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Structure and assembly cues for rhizospheric *nirK*- and *nirS*-type denitrifier communities in long-term fertilized soils

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ABSTRACT

The stimulatory effect of plants on soil denitrification activity has been widely reported, and root-derived carbon (C) is considered a factor contributing to enhanced denitrification activity in the rhizosphere. However, the mechanisms through which root-derived C shapes the rhizospheric denitrifying community structure remains unclear, especially under different soil fertility levels. Here, DNA-based stable isotope probing (DNA-SIP) and Illumina MiSeq sequencing were employed to characterize root-associated denitrifier communities containing nitrite reductase genes (*nirS* and *nirK*) in the rhizosphere of wheat grown in soils with three distinct long-term (32-year) fertilization regimes. Fertilization showed a significant impact on the composition of denitrifying communities that actively utilized photosynthetically fixed C in the wheat rhizosphere. In soils amended with inorganic fertilizers, wheat root-derived ^{13}C was mainly assimilated by *nirS*-type denitrifiers affiliated to Alcaligenaceae and *nirK*-type denitrifiers affiliated to Phyllobacteriaceae. In contrast, organic fertilization resulted in larger diversity of ^{13}C-labeled denitrifier communities where *nirS*-type denitrifiers such as Rhodobacteraceae and unclassified Burkholderiales and some unknown *nirK*-type denitrifiers were more abundant. The *nirS*-type denitrifier community was found to be more sensitive to the rhizosphere effect than the *nirK*-type community. Approximately 31% of the ^{13}C-labeled *nirS*-type denitrifiers were more abundant in the rhizosphere than in the bulk soil, but only 2% of the ^{13}C-labeled *nirK*-type denitrifiers showed increased abundance in the rhizosphere. The results of this study present direct evidence that root exudates can act as inducible C sources for heterotrophic denitrifying bacteria, but this induction pattern differs between *nirS*- and *nirK*-type communities and is dependent on soil fertility level.

1. Introduction

Nitrous oxide (N_2O) is a major stratospheric ozone-depleting substance and one of the most important greenhouse gases on Earth (Prentice et al., 2012; Blunden et al., 2013). The atmospheric concentration of N_2O has been increasing at nearly 0.75 ppb $year^{-1}$ since 1970 (IPCC, 2014). The increasing N_2O levels in the atmosphere are significantly associated with high nitrogen (N) and manure application rates in agricultural ecosystems (Syakila and Kroeze, 2011), and soil microbial denitrification plays a key role in this process. Denitrifying bacteria reduce soluble nitrate (NO_3^-) or nitrite (NO_2^-) to dinitrogen (N_2) through a series of gaseous intermediates, including nitric oxide (NO) and N_2O. Among these, the key reaction is reduction of NO_2^- to NO, which is catalyzed by two structurally different, but functionally equivalent NO_2^- reductases; namely, cytochrome cd1-containing reductase (NirS) and copper-containing reductase (NirK) (Hochstein and Tomlinson, 1988; Cutruzzola et al., 2001). This step causes dissolved N to become gaseous N for the first time during the denitrification process. Therefore, *nirS* and *nirK* genes are usually used as molecular markers to investigate the ecological behavior of denitrifying microorganisms in the environment. These two genes are thought to be mutually exclusive among denitrifying species and to represent two ecologically distinct denitrifying groups (Jones and Hallin, 2010). Several studies have shown that the responses of the *nirS*-type denitrifier community to environmental gradients are markedly different from those of the *nirK*-type denitrifier community, which supports the possibility that the two communities occupy different ecological niches (Jones and Hallin, 2010; Wei et al., 2015; Azziz et al., 2017).

Terrestrial plants release an array of substrates (i.e. rhizodeposition) that have the potential to lead to interactions between plants and

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various soil microbiota in the rhizosphere (Dennis et al., 2010). The stimulatory effect of plants on soil denitrification activity has been widely reported (Philippot et al., 2007; Henry et al., 2008; Guyonnet et al., 2017), and root exudates are considered to be potential determinants of enhanced denitrification activity in the rhizosphere. Henry et al. (2008) reported that root exudate composition can influence N_2O production, with the $N_2O/(N_2O + N_2)$ ratio being 3-fold higher in microcosms amended with root exudates containing 50%–59.5% organic acids than in microcosms amended with root exudates containing 10%–19.5% organic acids. In the rhizospheres of wetland vegetation, plant species had a stronger effect on *nirK*-type denitrifier communities than *nirS*-type denitrifier communities (Bañeras et al., 2012). Hallin et al. (2009) also revealed a significantly higher *nirS/nirK* ratio (3- to 10-fold) in a bare fallow soil than in a planted soil. Based on the transcription of *nir* genes, only *nirK* was detected in the rhizosphere of all grain legumes (Sharma et al., 2005). However, different results have been found in other studies. In an acidified Norway spruce forest soil, for example, Bárta et al. (2010) reported that *nirS*-type denitrifiers were primarily located in the rhizosphere, while the *nirK*-type denitrifiers were more abundant in the bulk soil. These inconsistent results may be attributable to different soil conditions, and indicate selection for a *nir*-type denitrifier by habitats created by the presence or absence of plants. However, the mechanisms through which plants shape rhizospheric denitrifying community structures remain unclear.

The influence of inorganic and organic fertilization on the structure and activity of soil denitrifying communities has been emphasized over the past few decades (Hallin et al., 2009; Chen et al., 2010; Cui et al., 2016), but little is known about how root-related denitrifiers respond to different fertilization regimes in agroecosystems. Generally, root-released carbon (C) accounted for 10%–40% of the photosynthetically fixed C depending on soil nutrient status (Bais et al., 2006). In our previous study, we found that the high soil nutrient availability decreased the preference of wheat rhizospheric microbiomes for root-derived substrates, leading to a simpler relationship between wheat and soil microbes (Ai et al., 2015). Therefore, there is reason to suspect that the composition of root-related denitrifying communities would also be regulated by the different fertilization practices. The recent development of stable isotope probing (SIP) (Radajewski et al., 2000) and high-throughput sequencing provide an opportunity to directly dissect the diversity of plant-associated denitrifiers by tracking plant-derived C into microbial nucleic acids.

In this study, we used *in situ* external $^{13}CO_2$ pulse labeling of wheat plants growing in three long-term (32-year) fertilized soils (i.e. no-fertilization (control); inorganic nitrogen, phosphorus, and potassium (NPK); and organic manure plus NPK (MNPK)) to track ^{13}C movement from aboveground to the rhizospheric microbiome through DNA-SIP, and then characterized the *nirS*- and *nirK*-type denitrifier communities by Illumina MiSeq sequencing. The aims of this study were to (i) identify the *nirS*- and *nirK*-type denitrifier communities actively utilizing root-derived C in wheat rhizospheres, and (ii) assess how root-associated denitrifiers respond to different fertilization regimes.

2. Materials and methods

2.1. Soil collection

Soils were collected from three fertilization treatments in a long-term fertilization experiment initiated in 1979 at Malan Farm (37°55′N, 115°13′E; 37 m above sea level), Hebei, China. This region has a temperate and monsoonal 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 fertilization treatments (control, NPK, and MNPK) were implemented in 12 m × 6.7 m plots with three replicates each. After 32 years of annual fertilization, the soil properties changed drastically among these three treatments (Ai et al., 2015). The field has been cropped with a rotation of winter wheat and summer maize since study establishment. Fertilization treatments were performed annually. For the NPK treatment, fertilizer N, P and K were applied in the form of urea (300 kg N ha^{-1} $year^{-1}$), superphosphate (150 kg P_2O_5 ha^{-1} $year^{-1}$), and potassium chloride (150 kg K_2O ha^{-1} $year^{-1}$). All fertilizer P and K and manure were applied once as basal dressing during the wheat season. Manure and mineral fertilizers were evenly broadcast onto the soil surface and immediately incorporated into the plowed soil (0–20 cm depth) by tillage before sowing. For the N fertilizer, 20% of the urea was used as a basal dressing before sowing wheat, 30% was top-dressed at the reviving stage of wheat, and 50% was top-dressed at the 10-leaf stage of maize. The organic manure (3.75×10^4 kg ha^{-1}) consisted of straw bedding impregnated with liquid and solid horse manure, which contained 120 g kg^{-1} organic matter, 5.0 and 2.2 g kg^{-1} total N and P, respectively, and about 50% water (5-year average concentration from 2006 to 2010).

To conduct the ^{13}C labeling experiment, ten soil cores (6 cm diameter) were taken from each plot as a composite sample at depths of 0–20 cm in October 2011. A total of nine composite samples were transported to the laboratory on ice, sieved (2 mm pore filter), and then stored at 4 °C until initiation of the greenhouse experiment.

2.2. Wheat growth, ^{13}C-CO_2 labeling, and identification of ^{13}C-labeled rhizospheric DNA

This study is a continuation of our previous work (Ai et al., 2015) in which the total rhizosphere bacterial community associated with wheat root-derived C was studied by DNA-SIP and 16S rRNA sequencing. Therefore, the workflows of a pot experiment, ^{13}C-CO_2 labeling, and identification of ^{13}C-labeled DNA have previously been described in detail (Ai et al., 2015). Briefly, seeds of wheat (*Triticum aestivum* cv. Shimai 18) were sown in the middle chamber of three-chambered growth pots (Ai et al., 2015) at a density of seven plants per pot. The pots were filled with distinctive soils from the control, NPK, or MNPK fertilization treatments at a bulk density of 1.21 g cm^{-3}, which was similar to that observed in the field. Pots were spatially randomized and plants were grown in a greenhouse under conditions of 16/8 h and 23 °C/18 °C (day/night). Soil moisture was maintained at 40%–60% of the water-holding capacity by weighing and adding deionized water every two days. The ^{13}C labeling started 40 days later, when plants were in an active vegetative growth phase. The wheat grown in the rhizobox system was labeled with $^{13}CO_2$ (98 atom % ^{13}C) between 9 a.m. and 5 p.m. (8 h) for seven consecutive days (Lu and Conrad, 2005). The parallel microcosms were also constructed with equal $^{12}CO_2$ as controls. At the end of labeling, the loose soil lumps were first removed from the root surfaces by kneading and shaking, after which the tightly adhering soil within approximately 2 mm of the root surface (i.e., rhizosphere soil) was carefully collected with a sterilized brush. The bulk soil was subsequently sampled from the center of the root-free compartments. All soil samples were immediately frozen with liquid nitrogen and stored at −80 °C until required for molecular analysis.

After labeling, the $\delta^{13}C$ value of wheat rhizosphere soil was on average 203‰, which was significantly greater than that of the bulk soil ($\delta^{13}C = -19$‰), implying incorporation of a large amount of ^{13}C-labeled rhizodeposits into the rhizosphere soil. The genomic DNA of rhizosphere and bulk soils was extracted using a Fast DNA SPIN Kit for soil (MP Biomedicals, Illkirch, France) according to the manufacturer's instructions. The total rhizospheric DNA was further fractionated by cesium chloride (CsCl) equilibrium density gradient centrifugation. The ultracentrifugation conditions were 56,200 rpm (228,166 g_{av}) for 24 h at 20 °C (Zhang et al., 2012). The centrifuged gradient was fractionated into 24 equal volumes (~220 μL) by displacing CsCl solution with sterile water. Based on the $\delta^{13}C$ (‰) value of DNA from gradient fractions and the DGGE analysis of 16S rRNA PCR products, DNA in the