Bacteria utilising plant-derived carbon in the rhizosphere of *Triticum aestivum* change in different depths of an arable soil

2nd revised version, August 28th, 2017

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as an 'Accepted Article', doi: 10.1111/1758-2229.12588

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Running title: Spatial distribution of rhizosphere bacteria

Keywords:

Rhizosphere, subsoil, topsoil, root exudates, soil microbiome, DNA stable isotope probing

Summary

Root exudates shape microbial communities at the plant soil interface. Here we compared bacterial communities that utilise plant-derived carbon in the rhizosphere of wheat in different soil depths, including topsoil, as well as two subsoil layers up to 1 m depth. The experiment was performed in a green house using soil monoliths with intact soil structure taken from an agricultural field. To identify bacteria utilizing plant derived carbon, ^{13}C-CO_2 labelling of plants was performed for two weeks at the EC50 stage, followed by stable isotope probing of extracted DNA from the rhizosphere combined with 16S rRNA gene-based amplicon sequencing.

Our findings suggest substantially different bacterial key players and interaction mechanisms between plants and bacteria utilising plant-derived carbon in the rhizosphere of subsoils and topsoil. Among the three soil depths, clear differences were found in ^{13}C enrichment pattern across abundant operational taxonomic units (OTUs). Whereas OTUs linked to Proteobacteria were enriched in ^{13}C mainly in the topsoil, in both subsoil layers OTUs related to Cohnella, Paenibacillus, Flavobacterium showed a clear ^{13}C signal, indicating an important, so far overseen role of Firmicutes and Bacteriodetes in the subsoil rhizosphere.

Introduction

The microbiome of the rhizosphere has been considered as an important driver of functions contributing to plant health- -growth and yield (Berg *et al.*, 2014). Thus, microbes in this compartment have been intensively studied in the last decades and enormous efforts have been made to unravel the complex processes taking place (Berendsen *et al.*, 2012; Philippot *et al.*, 2013; Lareen *et al.*, 2016). Today it is well accepted that bacteria promote plant growth mainly via nutrient mobilisation from the soil, phytohormone production, stimulation of the plant immune system or biocontrol of phyto-pathogens (Berg, 2009; Hartmann *et al.*, 2009). Studies on defined bacterial components have emphasised the special relevance of Proteobacteria (*Pseudomonas, Rhizobium, Burkholderia, Lysobacter*), Actinobacteria (*Streptomyces*), Bacteroidetes (*Flavobacterium, Cytophaga*), and Firmicutes (*Bacillus, Paenibacillus*) in the rhizosphere of diverse plant species (Haichar et al., 2008, 2012; Buée et al., 2009). Also other microbes colonizing the rhizosphere, mainly fungi, are considered to influence plant health (Malik *et al.*, 2015), although their plant growth promoting abilities in non-mycorrhizal interactions is far less investigated.

Today it is obvious, that the microbial community composition of the rhizosphere is mainly driven by the plant species, the plant development stage and the soil type (Marschner *et al.*, 2001; Berg and Smalla, 2009). A major mechanism how plants select for their specific microbiomes belowground is the specific composition of root exudates, which are used by microbes as an easily available carbon source (Haichar *et al.*, 2008). The quality and quantity of exudates varies with changes in soil physical and chemical parameters, plant developmental status and at different root zones (Jones *et al.*, 2004; Haichar *et al.*, 2008, 2014; Chaparro *et al.*, 2014). Highest root exudation rates were measured close to the root tip and during plant growth until flowering (Lynch and Whipps, 1990; Haichar *et al.*, 2014; Neumann *et al.*, 2014).

However, although previous studies have investigated the nature and composition of

rhizosphere microbes in detail, the transferability of findings to natural plant-microbe-soil systems may not always be straightforward due to the following considerations: (i) experiments are often conducted with disturbed or sieved soils, where the soil structure and compartments have been homogenised thus neglecting the influence of small-scale soil heterogeneity on root development (Luster *et al.*, 2009; Han *et al.*, 2015), (ii) studies are often limited to nutrient rich topsoil, although roots of agricultural crops can easily grow down to 2 m (Kautz *et al.*, 2013; Perkons *et al.*, 2014) and soil depth is recognised as a further important driver of soil microbial community composition (Berg and Smalla, 2009; Scharroba *et al.*, 2012), and (iii) rhizosphere microbiomes are often investigated at the level of presence or the relative abundance of taxa, but not their direct involvement in rhizosphere carbon flows.

In this study, we investigated bacteria utilizing plant derived carbon in the rhizosphere of *Triticum aestivum* in different soil depths. To reach our goals, we investigated intact soil columns planted with wheat over a soil depth of 1 m. Thus, the natural covariation of soil structure, pore network and root developmental stage over depth was conserved. We applied ^{13}C-CO_2 fumigation to the plant shortly before sampling and used DNA-based stable isotope probing (SIP) combined with barcoding of the 16S rRNA gene amplicons by high throughput sequencing to reveal a high resolution of key rhizosphere bacteria utilising plant-derived carbon (Haichar *et al.*, 2016).

Based on recent measurements of hydrolytic enzyme activities in the rhizosphere of wheat using samples from the same field (Uksa *et al.*, 2015b), we hypothesise that the degree of substrate assimilation and microbial activity in the subsoil rhizosphere is comparable to that of the topsoil rhizosphere. However, as a pre-study with soil from the same field trial demonstrated a substantial change of abundant bacterial phyla from topsoil to subsoil with a decrease of Proteobacteria and an increase of Firmicutes (Uksa *et al.*, 2015a), we postulate, that rhizosphere bacteria, which utilise the plant-derived carbon, will differ in the different

soil depths under investigation. As most microbes colonizing the rhizosphere are acquired from the soil microbiome, we expect a dominance of Firmicutes in the subsoil utilising plant-derived carbon in the subsoil rhizosphere, whereas in topsoil a dominance of Proteobacteria occurs.

Results

^{13}C distribution pattern in the different soil and plant compartments

At the end of the experimental period (90 days) *Triticum aestivum* formed a dense rooting network in all three analysed soil depths. However, a sharp decrease of root biomass with soil depth (ANOVA; $P < 0.001$) was clearly visible (supplementary Figure S1). The labelling of the plant with ^{13}C-CO_2 resulted in a significant ($P < 0.001$) ^{13}C enrichment in the plant shoot biomass (46.4 atom-%) as well as in the rhizosphere (6.1 atom-%) and bulk soil (1.3 atom-%) independently of the soil depth (Figure S1).

Bacterial community composition and ^{13}C enrichment of OTUs in the rhizosphere at different soil depths

Seven consecutive fractions of each DNA gradient known to span the range of buoyant densities (BDs) typical for light and heavy DNA were selected for downstream analyses. 16S rRNA gene-targeted qPCR indicated elevated gene counts in heavy fractions of ^{13}C-labelled treatments compared to the controls, *i.e.* at BDs of 1.715 $g \cdot ml^{-1}$ CsCl or higher (Figure 1). This was a first indication of the successful incorporation of ^{13}C-label into the DNA of rhizosphere microbiomes in our experiment.

The selected fractions were subjected to sequencing of bacterial 16S rRNA gene amplicons. After quality filtering 2,541,504 reads were obtained from all fractions, resulting in 29,188 OTUs at a level of 95% similarity. For further analysis, reads from all samples were rarefied to 30,256 reads per sample.

In a first analysis, sequencing data from the 7 fractions per sample were combined by weighing the relative abundance of each OTU according to the proportion of 16S rRNA gene abundance in each fraction. With this, we first compared overall depth-resolved rhizosphere communities, without differentiation between OTUs of labelled and control samples. Richness and Shannon diversity (H) were significantly higher in rhizosphere samples of the topsoil (ANOVA; $P < 0.001$; H = 7.47) as compared with subsoil U (H = 6.36) and subsoil L (H = 6.31). Clustering of the samples and relative abundance of the 100 most abundant OTUs is displayed in Figure 2. The relative abundance of bacterial phyla is provided in supplementary Table S2 and Figure S3. As expected the following phyla were major parts of the bacterial community: Actinobacteria (18-44%), Proteobacteria (15-27% [42-63% Beta-, 12-23% Gamma-, 11-21% Deltaproteobacteria]), Acidobacteria (8-21%), Firmicutes (3-16%), Bacteroidetes (3-14%), Nitrospirae (1-3%), and Gemmatimonadetes (1-2%). Already at the phylum level, significant differences were found between the soil depths: a significantly higher abundance was observed for Acidobacteria in topsoil (ANOVA; $P = 0.001$), for Actinobacteria in subsoil U ($P = 0.004$), and for Nitrospirae in subsoil L ($P = 0.020$). In addition, the overall relative abundance of Proteobacteria was reduced in the rhizosphere of the upper subsoil (subsoil U; $P = 0.001$). As expected, Firmicutes were generally more abundant in the of the lower subsoil rhizosphere. Similar to the T-RFLP fingerprints (supplementary results and Figure S4), variations between the 4 soil columns increased with soil depth. Whereas topsoil and subsoil U samples formed condensed clusters, the subsoil L bacterial community exhibited considerable variability mainly due to the occurrence of single, highly abundant OTUs in only one or two samples, e.g. Nocardiaceae, *Achromobacter*, *Microbacterium, Flavobacterium, Pedobacter*, *Janthinobacterium* or *Steroidobacter* (Figure 2).

In a second step, ^{13}C enrichment was estimated for bacterial OTUs as an indication of their direct involvement carbon flow at the plant – soil interface of different soil depths, (Figure

3A-C). Labelling was inferred via taxon-specific buoyant density shifts and interpreted as ^{13}C atom-% enrichment (Hungate *et al.*, 2015). Up to 35 ^{13}C atom-% enrichment were observed for specific OTUs, while the uncertainty thresholds increased with soil depth (topsoil – 1.0; subsoil U – 5.2; subsoil L – 16.0 ^{13}C atom-%). ^{13}C enrichment values were lower in average in the upper subsoil U compared to topsoil or subsoil L. However, overall relative abundance of ^{13}C-enriched OTUs was found to be highest in subsoil U and subsoil L rhizosphere. Among the three soil depths, clear differences were found in the ^{13}C enrichment pattern across abundant OTUs. Enrichment of ^{13}C was highly pronounced for OTUs related to *Cohnella*, *Paenibacillus*, *Flavobacterium*, and Chitinophagaceae in subsoil U and especially subsoil L. These OTUs also were of high relative abundance compared with the topsoil. For OTUs classified as Actinobacteria, e.g. *Agromyces*, *Arthrobacter*, *Glycomyces*, *Kitatospora*, *Lentzea*, and *Promicromonospora*, both, their relative abundance and ^{13}C-labelling were highest in the upper subsoil. In turn, reads which could be assigned to *Streptomyces* sppwere ^{13}C-enriched and highly abundant in all depths. Interestingly, different OTUs related to *Streptomyces* were contributing to this observation in different soil depths (Figure 2, 3).

In contrast to Actinobacteria, proteobacterial OTUs were generally less abundant and had a lower ^{13}C-atom fraction excess in the subsoil U rhizosphere compared with the other soil depths. The most important proteobacterial OTU was closely related to *Duganella*, which appeared very important in rhizosphere of subsoil L. Other ^{13}C-enriched Proteobacteria were identified as *Ideonella*, *Lysobacter*, *Massilia*, *Polaromonas*, *Pseudoxanthomonas*, *Steroidobacter*, and *Variovorax* showing varying abundance and ^{13}C enrichment in dependency to soil depth.

Labelling of Acidobacteria was apparent only in topsoil. Here, the Gp4 class exhibited considerable ^{13}C enrichment in one OTU. The phyla Nitrospirae and Gemmatimonadetes, as well as most unclassified OTUs, showed no relevant ^{13}C enrichment and their relative contribution to the microbial community was rather low.

Discussion

Unravelling soil-microbe-plant interactions in undisturbed subsoil

Soil depth is a factor which is still rarely considered despite the fact that roots grow deep into subsoil. As with depth soil properties change and the bulk soil microbial community composition changes drastically towards oligotrophic, slow-growing microbes (Eilers *et al.*, 2012; Uksa *et al.*, 2015a), mechanisms of interactions between the plant root and the surrounding microbes and soil will be affected as well. There are still methodological limitations that impede direct ^{13}C labelling in the field and sampling down to subsoil. The use of undisturbed subsoil columns incubated under greenhouse conditions may be a good compromise. With undisturbed subsoil – overlaid by homogenised topsoil which mimics a ploughing event – we could preserve the naturally developed soil profile and its spatial heterogeneity including bulk density, soil structure, soil pore network, earthworm burrows, biogeochemical gradients as well as microbial community distribution patterns and niche-separation. Root growth, root development and deposition of root exudates were therefore as close to natural conditions as possible. The separation or co-localisation of microbes and substrates has been shown to be critical for carbon turnover on a small scale (Pinheiro *et al.*, 2015) and is a so far underestimated factor in studies using homogenised soil solely.

Detection of carbon utilising microbial pools via quantitative DNA-SIP

In this study, we investigated the bacterial communities utilising plant-derived carbon in the rhizosphere of top- and subsoil of wheat. For this aim, DNA-SIP was used as a powerful method to detect and quantify microbes that directly or indirectly take up ^{13}C-labelled carbon provided by plants (Haichar *et al.*, 2016).

The relatively long time span of our labelling experiment, which was needed to obtain sufficient amounts of ^{13}C labelled carbon, needed for subsequent SIP analysis, in the microbial DNA pool (15 d), made it difficult to differentiate between primary exudate consumers or secondary metabolite or biomass consumers in the investigated soils. Cross-feeding in natural food-webs can complicate the interpretation of SIP data and time-resolved SIP analyses can help to overcome this caveat (Coyotzi *et al.*, 2016). In our present analyses, microbes with a higher ^{13}C enrichment were considered more likely to be primary consumers of root exudates, however the simultaneous presence of slow-growing primary consumers can also not be excluded (Haichar *et al.*, 2008; Rettedal and Brözel, 2015).

Previous studies have emphasised the importance of sufficient ^{13}C-labelling for successful separation of heavy and light DNA and to resolve labelling from GC-effects (Neufeld *et al.*, 2007a; Uhlik *et al.*, 2009). Due to the simultaneous contribution of ^{13}C enrichment and GC content to the buoyant density of DNA, unlabeled genomes with high GC content may be found in the same gradient fractions as low-GC DNA with a high ^{13}C enrichment (Buckley *et al.*, 2007). In our present approach, this potential caveat was circumvented by high throughput-sequencing of 16S rRNA genes across all relevant gradient fractions, including ^{13}C treatments and unlabeled controls. Thus, we could (i) achieve a high phylogenetic resolution of labelled microbial taxa, (ii) define taxon-specific buoyant density shifts, and (iii) infer from that the degree of ^{13}C-labelling for single OTUs (Hungate *et al.*, 2015). At the same time, we are aware that the use of only one ^{12}C-control column vs. triplicate ^{13}C-labelled treatment columns per depth compromises a strictly quantitative and statistical evaluation of labelling effects.

The key players – rhizosphere bacteria growing on plant-derived carbon in different soil depths and their putative plant growth promotion abilities

As proposed, results indicated that both on phylum and genus level, utilisation patterns of

plant-derived carbon were highly dependent on soil depth. However, for all soil depths the dominance of ^{13}C-enriched genera attributed to Proteobacteria, Actinobacteria, Bacteroidetes, or Firmicutes pointed towards an import role of fast growing root-exudate metabolising bacteria. Particularly within the aforementioned phyla, bacteria have been isolated from diverse rhizosphere soils and characterised as fast growing microbes in the presence of labile carbon sources (Fierer *et al.*, 2007, 2012; Ramirez *et al.*, 2012). Nevertheless, the spectrum of physiological traits and lifestyles within a defined phylum can still be rather diverse (Goldfarb *et al.*, 2011). *Arthrobacter* for example was originally reported to be oligotrophic (Thompson *et al.*, 1992) but has been repeatedly identified in SIP studies to consume labile sugars in soils (Mau *et al.*, 2014; Kramer *et al.*, 2016). Also in our study, this taxon was highly ^{13}C-enriched in the subsoil U. In contrast, reads from e.g. *Nitrosospira* showed almost no atom fraction excess in all depths, not surprising, as this genus is known as an autotrophic nitrifier (Xia *et al.*, 2011). Also Acidobacteria and Gemmatimonadetes were almost not ^{13}C-labelled and therefore less involved in the turnover of plant-derived carbon. This result is in accordance with the expectation that most bacteria of those phyla are oligotrophic (Zhang *et al.*, 2003; Jones *et al.*, 2009; Foesel *et al.*, 2014). Generally, atom fraction excess variation within phyla and even within OTUs affiliated to the same genus was still high. Especially for the genus *Streptomyces*, different OTUs showed not only different relative abundances over depth, but also a high variation in ^{13}C-labelling indicating distinct growth rates and substrate usage spectra.

Actinobacteria and *Streptomyces* as well as *Duganella* and *Janthinobacterium*, all observed in our study in different soil depths with differing ^{13}C labelling intensity, are known for their ability to produce secondary metabolites with antimicrobial activities (Basilio *et al.*, 2003; Choi *et al.*, 2015; Viaene *et al.*, 2016). Many of these taxa are discussed in the context of biocontrol (Haesler *et al.*, 2014). Labelling intensities of these taxa varied strongly over depth, suggesting that key players involved in plant protection from phytopathogens in the

rhizosphere significantly differed over depth in our study. Donn *et al.* (2015) also observed a shift from *Proteobacteria* to *Actinobacteria* during wheat development, but at a larger temporal scale not focusing on roots of the same plant. They found *Oxalobacteraceae* and *Pseudomonadaceae* to be abundant at younger parts of roots, whereas at older parts or at senescent roots *Micromonospora* species and other Actinobacteria were enriched.

Also other microbes possibly related to plant growth promoting functions showed similar variability over depth. For example, strains of *Massilia*, *Duganella*, *Variovorax*, and *Pseudoxanthomonas* are known to produce siderophores (Aranda *et al.*, 2011; Ofek *et al.*, 2012; Madhaiyan *et al.*, 2013; Lampis *et al.*, 2015). These taxa were especially ^{13}C-labelled in the subsoil L rhizosphere, possibly providing an additional positive effect on deeper wheat roots in terms of iron and phosphorous supply (Sharma *et al.*, 2013). This is consistent with our previous report of highest phosphatase activities in deeper rhizosphere from the same site (Uksa *et al.*, 2015b).

In the above mentioned study (Uksa *et al.*, 2015b) also glycoside hydrolase activities were measured in the wheat rhizosphere from topsoil, subsoil U as well as subsoil L and were generally lower in the upper subsoil as compared to topsoil and the lower subsoil. Possibly, hydrolase activities in the lower subsoil are induced by easy available hydrocarbons released from young roots to increase further carbon mobilisation from the surrounding bulk soil. A putative producer of glycoside hydrolases in the subsoil L rhizosphere may be *Flavobacterium*. Bacteria of this genus are copiotrophs, living on easy available substrates, and were found to induce hydrolase activities in the wheat rhizosphere in former studies (Thompson *et al.*, 1992; Mawdsley and Burns, 1994; Heijnen *et al.*, 1995). Furthermore, at the early plant vegetative growth phase – as in our study – *Flavobacterium* is more abundant whereas Sphingobacteria take over at later plant developmental stages (Donn *et al.*, 2015). This may explain, why this family did not show up in our study, although other studies of the wheat rhizosphere found them in higher abundance (Haichar *et al.*, 2008; Stroobants *et al.*,

2014).

Similarly, members of the genera *Paenibacillus*, *Bacillus*, and *Cohnella* spp. (all Firmicutes) are known as free-living diazotrophs (Mavingui *et al.*, 1992; Rosado *et al.*, 1996; Behrendt *et al.*, 2010; Wang *et al.*, 2012). In our studies these genera were of highest abundance and showed highest ^{13}C enrichment not only in subsoil rhizosphere (this study) but generally in subsoils (Uksa *et al.*, 2015a). In contrast, an apparent lack of labelled Firmicutes with possible nitrogen fixing abilities in topsoil may indicate sufficient nitrogen supply or functional redundancy by other lineages there.

Finally also the production of phytohormones such as indol acetic acid might play a role for plant growth promotion in the deeper subsoil L, where roots are predominantly young. For *Massilia*, *Janthinobacterium*, *Arthrobacter* (Kuffner *et al.*, 2010), *Paenibacillus* (Hanak *et al.*, 2014), and even an acidobacterial strain (Kielak *et al.*, 2016) IAA production was documented. Different mechanisms of plant growth promotion might fall together in single species and other bacteria in turn benefit from those mutualistic relationships.

Lack of ^{13}C enrichment in bacterial DNA in the upper subsoil

Interestingly, OTUs in the upper soil depth (subsoil U) showed a lower atom fraction excess on average when compared with the deeper subsoil L, although the overall ^{13}C content of the soil was not lower in the rhizosphere of subsoil U. In addition, total carbon content increased in the rhizosphere with soil depth (data not shown). The following scenario – based on the depth and age-differential release of root exudates preferably in young roots (Haichar *et al.*, 2014) – could explain this observation: During labelling, root exudation could have been highest in the subsoil L, because average root age is expected to be lowest in the deepest soil and root exudation is expected to be highest in the early root developmental stage (Neumann *et al.*, 2014). In the upper subsoil U, root exudation could already have been gradually reduced as a result of increased average root age when labelling with ^{13}C-CO_2 occurred. These

assumptions are supported by a previous study on the same soil and soil depths in the field (Uksa *et al.*, 2015b), where potential hydrolytic enzyme activities in the rhizosphere showed a similar decrease in the upper subsoil U. The limitation of readily available carbon sources such as root exudates in this soil depth at this explicit time point of sampling can explain the gap and is supported by the high abundance of Actinobacteria, e.g. *Arthrobacter*, which can compete at nutrient limiting conditions.

Conclusion

We were able to show that, dependent on soil depth, distinct patterns of bacteria utilising plant-derived carbon occur that indicate shifts in plant growth promoting bacteria already at the phylum level. The composition of root exudates, the surrounding indigenous microbial community or other soil properties at specific soil depths are major drivers of the observed patterns, while their specific contributions remain unclear.

As postulated, the degree of assimilation of plant-derived carbon by single bacterial taxa in the rhizosphere of subsoil L is similar to the topsoil. Furthermore, the so far underestimated role of Firmicutes and Bacteroidetes as important bacteria, which utilise plant-derived carbon in the subsoil, is an outstanding result adding to other related findings from the wheat rhizosphere and residues (Bernard *et al.*, 2007; Ai *et al.*, 2015). This first investigation of the microbial communities, which utilise plant-derived carbon in an undisturbed subsoil via DNA-SIP shows that it is worth to take a 'deeper' look into the rhizosphere, otherwise carbon turnover processes and key players might be overlooked or underestimated. As most of the studies in the past, also here the focus has been put on the analysis of the bacterial part of the microbiome. As also indicated in the introduction, also other microbes than bacteria strongly contribute to plant health. Thus we propose to implement mainly fungi into future studies on the role of plant derived carbon to understand how this important group of microbes is

influenced by root exudates.

Experimental procedures

Soil properties and soil core excavation

The soil used for this study originated from an arable field at Campus Klein-Altendorf near Bonn (Germany, 50°37´21´´ N, 6°59´29´´ E) and has been classified as Haplic Luvisol. The Ap horizon (topsoil, 0-20 cm) has been classified as a silt loam with a pH 6.5 and was influenced by conventional tillage. Subsoil horizons Bt1 (upper subsoil U, 45-75 cm) and Bt2 (lower subsoil L, 75-105 cm) are characterised by a high bulk density and clay accumulation (silty clay loam) with pH values of 6.9; total carbon and nitrogen decreased with depth. The intermediate E/B horizon (20-45 cm) varies highly in the field and is therefore excluded from this study. For further details about soil properties consult Gaiser *et al.* (2012) and Kautz *et al.* (2014). In April 2012 before soil management and cultivation started, twelve undisturbed subsoil monoliths from 45-105 cm soil depth and 20 cm in diameter were obtained with a lysimeter excavation technology (Meißner *et al.*, 2007). The distance between the monoliths taken at the field was set to 1 m. The soil columns were deposited in a covered polystyrene box (60×180×100 cm) on a copper plate. The plate was set to 14°C for cooling the soil from the bottom continuously. To simulate the disturbed plough horizon, the undisturbed subsoil cores were covered with a 20 cm thick layer of homogenised, sieved topsoil (Ap horizon) from the same field. As the soil depth between 20 and 45 cm were excluded from this study, subsoil U and L (45-75 and 75-105 cm) refer to the root depths 20-50 and 50-80 cm, respectively.

Wheat cultivation, ^{13}C-CO_2 labelling and sampling

11 germinated seeds of *Triticum aestivum* L. (cultivar Scirocco) were sown in the topsoil which is equivalent to a seeding density of 350 seeds per m^2 typically used in the area of the

sampled soil. 75 days after sowing plants had reached the developmental stage EC50 providing the highest root exudation rates (Haichar *et al.*, 2014) and were labelled with ^{13}C-CO_2 (for details see the Supplemental Material).

After labelling, the soil columns were vertically dissected into three blocks using an electric saw: topsoil (0-20 cm), upper subsoil (U, 20-50 cm), and lower subsoil (L, 50-80 cm). The subsoil from 20-80 cm corresponded to the field soil depth of 45-105 cm. Each block was cut longitudinally into two halves. For the determination of the root biomass a representative cylinder segment was cut along the whole height of each block half from the midpoint to the edge. The roots for the determination of the root biomass were washed with deionised water. From the second half of each block, roots with adhering soil within max 2 mm distance to the root surface were sampled with sterile tweezers and designated as root-rhizosphere-complex. Bulk soil was sampled with a sterile spoon with highest possible distance to the roots which increased from topsoil to subsoil. Therefore the effect of root exudation on designated bulk soil samples from topsoil cannot be totally excluded. For DNA extraction, the root-rhizosphere complex and bulk soil material was stored at -80°C until further analysis. For dry weight and carbon measurement of the shoot and roots, rhizosphere and bulk soil, the sample material was dried at 40°C.

Microbial analysis

Details on the described experimental procedures can be found in the Supplemental Material. The analyses were limited to one control and three treatment soil columns resulting in 24 samples (4 soil columns × 3 soil depth × 2 compartments (root-rhizosphere complex and bulk soil)). DNA was extracted from samples using a modified nucleic acid extraction method according to Lueders *et al.* (2004). Since roots were intact after homogenisation for simplified reading, we further designated the DNA, which was extracted from the root-rhizosphere complex, as ‘rhizosphere DNA’.

In order to verify that the 4 soil columns used for DNA-SIP and 16S rRNA sequencing are comparable regarding their overall bacterial community composition, terminal restriction fragment length polymorphism (T-RFLP) was performed as a pre-analysis. A detailed description and results can be found in the supplementary material and Figure S4.

DNA-SIP was performed on the basis of density gradient centrifugation and fractionation according to Lueders *et al.* (2004) and Neufeld *et al.* (2007b). Due to insufficient ^{13}C enrichment in the bulk soil (Figure S1), density gradient centrifugation was limited to rhizosphere DNA.

For sequencing of bacterial 16S rRNA genes, 7 consecutive fractions that contained sufficient DNA amounts for downstream molecular analyses were chosen from each CsCl-gradient resulting in 84 samples (4 columns × 3 soil depth × 1 compartment (rhizosphere) × 7 fractions). Bacterial 16S rRNA gene abundance was determined in each of the fractions by quantitative real-time PCR using the 7300 Real-Time PCR System and the Power SYBR® Green PCR Master Mix (Applied Biosystems, Darmstadt, Germany) following the protocol described by Töwe *et al.* (2010). Barcoded amplicon sequencing was performed using the Illumina MiSeq platform (Illumina Inc., USA). A total of 11,618,658 reads were obtained which is equivalent to 59,528 and 285,456 reads per sample.

Fastq files were processed and the sequencing reads filtered using mothur software (release v.1.33.0; Schloss *et al.* (2009)) according to the SOP by (Schloss *et al.*, 2011). For the alignment and removal of chimeras and plant-derived 16S rRNA gene sequences from chloroplasts and mitochondria, the SILVA reference file (release 119; Quast *et al.* (2013)) was used. The RDP database (release 10; Cole *et al.* (2014)) was the reference for classification of OTUs, which were found at 95% similarity clustering of the reads using the furthest neighbouring method. Raw read sequences can be found at GenBank's Short Read Archive (SRA) under the accession number SRP101445 (BioProject PRJNA378229).

The ^{13}C enrichment for each OTU was determined on the basis of the publication by Hungate

et al. (2015), where a detailed description and formulas can be found. First, the weighted average mean density was calculated for each OTU across the 7 fractions in each gradient. In this study, an intrinsic correlation of the GC content to the density has been performed. OTUs from the control samples, which could be classified at the genus level, were summed up and the weighted average density was calculated from the 7 fractions accordingly for each genus in each depth. If available, the corresponding genomic GC content from the NCBI database (ftp://ftp.ncbi.nlm.nih.gov/pub/taxonomy/taxdump.tar.gz; 19.03.2016) was assigned to each genus taxon found in the unlabelled control datasets, which resulted in 234 data points. Multiple GC content entries for single genera in the NCBI database were averaged in advance. The correlation between average mean density and GC content was expressed in a linear model (Figure S5), which was used to determine the GC content for each OTU in the control samples.

To calculate the increase of ^{13}C content for each OTU, the density shift between the control and the ^{13}C-labelled sample in the corresponding soil depth was determined as the difference of the weighted mean average densities. The GC content of each OTU served as a basis to calculate the increase in molecular weight of the DNA by the density shift and thus the ^{13}C enrichment, which is expressed as atom fraction excess. The extension of atom fraction excess values below '0' was taken as uncertainty range also for the positive measurements. Above this threshold, ^{13}C enrichments were considered as confident.

Significant differences for single variables – root biomass, ^{13}C content, Shannon diversity, and relative abundance of bacterial phyla – were calculated with univariate analysis of variance (ANOVA, R package 'stats', R Core Team (2013)). Square root transformed relative abundance data from 16S rRNA gene sequencing and T-RFLP were used to compute permutational multivariate analysis of variance (PERMANOVA, R package 'vegan', Oksanen *et al.* (2015)). Heatmaps are based on the same data (R package 'gplots', Warnes *et al.* (2016)). For clustering of the dendrograms, the complete linkage method was applied on the

euclidean distance matrix.

Acknowledgements

This project was funded by DFG within the Research Unit FOR1320 (MU831/21-1) and by the BMBF in the frame of the BonaRes initiative (Soil^3). We are grateful for technical assistance by Arsin Sabunchi, Cornelia Galonska, Oliver Gefke, and Dominik Dannenbauer (Helmholtz Zentrum München). We thank the Umwelt-Geräte-Technik GmbH especially Sascha Reth for excavating the soil cores as well as Heike Schneider (Forschungszentrum Jülich) for providing the climate box. We acknowledge the insightful discussions with Andreas Hofmann (Helmholtz Zentrum München).

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Legends

Figure 1: 16S rRNA gene abundance in CsCl-gradient fractions. 7 consecutive fractions are displayed, which were selected for barcoded 16S rRNA gene amplicon sequencing. Topsoil (0-20 cm); Subsoil U (upper subsoil, 20-50 cm); Subsoil L (lower subsoil, 50-80 cm).

Figure 2: Bacterial community composition in control and ^{13}C-labelled rhizosphere samples. Sequencing reads in gradient fractions were combined on the basis of weighted relative abundances. The 100 most abundant OTUs were selected for clustering and ordered from top to bottom first by their phylum affiliation and secondly, by the mean relative abundance across all samples. PERMANOVA revealed significant differences between soil depths (P = 0.001). Top – Topsoil (0-20 cm); Sub U – upper subsoil (20-50 cm); Sub L – lower subsoil (50-80 cm); ^{13}C-lab – plants were labelled with ^{13}C-CO_2; control – no labelling; u – unclassified at 80% cutoff.

Figure 3: ^{13}C enrichment of bacterial OTUs in different soil depths. The mean density shift – expressed as ^{13}C atom fraction excess – for each OTU between the 3 labelled samples and the control (n = 3) was plotted according to its phylogenetic affiliation. OTUs with a minimum relative abundance of 0.1% in at least one sample were selected. Spot sizes represent the OTU mean relative abundance in the control and the three labelled samples (n = 4). The threshold of uncertainty (continuous line) was set according to negative values of OTUs with a relative abundance >0.1%. Dashed circles include important OTUs above the threshold of uncertainty for easier recognition. (A) – Topsoil (0-20 cm); (B) – Subsoil U (upper subsoil, 20-50 cm); (C) – Subsoil L (lower subsoil, 50-80 cm).