β-mercaptoethanol (Sigma-Aldrich, St Louis, MO, USA) were added into the bead solution. Following the addition of solution SR4, the incubation took place at room temperature for 40 min. Total nucleic acids were subjected to DNAse (Sigma-Aldrich, St Louis, MO, USA) treatment. Approximate RNA concentrations were determined using NanoDrop ND1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Complete removal of DNA was verified by PCR. Complementary DNA (cDNA) was prepared from the RNA with random-primed reverse transcription reactions performed. This was done using the ImProm-II™ Reverse Transcription System (Promega Corporation, Madison, WI, USA), according to the manufacturer's instructions. RNA, DNA and cDNA samples were stored at −80°C. The synthesized cDNA, as well as total DNA, were used as templates for high-throughput sequencing of 16S rRNA amplicons using tag-encoded FLX amplicon pyrosequencing by the Research and Testing Laboratory (Lubbock, TX, USA), as described by Dowd and colleagues (2008). Amplicons sequencing was obtained from the 530 region numbered in relation to *Escherichia coli* 16S rRNA gene. Sequences were deposited in the EBI SRA database, under the accession number ERP001999.

16S rRNA gene/transcript sequences analysis

Deoxyribonucleic acid of a total of 64 samples was sequenced, yielding 428 750 raw partial 16S rRNA sequences. Sequences were trimmed (quality > 30, min length 250 bases, maximum homopolymer length of six bases) and then aligned using the Silva consensus sequences database provided by the MOTHUR website (http://www.mothur.org/wiki/Alignment_database) using the MOTHUR software (Schloss *et al*., 2009). Following alignment, the sequences were screened for chimeric sequences by applying the Chimera.uchim function on the dataset. A distance matrix was calculated for all high-quality, non-chimeric sequences and OTUs were defined using a 97% sequence similarity threshold. Following taxonomic classification of the OTUs, based on the Silva seed reference alignment database, those OTUs arising from plant plastid sequences were removed using the Remove.lineage command. Following these steps, a total of 296 905 high-quality, non-plastid, partial sequences were queried (69% of total reads), distributed among 2556 OTUs (using a 97% sequence similarity threshold). In order to obtain similar sizes for all samples, samples were rarefied each to 890 sequences. Subsequently, singletons and doubletons were removed. Finally, for each sample, 862 ± 21 sequences remained. The resulting table of OTU frequencies contained 1102 OTUs. Two replicate samples of tomato bulk soil-RNA were removed in the subsampling procedure due to low numbers of reads.

Taxonomic classification of the OTUs was verified by ARB-Silva and nucleotide basic local alignment search tool (BLASTN) analyses. AMOVA and ANOSIM statistical analyses were performed to test the effect of plant species, molecule type (DNA *vs*. RNA) and compartment (rhizoplane *vs*. bulk soil) on the composition of the bacterial communities. These analyses were performed using MOTHUR, based on Bray–Curtis, Jaccard and Yue and Clayton theta distance matrices, calculated from the rarefied OTUs table. A Bray–Curtis distance matrix of rhizoplane and bulk soil samples was used for non-metric multidimensional scaling ordination. Weighted Unifrac distances among samples and Unifrac-based neighbour-joining tree were also calculated. Community structure parameters were also calculated based on the rarefied OTU counts table. The Chao1 richness estimator (Chao, 1984) was calculated in Excel using the formula: $S_{chao1} = S_{obs} + F_1^2/2F_2$, where F_1 and F_2 are the numbers of singleton and doubleton OTUs respectively, and S_{obs} is the number of observed OTUs. The evenness index was calculated using the PAST software (Hammer *et al*., 2001). Comparison of community structure indices among plant species and compartments was done by factorial ANOVA and post-hoc Tukey honestly significant difference (HSD) test ($P < 0.05$) using software package STATISTICA® (StatSoft, Tulsa, OK, USA). In addition, critical range ($P < 0.05$) was determined by the Newman–Keuls multiple step test. In order to detect OTUs for which activity was stimulated, i.e. relative abundance was higher in RNA compared to corresponding DNA in the sample; we have used a binomial model, implemented in the DEGseq R package. The criteria chosen for a stimulated OTU call was a $\log_2$ value of ≥ 2 (in favour of RNA relative abundance) and false discovery rate (Benjamini adjusted q value) of < 0.001 (Benjamini and Hochberg, 1995). In order to assess plant species-specific stimulations, the relative abundance levels (arcsine normalized) of the stimulated OTUs were compared among plant species by non-parametric Kruskal–Wallis ANOVA using the STATISTICA software. Non-parametric Kruskal–Wallis ANOVA was also used for between plant comparison of relative abundance levels of the different bacterial classes detected in rhizoplane DNA and RNA data. Correlation coefficients between RNA and DNA profiles were calculated from the rarefied OTUs data set using STATISTICA. For each plant species and compartment, correlation coefficients were calculated for all possible RNA–DNA pairs. Then, the means were compared by non-parametric Kruskal–Wallis ANOVA, followed by multiple comparisons of mean ranks for all groups. For the most dominant active populations in each plant species rhizoplane, the rarified OTU counts were used for RNA–DNA ratio calculation. The read counts were normalized by dividing the counts by the average number of rRNA operons of closest taxon of each OTU based on the ribosomal RNA database (rrnDB; http://rrndb.mmg.msu.edu/index.php). Means and standard deviations of the RNA-based normalized counts were plotted against the means and standard deviations of the DNA-based normalized counts.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Analysis of molecular variance (AMOVA) and analysis of similarity (ANOSIM) statistics calculated for soil and rhizoplane bacterial communities. 16S rRNA gene and transcript sequences clustered into OTUs with a 97% sequence similarity threshold. A rarified OTUs table was used for construction of three different distance matrices using the Bray-Curtis, Jacard (Jest) and Yue and Clayton theta (Thetayc) similarity measures. The R value represents the degree of deviation between the groups tested. C: cucumber; T: Tomato; M: Maize; W: Wheat.

Table S2. Composition of plant species-specific stimulated active (rRNA based) bacterial OTUs in the bulk soil. Relative abundances (means of four replicate) are presented.