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Author

Goldfarb, K.C.

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Differential growth responses of soil bacterial taxa to carbon substrates of varying chemical recalcitrance

Katherine C. Goldfarb[1‡], Ulas Karaoz[1], China A. Hanson[2], Clark A. Santee[1], Mark A. Bradford[4], Kathleen K. Treseder[2], Matthew D. Wallenstein[3], and Eoin L. Brodie[1*].

[1]Ecology Department, Earth Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA, USA, [2]Ecology and Evolutionary Biology, University of California, Irvine, CA, USA, [3]Natural Resource Ecology Laboratory, Colorado State University, Fort Collins, CO, USA, [4]School of Forestry and Environmental Studies, Yale University, New Haven, CT, USA.

* Corresponding author
Dr. Eoin L. Brodie
Lawrence Berkeley National Laboratory,
1 Cyclotron Rd, MS70A-3317,
Berkeley, CA 94720, USA
elbrodie@lbl.gov

[‡]Current address:
Department of Chemistry and Biochemistry, University of Colorado, Boulder, CO, USA.

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Abstract
Soils are immensely diverse microbial habitats with thousands of co-existing bacterial, archaeal and fungal species. Across broad spatial scales, factors such as pH and soil moisture appear to determine the diversity and structure of soil bacterial communities. Within any one site however, bacterial taxon diversity is high and factors maintaining this diversity are poorly resolved. Candidate factors include organic substrate availability and chemical recalcitrance, and given that they appear to structure bacterial communities at the phylum-level, we examine whether these factors might structure bacterial communities at finer levels of taxonomic resolution. Analyzing 16S rRNA gene composition of nucleotide analog-labeled DNA by PhyloChip microarrays, we compare relative growth rates on organic substrates of increasing chemical recalcitrance of >2,200 bacterial taxa across 43 divisions/phyla. Taxa that increase in relative abundance with labile organic substrates (i.e. glycine, sucrose) are numerous (>500), phylogenetically-clustered, and occur predominantly in two phyla (Proteobacteria and Actinobacteria) including orders Actinomycetales, Enterobacterales, Burkholderiales, Rhodocyclales, Alteromonadales and Pseudomonadales. Taxa increasing in relative abundance with more chemically recalcitrant substrates (i.e. cellulose, lignin or tannin-protein) are fewer (168) but more phylogenetically-dispersed, occurring across 8 phyla and including Clostridiales, Sphingomonadalaes, Desulfovibrionales. Just over 6% of detected taxa, including many Burkholderiales increase in relative abundance with both labile and chemically recalcitrant substrates. Estimates of median rRNA copy number per genome of responding taxa demonstrate that these patterns are broadly consistent with bacterial growth strategies. Taken together, these data suggest that changes in availability of intrinsically labile substrates may result in predictable shifts in soil bacterial composition.

Introduction

With over a billion individual cells and estimates of 10^4 to 10^5 distinct genomes per gram of soil (Fierer *et al.* 2007b; Tringe *et al.* 2005; Gans *et al.* 2005), bacteria in soil are the reservoirs for much of Earth's genetic biodiversity. This vast phylogenetic and functional diversity can be attributed in part to the dynamic physical and chemical heterogeneity of soil, which results in spatial and temporal separation of microorganisms (Papke and Ward 2004). Given the high diversity of carbon (C) – rich compounds in soils, the ability of each taxon to compete for only a subset of resources could also contribute to the high diversity of bacteria in soils through resource partitioning (Zhou *et al.* 2002). Indeed, Waldrop and Firestone (2004) have demonstrated distinct substrate preferences by broad microbial groups in grassland soils and C resource partitioning has been demonstrated to be a key contributor to patterns of bacterial co-existence in model communities on plant surfaces (Wilson and Lindow 1994). Whether resource partitioning occurs in soil bacterial communities has not yet been addressed in a comprehensive manner, largely due to methodological limitations in sampling the enormous diversity and in measuring functional responses.

The development of high-throughput tools to assess the composition of soil bacterial communities is rapidly contributing to an improved understanding of bacterial diversity and biogeographical distribution (Chu *et al.* 2010; Drenovsky *et al.* 2009; Fierer *et al.* 2007c; Lauber *et al.* 2009). However, our ability to assess the functions of different bacterial taxa has not kept pace (Green *et al.* 2008). This limits our ability to interpret the functional consequences of shifts in community composition in response to environmental changes (Stein and Nicol 2011). For example, most of our knowledge of the metabolic capabilities of bacterial taxonomic groups is derived from culture-based studies, which may not represent their activities in natural environments and is a small sub-set of the total diversity of bacteria in soils. Improving our understanding of the metabolic plasticity/rigidity of soil bacteria and determining their ability to utilize different types of C substrates, will enable us to better predict the consequences of changes in substrate availability on soil microbial community composition and diversity.

Phylogenetic approaches such as those based upon 16S rRNA genes are limited in their ability to extrapolate function since many functional traits are phylogenetically-dispersed. For this reason, the use of tracer molecules such as stable-isotopes and the thymidine analog, 3-bromo-deoxyuridine (BrdU), have been widely adopted in an effort to connect phylogeny to function. Stable-isotopes, particularly the heavy carbon isotope ^{13}C, have been frequently used to identify microbial community members capable of catabolizing particular substrates (Buckley *et al.* 2007; Feth El Zahar *et al.* 2007; Griffiths *et al.* 2004; Radajewski *et al.* 2000; Schwartz 2007). This technique requires separation of nucleic acids based on buoyant density, so high concentrations of isotopically-labeled substrate are needed. Thus, this approach is costly and impractical for many complex organic compounds that are not commercially available. An alternative is the use of BrdU to monitor cell division following substrate addition. This approach was first applied to the study of bacterial populations over a decade ago (Urbach *et al.* 1999) and it has since been used to identify soil bacterial taxa that respond to various environmental stimuli (Artursson *et al.* 2005; Artursson and Jansson 2003; Borneman 1999; Yin *et al.* 2000). Recently, BrdU incorporation has been shown to

detect a broad diversity of bacterial phyla in marine systems (Edlund *et al.* 2008) and fungal taxa in boreal forest soils (Hanson *et al.* 2008).

In this study, we incubated soils from Harvard Forest (Massachusetts, USA) in the presence of BrdU and with a range of C substrates varying in chemical recalcitrance due to their molecular weight, structure, and the need for enzymatic digestion prior to microbial assimilation (glycine, sucrose, cellulose, lignin, tannin-protein). Next, we analyzed immunocaptured DNA by 16S rRNA PhyloChip (Brodie *et al.* 2006; Brodie *et al.* 2007; DeSantis *et al.* 2007) to determine which members of the soil bacterial community increased their growth rates in response to substrate additions. We hypothesized that the soil bacterial community is comprised of relatively distinct phylogenetic subsets that are either metabolically versatile with regard to their C substrate preferences or display specific C substrate utilization profiles and that response to substrates can be related to rRNA copy number as a proxy for growth strategy (Klappenbach *et al.* 2000; Lee *et al.* 2009).

Materials and Methods

Soil incubations, DNA extraction and BrdU immunocapture. Soil cores were collected from the Harvard Forest Warming Experimental Site in Petersham, Massachusetts in September 2005 according to Hanson *et al.* (2008). Soil moisture was 16.1% (w/v) and mean water holding capacity was 60.9%, this is within the range of previous laboratory incubations (Paul *et al*., 2001, Fierer and Schimel, 2002, Bradford *et al.*, 2010), therefore soil moisture was not adjusted prior to substrate addition. From each of four replicate soil samples two grams were incubated for 48 hours at room temperature in the dark with 260 µl of a solution containing 7.69 mM 3-bromodeoxyuridine (BrdU) and 20 mg of one of 5 substrates (glycine, sucrose, cellulose, lignin and tannin-protein). A BrdU-only control incubation was also performed for each soil replicate. Soil DNA extraction and immunocapture of BrdU-labeled DNA was performed according to Hanson *et al.* (2008). Substrate concentrations were provided in excess of demand (at 1% w/w) across the 48 h, as demonstrated with preliminary trials (data not shown). Addition of substrate concentrations in excess of demand is a common approach for assessing both the potential respiration and microbial community responses of substrate utilization in soils (e.g. Bradford *et al*. 2010). However, soil microbial communities are typically considered substrate limited, and so the caveat of the approach is that substrate concentrations are above what would be observed *in situ* (e.g. van Hees *et al*., 2005). This should be considered when extrapolating patterns observed in this study to those observed in the field.

PCR amplification of 16S rRNA genes. 0.5 µl of BrdU-labeled genomic DNA was used as template for PCR amplification of 16S rRNA genes. Eight replicate reactions containing 0.02 U/µL ExTaq (Takara Bio Inc., Japan), 1X ExTaq buffer, 0.2 mM dNTP mixture, 1 µg/µL Bovine Serum Albumin (BSA), and 300 pM each of universal bacterial primers: 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') were performed for each sample. To minimize PCR bias due to variable template annealing efficiencies and random priming effects, PCR was performed with an eight temperature annealing gradient (48-58˚C) and the following conditions: 95˚C (3 min), followed by 30 cycles of 95˚C (30 sec), annealing (30 sec),

72˚C (2 min), and a final extension at 72˚C (10 min). Reactions were combined for each sample and concentrated by precipitation with isopropanol, washed twice with ice-cold 70% ethanol and resuspended in 50 µL nuclease-free water.

PhyloChip microarray analysis of 16S rRNA gene diversity. 500 ng of pooled PCR amplicons from each sample were spiked with known concentrations of control amplicons derived from yeast and bacterial metabolic genes. This mix was subject to fragmentation, biotin labeling and hybridization to G2 PhyloChip microarrays according to manufacturer's protocols and as described previously (Brodie *et al.* 2007).

Each PhyloChip was scanned and recorded as a pixel image, and initial data acquisition and intensity determination were performed using standard Affymetrix software (GeneChip microarray analysis suite, version 5.1). Background subtraction and probe-pair scoring were performed as reported previously (Brodie *et al.* 2006; Brodie *et al.* 2007; DeSantis *et al.* 2007). The positive fraction (pf) was calculated for each probe set as the number of positive probe-pairs divided by the total number of probe-pairs in a probe set. Taxa were deemed present when the positive fraction value met or exceeded 0.90. Intensities were summarized for each taxon/probe-set using a trimmed mean (highest and lowest values removed before averaging) of the intensities of the perfect match probes (PM) minus their corresponding mismatch probes (PM).

Quantitative PCR. qPCR assays were performed on BrdU-captured DNA from each sample following Fierer *et al*. (2005, 2007a). Separate qPCR assays were conducted to quantify the abundance of 16S ribosomal RNA gene copies from total bacterial ("all bacteria" assay), in addition to rRNA gene copy numbers belonging to the following sub-groups of bacteria: Acidobacteria, Beta-proteobacteria, and Bacteriodetes. These assays were performed in triplicate for each sample in 96-well plates in a BioRad iCycler single-color real-time PCR detection system (BioRad, CA) using SYBR Green I dye (BioRad, CA). All primers, reaction conditions and reaction concentrations were identical to those used by Fierer *et al*. (2005, 2007a) with one modification: the annealing temperature for the Acidobacteria qPCR assay was increased to 52°C, to improve PCR specificity. Standard curves for each of the assays were generated using triplicate 10-fold dilutions of known plasmid standards containing PCR products from an appropriate positive control generated using the qPCR primers. After confirming the presence of target PCR product in each reaction using a melting curve analysis with the MyIQ software (BioRad), standard curves were used to calculate target copy numbers for each reaction. Relative abundances of each bacterial subgroup in each DNA sample were calculated as a ratio between the measured copy numbers of each group-specific assay and the "all bacteria" assay. Because amplification efficiencies can vary across DNA samples, copy numbers expressed as a fractional value more accurately reflect a relative index of the abundances of each of the targeted bacterial groups (Fierer *et al*., 2005).

Statistical analysis of PhyloChip assays of bacterial community composition. All statistical analyses were carried out in the R programming environment (http://www.r-project.org/). To correct for variation associated with quantification of amplicon target (quantification variation), and downstream variation associated with target fragmentation,

labeling, hybridization, washing, staining and scanning (microarray technical variation), hybridization intensities were subject to the following two-step normalization procedure. First, for each PhyloChip experiment, a scaling factor best explaining the intensities of the spiked control probes under a multiplicative error model was estimated using a maximum-likelihood procedure. The intensities in each experiment were multiplied by their corresponding optimal scaling factor. Second, the intensities for each experiment were corrected for the variation in total array intensity by dividing the intensities by their corresponding total array intensity for bacterial probe sets. Normalized intensities were then log10 transformed and only bacterial taxa present (positive fraction ≥ 0.90) in at least one replicate soil incubation were considered. A distance matrix was calculated from the normalized transformed intensity values using the Bray-Curtis distance metric within the function 'vegdist' in the R package 'vegan'. The distance matrix was represented as a nonmetric multidimensional scaling plot using the function 'metaMDS' and variance partitioning was calculated with the function 'adonis'. Bacterial taxa that were significantly enriched in abundance for a C substrate type relative to the BrdU-only control were determined by ANOVA ($p<0.05$ after correction for multiple observations using the Benjamini-Hochberg procedure (Benjamini and Hochberg 1995). Differences in distributions of pairwise distances between samples receiving substrate additions and BrdU-only controls were tested for significance using a Mann-Whitney test.

Phylogenetic tree construction. To display the phylogenetic breadth of organisms detected as capable of incorporating BrdU, a phylogenetic tree was generated using representative 16S rRNA sequences for those bacterial taxa detected in at least one sample following BrdU immunocapture. Representative sequences were exported aligned (7682 character NAST alignment; DeSantis *et al.* 2006b) from the greengenes database (DeSantis *et al.* 2006a) and local bootstrapped neighbor-joining trees constructed using FastTree (http://www.microbesonline.org/fasttree/) with default settings (Price *et al.* 2009). Dendrogram branches were annotated according to phylum and according to substrate use range using the Interactive Tree of Life (ITOL) web server (http://itol.embl.de/) (Letunic and Bork 2007).

Analysis of phylogenetic clustering and dispersion within bacterial communities responding to carbon substrate addition. To determine whether additions of a given substrate enriched bacterial taxa that were more or less phylogenetically-related than expected by chance, we calculated the Net Relatedness Index (NRI) and Nearest Taxon Index (NTI) using the functions 'ses.mpd' and 'ses.mnnd' respectively in the R package 'picante' (http://picante.r-forge.r-project.org/). NRI measures the distance between all pairs of taxa in a community to establish the overall phylogenetic relatedness, and NTI measures the distance between each taxon and its nearest neighbor to determine terminal phylogenetic relatedness. Both measures were compared with a random community drawn from the entire pool of taxa observed to incorporate BrdU. Positive values indicate phylogenetic clustering while negative values demonstrate more even dispersion than would be expected by a random sampling across a phylogeny (Horner-Devine and Bohannan 2006; Webb *et al.* 2008).

Relationship between growth response and rRNA copy number.

To asses whether the growth response of bacteria following substrate addition was related to rRNA copy number as a proxy for ecological strategy we calculated median rRNA copy numbers for individual response groups which we have defined as follows: organisms that increase in relative abundance in response to any C substrate (1), organisms that decrease in relative abundance in response to any C substrate (2), organisms that increase relative abundance in response to labile C (3), or chemically recalcitrant C (4), and organisms increasing in relative abundance in response to each of the substrates glycine (5), sucrose (6), cellulose (7), lignin (8), tannin-protein (9). As rRNA copy numbers are typically similar across genera, rRNA copy numbers for taxa detected in this study were inferred from genome sequenced relatives found in the rrnDB (http://ribosome.mmg.msu.edu/) as of April 12th 2010 (Lee *et al.* 2009).

Results

BrdU was incorporated into the genomes of a broad diversity of soil bacteria. Across all BrdU-labeled incubations, we detected 2,233 bacterial taxa (1,938 taxa with sequence lengths > 600bp are displayed in the phylogenetic tree) (Figure 1). These taxa were distributed across 43 of the 58 bacterial phyla detectable by the PhyloChip version G2 (available in supplemental file S2). The detected taxa represented the major lineages of soil bacteria (Proteobacteria, Firmicutes, Acidobacteria, Actinobacteria, Planctomycetes, Bacteroidetes, Verrucomicrobia, TM7, Spirochaetes, Gemmatimonadetes, Deinococcus, Nitrospira, Chloroflexi, and Cyanobacteria) and a strong phylogenetic signal for substrate utilization was apparent (Figures 1 & 3, Table S1).

The active bacterial community is impacted to a greater degree by labile organic C. Non-metric Multidimensional Scaling of inter-sample Bray-Curtis distances, based on PhyloChip probe-set intensities, demonstrated that more labile substrates such as glycine and sucrose resulted in a greater divergence of the active bacterial population from the BrdU-only controls than did more chemically recalcitrant substrates (Figure 2A). Many more bacterial taxa (>300) were significantly enriched by the addition of a labile substrate, whereas the addition of more chemically recalcitrant substrates stimulated 27 to 129 taxa (Figures 1 and 3).

The greater effect of labile substrates was apparent at taxonomy levels ranging from order to OTU/taxon (Fig. 1) and the order-level distribution of bacterial taxa responding to particular substrates varied substantially (Fig. 3). Nevertheless, strong patterns were apparent. Taxa that became enriched were primarily restricted to a limited number of bacterial orders primarily Enterobacteriales, Actinomycetales, Burkholderiales, Alteromonadales, Pseudomonadalaes, Rhodocyclales and Clostridiales and the Nearest Taxon Index (NTI) (Horner-Devine and Bohannan 2006; Swenson 2009; Webb *et al.* 2008) confirmed a phylogenetic clustering of the enriched taxa (Fig. 3). All substrates, with the exception of cellulose, resulted in enrichment of phylogenetically-clustered groups of bacteria. In other words, these substrates stimulated the growth of bacterial taxa that are more similar to each other than would be expected in a random sampling of taxa detected in Figure 1.

Comparison of the carbon substrate range of soil bacterial taxa. Many taxa (390) were enriched in response to labile substrates only, with 195 taxa only responding to glycine and 131 only to sucrose (Fig. 4). Sixty-four taxa responded to both glycine and sucrose but not to any chemically recalcitrant substrate. Fewer taxa responded only to chemically recalcitrant substrates. Notably, of the 27 taxa that became enriched in response to cellulose, 24 responded only to its addition. In contrast, 123 of the 129 lignin-enriched taxa were also enriched by the addition of labile substrates. Only one taxon (an unclassified γ-Proteobacterium) was enriched only by tannin-protein additions.

Most of the taxa responding only to labile-C were Actinobacteria (Actinomycetales), γ-Proteobacteria (Enterobacteriales, Alteromonadalaes, Pseudomonadalaes) and β-Proteobacteria (Burkholderiales). Organisms enriched by glycine only were mostly γ-Proteobacteria (Enterobacteriales, Alteromonadalaes) and Actinobacteria (Actinomycetales); those enriched by sucrose only were mostly Actinobacteria (Actinomycetales), β-Proteobacteria (Burkholderiales, Rhodocyclales) and γ-Proteobacteria (Pseudomonadales) (Fig. 1, Table S1). Although certain Burkholderiales taxa were enriched only by labile substrates, many more displayed broader substrate preferences as did many members of the Rhodocyclales and the Pseudomonadales. Some taxa within the Sphingomonadalaes responded positively to both sucrose and lignin, but declined in response to glycine (Table S1).

Very few taxa responded only to chemically recalcitrant substrates, with only 24 for cellulose (Clostridiales; termite gut clones, Deinococcus, Chloroflexi, Acetobacter, Desulfobacteraceae, Geobacteraceae, Syntrophobacteraceae, Treponema), 6 for lignin (Xanthamonadaceae, Desulfovibrionaceae, Entomoplastamaceae, Sphingomonadaceae), and 1 for tannin-protein (an unclassified γ-Proteobacterium) (Table S1). Organisms responding only to a specific substrate displayed a distinct taxonomic composition according to the C substrate applied (Fig. 4).

Substrate additions also elicited negative effects on certain taxa (Fig. 1 and Table S1). Specifically, glycine and sucrose additions reduced the relative abundance of approximately 300 taxa. Moreover, cellulose, lignin and tannin-protein decreased 69, 81 and 21 taxa, respectively. Although not universal, C substrates generally impacted a number of Clostridia and Bacilli families negatively. Glycine addition also negatively affected Acidobacteria and Bacteroidetes which was confirmed by qPCR (Table S3) as well as many α-Proteobacteria and other taxa. Sucrose addition also negatively affected Acidobacteria and Bacteroidetes (Fig. 1, Tables, S1 and S3) in addition to many δ-Proteobacteria, Planctomycetes and Gemmatimonadetes amongst others. Overall, qPCR estimates of changes in fractional abundance of specific bacterial groups agreed well with PhyloChip estimates ($r=0.77$, Table S3).

Table 1 shows the estimated rRNA copy numbers/genome for bacterial taxa responding to C substrate addition. Overall, taxa that increased in relative abundance in response to C addition had greater median rRNA copy numbers than those that decreased. Bacteria increasing in response to labile C had higher median rRNA copy numbers than those increasing in response to more chemically recalcitrant C. This latter trend is clear when comparing the median rRNA copy numbers by substrate applied.

Discussion

The microbial diversity of soils is vast, but the mechanisms maintaining this diversity are not fully understood. A major contributor to this diversity may be the development of distinct resource niches whereby many organisms can co-inhabit the same location by utilizing different substrates. BrdU labeling allowed us to characterize some of these niches by identifying the bacterial members who responded (through DNA replication) to the presence of specific C substrates.

Soil microbes, with the exception of those inhabiting the rhizosphere, are typically considered to be C-limited (Alden *et al.* 2001; Curtis *et al.* 1994; Garbeva and De Boer 2009; Zak *et al.* 1993). This together with the fact that soil C pools are heterogeneous and vary in chemical recalcitrance and turnover times (Dixon *et al.* 1994; Knorr *et al.* 2005; Lichtfouse *et al.* 1995) suggests that specialization on C substrates may contribute to the high phylogenetic diversity observed in soils. Indeed, it has been suggested that one of the main factors controlling bacterial species diversity is the diversity of C substrates (Zhou *et al.* 2002). Here, we identified hundreds of bacterial taxa that responded only to labile C additions (Fig. 4). In fact, addition of labile C (glycine or sucrose) resulted in a greater divergence of bacterial communities from controls than did chemically recalcitrant C (Fig. 2). This finding is supported by our estimates of rRNA copy numbers/genome of taxa responding to labile substrates, where we see a large number of fast-growing (copiotrophs) responding quickly to a pulsed resource. Conversely, slow-growing (oligotrophs) with lower rRNA copy numbers, such as some alpha-proteobacteria and Acidobacteria decline following substrate addition.

Labile C also significantly alters fungal community structure in this site, as does cellulose which Hanson *et al* (2008) observed to be mineralized rapidly. In contrast, cellulose did not induce a large change in bacterial community structure (Fig. 2), although we did not directly compare the relative abundances of bacteria and fungi in these data, suggesting that fungi—not bacteria—may be the main decomposers of cellulose in these soils. Numerous bacterial taxa were enriched by labile C (Fig. 3) but they were phylogenetically-clustered and represented few bacterial orders suggesting that labile C (such as that found in rhizosphere exudates) promotes outgrowth of low diversity assemblages, which results in a heavily skewed distribution of taxa abundance at the community level. In this study we detected significant increases in the relative abundances of many β-Proteobacteria (Burkholderiales and Rhodocyclales), γ-Proteobacteria (Enterobacteriales and Pseudomonadales) and Actinobacteria (Actinomycetales) in response to labile substrates. This does not necessarily indicate that other taxa are not capable of utilizing labile substrates, but does indicate that they were not competitive for these resources under these conditions. The rapid response of fast-growing bacteria (such as those that responded to glycine and/or sucrose) to labile C may partly explain the rhizosphere effect. Smalla *et al.,* (2001) reported an enrichment of Actinobacteria in the rhizosphere of several plant species and DeAngelis *et al.,* (2009) recently documented a higher abundance of both β-Proteobacteria and Actinobacteria in the rhizosphere of wild oats. In the latter study, of the 44 phyla detected in the grassland soil, root movement through soil only significantly affected ~7% of taxa within eight days. Drigo *et al*., (2009) found that increased rhizodeposition of sugars due to elevated CO_2 increased the abundance of Burkholderia and Pseudomonads. In our study, almost 50% of actively replicating bacterial taxa (1115 from 2233) were significantly altered by C substrate addition in two days, but as in the study by DeAngelis *et al.,* (2009) they

represented few phyla. Collectively, these findings suggest that in complex communities, labile substrates are primarily utilized by fast-growing copiotrophs, depleting these resources in time and space before oligotrophs can assimilate them.

Even though a limited number of phyla responded to labile C addition, substrate preference was still apparent. For example, glycine addition stimulated the growth of many γ-Proteobacteria (Enterobacteriales, Alteromonadalaes), whereas sucrose addition stimulated growth of many Actinobacteria (Actinomycetales), β-Proteobacteria (Burkholderiales, Rhodocyclales) and other γ-Proteobacteria (Pseudomonadales) (Fig. 1 and 3b). This substrate preference may allow co-existence between these functional groups, although our results also suggest that many organisms within these groups can compete for either form of labile C, which may be advantageous in a relatively C-limited environment such as mineral soil.

In contrast to the phylogenetic clustering observed among labile C users, chemically recalcitrant C users—primarily cellulose users—were less phylogenetically-related. Since relatively few bacterial taxa responded positively to chemically recalcitrant C, it appears that less functional redundancy exists in the turnover of these C pools. On the other hand, cellulose users were relatively phylogenetically-diverse (eight phyla), which may broaden the range of environmental conditions under which this metabolism may operate. Many of the taxa responding positively to cellulose have previously been shown to be capable of cellulose hydrolysis (e.g. bacteria from the families Clostridiaceae and Lachnospiraceae (Schwarz 2001), *Sphingomonas* spp., (Kurakake *et al.* 2007) and Spirochetes (Warnecke *et al.* 2007), while *Acetobacter aceti* (Moonmangmee *et al.* 2002) is typically considered a cellulose producer rather than a consumer. In contrast, other cellulose-stimulated taxa from our study, such as *Desulfobacterium* sp., Syntrophobacteraceae, Geobacteraceae, Coriobacteriaceae and Anaerolineae have not been previously implicated in cellulose hydrolysis directly.

Only six taxa were stimulated by lignin addition but no other substrate, these included two from the order Desulfovibrionales (δ-Proteobacteria), two from Sphingomonadalaes (α-Proteobacteria) and a Xanthomonadales (γ-Proteobacteria). Lignin hydrolysis has been documented under sulfate–reducing conditions (Dittmar and Lara 2001) and *Desulfovibrio desulfuricans* has been shown previously to oxidize lignin under anaerobic conditions, a process that impacts both the polyphenolic backbone and functional side groups on the compound (Ziomek and Williams 1989). *Sphingomonas* species have been reported to grow on several dimeric model compounds of lignin as a C and energy source using O demethylation systems (Sonoki *et al.* 2000) and Xanthomonas species have previously been demonstrated to use lignin as a C source for growth (Kern and Kirk 1987; Kirk and Farrell 1987). Only a single taxon was stimulated by tannin-protein alone; this probe-set represented an unclassified γ-Proteobacterium.

Almost 140 taxa responded to both labile and chemically recalcitrant C particularly from within the Burkholderiales families Alcaligenaceae (including *Alcaligenes* spp.), Comamonadaceae (including *Acidovorax* and *Variovorax* spp.) and Burkholderiaceae (mostly *Burkholderia* spp.) (Table S1). These versatile taxa responded mostly to sucrose, lignin and tannin-protein and this agrees with previous studies that demonstrated either an ability to metabolize lignin/lignin monomers (Kato *et al.* 1998; Krishna and Sunil 1993; Mitsui *et al.* 2003; Nigel *et al.* 1995) or presence of lignin peroxidase gene homologs (in the case of *Acidovorax avenae*). Many Rhodocyclales

(including *Azoarcus* and *Thauera* spp.) and Pseudomonadales (mostly *Pseudomonas* spp.) were also stimulated by multiple forms of C. Bacterial taxa such as *Burkholderia* and *Pseudomonas* species are known to be metabolically versatile (Yoder-Himes *et al.* 2009), a trait probably related to a capacity for genome re-arrangement (Lin *et al.* 2008; Silby *et al.* 2009) and maintenance of accessory genomes (Mahenthiralingam and Drevenik 2007; Mathee *et al.* 2008; Sim *et al.* 2008; Wolfgang *et al.* 2003). The ability of such organisms to catabolize labile and chemically recalcitrant C may make them efficient competitors in a frequently C-limited environment. They may also serve as contributors to rhizosphere priming, in which plant roots, through exudation, stimulate the mineralization of soil organic matter (Bader and Cheng 2007; Fu and Cheng 2002).

Changes in the abundance of taxa were relative within a community, so increases in relative abundance of one taxon were matched by decreases in relative abundance of others. Although a change in relative abundance does not necessarily imply a change in absolute number, it represents a shift in the rank order of an organism within a community. This shift could lead to a change in the ability of that organism to compete for resources and influence ecosystem processes. Specifically, we found a consistent decrease in relative abundance of more than 16 families of α-Proteobacteria in response to glycine (Fig 1 and Table S1). Similarly, DeAngelis *et al.,* (2009) found that α-Proteobacteria were significantly altered by root movement through the soil. Moreover, many taxa within this sub-phylum decrease in relative abundance at the root tips, where exudation peaks (Egeraat 1975; Personeni *et al.* 2007). Conversely, these bacteria are enriched near root hairs and mature roots compared to bulk soil populations. Glycine has been shown to have a high rate of efflux from roots of multiple plant species (Lesuffleur *et al.* 2007) which may explain lower relative abundances of α-Proteobacteria at the root tip. The mechanism for this observation is unclear. Glycine may inhibit these bacteria directly or through competitive exclusion by glycine users (e.g. the Enterobacteriales). Alternatively, it may simply be that the relative decline in α-Proteobacteria is not a product of an absolute decline. Future work is required to discern whether C substrate additions to soil provoke absolute declines in the abundance of specific taxa and, if so, the mechanisms underlying these declines.

The quality and quantity of C substrates is expected to be one of the primary drivers of microbial community composition in soils, at least at the phylum level (Fierer *et al.* 2007a). For example, Acidobacteria have been reported to show the highest relative abundance in soils where C mineralization rates are low and so classified as oligotrophs (Fierer *et al.* 2007a), while ß-Proteobacteria and Bacteriodetes are more abundant in soils with high C mineralization rates, and therefore classified as copiotrophs. Consistent with these global-scale patterns, ß-Proteobacteria increased in abundance following sucrose additions in our study. On the one hand, our results confirm that increases in the availability of specific substrates can stimulate the growth of the taxa that can best compete for those resources, which may quickly lead to changes in microbial community composition. On the other hand, the addition of substrates that are resistant to degradation (e.g. lignin and cellulose) did not change the overall composition of the active microbial community, although a small number of specialist taxa did show a growth response. Even though the abundance of these specialist taxa remained rare, they likely still play an important ecological role in these soils. Thus, we suggest that the function of microbial communities cannot be revealed by broad characterizations of

microbial community structure, but rather must also consider the functional contributions of the rare biosphere.

Here we demonstrated that by combining the BrdU labeling with a high-resolution microbial community analytical tool (16S rRNA PhyloChip), we could achieve a deep and broad coverage of soil bacterial diversity and its response to C substrates. Overall, 2,233 taxa from 43 phyla were detected following BrdU incorporation and these phyla include the predominant soil lineages (Hawkes *et al.* 2007; Janssen 2006; Lauber *et al.* 2009). Rare phyla (Urich *et al.* 2008) such as Chlorobi, Dictoglomi, SPAM, TM6, and Termite group 1 were also detected (Figure 1 & Table S1). Although a previous study has suggested that BrdU incorporation may not be as efficient in Gram-positive bacteria (Urbach *et al.* 1999). As our approach compares the response of individual taxa relative to a control, BrdU incorporation by Gram positive bacteria, even if it is inefficient, should be constant per taxon. Our data suggests this does not preclude the use of BrdU as an effective tool to monitor changes in bacterial replication.

The combination of BrdU incorporation and sensitive methods of community analysis such as high-density microarrays provides a powerful tool to investigate the response of soil microbes to changing resource availability. This technique could also have broad application for determining the effects of environmental perturbations, such as altered precipitation patterns and elevated temperature or CO_2. Indeed, almost 50% of the actively replicating bacterial taxa in the soil samples we investigated were significantly altered by C substrate addition, with labile C inducing the greatest effect on community structure. Our findings provide insight into the factors responsible for maintaining the high diversity of bacteria observed in soils and represent a platform for future analysis of the mechanisms underlying environmental-driven alterations in soil bacterial communities, and the potential implications for biogeochemical cycling.

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Titles and legends to tables and figures

Table 1. Estimated rRNA copy numbers/genome of bacterial taxa responding significantly to substrate addition.

Figure 1. Phylogeny of bacterial taxa detected following BrdU incorporation. Phyla are designated by branch colors and selected sub-phyla/classes are annotated. The outer rings display the taxa whose relative abundance increased (green) or decreased (red) significantly ($p<0.05$ following BH correction) in response to C substrate addition relative to the BrdU-only controls. Outer rings are arranged from the interior in order of expected substrate recalcitrance (glycine, sucrose, cellulose, lignin, tannin-protein).

Figure 2. Non-metric Multidimensional Scaling (NMDS) projection of a Bray-Curtis distance matrix showing the response of actively replicating bacterial communities to C substrate addition. Substrate incubations are depicted with colors and grouped by dotted lines.

Figure 3. Order level distributions of bacteria significantly ($p<0.05$) enriched in response to substrate addition relative to the BrdU-only control. Numbers of enriched taxa are given under each pie and Nearest Taxon Index (NTI) values are in parentheses. * denotes NTI value significantly different from the null community distribution ($p<0.05$).

Figure 4. Euler diagram showing substrate use range of bacterial taxa significantly enriched in response to substrate addition. Pies display the order level taxonomic composition of bacterial taxa enriched only by single substrates, by labile substrates only, and by both labile and chemically recalcitrant substrates. Only expanded pie wedges are included in the legend, a full color legend can be found as supplementary material (Fig. 5).

Supplementary material: (available at Frontiers in terrestrial Microbiology website).

Figure 5. Full color legend for pie charts in Figs. 3 & 4 that display the order level taxonomic composition of bacterial taxa enriched only by single substrates, by labile substrates only, and by both labile and chemically recalcitrant substrates.

Table S1. Bacterial taxa displaying significantly different relative abundance relative to the BrdU-only control following C substrate addition.

Supplemental file/Table S2. Excel spreadsheet containing taxon presence/absence data (probe-positive fraction; pf) and taxon intensity data (natural log transformed normalized array intensities) for all 8432 bacterial taxa represented on the PhyloChip version G2.

Table S3. Comparison of the relative impact addition of C substrates of varying chemical recalcitrance on three bacterial phyla/sub-phyla by microarray analysis and qPCR. For

each substrate, values shown for microarray analysis represent the sum of mean differences in log10 fluorescence intensity between BrdU-substrate incubations and BrdU-controls for those that were statistically significant in Table S1. For qPCR data, values represent mean differences in fractional abundance of groups within BrdU-substrate incubations relative to the BrdU-control. The correlation (*R*) between the PhyloChip measure of changes in relative abundance and qPCR measures of changes in fractional abundance is 0.77.

Table 1. Estimated rRNA copy numbers/genome of bacterial taxa responding significantly to substrate addition.

	Increased	Decreased	Labile	Recalcitrant	Glycine	Sucrose	Cellulose	Lignin	Tannin-Protein
Median	4.00	3.17	4.00	3.00	5.17	3.33	2.33	3.00	2.75
Max	15.00	11.97	15.00	10.13	15.00	15.00	9.22	10.13	6.00

Increased corresponds to rRNA copies/genome of taxa that increased significantly in response to any substrate, while decreased corresponds to those that declined. Labile corresponds to rRNA copies/genome of taxa that increased in relative abundance in response to either glycine or sucrose and recalcitrant relates to those increasing in response to either cellulose, lignin or tannin-protein. The rRNA copy numbers/genome of organisms with increased relative abundance in response to individual substrates are labeled by substrate.

Figure 1. Phylogeny of bacterial taxa detected following BrdU incorporation. Phyla are designated by branch colors and selected sub-phyla/classes are annotated. The outer rings display the taxa whose relative abundance increased (green) or decreased (red) significantly ($p<0.05$ following BH correction) in response to C substrate addition relative to the BrdU-only controls. Outer rings are arranged from the interior in order of expected substrate recalcitrance (glycine, sucrose, cellulose, lignin, tannin-protein).

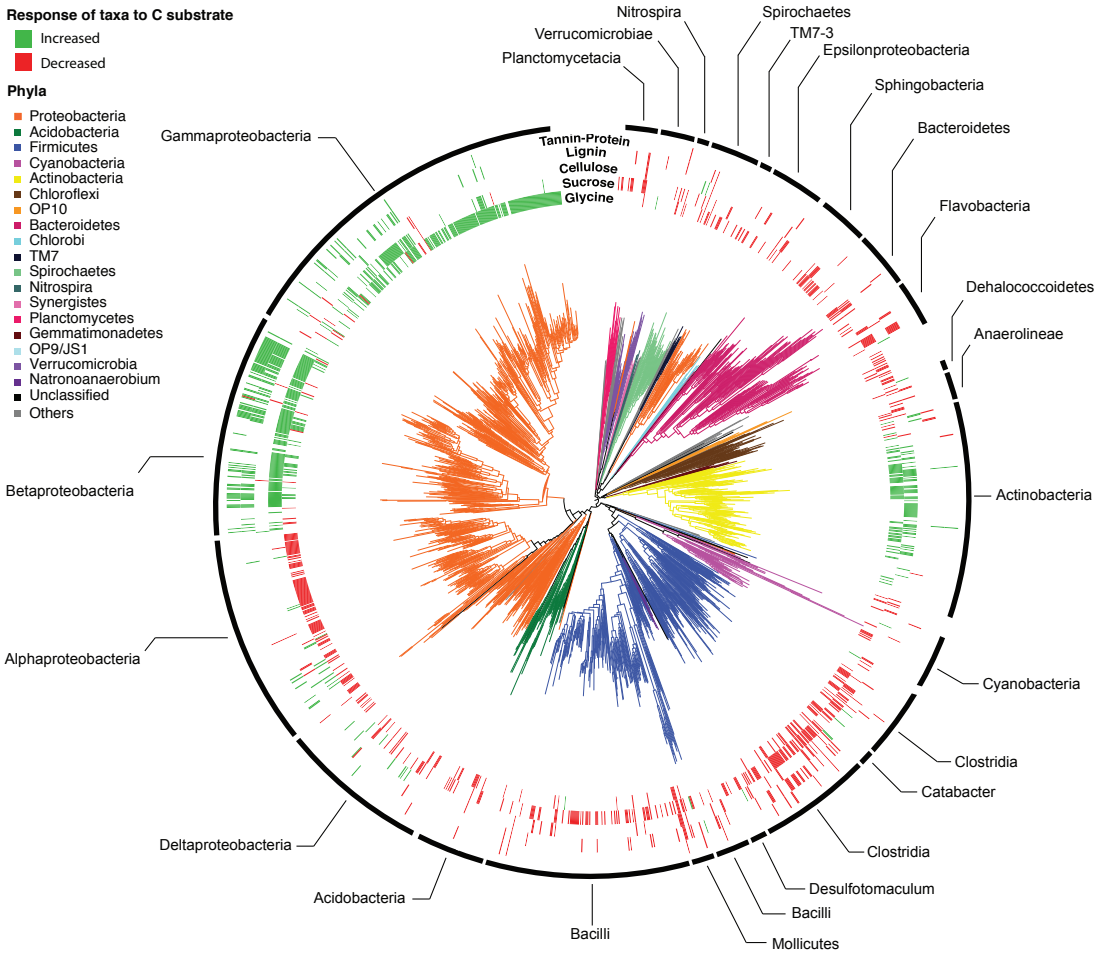


Figure 2. Non-metric Multidimensional Scaling (NMDS) projection of a Bray-Curtis distance matrix showing the response of actively replicating bacterial communities to C substrate addition. Substrate incubations are depicted with colors and grouped by dotted lines.

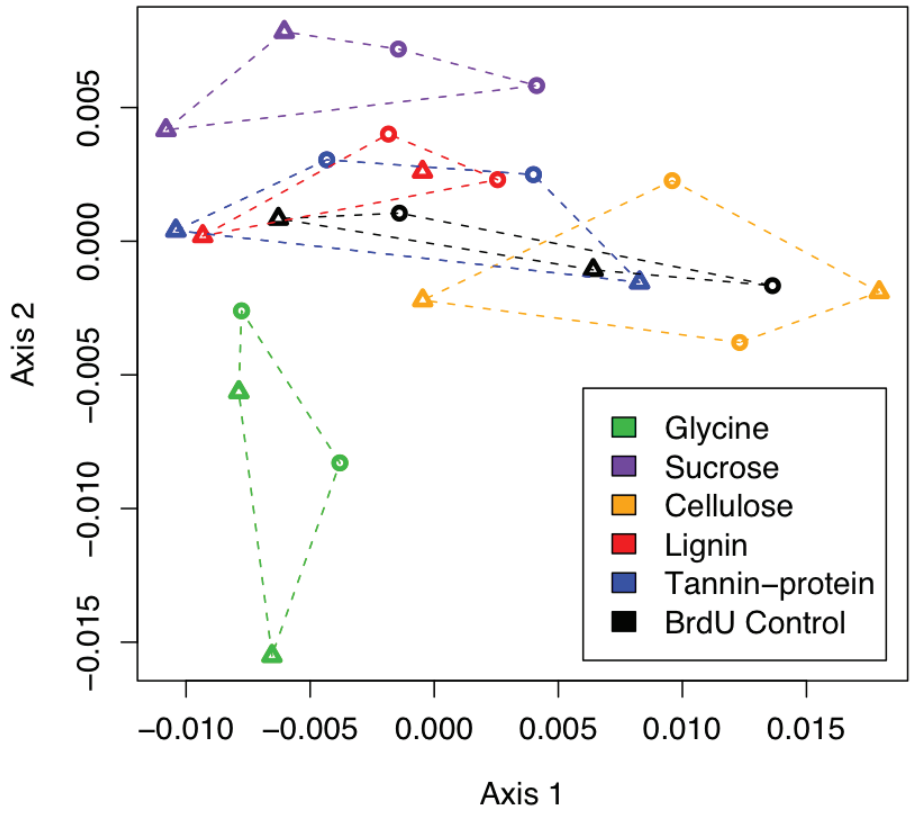


Figure 3. Order level distributions of bacteria significantly ($p<0.05$) enriched in response to substrate addition relative to the BrdU-only control. Numbers of enriched taxa are given under each pie and Nearest Taxon Index (NTI) values are in parentheses. * denotes NTI value significantly different from the null community distribution ($p<0.05$).

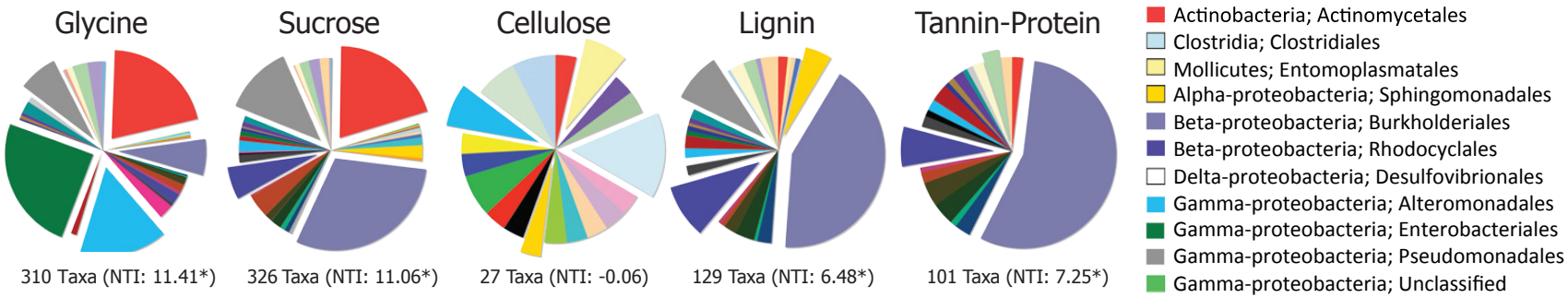
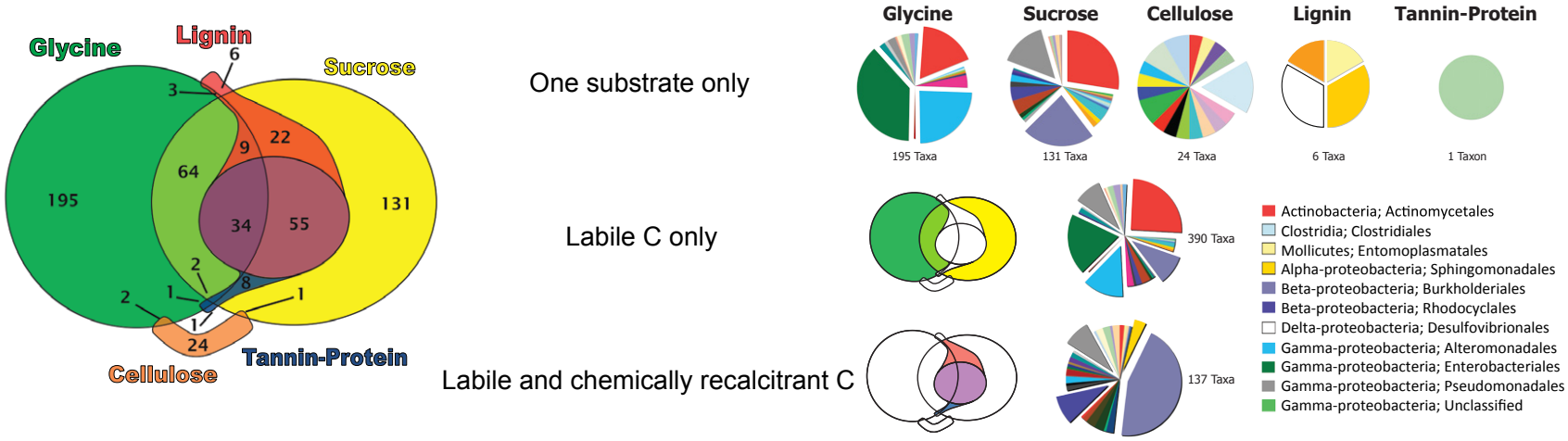


Figure 4. Euler diagram showing substrate use range of bacterial taxa significantly enriched in response to substrate addition. Pies display the order level taxonomic composition of bacterial taxa enriched only by single substrates, by labile substrates only, and by both labile and chemically recalcitrant substrates. Only expanded pie wedges are included in the legend, a full color legend can be found as supplementary material (Fig. 5).



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