



## Structural and functional differentiation of the root-associated bacterial microbiomes of perennial ryegrass



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### ARTICLE INFO

#### Article history:

Received 2 February 2016

Received in revised form

17 March 2016

Accepted 3 April 2016

#### Keywords:

Microbiome

Ryegrass

Bacterial community

Metagenome functions

Soil source

Elevated CO<sub>2</sub>

### ABSTRACT

The microbiomes inhabiting plant roots and their adjacent soil environment play significant roles in plant growth, health, and ecological services. Recent studies have provided detailed insights into many phytomicrobiomes, but our understanding of the root-associated microbiomes of perennial ryegrass is still very limited. Here we carried out a stratified rhizobox experiment involving a fluvo-aquic soil and red soil under elevated (750 ppm) and ambient (350–400 ppm) CO<sub>2</sub>, and used deep amplicon sequencing to investigate the ryegrass root-associated bacterial microbiomes across compartments (the bulk soil, 2-mm outer rhizosphere, rhizosphere, rhizoplane, and endosphere). We observed increased relative abundances of Proteobacteria (mainly Rhizobiaceae and Enterobacteriaceae) in the rhizosphere, rhizoplane, and endosphere compared with the bulk soil and outer rhizosphere. The dominant bacterial genera (mainly *Methylobacterium*, *Rhizobium*, *Pseudomonas*, *Stenotrophomonas*, and *Enterobacter*) which facilitate plant growth and soil nutrient cycling were enriched in the rhizosphere, rhizoplane, and endosphere compared with outer rhizosphere. PICRUSt predicted moderate functional differentiation of bacterial microbiomes across compartments. The bacterial  $\alpha$ -diversity showed a decreased trend along a spatial gradient from the bulk soil to the endosphere. The  $\beta$ -diversity and network analyses indicated significant differences in the root-associated bacterial microbiomes between the fluvo-aquic soil and red soil. In conclusion, our study suggests that the filtration and acquisition of the ryegrass root-associated bacterial microbiomes are largely controlled by indigenous soil microbial communities, rather than the changes in root rhizodeposition caused by elevated CO<sub>2</sub>.

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### 1. Introduction

A multitude of microorganisms residing closely outside, on, and in plant roots, which function as specific microbiomes (aka microbiota), contribute strongly to plant nutrient acquisition, primary productivity, disease suppression, phytoremediation, etc. (De Deyn et al., 2008; Rodriguez et al., 2008; Weyens et al., 2009; Lundberg et al., 2012; van der Heijden et al., 2016). In recent years, high-throughput sequencing approaches have provided detailed insights into the endophytic bacteria colonizing the roots of different plant species, including *Arabidopsis*, *Populus*, maize, and

rice (Gottel et al., 2011; Bulgarelli et al., 2012; Lundberg et al., 2012; Peiffer et al., 2013; Schlaeppi et al., 2014; Edwards et al., 2015). Using *Arabidopsis*, several studies reported that the dominant endophytic bacteria in the endosphere (inside the roots) are much less diverse than the bacteria in the rhizosphere, and a core root microbiome could be identified (Bulgarelli et al., 2012; Lundberg et al., 2012; Schlaeppi et al., 2014). A recent study presented a spatial-resolution on three rice root-associated compartments, the rhizosphere, rhizoplane (root surface), and endosphere, each of which was found to harbor a distinct microbiome (Edwards et al., 2015).

The microbiomes inhabiting different root-associated compartments show different structures and functions. The rhizosphere soil at approximately 1–2 mm distance from the roots is profoundly influenced by plant metabolism through the secretion

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of a great variety of exudates (and release of oxygen under flooded conditions), including carbon (C)-rich photosynthates and antimicrobial compounds. This makes the rhizosphere a 'hotspot' environment, which accordingly results in a differentiation of the rhizospheric microbiome from the bulk soil biome (Peiffer et al., 2013; Schreiter et al., 2014). The rhizoplane is usually defined as a separate microhabitat from the rhizosphere, and is colonized by the microorganisms firmly attached to the root surface. The rhizoplane functions as a transitional boundary that plays a critical gating role for controlling microbial entry into the host root tissue (Edwards et al., 2015; van der Heijden and Schlaeppi, 2015). In contrast to the bulk soil and rhizosphere, the endosphere features a highly specific microbiome, in which diversity is much lower than that estimated for microbiomes outside the roots. The endospheric microbiome is preferentially colonized by the phyla/classes Proteobacteria, Actinobacteria, Bacilli, and Bacteroidetes (Bulgarelli et al., 2012; Schlaeppi et al., 2014; Hardoim et al., 2015; Vandenkoornhuysen et al., 2015), and shows potential benefits for plant growth promotion, stress tolerance and resistance, and antagonistic effects on pathogens and parasites (Hardoim et al., 2008; Rodriguez et al., 2008; Sessitsch et al., 2012; Upreti and Thomas, 2015).

We still do not have a sufficient understanding of the structure and functions of the root-associated microbiomes of perennial ryegrass (*Lolium perenne*), which is commonly used as a model plant. A ryegrass <sup>13</sup>C- and <sup>15</sup>N-labelling experiment showed that C and nitrogen (N) derived from root rhizodeposition decreased rapidly with increasing distance from the roots, but both were still detectable up to 4.2 mm distance (zu Schweinsberg-Mickan et al., 2010). Due to the spatial heterogeneity of rhizodeposits in soil, there is likely to be changed composition and diversity of the soil microbial communities within 0–4 mm from the ryegrass root surface. Under climate change scenarios, the stimulated photosynthesis under an elevated atmospheric CO<sub>2</sub> concentration will increase root rhizodeposition (mainly root exudation), and will further affect soil microbial activity and community structure (Drigo et al., 2010). The rhizospheric microbiome of ryegrass in response to elevated CO<sub>2</sub> has been reported (e.g., Schortemeyer et al., 1996; Marilley et al., 1999; Jossi et al., 2006; Tarnawski et al., 2006). However, the responses of the microbiomes inhabiting the endosphere, rhizoplane, and other compartments associated with ryegrass roots to elevated CO<sub>2</sub> have not yet been investigated. Recent studies have shown that plant roots assemble their associated microbiomes by a two-step selection model, with the first step involving a general recruitment from the bulk soil biome and a second step to permit entry inside the roots (Bulgarelli et al., 2013; Edwards et al., 2015; Vandenkoornhuysen et al., 2015). Due to different indigenous microbial communities, the soil type and edaphic properties may have a strong influence on microbial colonization across the root-associated compartments. To address the above knowledge gaps, we conducted a stratified rhizobox experiment using two representative Chinese soils under elevated and ambient CO<sub>2</sub>, and explored the structural and functional differentiation of the ryegrass root-associated bacterial microbiomes.

In the present work, the following two hypotheses were tested: (i) variations in the composition, diversity, and functions of bacterial community occur across the ryegrass root-associated compartments, and (ii) soil type is a more important factor contributing to the root-associated bacterial microbiome variations than CO<sub>2</sub> concentration. To test our hypotheses, we grew ryegrass plants in stratified rhizoboxes under controlled greenhouse conditions. Two representative soils were sampled from the Fengqiu (35°00'N, 114°24'E) and Yingtan (28°15'N, 116°55'E) Agro-Ecological Experimental Stations of the Chinese Academy of Sciences, respectively. Ryegrass plants were exposed to elevated CO<sub>2</sub> at a concentration of

750 ppm, with ambient CO<sub>2</sub> (350–400 ppm) as the natural control. After tillering, the endosphere, rhizoplane, rhizosphere, 2-mm outer rhizosphere, and bulk soil were sampled. The composition and diversity of the bacterial community were investigated by means of deep MiSeq sequencing of the 16S rRNA gene amplicons. The metagenome functions were predicted from the 16S rRNA sequence data using PICRUSt (i.e., Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) (Langille et al., 2013).

## 2. Materials and methods

### 2.1. Site description and sampling

A field site was located at the Fengqiu Agro-Ecological Experimental Station of the Chinese Academy of Sciences in Fengqiu County (35°00'N, 114°24'E), Henan Province, Northern China. This region experiences a temperate monsoon climate, with an average annual rainfall and temperature of 615 mm and 13.9 °C, respectively. A sandy loam (sand 27%, silt 48%, and clay 25%) has developed from alluvial sediments of the Yellow River and is classified as an Aquic Inceptisol (a calcareous fluvo-aquic soil) according to the US Department of Agriculture (USDA) classification system. Fourteen treatments with three replicates in a randomized plot design were established in 2008. The planting pattern was a rotation of winter wheat (*Triticum aestivum* L.) and summer maize (*Zea mays* L.). The unfertilized fluvo-aquic soil was used in the work reported here.

The red soil was collected from the Yingtan Agro-Ecological Experimental Station (28°15'N, 116°55'E), Chinese Academy of Sciences, Jiangxi Province, Southern China. This region is characterized by a subtropical humid monsoon climate with an average annual rainfall and temperature of 1795 mm and 17.6 °C, respectively. The soil is an acid loamy clay (sand 21%, silt 43%, and clay 36%) derived from Quaternary red clay and classified as Ferric Acrisols (USDA classification). The monoculture maize crop was continuously planted. The soil without any input of manure was used.

Both soils were collected from the 0–20 cm tillage layer in July 2015. After visible stones and plant residues were removed, twelve samples from the split-plots were mixed, homogenized, and air-dried. The soils were sieved through a 2 mm mesh, and their general physicochemical characteristics were analyzed (Table 1). We selected unfertilized soils in order to exclude the influence of anthropogenic fertilization on the indigenous soil microbial communities.

### 2.2. Design of rhizobox experiment

500 g of the sieved soil (oven-dry basis) was homogeneously mixed with 180 mg of urea, 86 mg of superphosphate, and 56 mg of potassium sulfate (equivalent to 1 mg each of N, phosphorus (P), and potassium (K) applied to 6 g of soil), and then added to the stratified rhizobox. The rhizobox (length 10 cm, width 3 cm, and height 10 cm) was divided into seven compartments. Ryegrass seedlings were planted in the 2 mm width of the middle compartment, which was surrounded on each side by two symmetric 2 mm widths of compartments. Two 10 mm lateral compartments were also established. These compartments were separated by 0.25 mm nylon mesh (see Supplementary Fig. S1).

The ryegrass seeds were surface-disinfected using 30% H<sub>2</sub>O<sub>2</sub> for 30 min, and germinated under sterilized conditions on MS agar media in the dark for 48 h at 25 °C. After germination, similar sized ryegrass seedlings (0.2 g) were transplanted to the middle compartment of each rhizobox, and soil moisture adjusted to

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