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Reduced dependence of rhizosphere microbiome on plant-derived carbon in 32-year long-term inorganic and organic fertilized soils

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ABSTRACT

Root-derived carbon (C) is considered as critical fuel supporting the interaction between plant and rhizosphere microbiome, but knowledge of how plant–microbe association responds to soil fertility changes in the agroecosystem is lacking. We report an integrative methodology in which stable isotope probing (SIP) and high-throughput pyrosequencing are combined to completely characterize the root-feeding bacterial communities in the rhizosphere of wheat grown in historical soils under three long-term (32-year) fertilization regimes. Wheat root-derived ^{13}C was dominantly assimilated by Actinobacteria and Proteobacteria (notably Burkholderiales), accounting for nearly 70% of root-feeding microbiome. In contrast, rhizosphere bacteria utilizing original soil organic matter (SOM) possessed a higher diversity at phylum level. Some microbes (e.g. Bacteroidetes and Chloroflexi) enhancing in the rhizosphere were not actively recruited through selection by rhizodeposits, indicating a limited range of action of root exudates. Inorganic fertilization decreased the dependence of Actinobacteria on root-derived C, but significantly increased its proportion in SOM-feeding microbiome. Furthermore, significantly lower diversity of the root-feeding microbiome, but not the SOM-feeding microbiome, was observed under both organic and inorganic fertilizations. These results revealed that long-term fertilizations with increasing nutrients availability would decrease the preference of rhizosphere microbiome for root-derived substrates, leading to a simpler crop–microbe association.

1. Introduction

Terrestrial plants impact soil by producing an array of substrates that originate from sloughed-off root materials including cells, tissues and mucilages, as well as active root exudates such as organic acids, sugars, amino acids and phytohormones (Dennis et al., 2010). In general such plant-derived carbon (C) ranges from <10% photosynthetically fixed C to 44% total plant C under nutrient stress (Bais et al., 2006). This has the potential to lead to relationships between plants and various soil microbiota in the rhizosphere, because bacteria are often limited by available C sources in bulk soils. Microbes inhabiting such niches subsequently impart the plant with beneficial or detrimental traits; accordingly, altering this balance is of great interest in agronomy (Lundberg et al., 2012). However, the role of crop roots in the selection mechanism of the rhizosphere microbiome is less elucidated in agroecosystems (Hirsch and Mauchline, 2012), in part because of a lack of analytical methods (Cardon and Gage, 2006). The core rhizosphere-inhabiting microbiotas in *Arabidopsis thaliana*, soybean and wheat have been recently revealed in great detail (Bulgarelli et al., 2012; Lundberg et al., 2012; Donn et al., 2014; Mendes et al., 2014). These studies indicated that the rhizosphere or endophytic microbiome was a subset of the bulk soil community and dominated by Proteobacteria, Actinobacteria and Bacteroidetes. As a result, considering the less complexity of bacterial groups in the rhizosphere, a higher specialization of rhizosphere bacterial functions would be expected (Mendes et al., 2014). However, evidence of whether these dominant microorganisms thriving in the rhizosphere that is directly

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recruited by plant-derived C or via preferences for specific conditions such as soil pH, mineral nutrients and physical structure is still limited.

Soil organic matter (SOM) and nutrient availability have been increased in numerous agricultural lands over the last decades as a results of organic and inorganic fertilizations (Ai et al., 2012; Maillard and Angers, 2014) and enhanced nitrogen deposition (Liu et al., 2013). However, the ecosystem-level influences of these cumulative nutrients on soil–plant–microbe processes are unclear. Nutrient availability can impact the rhizosphere microbiotas either directly by limiting their nutrition or, more indirectly, by altering root exudation and root morphology (Rengel and Marschner, 2005). For example, inorganic fertilization suppressed microbial respiration by 36–46% in the rhizosphere of three hardwood trees, possibly via a decreased rhizosphere C flux (Phillips and Fahey, 2007). Nitrogen also has the potential to shift metabolic capabilities of microbial communities in decomposing soil C pools (Mack et al., 2004; Ramirez et al., 2012). In contrast to inorganic fertilizers, application of organic manure supports the accumulation of SOM and development of soil microbial communities with greater biodiversity (Mäder et al., 2002). Developing a predictive understanding of the role of fertilization practices in governing plant–microbe interaction would thus be a key step towards enhancing the environmental sustainability of crop production.

At present, limited information is available to address the plant-associated rhizosphere microbiome and their roles in root-derived C utilization, although the impacts of inorganic fertilizer and organic manure on soil microbial community have been widely reported in recent years (Sun et al., 2004; Ramirez et al., 2012; Coolon et al., 2013). As a result, the aims of this study were (*i*) to identify the bacterial community actively utilizing root-derived C in wheat rhizosphere, (*ii*) to determine whether the enhancement of rhizosphere microbes was directly driven by root-derived C, and (*iii*) to assess how soil microbial community and plant–microbe interaction responded to long-term fertilization practices. In doing so, we collected soils from a long-term fertilization field experiment with three treatments (i.e. no-fertilization control; inorganic nitrogen, phosphorus and potassium, NPK; and organic manure plus NPK, MNPK). With an *in situ* external $^{13}CO_2$ pulse labeling to wheat plants growing in the middle rhizosphere soil chamber in a 3-chambered-microcosm, we tracked ^{13}C movement from aboveground to belowground through DNA-SIP (stable isotope probing), and then characterized the bacterial communities through 454 pyrosequencing in wheat rhizosphere and root-free (bulk) soils. We hypothesized that: (*i*) microbial assimilation of wheat root-derived C would be dominated by a small subset of rhizosphere microbiome; (*ii*) a more diverse group of soil microbes would be activated under organic manure than under chemical fertilizers in the bulk soil; and (*iii*) long-term fertilization practices with increasing nutrients availability would reduce the dependence of rhizosphere microbiome on plant-derived C source.

2. Experimental procedures

2.1. Soil collection

Soil for the greenhouse experiment was collected in three fertilization fields from a long-term fertilization site initiated in 1979 at the Malan Farm (37°55′N, 115°13′E, 37 m above the sea level), Hebei, China. This region has a temperate and monsoonal type climate with an annual average temperature and precipitation of 12.6 °C and 490 mm, respectively. The experimental soil was classified as aquic inceptisol with a sandy loam texture according to U.S. soil taxonomy, which is typical in the North China Plain. Three fertilizations (three replicates each) as no fertilization (Control), chemical fertilizer N, P and K (NPK), and organic manure plus inorganic fertilizer N, P and K (MNPK) were respectively implemented in plots (12 m × 6.7 m) (Ai et al., 2013). The cropping system has been a rotation of winter wheat (*Triticum aestivum* cv. Shimai 18) and summer maize (*Zea mays* cv. Zhengdan 958). After 32-year fertilization, soil biochemical and biological properties are substantially different under these three fertilizations (Table S1) (Ai et al., 2012, 2013). In October 2011, ten random soil cores (6 cm diameter) from each plot as a composite sample were taken at 0–20 cm depth. A total of nine composite samples were transported to laboratory on ice, sieved (2-mm), and then stored at 4 °C until greenhouse experiments.

2.2. Soil microcosm construction, wheat growth and ^{13}C labeling

Seeds of wheat (*T. aestivum* cv. Shimai 18) were sown in the middle chamber of three-chambered growth pots (see Fig. S1) at a density of seven plants per pot, which were filled with distinctive soils from the Control, NPK or MNPK fertilization treatments with three replicates for each soil. Pots were spatially randomized and plants were grown in a greenhouse at the Institute under 16/8 h and 23/18 °C (day/night). Soil moisture was maintained at 40–60% of water-holding capacity. The ^{13}C labeling started 40 days later when plants were in an active vegetative growth state. Plants in the rhizobox system were labeled with $^{13}CO_2$ (98 atom % ^{13}C, Shanghai Research Institute of Chemical Industry, Shanghai, China) between 9 am and 5 pm (8 h) for 7 consecutive days (Lu and Conrad, 2005). During the labeling period, the total CO_2 concentration inside the chamber were maintained at 300–400 mg kg^{-1} with additional $^{13}CO_2$. It is very important that wheat plants were exposed to a normal CO_2 concentration level, because an elevated CO_2 level might affect rhizosphere C flow and associated microbiota (Drigo et al., 2010). Parallel microcosms as controls were also constructed without $^{13}CO_2$ labeling, i.e. under the ambient $^{12}CO_2$ condition (Thereafter we defined this as a $^{12}CO_2$ labeling condition). At the end of $^{13}CO_2$ labeling, the rhizosphere and bulk soils were sampled from the rhizobox (see Supplementary Information Methods S1 for details).

2.3. DNA extraction, gradient fractionation, denaturing gradient gel electrophoresis (DGGE) and ^{13}C analyses

Soil genomic DNA was extracted using a Fast DNA SPIN Kit for soil (MP Biomedicals, Illkirch, France) according to the manufacturer's instruction. In 5.1 ml quick-seal polyallomer tubes in a VTi 90 vertical rotor (Beckman Coulter, CA, USA), the rhizosphere DNA was fractionated by the cesium chloride (CsCl) equilibrium density gradient centrifugation (Lueders et al., 2004). Ultracentrifugation conditions were 56,200 r.p.m. (228 166 g_{av}) for 24 h at 20 °C (Zhang et al., 2012). DNA (4.0 μg) was mixed in CsCl gradients with an initial density of 1.725 g ml^{-1}. At a flow rate of 440 μl min^{-1}, centrifuged gradient was fractionated from bottom to top into 24 equal volumes (~220 μl) by displacing the CsCl solution with sterilized water at the top of the tube, using a programmable syringe pump (New Era Pump Systems Inc., NY, USA). Buoyant density of each collected fraction was determined by using an AR200 digital refractometer (Reichert, NY, USA). Recovery of nucleic acids from the CsCl salts was conducted by precipitation in two volumes of polyethylene glycol (PEG) 6000 solution (30% PEG 6000, 1.6 M NaCl) (Neufeld et al., 2007), and precipitates were washed twice with 70% ethanol and re-dissolved in 30 μl nuclease-free water. Quantification of nucleic acids was performed using the Picogreen assays (Molecular Probes, Invitrogen, Eugene, OR, USA). DGGE analyses (see Supplementary Information Methods S1) of the 16S rRNA PCR products derived from gradient fractions were performed using

samples from both $^{13}C–CO_2$ (^{13}C-labelling) and ambient CO_2 (^{12}C-labelling) growth microcosms.

The ^{13}C enrichment of DNA from gradient fractions was determined by an isotope ratio mass spectrometry (IRMS, IsoPrime 100, Cheadle, UK) coupled to an elemental analyser (Vario MICRO Cube, Elementar, Hanau, Germany) at the Institute of Environment and Sustainable Development in Agriculture, CAAS (Beijing, China), based on the method described by Haichar et al. (2008). $\delta^{13}C$ (‰) was calculated as: $\delta^{13}C\,(‰) = [(R_{sample}/R_{standard}) - 1] \times 1000$, where $R = {}^{13}C/{}^{12}C$, R_{sample} or $R_{standard}$ was the isotope ratio of the sample or a standard referenced to Pee Dee Belemnite.

2.4. 454 pyrosequencing

Bacterial DNA pyrosequencing was based on ~520 bp amplicons generated by the PCR primers 27F and 533R covering the hypervariable regions V1–V3 of the 16S rRNA gene. For multiplex pyrosequencing, the reverse primer (533R: 5′-TTA CCG CGG CTG CTG GCA C-3′) was extended at the 5′ end with an eight-base sample specific barcode sequence followed by the 454 Adaptor A. The sequence of forward primer (27F: 5′-AGA GTT TGA TCC TGG CTC AG-3′) was extended at the 5′ end by the 454 Adaptor B. All PCR reactions were carried out in triplicate 20 μl reactions with 1× PCR reaction buffer, 0.1 μM each of the primer, 0.25 mM of dNTPs, 1 U pfu DNA Polymerase (Promega, Madison, USA), and about 10 ng of template DNA. Thermal cycling consisted of initial denaturation at 95 °C for 2 min, followed by 25 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s, with a final extension of 5 min at 72 °C. Three replicate PCR products of the same sample were pooled and purified with a DNA gel extraction kit (Takara, Dalian, China). The purified DNA was quantified with Picogreen assays (Molecular Probes, Invitrogen, Eugene, OR, USA) and quality-controlled using an Agilent 2100 bioanalyzer (Agilent, Palo Alto, CA, USA). Amplicons were then pooled in equimolar ratios and subjected to emulsion PCR to generate amplicon libraries. Pyrosequencing was performed using primer A on the Roche/454 GS FLX platform with Titanium chemistry at the Majorbio Bio-Pharm Technology Co., Ltd., Shanghai, China.

2.5. Analyses of 16S rRNA gene sequences

Sequences were analyzed using the MOTHUR software package (Schloss et al., 2009). Sequence reads were assigned to each sample according to sample-specific barcodes. Low-quality sequences were removed if they did not meet the following criteria: (*i*) exact match to barcode and primers; (*ii*) at least 200 nucleotides in length with no ambiguous base pairs; (*iii*) their average quality score was >25. The potential chimeras were checked and eliminated from further analyses using the Chimera Slayer algorithm (Haas et al., 2011). In the final analysis of operational taxonomic unit (OTU) picking at 97% sequence identity, sequences that passed pre-processing were used and taxonomy was assigned using the SILVA bacterial database (Pruesse et al., 2007) by the MOTHUR classify.otu module (Schloss et al., 2009). Rarefaction curves were generated using the MOTHUR rarefaction.single module. The Simpson and Shannon diversity indexes were estimated by the MOTHUR software package. The obtained sequences have been deposited in the NCBI Sequence Read Archive (SRA) database (SRA099701).

2.6. Statistical analyses

The relative abundance of OTUs and phyla were reported as mean ± SEM ($n = 3$). For the relative abundance of phyla in different fractions or fertilization treatments, the data were analyzed by one-way ANOVA and significant differences in group means were compared using the Fisher's least significant difference (LSD) at $P = 0.05$. Two-way ANOVA was also performed using the soil fractions and the fertilization treatments as main effects. To detect differentially abundant OTUs in bacterial communities among fractions or fertilization treatments, we employed the Metastats program (White et al., 2009). Metastats was run using 1000 permutations to compute *P*-values in statistical tests, where the significant changes were considered if the generated *P*-values were less than 0.05. Ternary plots were prepared using the mean of the relative abundances of OTUs per treatment. The function "ternaryplot" from the R package "vcd" (Meyer et al., 2012) was employed to plot the point size of each OTU proportional to its weighted sum instead of the total sum of the relative abundances. Heatmaps were constructed based on Log_2 transformed data using the function "heatmap.2" from the R package "gplots" (Warnes et al., 2012), and the data were Log_2-transformed with the formula $Log_2(1000x + 1)$ (Lundberg et al., 2012), where x was the relative abundance of genus. For better visualization, only the top 30 most abundant genera from each treatment were shown in the heatmap. Dendrogram analysis, principal coordinate analysis and analysis of molecular variance and homogeneity of molecular variance were performed using the MOTHUR software package (Schloss et al., 2009).

3. Results

3.1. Microbial incorporation of wheat ^{13}C-rhizodeposits

The ^{13}C-label delivered by wheat roots was only detected in the rhizosphere soil (Fig. 1). The $\delta^{13}C$ values were significantly greater in the wheat rhizosphere soil (203‰) than in the bulk soil (−19‰). The $\delta^{13}C$ values were similar between the bulk soil in the $^{13}CO_2$-labeling chamber and the parallel rhizosphere (−22‰) or bulk soils (−23‰) under the ambient $^{12}CO_2$ condition (thereafter we defined this as a $^{12}CO_2$ labeling condition). These results indicated that the successful incorporation of ^{13}C-labelled rhizodeposits into rhizosphere soil.

DNA extracted from each ^{13}C-labelled rhizosphere soil was subjected to isopycnic ultracentrifugation, each fraction along the buoyant density gradient was then analyzed for DNA content and $\delta^{13}C$ to identify the heavy and light DNA (Fig. 2A). The DNA content profile showed a unique peak within 1.720–1.726 g ml^{-1} buoyant density (BD). The $\delta^{13}C$ values of DNA were slightly increased in the light CsCl fractions prior to a dramatic enhancement at a BD of 1.726–1.735 g ml^{-1}, indicating the presence of ^{13}C-labelled DNA. Similar BD distributions were obtained for DNA extracted from rhizosphere soils under different fertilization regimes (Fig. 2A).

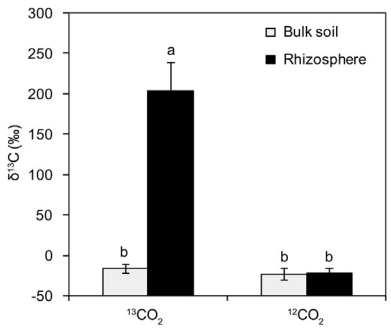

Fig. 1. Incorporation of ^{13}C-labelled rhizodeposits into rhizosphere soil of wheat. The ^{13}C enrichments (mean ± SEM, $n = 9$) in both rhizosphere and bulk soil were measured after 7 days of $^{13}CO_2$- or $^{12}CO_2$-labeling. Different letters indicate significant differences at $P < 0.05$, Fisher's LSD test.

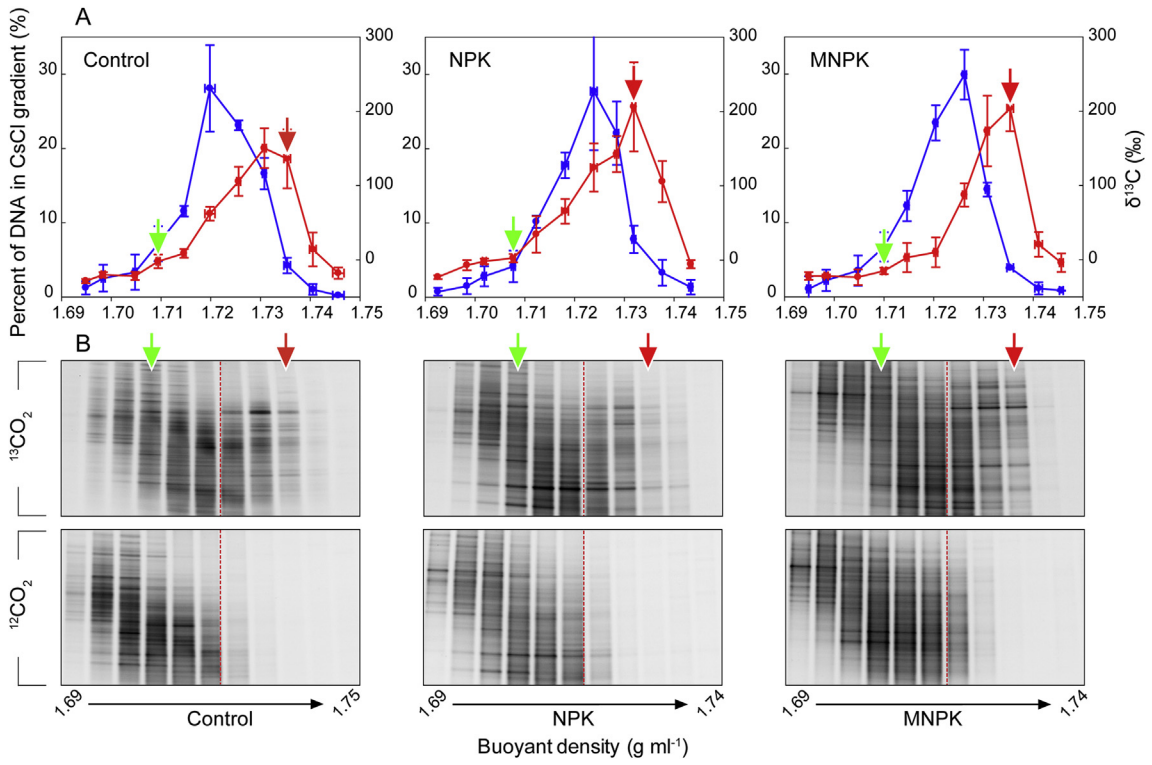

Fig. 2. Incorporation of plant-derived ^{13}C into DNA of rhizosphere microbiota. (A) DNA content (blue line, %) and $\delta^{13}C$ value (red line, ‰) of each gradient fraction obtained after the separation of labeled and unlabelled DNA from the rhizosphere soil of ^{13}C-labeled wheat by CsCl density-gradient centrifugation under the no-fertilization control, NPK and MNPK fertilization, respectively. The values of DNA content were converted to the proportion of total DNA throughout the gradient. The values given are the means ± SEM of three separate treatments. The red and green arrows indicate the locations of ^{13}C-labelled and unlabelled rhizosphere DNA, respectively, which are used to construct amplicon libraries for 454 pyrosequencing. (B) DGGE analyses of 16S rRNA PCR products derived from 11 density-gradient fractions was conducted using soil samples from microcosms with or without a 7-day period of $^{13}CO_2$ gas labeling. Significant differences in the DGGE patterns were observed in ^{13}C-labeled rhizosphere soil (at the right side of the red dotted line under 1.726–1.737 g ml^{-1} BD, buoyant density). Color arrows indicate the selected fractions as defined in A. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

To verify the separation of ^{13}C-labelled DNA from unlabelled DNA, DGGE analyses of the 16S rRNA PCR products derived from 11 density-gradient fractions (BD: 1.694–1.747 g ml^{-1}) were then performed using samples from microcosms where plants were grown under either $^{13}CO_2$ or $^{12}CO_2$ (Fig. 2B). The increase in relative intensity of 16S rRNA bands in heavy fractions with a BD of 1.726–1.737 g ml^{-1} was only observed in the ^{13}C-labeled chamber, whereas no obvious bacterial 16S rRNA bands could be detected in the heavy fractions from the $^{12}CO_2$ cultured chamber, which was consistent with the results observed for wheat rhizosphere soils under the three fertilization regimes (Fig. 2B).

Based on the above results, DNA in the heavy fraction with a BD of approximately 1.735 g ml^{-1} was considered to be a representative of the ^{13}C-labelled rhizosphere DNA (Fig. 2), indicating that bacteria with ^{13}C-DNA were the population members that most actively assimilated plant-derived C (i.e. root-feeding microbiome). DNA in the light fraction with a BD of approximately 1.710 g ml^{-1} was considered unlabelled rhizosphere DNA, indicating that microbiota with ^{12}C-DNA utilized original SOM as their main C source in the wheat rhizosphere (i.e. SOM-feeding microbiome).

3.2. Taxonomic profiling of the wheat rhizosphere microbiome

We subjected the 16S rRNA (V1–V3) gene amplicons of DNA prepared from ^{13}C-labelled and unlabelled rhizosphere DNA separated by the CsCl density-gradient centrifugation, and from whole-rhizosphere and bulk soil DNA extracted directly from corresponding compartments to 454 pyrosequencing. A dataset of 460 506 high-quality sequences with an average length of 501 bp and around 12 800 reads per sample was generated. At 97% similarity, the number of operational taxonomic units (OTUs) ranged from 3368 to 6291 depending on the DNA sample. Rarefaction analyses of individual samples revealed that new bacterial phylotypes continued to emerge, even after 15 000 reads, especially for the whole-rhizosphere and bulk soil samples (see Fig. S2).

To identify the main factors driving community composition, we conducted both the dendrogram analysis (Fig. 3) and principal coordinate analysis (PCoA) (see Fig. S3) based on the Yue–Clayton and Bray–Curtis distances, respectively, using all detected OTUs. The ^{13}C-labelled microbiota was significantly dissimilar from the communities recovered from unlabelled rhizosphere, whole-rhizosphere and bulk soil fractions (Fig. 3). These differences were confirmed by the heatmap (Fig. S4) generated using the top

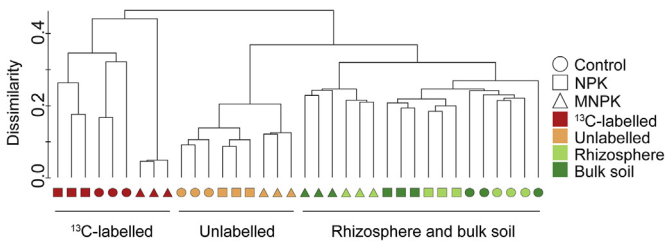

Fig. 3. Dendrogram analysis of samples using all OTUs identified under three fertilizations (no-fertilization control, NPK and MNPK) and four DNA fractions (^{13}C-labelled and unlabelled rhizosphere DNA, whole-rhizosphere and bulk soil DNA).

30 genera from each treatment. Specifically, the map demonstrated that the distribution of dominant genera in ^{13}C-labelled communities was distinct from that of unlabelled fractions. Closer examination revealed a marked fertilization effect (especially MNPK treatment) on bacterial communities of all four DNA fractions (Table S2a, Fig. 3). However, the statistical differentiation of the community structure between the whole-rhizosphere and bulk soil was not evident according to the analysis of molecular variance (AMOVA) of the Yue–Clayton distance (Table S2b), even though their chemical properties were obviously distinguishable between them (Table S1).

Taxonomic profiling showed that Proteobacteria and Actinobacteria were the dominant phyla (nearly 70%) in the ^{13}C-labelled rhizosphere communities and were significantly more abundant than in the unlabelled rhizosphere samples (Fig. 4A). The lower-order taxonomic analyses demonstrated that the enhancement of the rhizodeposits-stimulating Proteobacteria community was driven by the Alpha- and Beta-proteobacteria, notably Burkholderiales (accounting for 16% of ^{13}C-labelled rhizosphere microbiome) (Fig. 4B), whereas Gama- and Delta-proteobacteria in the ^{13}C-rhizosphere fraction were equally or less abundant than those in the unlabelled rhizosphere microbiome. All Actinobacteria were significantly enhanced at order level in the ^{13}C-labelled rhizosphere microbiome (Fig. 4C). In contrast, bacteria related to Acidobacteria, Chloroflexi, Firmicutes, Bacteroidetes and Verrucomicrobia were primarily identified in the unlabelled rhizosphere microbiome (Fig. 4A).

3.3. Wheat rhizosphere vs bulk soil

The soil microbial community was distributed across 11 dominant phyla and contained 93–95% of the sequences (Fig. 5A). Two-way ANOVA to compare the influence of rhizosphere effect and fertilization regime on the relative abundance of the major phyla demonstrated that most members in Group 2 (i.e. main unlabelled groups in Fig. 4A) were significantly affected by fertilization,

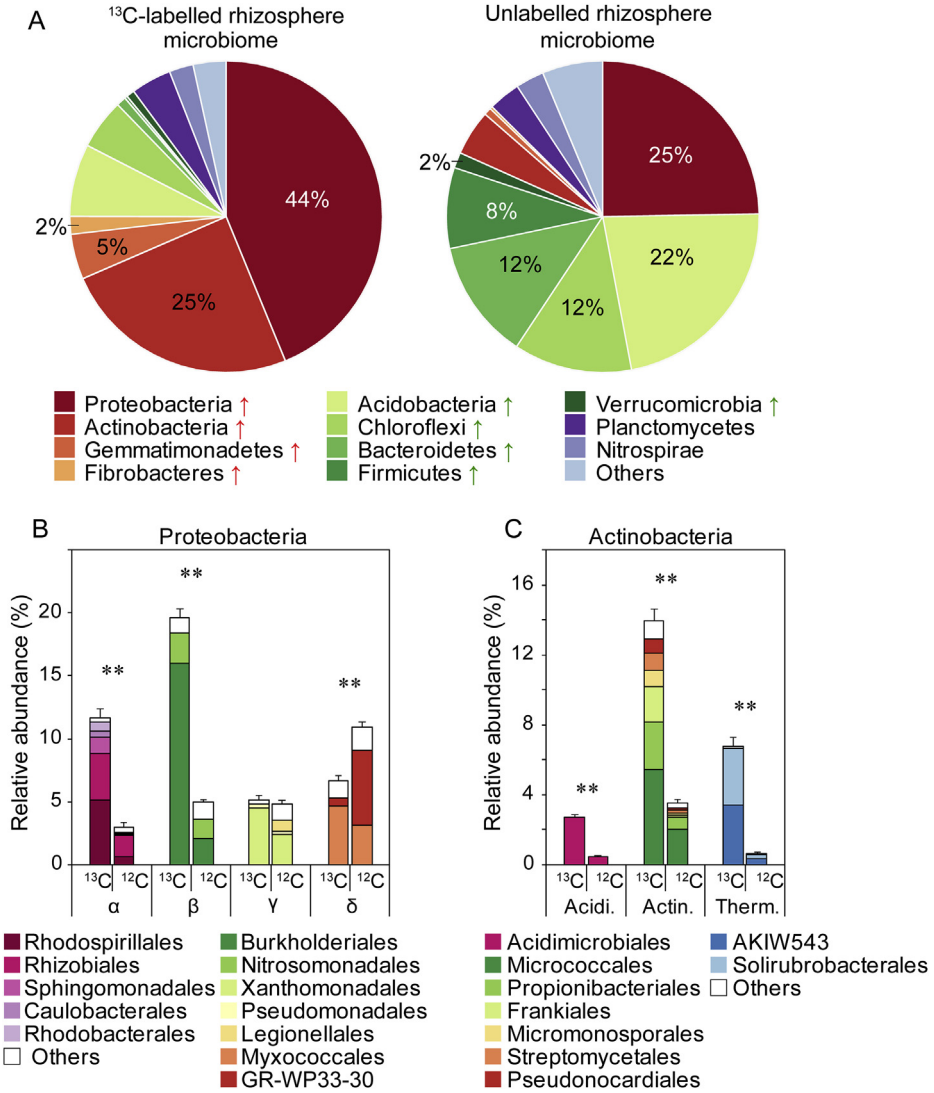

Fig. 4. Rhizodeposition assembles a distinct bacterial microbiota in wheat rhizosphere. (A) Taxonomic composition of the ^{13}C-labelled and unlabelled rhizosphere communities. The arrows indicate that the phyla are significantly enhanced in ^{13}C-labelled (red ↑) or unlabelled (green ↑) rhizosphere microbiome ($P < 0.01$, Fisher's LSD test). (B) Histograms showing the relative abundance of Proteobacteria orders in the ^{13}C-labelled (^{13}C) and unlabelled (^{12}C) rhizosphere microbiome. Four classes of the phylum Proteobacteria are (α) Alpha-, (β) Beta-, (γ) Gamma- and (δ) Delta-proteobacteria. Asterisks indicate a significant difference ($P < 0.01$, Fisher's LSD test) between ^{13}C-labelled and unlabelled rhizosphere microbiome. (C) Histograms showing the relative abundance of Actinobacteria orders in the ^{13}C-labelled and unlabelled rhizosphere microbiome. Three classes of the phylum Actinobacteria are Acidimicrobiia (Acidi), Actinobacteria (Actin), Thermoleophilia (Thermo). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

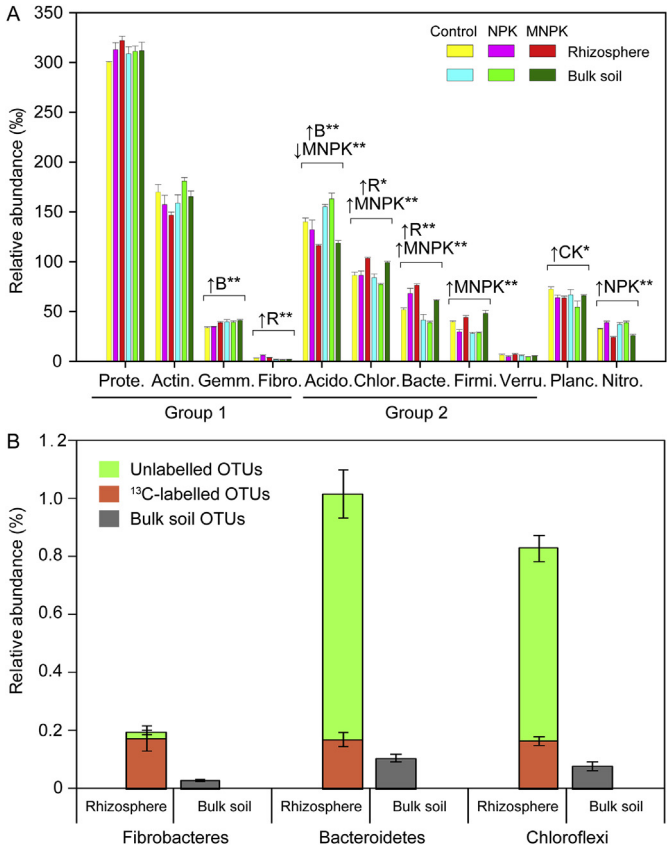

Fig. 5. Abundance of bacterial groups in the rhizosphere and bulk soil under different fertilizations (no-fertilization control, NPK and MNPK). (A) Relative abundance (mean + SEM, $n = 3$) of the most abundant bacterial phyla in the rhizosphere and bulk soil. Up and down arrows indicate significant changes in the relative abundance of bacterial phyla (↑, increased; ↓, decreased). Asterisks indicate significant differences between the rhizosphere (R) and bulk soil (B), or among fertilizations (two-way ANOVA, $^{*}P < 0.05$; $^{**}P < 0.01$). Group 1 and 2 represent the main ^{13}C-labelled and unlabelled groups, respectively, as shown in Fig. 4A. Abbreviations: Prote., Proteobacteria; Actin., Actinobacteria; Gemm., Gemmatimonadetes; Fibro., Fibrobacteres; Acido., Acidobacteria; Chlor., Chloroflexi; Bacte., Bacteroidetes; Firmi., Firmicutes; Verru., Verrucomicrobia; Planc., Planctomycetes; Nitro., Nitrospirae. (B) The distributions of rhizosphere-enhanced OTUs belonging to Fibrobacteres, Bacteroidetes and Chloroflexi, respectively, between ^{13}C-labelled and unlabelled rhizosphere fractions.

especially under MNPK (Fig. 5A). For instance, Chloroflexi, Bacteroidetes and Firmicutes were significantly enriched, while Acidobacteria were typically depleted under MNPK.

Significant quantitative differences in the relative abundance of five phyla were observed between the rhizosphere and bulk soil (Fig. 5A), with Fibrobacteres, Bacteroidetes and Chloroflexi, being more abundant in the rhizosphere. Results from the analyses with the Metastats program (White et al., 2009) revealed that enhancement of Fibrobacteres, Bacteroidetes and Chloroflexi in the rhizosphere was driven by a subset of OTUs. To evaluate potentially plant-derived assembly cues for this rhizosphere enrichment, we further assessed the distribution of these OTUs in the ^{13}C-labelled and unlabelled rhizosphere fractions (Fig. 5B). The rhizosphere-enhanced OTUs belonging to Fibrobacteres were primarily distributed in the ^{13}C-labelled rhizosphere fraction (90%) (Fig. 5B), and implying that wheat root-derived C served as a sufficient enrichment cue for Fibrobacteres in the rhizosphere. Surprisingly, other rhizosphere-enhanced OTUs belonging to Bacteroidetes and Chloroflexi were primarily distributed in unlabelled rhizosphere DNA (83% and 80%) (Fig. 5B). This result indicated that these subcommunities still utilized original SOM as their main C sources and thrived in the rhizosphere.

3.4. Rhizosphere microbiome under long-term inorganic vs organic fertilization

Abundances of OTUs were compared for three fertilization regimes (Control, NPK and MPK) in each DNA fraction (^{13}C-labelled and unlabelled rhizosphere DNA, whole-rhizosphere and bulk soil DNA) (Fig. S5A–D and Supplementary Information Data 1). The contribution of different fertilization regimes to microbial community composition changes was showed in Fig. 6A–D. Within the ^{13}C-labelled rhizosphere OTUs community (Figs. 6A and S5A), the NPK-enhanced microbiota was dominated by Proteobacteria and Fibrobacteres, while supporting fewer Actinobacteria. The lower Shannon diversity of the NPK-enhanced community (Fig. 6A) was consistent with enhancement of a small subset of dominant OTUs (Fig. S5A), whereas the Control-enhanced community possessed greater bacterial diversity than the NPK-enhanced and MNPK-enhanced communities (Fig. 6A). Surprisingly, an opposite trend of diversity was observed in the unlabelled rhizosphere fraction (Fig. 6B). Specifically, MNPK-enhanced and NPK-enhanced microbiota showed much greater diversity in unlabelled rhizosphere fraction, with a remarkable increase in Actinobacteria and Nitrospirae under NPK treatment.

The phyla distribution of the Control-enhanced OTUs was similar to that of MNPK-enhanced OTUs, but clearly differed from that of NPK-enhanced OTUs in both the rhizosphere and bulk soils (Fig. 6C and D). The results of Bartlett's test also showed that the homogeneity of variance between Control and MNPK was similar in both the rhizosphere and bulk soils (Table S3). Long-term application of inorganic fertilizers (NPK) remarkably increased the relative abundances of Actinobacteria and Nitrospirae, but decreased soil microbial diversity in the bulk soil (Fig. 6D).

4. Discussion

In the present study, we characterized the microbial community in both wheat rhizosphere and bulk soil under 32-year long-term fertilizations. The rhizosphere microbiome was then further divided into root-feeding and SOM-feeding communities by DNA-SIP. The objectives were to identify the core rhizosphere microbiome involved in root C dynamics, and to get an idea of whether the enhancement of rhizosphere microbes was directly driven by

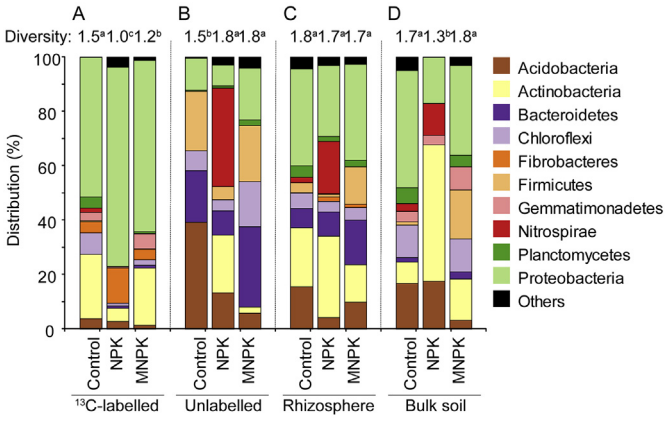

Fig. 6. Impact of fertilizer treatments on the bacterial community composition. Histogram showing the distribution of the phylum presented in OTUs that were significantly enhanced under Control, NPK and MNPK, respectively. The Shannon diversity (considering phyla as individuals) is given above each bar and different letters indicate significant differences among fertilizer treatments in the indicated fraction ($P < 0.05$, Fisher's LSD test). A, ^{13}C-labelled rhizosphere fraction; B, unlabelled rhizosphere fraction; C, whole-rhizosphere fraction; D, bulk soil fraction.

root-derived C, and how soil microbial community and plant–microbe interaction respond to various fertilizations.

4.1. The range of action of wheat root-derived substrates in shaping rhizosphere microbiome

Consistent with our Hypothesis 1 that microbial assimilation of wheat root-derived C would be dominated by a small subset of rhizosphere microbiome, the result showed that two major phyla Actinobacteria and Proteobacteria (notably Alpha- and Beta-Proteobacteria) make up nearly 70% of the ^{13}C-labelled rhizosphere community (Fig. 4A). Proteobacteria have previously been described as effective rhizosphere and root colonizers (Uroz et al., 2010), and the families and genera have been shown to actively assimilate C derived from labeled rice plants (Lu et al., 2006). Recent studies of maize (Peiffer et al., 2013), *A. thaliana* (Lundberg et al., 2012), switchgrass (Mao et al., 2014) and wheat (Donn et al., 2014) have further suggested that Beta-Proteobacteria may be enriched in diverse plant rhizosphere due to their high ability to utilize root exudates (Fierer et al., 2007). Although most Proteobacteria could be induced by plant-derived labile C, we observed an unlabelled Proteobacteria in the wheat rhizosphere (Fig. 4A), implying that this subset of Proteobacteria might also play roles in the degradation of refractory SOM in the wheat rhizosphere. This finding supports the idea that a portion of root-enhanced Proteobacteria could be saprophytic bacteria that had been generally adapted to the plant rhizosphere and across diverse plant species (Bulgarelli et al., 2012). In contrast to Proteobacteria, Actinobacteria used ^{13}C-labelled rhizodeposits almost entirely as their sole C source (Fig. 4A and C). This finding is consistent with the previous observation that selective enhancement of Actinobacteria in *Arabidopsis* root depends on cues from metabolically active host cells (Bulgarelli et al., 2012), providing further direct evidence that this host regulation of rhizosphere Actinobacteria is completed by fresh C released from plant roots. However, Mao et al. (2014) recently reported Actinobacteria were only weakly labelled by ^{13}C-root exudates in the rhizosphere of switchgrass. This discrepancy might be in part due to the selective responses of Actinobacteria to different root exudates.

Our results showed that Acidobacteria, Chloroflexi, Bacteroidetes and Firmicutes were mainly detected in the unlabelled rhizosphere fraction (Fig. 4A), indicating that they did not utilize much of the root-released ^{13}C. This result might explain recent observation that the community loosely associated with wheat roots possessed greater proportion of Acidobacteria and Firmicutes than the community tightly associated with roots (Donn et al., 2014). Acidobacteria and Chloroflexi have previously been considered soil oligotrophs (Fierer et al., 2007) and are likely specialized in the degradation of ancient or older SOM in the rhizosphere. However, Bacteroidetes and Firmicutes, which are generally characterized as copiotrophic soil bacteria or fast-growing microbiota when labile C sources are abundant (Cleveland et al., 2007; Fierer et al., 2007), do not directly use ^{13}C-labelled fresh C released from wheat roots as their main C source. This discrepancy might reflect their lower ability to compete for initially released and highly ^{13}C-enriched root exudates when compared with Actinobacteria and Proteobacteria. Thus, they may preferentially assimilate degraded products of SOM because the fresh root exudates allocated to the rhizosphere can accelerate SOM decomposition (Fontaine et al., 2003), leading to an abundance of alternative C sources. This interpretation was consistent with less root-released ^{13}C being incorporated into the branched chain fatty acids (potential biomarkers of Bacteroidetes) in a rice paddy microcosm (Lu et al., 2004).

4.2. Structure and assembly cues for wheat rhizosphere-inhabiting bacterial microbiotas

Selective responses of soil bacterial communities to plant roots have been widely reported (Lu et al., 2006; Paterson et al., 2007; Uroz et al., 2010), and root exudates are considered as key determinants of microbial abundance in the rhizosphere. However, we found that only the enhancement of Fibrobacteres was induced by wheat rhizodeposits, whereas the potential enhancement cues for Bacteroidetes and Chloroflexi in the rhizosphere were not directly associated with root-derived products (Fig. 5B). Dennis et al. (2010) suggested that the direct effects of rhizodeposits on bacterial communities were likely limited to small spatiotemporal scales, and that microbial exudates, degradation products of SOM and secondary metabolites of plant roots all could simultaneously stimulate or inhibit the growth of rhizosphere microbiota. Bulgarelli et al. (2012) found that about 40% of the *Arabidopsis* root-inhabiting bacterial microbiota were induced by the lignocellulose nature of the root surface rather than the active host cells. The significant alteration of chemical properties such as pH and redox potential in the rhizosphere (Husson, 2013) could also have considerably discriminatory effects on the rhizosphere microbiota. Thus, we suggest that structure and assembly of the rhizosphere bacterial community depend not only on root exudates, but also on other cues under specific rhizosphere conditions.

4.3. Fertilizer sources impacted the composition of soil microbial community

After 32 years application of inorganic or organic fertilizers, soil characteristics such as pH, organic C, and macronutrients were significantly changed compared with the Control (Table S1), which was in agreement with other long-term experiments in various ecosystems (Mäder et al., 2002; Mack et al., 2004; Coolon et al., 2013). Furthermore, we observed that the soil bacterial community was highly responsive to inorganic fertilizers and organic manure, but such responses differed among phyla (Fig. 6C and D). The phylogenetic groups within Nitrospirae are capable of nitrification, and are reported to be more abundant in soils amended with inorganic N fertilizer (Coolon et al., 2013). Recent pyrosequencing-based studies in a broad range of ecosystems have also revealed the enhancement of Actinobacteria in N-fertilized soil (Chaudhry et al., 2012; Ramirez et al., 2012). Thus, these results indicate that soil microbial responses to inorganic fertilizers, especially N, are likely controlled by similar mechanisms (Ramirez et al., 2012). The changes of microbial community structure in manure-amended soil were more closely similar to those in unamended soil than in soils amended with inorganic fertilizers (Fig. 6C and D). This result is closely consistent with previous observation in a century-old manure-treated agroecosystem (Sun et al., 2004), indicating that alterations of the soil bacterial community composition caused by the application of inorganic fertilizers could be resumed through the addition of organic manure. Long-term sole inorganic fertilization frequently decrease microbial biomass (Ramirez et al., 2012) and microbial diversity (Coolon et al., 2013), due to the disruption of the biogeochemical mechanism. When added to soils, organic manure enhances soil fertility (Chaudhry et al., 2012) and microbial diversity (Mäder et al., 2002) by increasing soil organic C, available N and P, micronutrients, soil aggregation, and water holding capacity, as well as leading to a high soil buffering capacity against external disturbances. The much more sustained positive responses of soil environment to organic fertilization fit with our Hypothesis 2 that organic manure would activate a more diverse group of soil microbes as compared with conventionally applied inorganic fertilizers in the bulk soil.

4.4. Fertilizer sources regulated the plant–microbe relationship

Our Hypothesis 3 predicted that long-term fertilization practices with increasing nutrients availability would reduce the dependence of rhizosphere microbiome on plant-derived C source. As expected, long-term inorganic fertilization altered not only the soil microbial community structure, but also the plant–microbe interaction. Inorganic fertilizers suppressed the utilization of plant-derived C by Actinobacteria, while increasing such plant-derived C utilization by Fibrobacteres (Fig. 6A). These findings are consistent with the previous report that the proportion of root-derived C recovered from Gram-negative biomarkers was greater in fertilized than in non-fertilized grassland (Paterson et al., 2007). Furthermore, we found that the NPK-enhanced and MNPK-enhanced communities possessed lower bacterial diversity than the Control-enhanced community in the ^{13}C-lebelled rhizosphere microbiome, while the opposite was true for the SOM-feeding rhizosphere microbiome (Fig. 6A and B). These results implied that rhizosphere bacteria could decrease the dependence on plant-derived C, but increase the preference for SOM when applying external fertilizers, leading to a simpler relationship between wheat and soil bacteria. This is a striking result, which means that long-term fertilization practices, inorganic fertilization in particular, are likely to decrease the probiotic functions of rhizosphere microbiota through simplifying the plant–microbe relationship, because the sub-community induced by the rhizodeposits contains diverse plant growth-promoting rhizobacteria (PGPR) (Lugtenberg and Kamilova, 2009). The effect of fertilizers on the diversity of bacteria assimilating plant-derived C might have been caused by the altered quality and quantity of plant rhizodeposition (Kuzyakov et al., 2002). The increase in nutrient availability also has the potential to change the microbial C use efficiency (Fisk and Fahey, 2001).

In summary, our results suggest that microbial assimilation of wheat rhizodeposits may be dominated by Actinobacteria and Proteobacteria (notably Alpha- and Beta-proteobacteria). We present evidence that root-derived C is not a sole determinant of microbial enrichment in the rhizosphere. Long-term (32-year) application of organic manure facilitates the development of microbial communities with high biodiversity in the bulk soil, whereas inorganic fertilizers selectively activate specific populations such as Actinobacteria and Nitrospirae. The results further demonstrate that the plant–microbe relationship has been changed by the long-term fertilization practices. The increase in soil nutrient availability probably reduced the dependence of rhizosphere microbiome on root-derived substrates, but increased the preference for SOM. Future studies are needed to better understand how changes of plant–microbe relationships observed under various fertilizations influence the ecological functions of rhizosphere microbiome.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2014.09.028.

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