Soil Biology & Biochemistry 42 (2010) 896–903

Contents lists available at ScienceDirect

Soil Biology & Biochemistry

journal homepage: www.elsevier.com/locate/soilbio

Shifts in bacterial community structure associated with inputs of low molecular weight carbon compounds to soil

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ARTICLE INFO

Article history:
Received 5 October 2009
Received in revised form
28 January 2010
Accepted 4 February 2010
Available online 20 February 2010

Keywords:
Soil bacteria
Bacterial communities
Pyrosequencing
UniFrac
Soil carbon dynamics
Microbial community structure

ABSTRACT

Low molecular weight carbon (C) substrates are major drivers of bacterial activity and diversity in the soil environment. However, it is not well understood how specific low molecular weight C compounds, which are frequently found in root exudates and litter leachates, influence bacterial community structure or if there are specific groups of soil bacteria that preferentially respond to these C inputs. To address these knowledge gaps, we added three simple C substrates representative of common root exudate compounds (glucose, glycine, and citric acid) to microcosms containing three distinct soils from a grassland, hardwood forest, and coniferous forest. CO_2 production was assessed over a 24 h incubation period and, at the end of the incubation, DNA was extracted from the samples for assessment of bacterial community structure via bar-coded pyrosequencing of the 16S rRNA gene. All three C substrates significantly increased CO_2 production in all soils; however, there was no relationship between the magnitude of the increase in CO_2 production and the shift in bacterial community composition. All three substrates had significant effects on overall community structure with the changes primarily driven by relative increases in β-Proteobacteria, γ-Proteobacteria, and Actinobacteria. Citric acid additions had a particularly strong influence on bacterial communities, producing a 2–5-fold increase in the relative abundance of the β-Proteobacteria subphylum. These results suggest that although community-level responses to substrate additions vary depending on the substrate and soil in question, there are specific bacterial taxa that preferentially respond to the substrate additions across soil types.

1. Introduction

Linking members of bacterial communities in soil with their function has long been a goal of microbial ecologists, yet this task has proven to be difficult for a variety of reasons. First, soil bacterial communities are phylogenetically diverse (Torsvik and Ovreas, 2002; Fierer et al., 2007a; Fierer et al., 2007b) making it difficult to accurately survey and document changes in bacterial community composition. Second, since most soil microbial taxa, including dominant taxa (e.g. the Acidobacteria phylum), cannot be readily isolated and cultivated in the laboratory, the metabolic capabilities of many taxa are not well known (Torsvik and Ovreas, 2002; Jones et al., 2009). Third, microbes exhibit a broad array of 'functions' in soil and, for certain microbial processes there is likely to be a high degree of functional redundancy among bacterial taxa, with phylogenetically dissimilar taxa carrying out similar processes (Allison and Martiny, 2008). This functional redundancy within communities (and functional similarity between different soil communities) can often obscure linkages between bacterial taxonomy and functional traits (Schimel, 1995; Allison and Martiny, 2008; Green et al., 2008) particularly when examining more broadly-defined biogeochemical processes (e.g. metabolism of root exudate compounds) where many taxa may be responsible (directly or indirectly) for the same biogeochemical function (Schimel, 1995).

One important category of these 'broad' microbial processes is the range of processes associated with the mineralization of low molecular weight organic carbon (LMW-C) compounds in soils. LMW-C compounds are released into soil in large quantities through root exudation (rhizodeposition) and via leaching of litter, with the inputs of these compounds having an important influence on both long-term and short-term C dynamics (Lynch and Whipps, 1990; van Hees et al., 2005; Hartmann et al., 2009).

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doi:10.1016/j.soilbio.2010.02.003

The major types of LMW-C compounds released from roots include simple sugars (e.g. glucose, fructose), amino acids (e.g. glycine, alanine), and carboxylic acids (e.g. citric acid, lactic acid) (Lynch and Whipps, 1990; Baudoin et al., 2003; van Hees et al., 2005; Hartmann et al., 2009), many of which can be readily metabolized by soil microbes.

A number of studies have shown, directly and indirectly, that the addition of LMW-C substrates to soil (often simulated root exudates or leaf leachates) can increase microbial biomass and CO_2 production, and also cause distinct shifts in the composition of bacterial communities (Bernard et al., 2007; Cleveland et al., 2007; Paterson et al., 2007; Chen et al., 2008). Likewise, inputs of LMW-C compounds via root exudation may, in part, contribute to observed differences between rhizosphere and bulk soil microbial communities, where rhizosphere soils often have lower bacterial diversity (Kowalchuk et al., 2002) and harbor communities that are distinct from the surrounding bulk soils (de Boer et al., 2006; de Ridder-Duine et al., 2005; Kowalchuk et al., 2002). Based on these previous studies, we would expect that particular bacterial taxa preferentially metabolize LMW-C compounds and that bacterial communities will respond (e.g. increase in relative abundance) in a predictable manner to inputs of LMW-C substrates. Recent work recognizing broad, ecologically-based, classes of bacteria (Bernard et al., 2007; Fierer et al., 2007a) supports this expectation: 'copiotrophic' bacteria are described as thriving under conditions where resource availability is high (e.g., soils with labile rhizodeposited carbon), and oligotrophs are relatively more abundant under resource-limited conditions (e.g. bulk soil). More specifically, Fierer et al. (2007a) showed, using experimental, observation, and meta-analytical approaches, that the members of the Bacteroidetes phylum and β-Proteobacteria subphylum are copiotrophic and therefore would be expected to preferentially metabolize LMW-C compounds in soil (Fierer et al., 2007a).

Despite this previous work, key knowledge gaps remain. In particular, we have a limited understanding of the specific bacterial community shifts associated with the addition of specific LMW-C substrates, and whether these shifts are consistent across soil types, as most previous C-addition studies have used only a single soil or defined substrate (e.g. glucose (Baudoin et al., 2003; Paterson et al., 2007; Chen et al., 2008)), or an undefined mix of C substrates (e.g. leaf leachate (Bernard et al., 2007; Cleveland et al., 2007)). In addition, many previous studies have used low resolution analyses (e.g. DNA-fingerprinting techniques and phospholipid fatty acid analysis) to document how C additions influence microbial communities, but such techniques provide very limited information on the specific shifts in bacterial community structure associated with the C additions. In this study, we separately added three LMW-C substrates (glucose, glycine, and citric acid) that are representative of the dominant classes of root exudates (and thus their degradation represents a soil function) to three distinct soil types. We then used a bar-coded pyrosequencing method to characterize the specific changes in bacterial community phylogenetic structure associated with the various treatments, and to identify the individual bacterial taxa that respond to additions of specific substrates in soils. We hypothesized (1) that specific bacterial taxa would increase in relative abundance in response to additions of LMW-C substrate, (2) that these responses would be consistent across soil types, indicating that bacterial taxa fulfill similar functions regardless of soil type, and (3) that the community responses would be dependent on the specific LMW-C compound added to soil, which is to say, that not all LMW-C compounds have an equivalent effect on bacterial communities. This study presents a method for experimentally connecting specific soil functions, e.g. citric acid metabolism, to specific bacterial taxa in soil.

2. Methods

2.1. Soil collection

Soils were collected from under three distinct vegetation types (grassland, hardwood forest, and coniferous forest) located within the Cedar Creek Natural History Area in Minnesota, USA (45° 24′N 93° 12′W). At each of the three locations, 20 soil cores (2 cm in diameter, 5 cm in depth) were collected from an area of 20 m^2, composited together, sieved to 2 mm, and thoroughly homogenized. The soils were stored at 4 °C for 23 days prior to the start of the experiment. The soils from the three different vegetation types (henceforth referred to as 'grassland', 'hardwood forest' and 'coniferous forest' soils) were distinct with respect to their organic C concentrations, percent total N, C:N ratios, and pH levels (Table 1).

2.2. Microcosm preparation

Microcosms were prepared by adding 5 g (fresh weight) of grassland, hardwood, or coniferous forest soil to a 50 mL glass vial equipped with a gas-tight screw cap lid and septa. Microcosm soils were adjusted to 50% of water holding capacity (see Table 1), then capped and incubated at 27 °C for 4 days. Microcosms were then removed from the incubator, uncapped, and vented for approximately 10 min prior to substrate addition.

2.3. Substrate addition and incubation

Each microcosm received 500 μL of a sterile glucose, glycine, or citric acid solution with each solution pH balanced to pH 7 (using NaOH and HCl) and adjusted to a standard carbon (C) concentration (200 mM C, or 240 μg C g $soil^{-1}$) which was determined to be optimal for maximizing soil respiration rates (data not shown). The control treatments received 500 μl of sterile H_2O alone. For each substrate and soil combination (3 LMW-C substrates, 3 soils), four replicated microcosms were used, resulting in a total of 48 microcosms including the no-substrate water controls. Microcosms were incubated at 27 °C for 24 h with headspace CO_2 accumulation measured at 0, 2, 4, 8, 12, 18, and 24 h using an infrared gas analyzer (IRGA) (CA-10a, Sable Systems, Inc., Las Vegas, NV, USA). In all cases and all timepoints, headspace CO_2 concentrations did not exceed 2%. At the end of the 24 h period, the soil from each microcosm was thoroughly homogenized and frozen at −80 °C prior to DNA extraction.

2.4. DNA extraction and sequencing

DNA was extracted from 0.25 g soil from each microcosm using a MoBio PowerSoil DNA extraction kit (MoBio Laboratories, Carlsbad, CA, USA) following manufacturer's instructions. We then amplified the 16S rRNA gene using a bar-coded primer set that is well suited for community analyses via pyrosequencing (Liu et al., 2007; Hamady et al., 2008; Lauber et al., 2009) following a protocol described previously (Fierer et al., 2008; Hamady et al., 2008; Lauber et al., 2009). Each PCR reaction consisted of 0.25 μl (30 mM) of each primer, 3 μl of template, and 22.5 μl Platinum PCR

Table 1
Soil characteristics.

Soil	% Sand	% Silt	% Clay	pH	C:N	%C	%N
Grassland	90	6.5	3.5	5.6	12.8	2.77	0.22
Hardwood Forest	86	10	4.0	5.0	14.9	1.81	0.12
Coniferous forest	90	6.9	3.1	5.8	14.3	0.89	0.06

Mean values within vegetation type for measured soil properties.

SuperMix (Invitrogen, Carlsbad, CA, USA) with PCR reactions conducted in triplicate for each sample. The PCR program was as follows: denature at 94 °C for 3 min, 35 cycles of (94 °C for 45 s, 50 °C for 30 s, 72 °C for 90 s), and a final extension of 72 °C for 10 min. Triplicate reactions were pooled for each sample and cleaned with a Qiaquick kit (Qiagen, Valencia, CA, USA). The concentration of DNA in each sample was determined with Pico-Green dsDNA reagent (Invitrogen, Carlsbad, CA, USA) and approximately equal amounts of amplicon from each sample were pooled together. The pooled sample was cleaned via ethanol precipitation and re-suspended in nuclease-free water. An aliquot of the concentrated sample shipped on dry ice to the Environmental Genomics Core Facility at the University of South Carolina (Columbia) for pyrosequencing on a 454 Life Sciences Genome Sequencer FLX (Roche) machine.

2.5. Sequence analysis

Processing and analysis of sequences were conducted as described previously (Fierer et al., 2008; Hamady et al., 2008). Sequences without ambiguous characters, >200 bp in length, and a quality score >25 were included in the analysis and sequences were assigned to samples by the 12 bp barcode. Phylotypes were identified by using Megablast to identify connected components (nearest neighbor) sets of similar sequences. Parameters for Megablast were as follows: *E* value, 1e−8; minimum coverage, 99%; minimum pairwise identity, 97%. For each phylotype, the most highly connected sequences with the most hits more significant than the BLAST threshold to other sequences in the dataset were chosen as representative sequences. The set of all representative sequences were aligned through NAST (DeSantis et al., 2006) using a minimum alignment length of 190 nt and sequence identity of 70% as parameters and with lanemask PH to screen out hypervariable regions of sequences (http://greengenes.lbl.gov/). A relaxed neighbor-joining tree was built using Clearcut (Sheneman et al., 2006) with Kimura correction and the resulting tree was analyzed using the weighted UniFrac metric with sequences annotated by sample. The taxonomic identity of phylotypes was determined by BLAST against the GreenGenes database (*E* value cutoff of 1e−10) using the Hugenholtz taxonomy.

2.6. Statistical analysis

Community composition was quantitatively compared across soils using the weighted UniFrac algorithm. This method takes into account phylogenetic relationships between community members, incorporating the abundances of phylotypes into the pairwise community comparisons. This allows us to quantify changes in the relative abundances of phylotypes already present in the sample following substrate addition, not simply changes in the presence or absence of phylotypes (Lozupone and Knight, 2005; Lozupone and Knight, 2008). The UniFrac algorithm estimates the phylogenetic overlap between pairs of communities and avoids some of the pitfalls associated with comparing communities at a single level of taxonomic resolution (Lozupone and Knight, 2005; Lozupone and Knight, 2008; Hamady and Knight, 2009). To describe the changes in the relative abundances of specific bacterial taxonomic groups we binned the phylotypes by phylum/sub-phylum and calculated the percent abundance of each taxa as (total sequences of taxa in sample/total sequences in sample) for each replicate and then the percent abundances of replicates for each treatment were averaged. The change in percent abundance of taxa was calculated as:

$$(A_T - A_C)/A_C$$

where A_T is the average percent abundance of the treatment and A_C is the average percent abundance for the control for that soil. *T*-tests were used to test for significance of individual group changes between treatments and control with ANOSIM (analysis of similarity) tests used to determine the statistical significance of overall community changes between sample categories within and among soils using the pairwise UniFrac distances. Mantel tests were used to test the significance of the relationships between CO_2 production responses to substrate additions and the magnitude of changes in community composition measured using the UniFrac algorithm.

3. Results

3.1. CO_2 production

All three substrates significantly increased respiration in each of the soils above the water-only controls ($P < 0.01$ in all cases) over the 24-h incubation (Fig. 1). However, no one substrate induced the largest increase in CO_2 production in all three soils (Fig. 1). The addition of glucose induced the greatest change in CO_2 production in the coniferous forest soil (555% above controls) after 24 h (Fig. 1) while citric acid addition induced the highest observed CO_2 production in the grassland and hardwood forest soils (391% and 82% above controls respectively) (Fig. 1). As explained in more detail below, the magnitude of the increase in CO_2 production from the individual substrates did not strongly correlate with the magnitude of the community response (Mantel $R = 0.19$, 0.004, and 0.20 with *P* values of 0.07, 0.45, and 0.04 for grassland, hardwood forest, and coniferous forest soils respectively). This is evident by comparing the CO_2 production in Fig. 1 to the average community structures for each soil and substrate in Fig. 2; the substrates that induced the largest increase in CO_2 production did not necessarily lead to the largest shifts in bacterial community structure (e.g. the addition of glucose to the grassland soil).

3.2. Community composition

After removing short and low quality sequences, pyrosequencing yielded 118,407 individual sequences, comprising 19,630 unique phylotypes (with phylotypes defined as those sequences that are 97% similar) and an average of 2467 sequences and 1185 unique phylotypes per sample. One replicate of the grassland soil that received glycine was excluded from all statistical analyses due to errors in sample processing. The soils were, on average, dominated by the following phyla: Acidobacteria (38% of all sequences), Actinobacteria (5% of all sequences), Bacteroidetes (15% of all sequences), and Proteobacteria (α, β, and γ-Proteobacteria subphyla with 16%, 9%, and 8% for each subphylum respectively) (Fig. 2 and Table 2).

The communities in the control treatments of each of the three soils were distinct from one another (ANOSIM global $R = 0.534$, $P < 0.05$) as reflected by their phylogenetically distinct community structures (Table 3 and Fig. 3). Although all three soils were dominated by the phylum Acidobacteria, members of this phylum were relatively more abundant in the hardwood forest soil with an average relative abundance of 44.9%. In contrast, the grassland and coniferous forest soils contained only 29.2% and 28.2% Acidobacteria, respectively (Table 2). Proteobacteria were the next most abundant phylum, with α-Proteobacteria being the most abundant subphylum in all three soils (relative abundances of 14.6%, 18.7%, and 16.5% for grassland, hardwood forest, and coniferous forest soils, respectively). The abundance of the phylum Bacteroidetes varied among soils, with the coniferous forest soil having the highest relative abundance at 23.9% (Table 2).

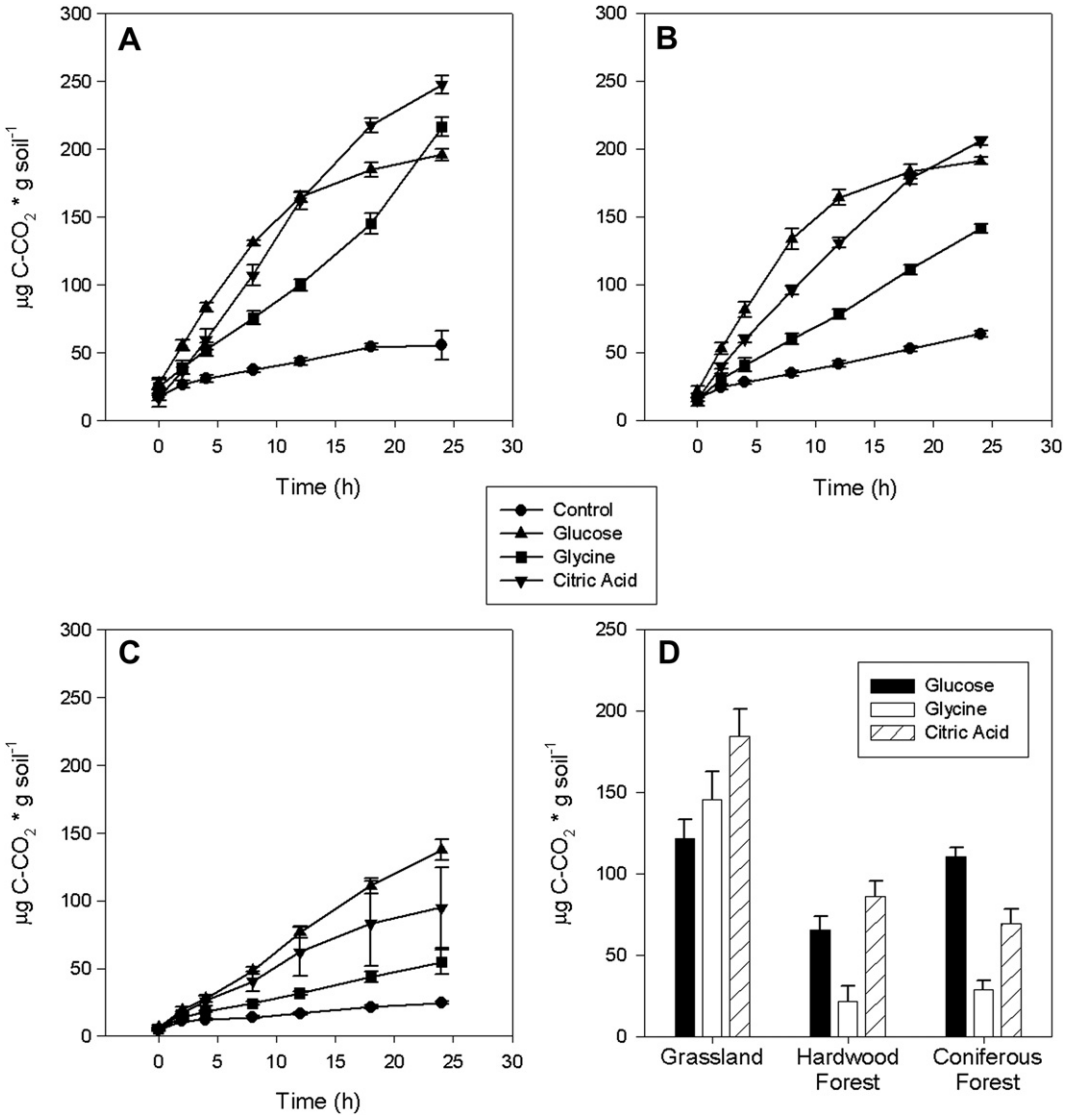

Fig. 1. CO_2 production. CO_2 accumulation over a 24 h period (per g of dry soil) after the addition of a carbon substrate, glucose (▲), glycine (■), or citric acid (▼), or a water-only control (●) to grassland (A), hardwood (B), and coniferous forest (C) soils. (D) Increase in CO_2 production in microcosms amended with glucose, glycine, or citric acid over control treatments for grassland, hardwood, and coniferous forest soils. The addition of a substrate resulted in a significant increase in CO_2 production for all three substrates and soils ($P < 0.05$ in all cases).

Except for the addition of glucose to the grassland soil (Table 3), the addition of the LMW-C substrates to the soils did alter bacterial community composition (Figs. 2 and 3, ANOSIM global $R = 0.78$, $P < 0.05$ for the substrate effects). However, the differences between the soils were always greater than the community differences within an individual soil that received the LMW-C substrates (Fig. 3), i.e. the soils harbored distinct bacterial communities regardless of the substrate being added (ANOSIM global $R = 0.534$, $P < 0.05$ for soil type).

The addition of glucose had a significant effect on bacterial communities in the hardwood and coniferous forest soils (Table 3), but different groups responded to the substrate additions in the two soils. In the hardwood forest soil, only the phylum Bacteroidetes (76% above control soil) significantly increased in relative abundance ($P < 0.05$) relative to the control. As evident in Fig. 4, the community shifts in the coniferous forest soil receiving glucose were largely driven by slight increases in the relative abundances of α-Proteobacteria and γ-Proteobacteria (35% and 34% increase over the control soils, respectively), and larger relative increases in the abundances of β-Proteobacteria and Actinobacteria (114% and 273% increase relative to controls, respectively). Within the β-Proteobacteria and Actinobacteria groups, the Burkholderiales, Actinobacteridae, and Rubrobacteridae had the largest increase in relative abundance with the addition of glucose to the coniferous forest soil (Table 2). The addition of glucose did not significantly affect community structure in grassland soil according to the ANOSIM (Table 3), and no dominant phyla or subphyla increased significantly ($P > 0.05$) over the control soils (Fig. 4).

The addition of glycine caused a significant change in community structure compared to controls in all three soils (Table 3). However, the community response varied across soil type, with multiple taxa increasing in relative abundance relative to controls in all three soils. In both grassland and coniferous forest soils, there were modest increases in the relative abundance of the α-Proteobacteria subphylum (24 and 35% above controls, respectively) and larger increases in the relative abundance of the phylum Actinobacteria (79 and 266%, respectively). However, in the hardwood

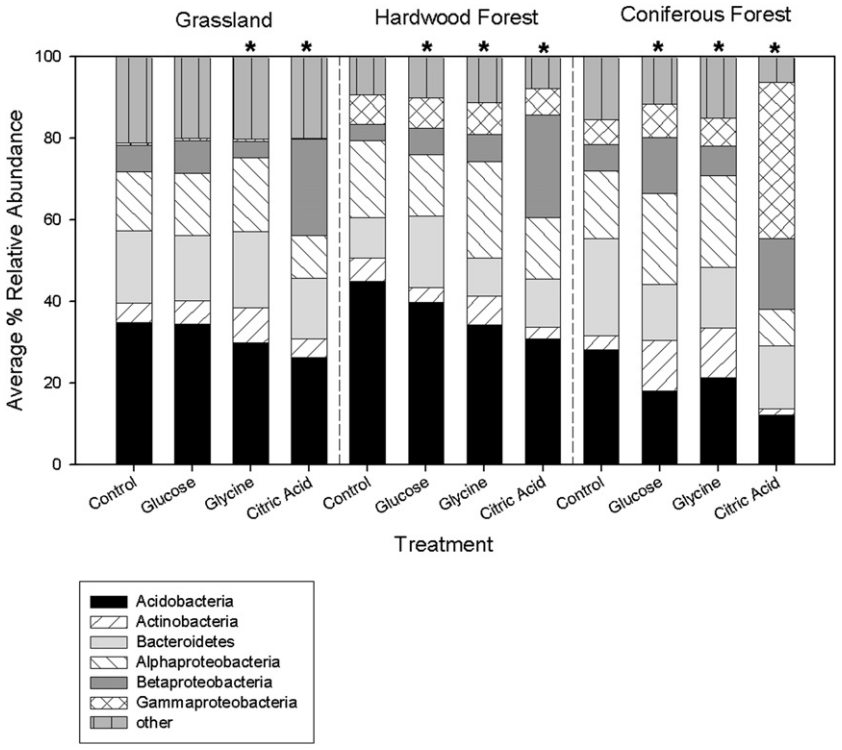

Fig. 2. Community composition profiles. Average relative abundances of dominant taxa (phyla Acidobacteria, Actinobacteria, and Bacteroidetes and the α-, β-, and γ-Proteobacteria subphyla) in the control and substrate addition treatments for the three soil types.

Table 2
Taxonomic community compositions.

Phylum/Sub-Phylum Order	Grassland				Hardwood forest				Coniferous forest			
	Control	Glucose	Glycine	Citric acid	Control	Glucose	Glycine	Citric acid	Control	Glucose	Glycine	Citric acid
Acidobacteria	**29.2 (1.1)**	**34.4 (2.2)**	**29.8 (0.4)**	**26.3 (1.4)**	**44.9 (2.0)**	**39.9 (1.4)**	**34.2 (2.0)**	**30.7 (1.4)**	**28.2 (0.9)**	**18.2 (2.3)**	**21.3 (0.8)**	**12.1 (0.3)**
Actinobacteria	**4.8 (0.3)**	**5.8 (1.1)**	**8.6 (1.0)**	**4.5 (0.8)**	**5.8 (1.0)**	**3.6 (0.5)**	**7.1 (1.5)**	**2.9 (0.3)**	**3.3 (0.5)**	**12.4 (2.3)**	**12.2 (1.6)**	**1.6 (0.3)**
Acidimicrobidae	0.8 (0.1)	1.0 (0.1)	0.6 (0.0)	0.6 (0.1)	1.2 (0.3)	0.8 (0.1)	1.2 (0.2)	0.8 (0.2)	0.6 (0.1)	1.0 (0.1)	1.1 (0.0)	0.3 (0.1)
Actinobacteridae	2.9 (0.2)	3.6 (0.6)	6.9 (1.0)	2.9 (0.6)	3.2 (0.6)	2.0 (0.5)	4.3 (1.1)	1.5 (0.3)	2.0 (0.3)	8.8 (1.6)	8.8 (1.6)	1.0 (0.1)
Rubrobacteridae	1.0 (0.1)	1.1 (0.4)	1.0 (0.1)	1.0 (0.2)	1.4 (0.3)	0.8 (0.2)	1.6 (0.3)	0.6 (0.2)	0.7 (0.1)	2.5 (0.7)	2.3 (0.3)	0.4 (0.1)
Other	0.1 (0.0)	0.1 (0.0)	0.1 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.1 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Bacteroidetes	**17.6 (0.7)**	**15.9 (0.5)**	**18.7 (1.2)**	**14.8 (1.4)**	**10.0 (0.6)**	**17.6 (1.3)**	**9.4 (1.3)**	**11.9 (0.6)**	**23.9 (1.4)**	**13.5 (1.5)**	**14.9 (1.7)**	**15.3 (0.9)**
Flavobacteriales	0.7 (0.1)	0.6 (0.1)	0.7 (0.0)	0.5 (0.1)	0.3 (0.1)	0.4 (0.1)	0.5 (0.1)	0.5 (0.1)	1.1 (0.2)	0.6 (0.1)	1.0 (0.3)	1.1 (0.1)
Flexibacterales	1.2 (0.2)	1.3 (0.1)	1.4 (0.3)	1.0 (0.1)	0.6 (0.2)	0.7 (0.1)	0.6 (0.2)	0.6 (0.1)	1.3 (0.2)	0.7 (0.2)	0.7 (0.2)	0.6 (0.1)
PC1-9	1.1 (0.2)	1.3 (0.2)	1.0 (0.2)	0.9 (0.1)	2.6 (0.2)	6.9 (0.7)	2.8 (0.4)	3.7 (0.2)	5.9 (0.5)	4.1 (0.7)	3.7 (0.5)	3.5 (0.5)
Saprospirales	12.7 (0.5)	11.3 (0.6)	13.7 (1.5)	10.7 (0.8)	5.6 (0.3)	8.1 (0.9)	4.7 (0.7)	6.4 (0.3)	13.5 (1.0)	7.0 (0.5)	8.3 (0.7)	8.5 (0.7)
Other	1.9 (0.0)	1.5 (0.0)	1.9 (0.0)	1.7 (0.2)	0.8 (0.1)	1.6 (0.1)	0.8 (0.0)	0.8 (0.1)	2.2 (0.1)	1.2 (0.1)	1.4 (0.2)	1.6 (0.0)
α-Proteobacteria	**14.6 (0.8)**	**15.3 (0.6)**	**18.1 (0.5)**	**10.5 (1.6)**	**18.7 (1.6)**	**15.0 (1.0)**	**23.5 (3.2)**	**14.9 (0.7)**	**16.5 (1.1)**	**22.4 (0.8)**	**22.4 (0.9)**	**9.0 (0.6)**
Acetobacterales	0.4 (0.0)	0.3 (0.0)	0.2 (0.0)	0.2 (0.1)	1.0 (0.2)	0.7 (0.1)	0.8 (0.1)	0.6 (0.2)	0.8 (0.1)	1.0 (0.1)	0.9 (0.3)	0.2 (0.1)
Bradyrhizobiales	6.0 (0.5)	7.1 (0.6)	9.5 (0.5)	5.0 (0.7)	11.0 (1.2)	7.7 (0.9)	16.2 (3.0)	8.9 (0.4)	7.7 (0.5)	13.3 (1.0)	12.2 (0.6)	4.9 (0.4)
Caulobacterales	0.9 (0.3)	0.8 (0.1)	0.8 (0.2)	0.3 (0.1)	1.0 (0.2)	1.0 (0.0)	0.9 (0.1)	1.0 (0.2)	1.1 (0.2)	1.2 (0.1)	1.5 (0.2)	0.7 (0.2)
Ellin329	1.1 (0.1)	1.1 (0.1)	0.9 (0.2)	0.6 (0.1)	1.2 (0.2)	1.2 (0.1)	1.4 (0.2)	1.1 (0.1)	1.4 (0.2)	1.0 (0.2)	1.1 (0.1)	0.5 (0.1)
Sphingomonadales	1.8 (0.3)	1.5 (0.2)	1.9 (0.2)	1.1 (0.3)	0.4 (0.1)	0.2 (0.1)	0.3 (0.1)	0.2 (0.1)	1.1 (0.1)	1.5 (0.2)	1.5 (0.2)	0.7 (0.1)
Other	4.4 (0.2)	4.6 (0.2)	4.8 (0.1)	3.3 (0.4)	4.1 (0.4)	4.3 (0.4)	3.9 (0.4)	3.1 (0.4)	4.4 (0.3)	4.4 (0.2)	5.1 (0.4)	2.0 (0.3)
β-Proteobacteria	**6.4 (0.1)**	**8.1 (0.9)**	**4.1 (0.7)**	**23.6 (2.5)**	**4.0 (0.2)**	**6.4 (1.0)**	**6.8 (0.5)**	**25.3 (1.8)**	**6.4 (0.1)**	**13.7 (1.4)**	**7.3 (0.6)**	**17.4 (1.3)**
Burkholderiales	3.4 (0.3)	5.6 (0.7)	2.3 (0.3)	21.3 (2.4)	1.9 (0.3)	4.2 (0.9)	4.0 (0.3)	22.1 (1.6)	4.0 (0.3)	10.8 (1.2)	4.9 (0.4)	15.5 (1.2)
MNn1	1.2 (0.1)	1.1 (0.1)	0.7 (0.2)	0.9 (0.1)	0.8 (0.1)	0.8 (0.1)	1.2 (0.1)	0.9 (0.2)	1.1 (0.2)	0.9 (0.1)	0.8 (0.3)	0.5 (0.1)
Other	1.8 (0.1)	1.5 (0.1)	1.0 (0.1)	1.4 (0.1)	1.2 (0.1)	1.3 (0.2)	1.6 (0.3)	2.3 (0.4)	1.4 (0.1)	2.0 (0.3)	1.6 (0.3)	1.4 (0.1)
γ-Proteobacteria	**4.6 (0.4)**	**4.6 (0.2)**	**4.8 (0.3)**	**6.8 (0.9)**	**7.3 (0.4)**	**7.6 (0.5)**	**7.7 (0.2)**	**6.5 (0.7)**	**6.1 (0.4)**	**8.2 (0.5)**	**6.9 (0.5)**	**38.3 (2.3)**
CCD24	2.3 (0.3)	2.0 (0.1)	2.0 (0.2)	1.8 (0.0)	3.2 (0.1)	2.5 (0.2)	3.2 (0.2)	2.3 (0.4)	1.7 (0.2)	0.9 (0.3)	1.0 (0.2)	0.5 (0.1)
Legionellales	0.9 (0.2)	0.8 (0.2)	0.9 (0.2)	0.8 (0.2)	3.0 (0.4)	3.7 (0.4)	3.2 (0.3)	2.8 (0.3)	2.0 (0.2)	0.8 (0.2)	1.5 (0.1)	0.8 (0.1)
Pseudomonadaceae	0.1 (0.0)	0.4 (0.2)	0.7 (0.2)	2.9 (1.1)	0.2 (0.1)	0.5 (0.1)	0.4 (0.1)	0.5 (0.2)	0.4 (0.1)	2.4 (0.5)	1.1 (0.4)	34.9 (2.6)
Xanthomonadales	0.9 (0.0)	1.0 (0.1)	0.9 (0.1)	0.7 (0.2)	0.6 (0.1)	0.5 (0.1)	0.6 (0.1)	0.7 (0.3)	1.3 (0.3)	1.6 (0.3)	2.5 (0.2)	0.8 (0.1)
Other	0.4 (0.0)	0.4 (0.0)	0.3 (0.1)	0.7 (0.2)	0.3 (0.1)	0.3 (0.1)	0.3 (0.1)	0.2 (0.1)	0.7 (0.1)	2.6 (0.6)	0.9 (0.2)	1.2 (0.5)
δ-Proteobacteria	**4.3 (0.3)**	**3.2 (0.2)**	**2.8 (0.1)**	**2.8 (0.1)**	**2.1 (0.3)**	**2.0 (0.3)**	**2.3 (0.1)**	**1.6 (0.3)**	**3.0 (0.2)**	**2.2 (0.2)**	**2.8 (0.4)**	**1.2 (0.1)**
Myxococcales	2.9 (0.2)	2.2 (0.1)	1.6 (0.1)	1.7 (0.2)	1.0 (0.1)	1.2 (0.3)	1.3 (0.1)	0.9 (0.3)	1.8 (0.2)	1.6 (0.2)	1.8 (0.2)	0.6 (0.0)
Other	1.4 (0.0)	1.0 (0.1)	1.2 (0.1)	1.0 (0.0)	1.1 (0.1)	0.8 (0.0)	1.0 (0.1)	0.7 (0.0)	1.2 (0.1)	0.6 (0.1)	1.0 (0.1)	0.6 (0.0)
Gemmatimonadetes	**6.4 (0.3)**	**5.6 (0.5)**	**5.1 (0.2)**	**4.6 (0.2)**	**2.7 (0.3)**	**2.7 (0.1)**	**3.5 (0.4)**	**2.9 (0.3)**	**4.1 (0.3)**	**3.1 (0.3)**	**4.9 (0.5)**	**1.8 (0.4)**
Gemmatimonadales	2.7 (0.2)	2.2 (0.3)	2.3 (0.2)	1.9 (0.2)	0.7 (0.2)	0.9 (0.1)	0.8 (0.1)	0.9 (0.1)	2.6 (0.2)	1.6 (0.3)	2.8 (0.4)	1.3 (0.2)
Other	0.9 (0.1)	0.7 (0.0)	0.7 (0.1)	0.7 (0.1)	0.1 (0.0)	0.1 (0.0)	0.3 (0.0)	0.2 (0.1)	0.2 (0.0)	0.3 (0.1)	0.4 (0.1)	0.0 (0.0)
Other	**9.2 (0.4)**	**9.8 (0.5)**	**10.2 (0.4)**	**8.0 (0.4)**	**6.5 (0.5)**	**7.1 (1.0)**	**7.9 (0.9)**	**5.1 (0.3)**	**9.7 (0.6)**	**7.4 (0.4)**	**9.1 (0.4)**	**3.8 (0.2)**

Average relative abundances of dominant phyla and subphyla as calculated for each of the 4 replicates for each treatment. Dominant phyla and subphyla are represented in **Bold**, orders are in plain type and standard errors are enclosed in parentheses.

Table 3
ANOSIM results.

Soil	All substrates vs. control	Substrate vs. control	*R*-Value
Grassland*	R-Value	Glucose	0.047
	0.534	Glycine*	0.43
		Citric Acid*	0.85
Hardwood forest*	R-Value	Glucose*	0.75
	0.82	Glycine*	0.67
		Citric Acid*	1.0
Coniferous forest*	R-Value	Glucose*	0.97
	0.94	Glycine*	0.90
		Citric Acid*	1.0

ANOSIM results comparing community composition between soil types (first column) and between substrate additions and control treatments within soil type. * indicates significant result with $P < 0.05$. The overall soil type effect was found to have a global R of 0.534 ($P < 0.05$).

forest soil, only the β-Proteobacteria subphylum (71%) increased in relative abundance.

Of the three substrates, the microbial community response to the addition of citric acid was most pronounced. In the grassland soil, β-Proteobacteria increased in average relative abundance by 268% relative to controls. In the hardwood soil, there was a large increase in the relative abundance of β-Proteobacteria (539%) and a modest increase in Bacteroidetes (19%). In the coniferous forest soil, β-Proteobacteria and γ-Proteobacteria increased in average relative abundance by 171% and 523%, respectively. In the coniferous forest soil, the increase in γ-Proteobacteria was primarily driven by Pseudomonadaceae, and in all three soil types, the increase in β-Proteobacteria was driven by the Burkholderiales.

4. Discussion

As expected, the addition of the LMW-C substrates led to significant increases in CO_2 production. However, the magnitude of the CO_2 response did not correspond to the shift in bacterial community structure. There are three possible reasons for this disconnect between the catabolic and community responses. First, substrate use efficiencies likely vary across substrates (or across soils within a given soil type), with some substrates being preferentially catabolized with no apparent net microbial biomass accumulation (Devevre and Horwath, 2000). Second, if all taxa increased equally in response to the addition of certain substrates, relative shifts in community composition would be undetectable. This is unlikely given that bacterial community composition was significantly altered by substrate additions in nearly all cases (Fig. 2) and we would expect that LMW-C compounds should preferentially enrich some microbial taxa over others (Dunbar et al., 2002; Bernard et al., 2007; Cleveland et al., 2007). Third, it is possible

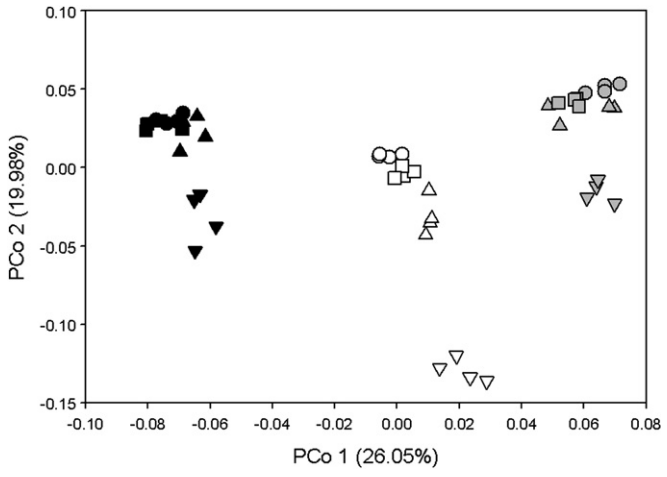

Fig. 3. Weighted UniFrac principle coordinates analysis of microbial communities from grassland (●▲■▼), hardwood forest (●▲■▼), and coniferous forest (○△□▽) soils receiving only water (●), glucose (▲), glycine (■), and citric acid (▼) amendments.

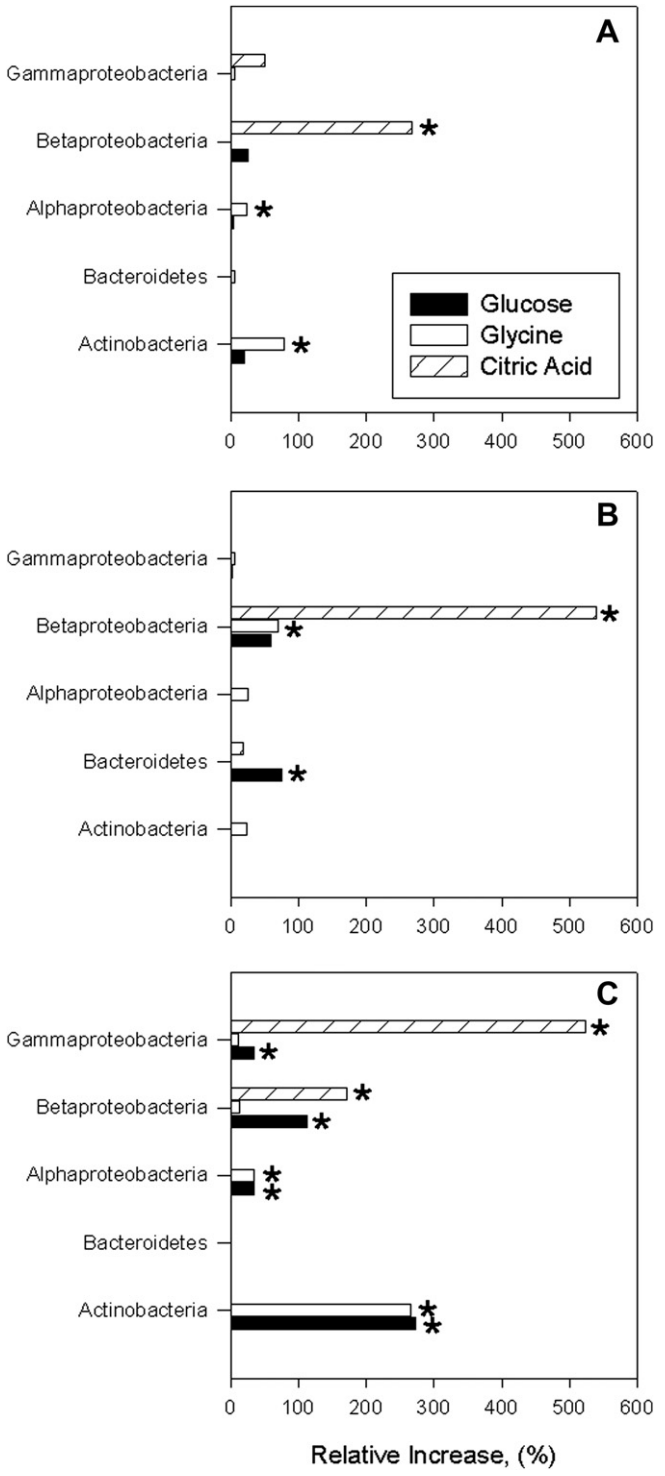

Fig. 4. Percent increases in the relative abundances of dominant taxa in response to substrate additions to (A) grassland, (B) hardwood forest, and (C) coniferous forest soils. (*) indicates a significant increase (t test, $P < 0.05$) in relative abundance in the treatment over the control for that soil.

that, even with more than 1500 sequences per sample, the shifts in the relative abundances of individual taxa were too small to be detectable at this level of surveying effort.

Where we did observe significant responses to the substrate additions, only a subset of the bacterial community—namely taxa within the β-Proteobacteria, γ-Proteobacteria, and to a lesser extent α-Proteobacteria, Bacteroidetes, and Actinobacteria groups—increased in relative abundance. It is important to recognize that multiple bacterial taxa increased in response to substrate additions (and different groups increased in different soils). However, there were many taxa (including the Acidobacteria and the Gemmatimonadates) that were abundant in the soils examined but exhibited little to no response to substrate additions. Furthermore, it is not known why the addition of glucose did not result in a community shift in the grassland soil, possibly due to more anabolism than catabolism of the substrate in that soil. In this study, it is unknown if the taxa which responded to the substrate additions were directly metabolizing the added substrates and isotope probing techniques may be more effective for resolving this issue (Padmanabhan et al., 2003; Rangel-Castro et al., 2005; Bernard et al., 2007). However, those techniques also face a similar problem when used with readily metabolized substrates given that it is impossible to distinguish between the organisms that originally consumed the substrate and the organisms which consumed the substrate C or N indirectly via uptake of cellular products (Morris et al., 2002).

The specific taxonomic groups that responded to the addition of a single substrate (e.g. citric acid) in this study differed across soils—there was no single, LMW-C metabolizing taxon in these soils. Thus, this apparent functional redundancy and the immense diversity of microbial communities highlights the difficulties in defining functional groups of bacteria at more detailed levels of taxonomic resolution. The large variability in the responses to the substrate additions also highlights the difficulty of defining broad ecological functions at a more detailed level of taxonomic resolution. For example, while β-Proteobacteria responded to the addition of citric acid in all three soils, the same β-Proteobacterial phylotypes did not respond in each of the soils (Table 2). However, as observed in previous studies of bulk soil and rhizosphere communities (Chow et al., 2002; Dunbar et al., 2002; Bernard et al., 2007), Proteobacteria increased in abundance in response to LMW-C additions in nearly all treatments. Likewise, Fierer et al. (2007a) found Proteobacteria to be more abundant in soils with higher C availability. Of the Proteobacteria subphyla, it was the β-Proteobacteria that responded most consistently across all three soil types. The β-Proteobacteria have also been found to increase in relative abundance with C additions in studies using clone libraries (Chow et al., 2002), and DGGE (Langenheder and Prosser, 2008), as well as in the heavy fractions of stable isotope probing LMW-C additions (Padmanabhan et al., 2003; Bernard et al., 2007). Additionally, members of the γ-Proteobacteria subphylum also responded strongly to the addition of citric acid in the coniferous forest soil. Several studies have also found the γ-Proteobacteria to respond to LMW-C substrate additions (Padmanabhan et al., 2003; Cleveland et al., 2007) and Dunbar et al. (2002) found an increased abundance of γ-Proteobacteria in rhizosphere soil compared to interspace soil at two locations (Dunbar et al., 2002). Thus, these results and the results from other studies suggest that the β- and γ-Proteobacteria represent important copiotrophic taxa in soil, with increases in their relative abundance potentially tied to increases in the supply of labile C substrates to soil.

It is notable that members of the Acidobacteria phylum, although numerically dominant in all three soils, did not respond appreciably to any of the substrate additions. Acidobacteria have been observed to be abundant in rhizosphere soils (Chow et al., 2002; Lee et al., 2008), but large decreases in Acidobacteria abundances have been observed after the addition of C substrates (Cleveland et al., 2007). Due to their limited culturability, little is known about the physiology of Acidobacteria. However, a meta-analysis of rhizosphere and bulk soil studies found that Acidobacteria tend to be more abundant in soils with low C availability (Fierer et al., 2007a), which is consistent with the results from this study and the hypothesis that this taxonomic group is predominately oligotrophic.

While we were able to observe fairly predictable responses across soil types with only a few taxa typically responding to substrate additions, it is important to note that the three substrates were added one at a time to the soils, whereas microbes encounter a complex cocktail of many different C compounds released into the soil through root exudation and litter leaching (Hartmann et al., 2009). Thus, it is possible that communities could behave differently when given a cocktail as opposed to single substrates in isolation. Likewise, the concentrations of the substrates that were added were relatively high, so longer-term responses observed under field conditions may not equate with the shorter-term responses observed here. Additional work is required to determine how these LMW-C compounds may alter soil microbial communities *in situ*, using lower concentrations as well as adding mixtures of different substrates.

This study demonstrates the utility of using pyrosequencing for determining lineages associated with specific community responses to changes in substrate availability. By using bar-coded primers, we were able to analyze a sufficient number of samples to replicate experimental treatments, while still surveying each bacterial community in depth—something that has been difficult to do with other sequence-based approaches due to the great expense and time-consuming nature of producing large clone libraries. The ability to replicate experimental treatments is of particular importance as it allows us to attribute community responses to environmental conditions rather than to methodological variation. We also demonstrate that we were able to detect differences between microbial communities, using both taxonomic and phylogenetic methods, as well as shifts in microbial community structures within a relatively short time period, potentially allowing many different soils to be surveyed for functional groups in a reasonable amount of time.

This study shows that specific phylogenetic groups respond to LMW-C additions at a relatively coarse level of phylogenetic resolution. We also observed similar taxa responding to the same substrate in different soil types that differ with respect to their initial bacterial community composition. The magnitude of the community response was dependent on both substrate type and soil type, but in nearly all cases, we could resolve changes in bacterial community structure after a relatively short incubation period. The results of this study indicate that we can begin to link the taxonomic composition of microbial communities to functional attributes, such as the processing of LMW-C substrates. These linkages can be further resolved by adding a wider variety of LMW-C compounds (and mixtures of these compounds) to a broader array of soil types in order to build a predictive understanding of how soil edaphic properties may influence which specific bacterial taxa respond to substrate additions.

Acknowledgements

We gratefully acknowledge the Andrew W. Mellon Foundation and the National Science Foundation for funding. Thanks to Micah Hamady for assistance in bioinformatics.

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