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Selective progressive response of soil microbial community to wild oat roots

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Roots moving through soil induce physical and chemical changes that differentiate rhizosphere from bulk soil, and the effects of these changes on soil microorganisms have long been a topic of interest. The use of a high-density 16S rRNA microarray (PhyloChip) for bacterial and archaeal community analysis has allowed definition of the populations that respond to the root within the complex grassland soil community; this research accompanies compositional changes reported earlier, including increases in chitinase- and protease-specific activity, cell numbers and quorum sensing signal. PhyloChip results showed a significant change compared with bulk soil in relative abundance for 7% of the total rhizosphere microbial community (147 of 1917 taxa); the 7% response value was confirmed by16S rRNA terminal restriction fragment length polymorphism analysis. This PhyloChip-defined dynamic subset was comprised of taxa in 17 of the 44 phyla detected in all soil samples. Expected rhizosphere-competent phyla, such as Proteobacteria and Firmicutes, were well represented, as were less-well-documented rhizosphere colonizers including Actinobacteria, Verrucomicrobia and Nitrospira. Richness of Bacteroidetes and Actinobacteria decreased in soil near the root tip compared with bulk soil, but then increased in older root zones. Quantitative PCR revealed rhizosphere abundance of β-Proteobacteria and Actinobacteria at about 10^8 copies of 16S rRNA genes per g soil, with Nitrospira having about 10^5 copies per g soil. This report demonstrates that changes in a relatively small subset of the soil microbial community are sufficient to produce substantial changes in functions observed earlier in progressively more mature rhizosphere zones.

Introduction

As roots move through soil, they impact its physical, chemical and biotic characteristics, and these changes are accompanied by alterations in microbial community activity (Bringhurst *et al.*, 2001; DeAngelis *et al.*, 2008). Soil that is directly influenced by roots, the rhizosphere, can make up a substantial volume of temperate zone soils in the top 10–15 cm, though root influence may extend to meters of depth (Lynch and Whipps, 1990). Root movement through soil creates dynamic environmental gradients that are constantly reiterated with new root growth. A root moving through 'bulk soil' introduces labile carbon and nutrients, creates water conduits, and deposits antimicrobial compounds and hormones (Hawes *et al.*, 1998; Brimecombe *et al.*, 2001; Bringhurst *et al.*, 2001; DeAngelis *et al.*, 2005; Hawkes *et al.*, 2007) across temporal scales of hours to days (Jaeger *et al.*, 1999; Lubeck *et al.*, 2000). As many soil microbes are carbon limited (Paul and Clark, 1996), they may be expected to respond quickly to root-induced changes in soil chemistry and nutrient status by reproducing and increasing in activity (Heijnen *et al.*, 1995; Jaeger *et al.*, 1999; Herman *et al.*, 2006). This enhanced microbial activity may accelerate nutrient cycling as well as other functions such as pathogenesis.

Though there is little direct evidence for changes occurring in specific indigenous soil populations outside of specific pathogenic or symbiotic interactions, studies of microbial community profiles posit that there may be substantial impacts of roots on bacterial and archaeal communities in soil (Lynch and Whipps, 1990). Earlier studies on rhizosphere microbial community dynamics have used fingerprinting methods such as denaturing

gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP) along with diversity indices such as Shannon–Simpson's to describe these communities (Yang and Crowley, 2000; Pett-Ridge and Firestone, 2005; Houlden et al., 2008); using these methods, Yang and Crowley (2000) found differences between barley rhizosphere and bulk soil microbial communities, but observed only under iron-limiting soil conditions. Some taxonomic resolution has been achieved by primer-specific DGGE targeting α-Proteobacteria, β-Proteobacteria and Actinomycetes, where the relative abundance of several Proteobacteria and some Actinobacteria was observed to increase in the rhizosphere (Costa et al., 2006). Although such studies suggest that a selective growth of specific groups of bacteria occurs in the rhizosphere, the sheer diversity of soil microbiota and the limited resolution of available methods have constrained such assessments.

Soil genomic DNA re-association kinetics predict that about 10^6 different genomes per g soil comprise temperate uncontaminated soil microbial communities (Torsvik et al., 2002; Curtis and Sloan, 2005), but most methods are only capable of resolving fewer orders of magnitude of taxa. T-RFLP of 16S rRNA can resolve about 10^2 operational taxonomic units; operational taxonomic units are usually assumed to be distinct phylotypes, or taxa, for methodological purposes. Although this is a robust and reproducible method, it yields relatively low-resolution information about microbial community composition (Osborn et al., 2000). Clone library analysis has expanded our knowledge of rhizosphere community composition and allowed the documentation of uncultivable taxa, but is limited to describing only those taxa that are most common in the sample. In a recent meta-analysis of 19 clone libraries from 14 plant species, over 1200 distinguishable taxa from 35 different taxonomic orders were revealed (Hawkes et al., 2007). Proteobacteria dominated the rhizosphere in 16 of 19 studies included, which is in keeping with other suppositions of proteobacterial dominance of rhizosphere communities, presumably due to their relatively rapid growth rates (Atlas and Bartha, 1993). However, this analysis also uncovered a large number of other phyla, suggesting that root stimulation of microbial communities may be more complex than that predicted by the model of 'hungry heterotrophs' simply responding to rhizosphere carbon deposition.

This research employs a method capable of documenting community composition with resolution that more closely approaches the expected diversity in soil. High-density 16S rRNA PhyloChip has the resolution of almost 10^4 taxa, and the ability to identify individual taxon varying by over five orders of magnitude in abundance (Brodie et al., 2006). This PhyloChip community analysis examines how microbial community composition changes in response to the environmental changes accompanying root movement through soil. The exploration of soil community dynamics in response to roots complements our earlier reports indicating that roots moving through soil result in substantial changes in microbial function (Herman et al., 2006; DeAngelis et al., 2008). These data may provide the most comprehensive analysis, to date, of the succession of a rhizosphere microbial community, additionally articulating the magnitude and identity of changing component populations within the rhizosphere communities.

Materials and methods

Preparation of soils, plants and microcosms
Soils were collected at the University of California Hopland Research and Extension Center, from permanent managed mixed grassland in areas where the annual graminoid Avena fatua was dominant (Hopland, CA, USA). This soil is a medium-texture loam derived from hard sandstone and shale, classified as an ultic haploxeralf (Waldrop et al., 2000). Soils were collected from up to a depth of 10 cm and immediately transported to the lab, where they were sieved to 2 mm, rocks and plant materials removed, homogenized and hydrated to 50% water-holding capacity just before packing into microcosms.

Microcosms (Figure 1) were employed as described earlier (Jaeger et al., 1999; DeAngelis et al., 2008) and were established as three biological replicates that were individual microcosms. Briefly, seeds of Avena fatua (Valley Seed Service, Fresno, CA, USA) were pre-germinated before planting under a slow drip of tap water for 4 days in darkness. To emulate field conditions in the greenhouse, plants were watered until soil reached the field water-holding capacity (which was about 50% saturation) with tap water every second or third day, depending upon the weather to avoid saturation, and incubated in the greenhouse under ambient light conditions. After 8 weeks of growth, the experimental side chambers were filled with soil and the microcosms were inclined at an angle of 45° so that the roots would grow along one face of the microcosm. After 8 days of growth, the front plate was carefully removed to expose the roots, and samples of four types of soil were harvested. Bulk soil was excised at least 4 mm away from any roots, and soils within 2 mm of the root surface were considered rhizosphere soil and extracted with a scalpel from three rhizosphere zones: root-tip rhizosphere soil was located 0–4 cm from the root tip, root-hair rhizosphere soil was 4–8 cm from the root tip and mature root rhizosphere soil was 8–16 cm from the root tip. Roots shorter than 16 cm were sampled up as far up as possible, and roots longer than 16 cm were sampled up to 16 cm unless the root had outgrown the chamber.

Determination of live-cell abundance by direct count
The number of live bacteria was determined using the BacLight Bacterial Viability Kit (Molecular
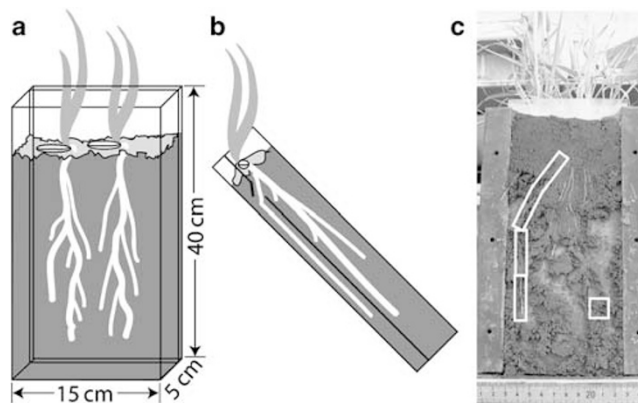
Figure 1 (a) Microcosms used in this study were packed with sieved (2 mm) homogenized soil in the main chamber, and plants are grown from seedlings to maturity as shown. (b) After 6–8 weeks of growth, the microcosms are tipped to an angle of 45°, and the experimental chamber is opened with a slotted divider, so that the roots will grow along the outside face of the microcosm. (c) A photograph of the microcosm shows the experimental chamber, with the roots visible; white lines circle the four soil types sampled: root tip, root hairs, mature root rhizosphere soil and bulk soil. Apparent gaps in soil are a result of aggregates that adhered to the faceplate when it was removed for visualization and excavation of experimental soil.

Table 1 Primers used in this study

Primer name	Target group	Sequence 5'-3'	16S location	Q-PCR pair	Reference
8F*	Bacteria	AGAGTTTGATCCTGGCTCAG	8		EL Brodie, personal communication
4Fa	Archaea	TCCGGTTGATCCTGCCRG	4		Hershberger et al. (1996)
1392R	Universal	ACGGGCGGTGTGTACA	1392		Amann et al. (1995)
1492R	Universal	GGTTACCTTGTTACGACTT	1492		Wilson et al. (1990)
Bet680R	ß-Proteobacteria	TCACTGCTACACGYG	680	Eub338F	Fierer et al. (2005)
Eub338F	Bacteria	ACTCCTACGGGAGGCAGCAG	338		Fierer et al. (2005)
Actino1175F	Actinobacteria	GGTACAGAGGGCTGCGATAC	1175	1392R	This study
Nitrosp1225F	Nitrospira	GGCGACACACGTGCAAC	1225	1392R	This study

Probes Inc., Eugene, OR, USA) (Boulos et al., 1999). Serial dilutions of fresh soil were immediately made in phosphate-buffered saline, sonicated, stained and counted within 48 h of harvest. Cells were stained with SYTO9 and propidium iodide nucleic acid stains; SYTO9 stains only cells with intact membranes in the presence of propidium iodide, indicating living cells. Live cells were counted using epiflourescence microscopy on a Leica DMRX with a ×630 fluorescence oil-immersion lens (Leica Microsystems, Bannockburn, IL, USA). At least two slides were prepared per sample, for different dilutions, and 10 fields of view were counted per slide. Numbers of live bacteria are presented as cells per g dry soil.

PCR amplification of 16S ribosomal RNA genes
DNA was pooled from three separate extractions from about 250 mg (fresh weight) of soil using a modified bead-beating method (Griffiths et al., 2000; Brodie et al., 2002). Soils were added to hexadecyltrimethylammonium bromide extraction buffer, consisting of equal volumes of 10% hexadecyltrimethylammonium bromide in 0.7 M NaCl and 240 mM potassium phosphate buffer (pH 8.0), and then bead-beaten by adding 0.1 mm glass, 0.5 mm zirconia/silica beads (Biospec Products Inc., Bartlesville, OK, USA) and 0.5 ml phenol:chloroform:isoamylalcohol (25:24:1) and shaken in a FastPrep Instrument (Qbiogene Inc., Irvine, CA, USA) at 5.5 m s⁻¹ for 30 s. Following bead-beating, soils were extracted with an equal volume of chloroform:isoamylalcohol (24:1), DNA was precipitated with PEG6000/NaCl solution and, following ethanol washing, was resuspended in water. One-tenth dilutions of soil DNA extracts were used as a template for amplification of bacteria and archaea 16S rRNA genes for T-RFLP and PhyloChip analyses. The primers 8F and 1492R were used for T-RFLP, whereas the primers 8F* and 1492R for bacteria and 4Fa and 1492R were used for archaea for PhyloChip analysis (Table 1). PCRs were performed in 50 μl using Takara ExTaq with 3 μM of each primer, 50 μg bovine serum albumin and 2 U of DNA polymerase (Takara Mirus Bio Inc., Madison WI, USA). Eight replicate PCR amplifications were performed at a range of annealing temperatures from 52 to 62 °C in a BioRad iCycler (BioRad, Hercules, CA, USA) with an initial denaturation (5 min) followed by 30 cycles for T-RFLP and 25 cycles for PhyloChip of 95 °C (30 s), annealing (30 s) and 72 °C

(90 s), and a final extension of 72 °C (8 min). PCRs were run on 1% Tris-acetate-EDTA agarose gel to check for products. Bacterial 16S rRNA PCR product was cleaned up using Qiagen PCR Miniprep Kit (Qiagen Sciences, Valencia, CA, USA), whereas archaeal 16S rRNA PCR product was gel purified and cleaned up using the MoBio Gel Purification Kit (MoBio Laboratories Inc., Solana Beach, CA, USA).

Microbial community analysis by 16S rRNA PhyloChip
For application onto the PhyloChip, PCR products were concentrated and 1000 ng (bacterial) or 200 ng (archaeal), was then fragmented, biotin labeled and hybridized to PhyloChips as described earlier (Brodie *et al.*, 2006). The microbial community analysis was resolved as a subset of 8743 taxa on the PhyloChip, in which each taxon is represented by a set of an average of 24 perfect match–mismatch probe pairs (minimum 11, maximum 30). For a taxon to be reported in this analysis, 90% of probe pairs in its set (probe fraction or pf) must have (1) a perfect match intensity at least 1.3 times the mismatch and (2) differences between perfect match and mismatch intensity that are 130 times the square of background intensity. Hybridization scores for a taxon are reported for all samples if at least one sample out of the 12 has a pf-value > 0.9; for inclusion in the dynamic subset, a taxon had to have a hybe score in 3 out of 12 replicated samples that passed the pf cutoff of 0.9, which is analogous to stringent terminal restriction fragment (TRF) culling suggested earlier (Blackwood *et al.*, 2003). Hybridization scores are an average of the differences between perfect match and mismatch fluorescent intensity of all probe pairs excluding the highest and the lowest; they were normalized to an average of 2500 arbitrary units based on internal standards and are reported as arbitrary units. On presenting relative abundances of reported taxa, hybridization scores were converted to 16S copy number based on the empirically determined log-linear relationship between copy number of applied 16S rRNA PCR product and hybridization score (Brodie *et al.*, 2007).

Microbial community analysis by 16S rRNA T-RFLP
Bacterial 16S rRNA gene products were amplified by PCR as above, with the forward primer modified to contain a 6-carboxyfluorescein (Sigma-Genosys, The Woodlands, TX, USA) for detection in capillary electrophoresis. About 0.5 μg of PCR product was digested overnight with the restriction enzyme *Msp*I in a reaction mixture containing 2 U enzyme and appropriate buffers. Digested DNA was precipitated, resuspended in formamide and mixed with GeneScan 500-ROX size standards (Applied Biosystems, Foster City, CA, USA) (Brodie *et al.*, 2002). Immediately before electrophoresis, amplicons were denatured and electrophoresis was performed on an

ABI 3100 automated capillary sequencer (Applied Biosystems). In culling TRFs, for use in the final data analysis, peaks were eliminated that were present in only one replicate, that had peak heights below 50 fluorescent units or sizes outside of the range of the ROX size standard (Blackwood *et al.*, 2003). TRF peak heights were normalized, and TRF sizes were expressed in base pairs to the nearest base.

Quantitative PCR of dynamic taxa and groups
Primers were chosen or designed to target specific groups and taxa identified on the PhyloChip (Table 1). Designed quantitative real-time PCR probes were based on PhyloChip probes using the Greengenes web application (DeSantis *et al.*, 2006), checked for utility using Primer3 (Rozen and Saletsky, 2000), checked for specificity using RDP Probe Match (Cole *et al.*, 2005) and manufactured by IDT DNA (Integrated DNA Technologies DNA, Skokie, IL, USA). All quantitative real-time PCRs were performed on an iCycler iQ real-time detection system (Applied Biosystems) using the Qiagen SYBR green kit (Qiagen Sciences). A volume of 20 μl reaction contained SYBR green 2 × master mix, 6 pmol each of forward and reverse primers, 10 nM fluorescein and one-tenth dilutions of soil DNA extract in nuclease-free water. Conditions began with hot-start activation at 95 °C (7 min), followed by 40 cycles of 95 °C (30 s), 55 °C (30 s), 72 °C (75 s) and a data-acquisition step at 78 °C (10 s) empirically determined and optimized using melt curve analysis. Standard curves were run in parallel corresponding to a range of 10^8–10^1 copies per μl. Standard curve regression coefficients were consistently above 0.99, and melt curve analysis verified a single amplicon per reaction.

Statistical analysis
Descriptive statistics were performed using JMPIN (SAS Institute Inc., Cary, NC, USA), and multivariate statistics were performed using PCOrd (MjM Software, Gleneden Beach, OR, USA). In all cases, the number of replicates is three, representing separate microcosms. The array intensities for all taxa were normally distributed based on the Kolmogorov–Smirnov–Lillifors goodness-of-fit test ($P < 0.01$). Application of an adjusted P-value is too strong a restriction on the community data for these purposes (Yang and Speed, 2003), and because a stricter definition of the dynamic community does not substantially change the results or the variance explained, statistics were performed using a P-value of 0.05. To examine only the taxa that were major contributors to the change in community, we used two different methods because of the differences in size of the data sets. For T-RFLP, 132 analysis of variances were performed in JMPIN; this method was not possible for the larger PhyloChip data set for which three two-tailed paired t-tests were executed

between the bulk soil and each rhizosphere root zone (root tip, root hairs or mature root). We defined the 'dynamic subset' of a community as comprised of taxa having at least one significant paired t-test when three paired t-tests were performed. For ordination of whole communities, principle component analysis was chosen because it is best suited for data with an approximate linear relationship (McCune and Grace, 2002); in this case, the gradient is that of root exudate deposition with root age (Jaeger et al., 1999). To estimate richness (S), we used a pf-value of 0.9 as a cutoff, below which the taxa were deemed absent. Earlier, pf was found to correlate well with richness patterns displayed by clone library analysis (DeSantis et al., 2007). For each root zone, we calculated differences in hybridization scores for each rhizosphere root zone from bulk soil and then converted the difference to rRNA gene copies. Numbers of individuals (N) are based on live-cell counts.

Results

Cell density in the rhizosphere compared with bulk soil
Significantly more live cells were detected in the rhizosphere compared with bulk soil (Figure 2). As many as 10-fold more cells were detected in the root hairs and the root tip rhizosphere compared with bulk soil, with mature root rhizosphere soil having significantly higher cell density than bulk but less than root hairs or root tips ($P < 0.0001$). The rate of *Avena fatua* root growth in experimental microcosms was approximately $2 \, \text{cm} \, \text{day}^{-1}$ (Herman et al., 2006), and roots up to 16 cm were surveyed, assessing changes in the rhizosphere over about 8 days.
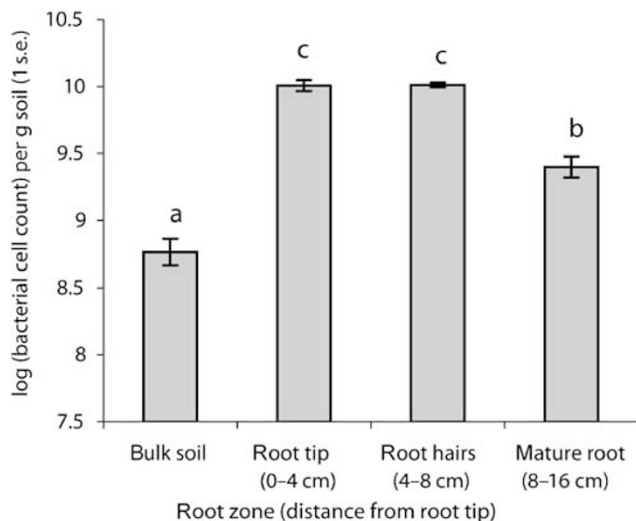

Figure 2 Log of live bacterial cell counts is shown, where differences between root zones (treatments) were calculated using one-way analysis of variance (ANOVA). The Tukey–Kramer HSD test was applied to rank the differences in magnitudes of means; lowercase letters indicate means that are not significantly different using a P-value cutoff of 0.05. For live cells, $P < 0.0001$, indicating significant differences between root zones.

16S rRNA PhyloChip community analysis
Of the possible 8743 resolvable taxa on the Phylo-Chip, we detected 2595 that had a positive pf-value (pf > 0.9) in at least one of the 12 samples. For the whole-community analysis, we examined only taxa that were present (had a positive pf-value) in all three replicates in any one root zone, defined as replicated taxa; 1917 taxa fit this criteria and comprised the total community (Supplementary Table S1). The fact that more than 600 taxa were eliminated due to non-replication suggests the high biological variability, and more replicates would likely put the total richness of these soils well above the 1917 taxa observed.

For each of the observed 1917 taxa, we examined the change in relative abundance compared with the background bulk soil. The fold difference in 16S rRNA gene copy number of each taxon in the root tip, root hairs or mature root zone rhizosphere relative to the bulk soil background is displayed in an ordered histogram in which the taxa are ranked by decreasing relative abundance (Figure 3a). Most populations responded positively to the presence of the root in all root zones compared with bulk soil. A small portion of the taxa exhibited more than a twofold increase compared with bulk soil, whereas a few taxa exhibited large (up to 10-fold) increases relative to bulk soil. The dynamic subset of the community contains all taxa that showed significant response to the presence of the root ($P < 0.05$) and are summarized in Table 2. The dynamic subset is comprised of 147 significant taxa and represents 7% of the total community, falling into 17 phyla of the 44 initially detected phyla. Seven of these phyla contain taxa that significantly decreased in the root tip rhizosphere zone compared with bulk soil (Figure 3b). Taxa in the dynamic subset all eventually become successful root colonizers, as in general, the longer the community was exposed to the root, the greater the increase in relative abundance of the taxa. Representative response patterns of individual taxa can be discerned by examining the 10 individual taxa that changed the most within the dynamic subset (Figure 3c). The different response patterns of these taxa show that distinct soil populations react quite differently to incoming roots, with the response to the root tip appearing to range from positive to negative. Only three taxa, TM7 AB100499, Acidobacteria AF498753, and Bacteroidetes AB023506, show a progressively positive response to the aging root zones.

Principle component analysis performed for the dynamic subgroup of 147 taxa explained 78% of the data set variance in two axes (Figure 4a). Differences in the root zone communities could be discerned, indicating that the dynamic subset is comprised of taxa that responded strongly to the presence of the root. Multiresponse permutation procedure determined that there is greater than 90% chance that there are differences between the microbial communities in the four soil zones ($P = 0.099$).
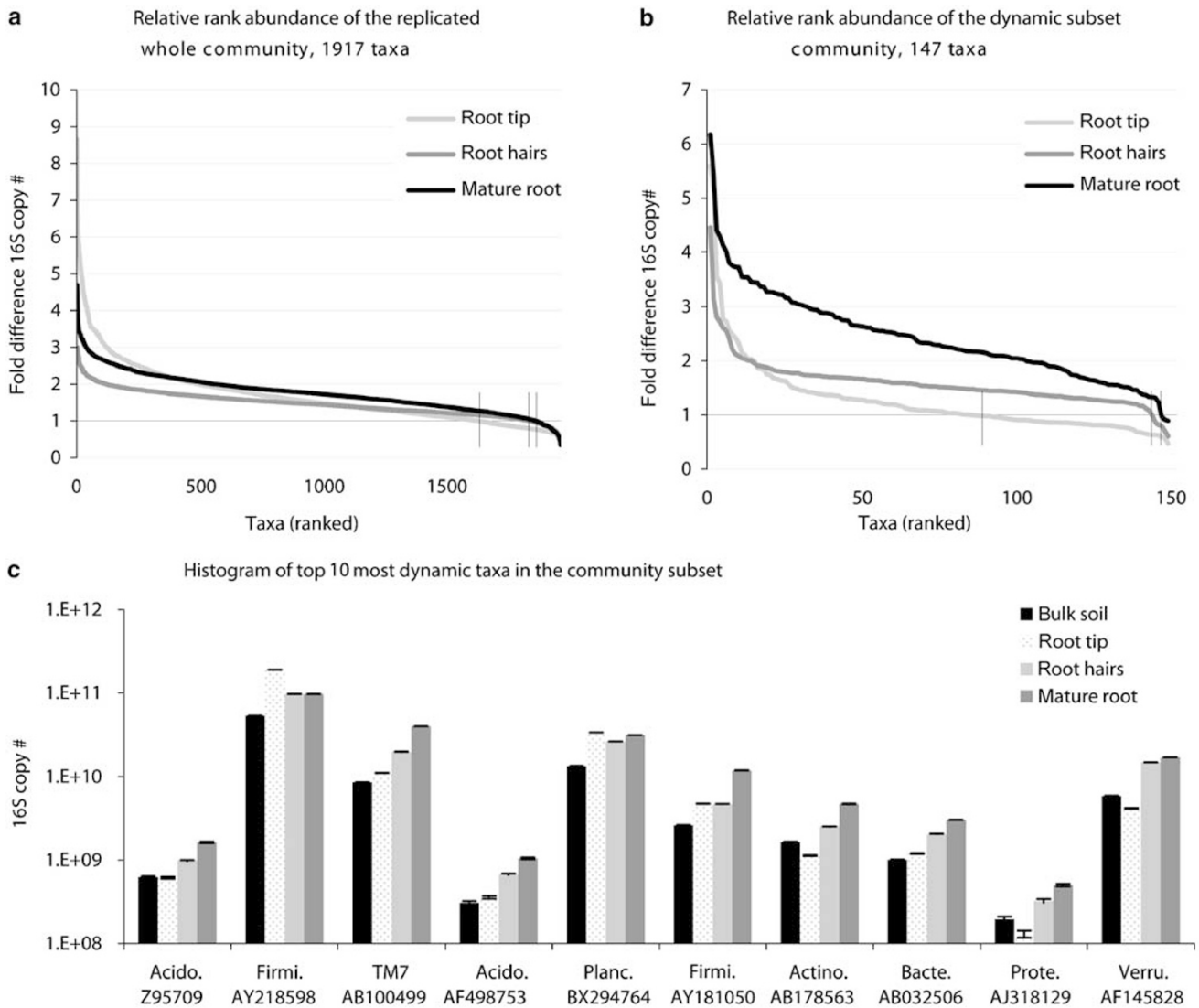
Figure 3 In (**a**) and (**b**), ordered histograms display the relative fold differences in average 16S rRNA copy numbers for each taxa in the root tip, root hairs or mature root compared with bulk soil. The whole community (1917 taxa (**a**)) is shown along side the dynamic community only (147 taxa (**b**)). The y axis value is the fold difference in 16S rRNA copy number for each taxon, calculated as the ratio of average 16S copy number in the rhizosphere divided by the average 16S copy number in the bulk soil background. In this display, a value of one indicates that there is no fold difference over the background, and a vertical line marks this point for each root zone. For all taxa, $n = 3$. (**c**) Average hybridization scores for the most dynamic taxa from the dynamic subgroup in (**b**) are shown. Error bars represent standard errors, and in many cases are too small to be seen on the graph. Taxa are labeled with phyla and reference identification number, with phyla abbreviations as 'Acido.', Acidobacteria; 'Actino.', Actinomycetes; 'Bacte.', Bacteroidetes; 'Firmi.', Firmicutes; 'Planc.', Planctomycetes; 'Prote.', Proteobacteria; and 'Verru.', Verrucomicrobia.

Twenty of a possible 309 archaeal taxa were detected by PhyloChip analysis of these soils and, of these, 19 were not present with pf-value > 0.90 in all replicates, thus excluding them from the replicated data set of 1917 taxa (Supplementary Table S1). However, the archaea examined at all pf-values revealed a strong positive response to the presence of the root (Figure 4b).

To understand whether the changing relative abundances in taxa between different root zones were accompanied by changes in richness, we enforced a cutoff pf-value of 0.9, below which we assumed that the taxon was not present. This revealed differences

in richness between root zones, as there were more taxa in the root hairs and mature root compared with bulk soil. The root tip actually shows a slight decrease in richness compared with bulk soil (Figure 5); these differences were not significant overall, but were manifested in a decrease in richness for the Bacteroidetes overall ($P = 0.083$) and the Actinobacteria in the dynamic subset community ($P = 0.049$). In addition, it was the sum of many diverse phyla ('all others', Figure 5) more than the major phyla that appeared to contribute most to the increased whole-community richness in the root hairs ($P = 0.065$) (Figure 5, Supplementary Table S1).

Table 2 Relative contribution of taxa[a] to dynamic subset community by phylum

Phylum	Total taxa detected	Number of significant taxa in the dynamic subset	% Significant taxa in the dynamic subset of total
Crenarchaeota	8	2	25.0
Nitrospira	5	1	20.0
Cyanobacteria	21	4	19.0
Planctomycetes	21	4	19.0
Bacteroidetes	105	19	18.1
Actinobacteria	225	38	16.9
Verrucomicrobia	33	5	15.2
δ-Proteobacteria	35	5	14.3
α-Proteobacteria	246	28	11.4
γ-Proteobacteria	150	17	11.3
Acidobacteria	65	7	10.8
β-Proteobacteria	128	12	9.4
Unclassified	34	3	8.8
Total (all phyla)	1917	147	7.7
Firmicutes	346	24	6.9
ε-Proteobacteria	42	2	4.8
Spirochaetes	31	1	3.2
Chloroflexi	41	1	2.4

[a]Only groups with more than five taxa are included in this table for clarity.

T-RFLP community analysis of rhizosphere diversity

A total of 132 TRFs were resolved among all samples by 16S rRNA T-RFLP. Of these TRFs, nine had a significantly different relative abundance as determined by analysis of variance ($P < 0.05$) in the presence of the root (data not shown). It is likely that each TRF represents more than one bacterial species or taxa (Nocker *et al.*, 2007), but we can assume that all TRFs represent, on average, the same number of taxa. About 7% of species present in this analysis (9 of 132 TRFs) had an abundance that was significantly affected by the root.

Quantitative PCR of dynamic taxa groups

Quantitative PCR was performed to assess the actual abundance of certain taxa that were observed by PhyloChip analysis to be most responsive to the root (Supplementary Table S1, Table 2). We used specific primer sets to examine the β-Proteobacteria and Actinobacteria, which were commonly represented, and Nitrospira, having only one represented taxon (Supplementary Table S1). Although the variance in estimate of taxa abundance was too large to resolve differences of any of the groups between the different root zones, the absolute abundances of
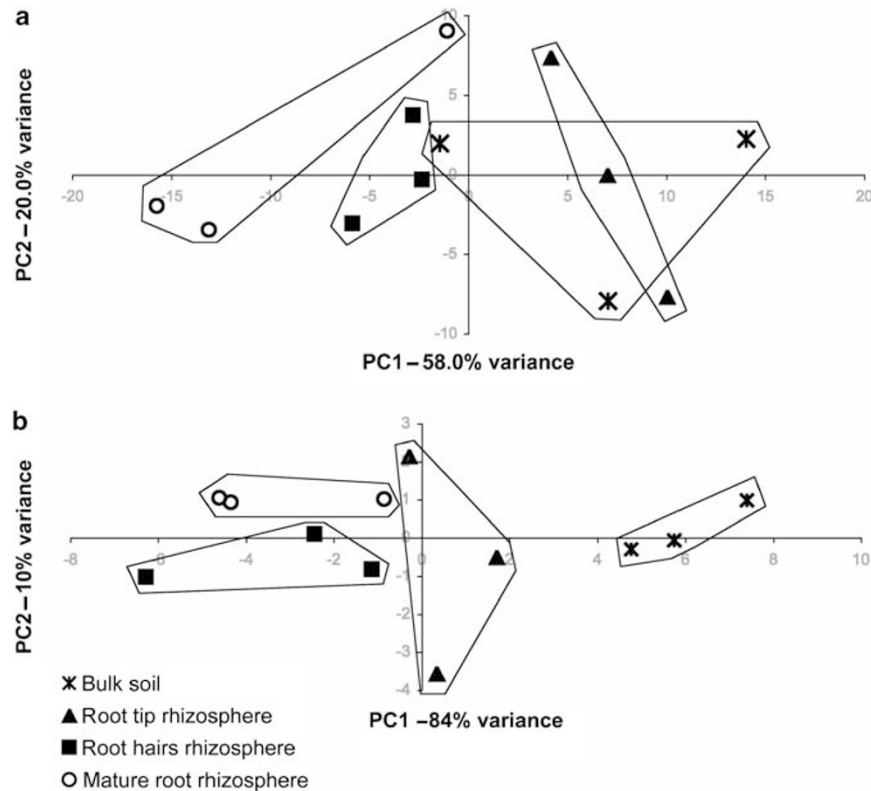
Figure 4 Principle component analysis is shown for (**a**) the dynamic subset of the microbial community, 147 taxa and (**b**) 20 Archaeal taxa out of the possible 309 on the PhyloChip that were detected in our soils. In this analysis, principle component axes 1 and 2 explain most of the variance in the data cumulatively. Polygons drawn around the three samples, which are replicates for each soil type, are intended as guides and are not statistically supported.
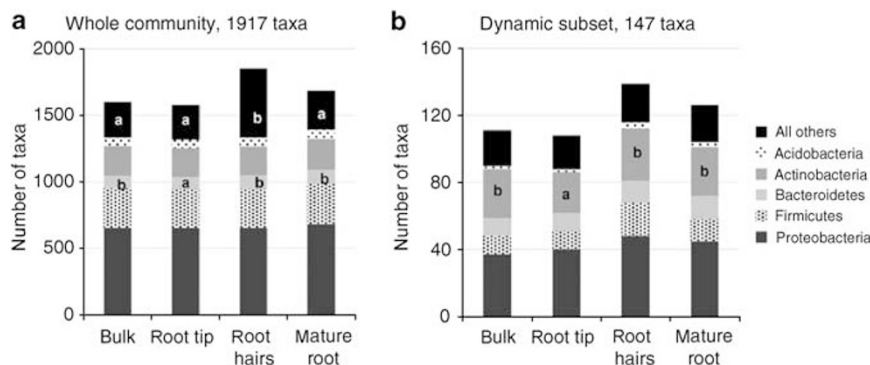
Figure 5 Relative contribution of major phyla to richness in (**a**) the entire community and (**b**) the dynamic subset community. Richness is determined by the presence or absence as defined by probe fraction; see Materials and methods for details. Stacked bars with letters were significant by one-way ANOVA to the following *P*-values: (**a**) the entire community, Bacteroidetes ($P = 0.0837$) and all others ($P = 0.0650$) and (**b**) the dynamic subset, Actinobacteria ($P = 0.049$). ANOVA, analysis of variance.

β-Proteobacteria and Actinobacteria were both about 10^8 16S copies per g soil, whereas those for Nitrospira were about 10^5 copies per g soil.

Discussion

PhyloChip analysis reveals that a diverse array (17 phyla) of bacterial and archaeal populations changed in relative abundance in the rhizosphere, which is somewhat at odds with the long-held assumption that the fast-growing, easily cultivable Proteobacteria are the dominant rhizosphere colonizers (Lynch and Whipps, 1990; Paul and Clark, 1996; Hawkes *et al.*, 2007). We have detected earlier an increased acyl-homoserine lactone abundance in the rhizosphere compared with bulk soil (DeAngelis *et al.*, 2007, 2008); acyl-homoserine lactone-mediated quorum sensing occurs exclusively within the phylum Proteobacteria (Loh *et al.*, 2002). Although this suggests that proteobacteria increase in numbers in the rhizosphere compared with bulk soil, the proteobacteria as a group were only one phylum among many represented in the dynamic subset of 147 taxa. Our data suggest that there is a diverse dynamic subset of the soil bacterial and archaeal community that is specifically stimulated by the root; the richness of the responsive subset spans about 7% of the total community and encompasses up to 17 different phyla.

On the basis of richness estimates from the PhyloChip, the Firmicutes, α-Proteobacteria and Actinobacteria comprised the greatest portion of taxa that changed significantly in relative abundance in response to the root (Table 2). A greater portion of the Actinomycetes (16.9%) and α-Proteobacteria (11.4%) changed in response to the root than the overall percentage of taxa in the dynamic subset (7.7%); the Firmicutes were about as responsive to the root as the dynamic taxa as a whole (6.9% compared with 7.7% overall; Table 2). The Actinobacteria are considered prototypical microbial *k*-strategists (Atlas and Bartha, 1993); as such, it is

somewhat surprising that so many members of this group would increase in rhizosphere dominance. Well-known decomposers, Actinobacteria, responding strongly to the *Avena* root may explain some of the increase in exoenzyme activity that we have recently reported (DeAngelis *et al.*, 2008). Our results suggest that the Actinomycetes and α-Proteobacteria include taxa that are exceptionally rhizosphere competent.

Many microorganisms remain known only by 16S rRNA as they resist culturing efforts (Macrae *et al.*, 2000), and community analysis by PhyloChip yields an insight into the lifestyle of such organisms. Verrucomicrobia generally resists culturing (Sangwan *et al.*, 2005), but culture-independent analyses reveal that this group is present in many soils. This group has been occasionally observed in rhizosphere soils from aerated systems (Ulrich and Becker, 2006) and seems to have representatives that respond positively to the rhizosphere in this study (Table 2). A recent study used ^{13}C-CO_2 to pulse-label plant root exudates and thus identify the primary recipients of root exudates in the rhizosphere, and found mostly Proteobacteria with many fewer Actinobacteria and Acidobacteria (Vandenkoornhuyse *et al.*, 2007). Though the proteobacteria as a group are often considered prototypical fast growers, only the β- and γ-Proteobacteria are well represented by culturing efforts as known fast growers (Paul and Clark, 1996; Schmidt *et al.*, 2007). The functionally diverse Acidobacteria phylum also seems to have a few members that are strongly rhizosphere competent (Vandenkoornhuyse *et al.*, 2007), but the role of these in rhizosphere processes remains to be demonstrated. A related study of cultivated strains from *Avena* rhizosphere soil uncovered many diverse and previously uncultured α-Proteobacteria (DeAngelis *et al.*, 2008), further suggesting that the α-Proteobacteria may contain many rhizosphere-competent taxa. Vandenkoornhuyse *et al.* (2007) also found five potentially new phylotypes by stable isotope probing. Taken together, these observations suggest that roots

stimulate a broad diversity of the soil microbial community, influencing taxa that are as yet unknown and undefined.

Although the older root zones exhibited a fairly typical rhizosphere effect, having increasing taxa abundance, the root tip was somewhat of an anomaly. The number of cells in soil near the root tip was significantly higher than that in bulk soil (Figure 2); however, the number of taxa present was not higher (Figure 5). Production of plant defense compounds by border cells in the root tip may be responsible for the somewhat decreased richness in this zone (Hawes et al., 1998). The phyla that include typical plant pathogens and symbionts, such as the Pseudomonadales, Rhizobiales and Bradyrhizobiales, were among taxa that decreased in relative abundance in the root tip (Table 2), as well as many taxa in the Actinobacteria, Bacteroidetes and α-Proteobacteria. However, as the root tip moved through soil, these populations all eventually recovered, as almost the entire community responded positively to the presence of the mature root (Figure 3); exceptions were all four Bacilli (phylum Firmicutes) whose population sizes remained low (Table 2). The effect of root defense compounds produced in the root tip on selective microbial populations merits further investigation.

A few taxa that showed a positive response to the root have not been found earlier to be competent for rhizosphere growth because they are present only in low relative abundance; the ability to detect such taxa is one of the major advantages of community analysis by PhyloChip. Nitrospira accounted for about 10^5 cells per g soil compared with the 10^{9-10} total bacteria, and one taxon of Nitrospira was identified as responding strongly positively to the presence of the root in this study. To our knowledge, all studies on Nitrospira are from water-saturated (wetland or rice) rhizosphere soil (Kowalchuk et al., 1998; Briones et al., 2003; Ikenaga et al., 2003). Our results also suggest a Crenarchaeal population in soil that responds strongly to the root (Table 2, Figure 4). The Crenarchaeota include ammonia oxidizers that may be functional in mesophilic, aerobic soil environments (Treusch et al., 2005). A more focused investigation is required before concluding that Nitrospira or Archaea contain taxa that are strong rhizosphere responders. PhyloChip detection of Nitrospira, verified by quantitative real-time PCR to be present at about 10^5 per g soil, confirms the capacity of this method to reliably detect taxa in low relative abundance, down to a detection limit of about 0.01% of the total community (Brodie et al., 2007).

Changes in 16S rRNA gene copy numbers suggest that a large fraction of the rhizosphere community is 2–10 times the relative abundance of the bulk soil (Figure 3), and community analysis reveals that the root affects a dynamic subset community in a coordinated way (Figure 4). The dynamic subset is not only increasing in relative abundance, but taxa in a diverse, yet-defined, subset differentially respond to the characteristics of specific root zones as the root moves through soil. Compositional differences in the rhizosphere community compared with bulk soil and between different root zones point to a fraction of the soil microbial community that is especially rhizosphere competent (Folman et al., 2001; DeAngelis et al., 2005; Nunan et al., 2005; DeAngelis, 2006). These compositional changes accompany large changes in soil function associated with rhizosphere N cycling (DeAngelis, 2006; DeAngelis et al., 2008). The linkage suggested between the changes in community composition reported here and the changes in N-cycling reported earlier clearly deserve further exploration.

Acknowledgements

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