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Probing potential microbial coupling of carbon and nitrogen cycling during decomposition of maize residue by ^{13}C-DNA-SIP

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ABSTRACT

The links between microbial taxa, *in situ* organic matter decomposition, and coupling of carbon (C) and nitrogen (N) cycles remain unresolved. Here, we used stable isotope probing (SIP) technique to investigate bacterial carbon assimilation and C and N coupling during decomposition of ^{13}C-labeled maize residue in a black soil from Northeast China. Bacteria assimilating carbon from maize residue (16S rRNA analysis) were primarily distributed in the Phyla Actinobacteria, Firmicutes and Proteobacteria. These include the recognized stubble decomposing lineages of *Arthrobacter*, *Streptomyces*, *Bacillus* and *Rhizobium*, but also lineages not previously reported (*Agromyces*, *Blastococcus*, *Gemmatimonas*, *Glycomyces*, *Heliobacillus*, *Lysobacter*, *Microlunatus*, *Mycoplasma*, *Natronocella*, *Ohtaekwangia*, *Paenibacillus*, *Schlegelella*, *Sorangium*, *Steroidobacter* and *Thermacetogenium*). Analysis of nitrogen fixation (*nifH*) and denitrification (*nirS*) genes in heavy-fraction DNA was used to link microbial taxa involved in N cycling to C transformation of the maize residue. A cluster of *nifH* genotypes affiliated with Rhizobium and two other 'uncultured' clusters dominated the N-fixing clone library, and genotypes affiliated with *Kocuria varians* and an uncultured cluster dominated the library of nitrite reducing (*nirS*) taxa. The results suggest that plant residue decomposition may stimulate both N-fixation and denitrification through direct C-feeding of related microbes in soil.

1. Introduction

Globally it is estimated that 3.75 billion tons of crop residues are produced annually (Smil, 1999), and most of these are incorporated into arable soils. The decomposition of crop residues not only affects the soil C balance, but also greatly influences the cycling of other elements such as nitrogen (N), phosphorus (P), and sulfur (S), and thereby impacts overall soil fertility (Smil, 1999; Lu et al., 2009). Efforts have been made to better understand the processes of residue decomposition and coupling of nutrient cycles. For instances, abiotic factors, such as residue chemistry, soil properties (e.g. N availability) and climate, have been identified as the major environmental factors controlling residue decomposition (Melillo et al., 1982; Aerts, 1997). The diversity and succession of soil microbes, the major drivers of residue decomposition, have also been frequently monitored during this process (Rui et al., 2009; Marschner et al., 2011). However, the linking of microbial taxa with function is not well understood and remains a key area for investigation.

Recently, stable isotope probing (SIP) has been used to bridge the gap between microbial identity and function during processes such as residue decomposition. Bernard et al. (2007) used this technique to identify bacteria associated with wheat-residue cycling and found that C from ^{13}C-labeled wheat was mainly assimilated by the Betaproteobacteria taxa *Janthinobacterium*, *Massilia* and *Variovorax*, and the Gammaproteobacteria taxa *Xanthomonas* and *Pseudomonas*. Subsequently, several studies using ^{13}C-labeled fresh potato, alfalfa and rice or ^{15}N-labeled maize and soybean have demonstrated that active residue-decomposing microbes were unevenly distributed among the phyla Actinobacteria and Proteobacteria, and the taxa assimilating the labeled residues differed with residue chemistry, soil moisture and soil types (España et al., 2011a; Shrestha et al., 2011; Li et al., 2012; Semenov

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et al., 2012; Pascault et al., 2013). To date, the range of studies linking microbial taxa to residue decomposition has limited to a few Japanese and Italian rice paddy soils and American upland soils; little information is available for many other arable ecosystems.

Approaches using SIP have been able to provide novel insights into microbial coupling of C and N cycles. For example, several subgroups of N-cycling Rhizobiales such as Mesorhizobium and Bradyrhizobiaceae, were commonly detected in heavy DNA fractions of ^{13}C-labeled residue or residue component (i.e. cellulose) (Lee et al., 2011; Eichorst and Kuske, 2012; Semenov et al., 2012). These studies have not yet been widely extended to other N cycling processes, such as denitrification. Although available C is a key driver of denitrification at a gross process level, the specific C requirements of denitrifying bacteria remain largely uninvestigated. Selected bacterial taxa, such as *Methylophilales*, *Comamonadaceae*, and *Rhodocyclaceae*, have been identified as denitrifying bacteria in methanol- and acetate-fed batch reactors (Ginige et al., 2004, 2005), however studies on utilization of other carbon sources by denitrifiers in soils are lacking.

Northeast China is a major cereal producing region. Soil biogeochemical cycling in this region has received increasing attention in recent years (Fan et al., 2011, 2012; Xu et al., 2012; Li et al., 2013), however the main focus has been on soil N cycling and little on soil C transformation (Fan et al., 2012). Maize residue is the major fraction of organic C entering into the arable soils in this region. Thus, the main objectives of the present study were to (1) identify the bacterial taxa associated cycling (assimilation) of C from ^{13}C-labeled maize residue, and (2) test the hypothesis that C transformation is coupled with the taxa involved in N-cycling in the black soil.

2. Materials and methods

2.1. Preparation and characterization of ^{13}C-labeled maize residue

Seeds of maize (*Zea mays* L. cv. Zhengdan 958) were surface sterilized with H_2O_2 and planted into vermiculite which had been sterilized in a muffle furnace at 500 °C for 5 h. At five days after emergence, the pots were thinned to contain a single uniform-sized seedling. The pots were then transferred to 40-L transparent polymethyl chambers supplied with either $^{12}CO_2$ (natural abundance) or $^{13}CO_2$ (^{13}C atom% = 98, Shanghai Research Institute of Chemical Industry, China). The maize plants were supplied with half-strength Hoagland nutrient solution during the first 10 days and with full-strength Hoagland nutrient solution afterward. Maize photosynthesis was monitored with an infrared CO_2 analyzer (GXH-3010E1, Huayun) and at forty-two day after C labeling the whole maize plants (including shoot and root material) were destructively sampled, oven-dried, and grounded to pass a 0.5-mm sieve. Abundances of ^{13}C of the labeled and non-labeled residue samples were determined using an isotope ratio mass spectrometer (MAT253, Finnigan).

The chemical compositions of labeled and non-labeled residues were analyzed on a Bruker 400M spectrometer (Bruker) with a 4-mm sample rotor. Solid-state ^{13}C nuclear magnetic resonance (NMR) spectra were recorded using the cross polarization/total sideband suppression magic angle spinning (CP/TOSS MAS) technique, with a ^{13}C signal at 100 MHz, a spinning speed of 5 kHz and a CP time of 1 ms, and pulse interval of 4 μs. The NMR spectrum was divided into five chemical shift proportions: 0–48 ppm (alkyl C), 48–63 ppm (N-alkyl or methoxyl C), 63–112 (O-alkyl or di-O-alkyl C), 112–164 ppm (aromatic C), 164–210 ppm (Carbonyl C). The proportion by each C type was determined by integration of the spectral regions.

2.2. Soil incubation and gas analyses

A typical black soil in northeastern China was collected from Gongzhuling long-term maize monoculture experiment station, Jilin province (43°30′23″N, 124°48′33.9″E). The soil had a pH of 8.06 (in water), 16.88 g kg^{-1} organic C, 1.43 g kg^{-1} total N, and 0.96 g kg^{-1} total phosphorus (P). Ten grams (dry weight equivalent) of soil were amended with either 50 mg ^{12}C- or ^{13}C-labeled ground maize residue in a 50 ml glass bottle. A control treatment consisting of soil without maize residue addition was also included. All treatments were replicated six times, with 3 technical replicates for each sampling point. The samples were hermetically sealed with a rubber plug and incubated at 25 °C in the dark for 31 days. Soil moisture was maintained at 60% of water holding capacity over the incubation period. Headspace gases were sampled at 3, 8, 16 and 31 d after incubation. Concentrations of total headspace CO_2 and N_2O were measured via gas chromatography (HP7890A, Agilent Technologies) as used previously (Lam et al., 2011). Relative abundances of $^{12/13}CO_2$ were determined with an isotope ratio mass spectrometer (MAT253, Finnigan). After gas sampling, all bottles were open in a clean bench for 15 min to refresh headspace oxygen and then resealed with sterilized plugs. One-way analysis of variance (ANOVA) was performed to examine the significance of differences between treatments using SAS 8.0 (SAS Institute Inc.).

2.3. Sampling, DNA extraction and gradient centrifugation

Triplicate soils of each treatment were destructively sampled at 16 and 31 d. DNA was extracted from 0.5 g soil with Fast DNA spin kit for soil and the FastPrep-24 instrument (MP Biomedicals) according to the manufacturer's instructions. Density gradient centrifugation was performed following a previous protocol with some modifications (Gan et al., 2012). Briefly, 5 μg soil DNA was mixed with 2.0 g ml^{-1} cesium trifluoroacetate (CsTFA) (Amersham Pharmacia Biotech) and gradient buffer (0.1 M Tris–HCl, 0.1 M KCl, 1 mM EDTA, pH = 8.0) to reach a density of 1.5 g ml^{-1}. The sample was centrifuged in a Beckman coulter Optima L-XP ultracentrifuge on a 100 Ti rotor (Beckman Coulter) at 177,000 g for 32 h at 20 °C. Sample from each centrifuged tube was separated into twenty-five 220 μL fractions. The buoyant density of each fraction was determined with AR200 digital refractometer (Reichert). DNA in each fraction was precipitated with 2 volumes of isopropanol, washed with 70% ethanol and re-suspended in sterilized ultrapure water for further use.

2.4. Bacteria community analysis

Bacterial 16S rRNA genes in soil DNA were amplified using the primer pair 357fgc and 518r according to Muyzer et al. (1993). Denaturing gradient gel electrophoresis (DGGE) of PCR product was performed using the D-Code Universal Mutation Detection System (Bio-Rad). Briefly, electrophoresis was run for 16 h at 75 V on 8% polyacrylamide gels containing a denaturing gradient of 30–60% urea/formamide.

The bacterial community was characterized by shotgun cloning and Sanger sequencing. The heavy fraction was defined according to DGGE analysis and density of each fraction of DNA. The heavy and light DGGE profiles differentiated around 1.60 g mL^{-1}; fractions heavier than this density were considered as heavy fractions. Difference in labeled and unlabeled DNA was also predominant around this density centrifuged with CsTFA (Liu et al., 2011; Gan et al., 2012). Heavy-fraction DNA from ^{12}C- or ^{13}C-maize treated soils (hereafter designated as ^{12}C-DNA and ^{13}C-DNA, respectively) was respectively pooled and then subjected to PCR using primers