Chapter 30
Microbial Communities in the Rhizosphere Analyzed by Cultivation-Independent DNA-Based Methods

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Abstract The development of methods to extract nucleic acids directly from the rhizosphere or from microbial cells detached by a mechanical treatment from roots opened new dimensions to study the rhizosphere microbiome and to overcome limitations of cultivation-dependent methods. This chapter summarizes the potentials and limitations of cultivation-independent methods used by our group in the last 15 years to investigate microbial communities in the rhizosphere and their response to changing environmental conditions. We showed that rhizosphere microbial communities are highly dynamic, and that their composition is mainly shaped by the plant and the soil type and factors influencing these drivers of microbial diversity in the rhizosphere.

30.1 Introduction

The importance of the plant microbiome for plant growth and health is increasingly recognized. The fraction of soil influenced by the plant, termed rhizosphere, is an interface that connects the soil with the plant. Understanding the complex interactions in the rhizosphere remained a challenge until tools allowing cultivation-independent analysis of DNA or RNA extracted directly from the rhizosphere became available. Here we provide a short overview of some of these tools which were used to study the influence of different factors on the microbial community compositions in the rhizosphere. The chapter is biased towards our own work and for more comprehensive compilation the reader is referred to recent reviews

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(Berg and Smalla 2009; Berendsen et al. 2012; Bulgarelli et al. 2013). Before discussing the different nucleic acid-based methods and the major findings we would like to draw the reader's attention to critical prerequisites for obtaining meaningful data.

30.2 Experimental Design and Sampling

The adequate experimental design and sampling strategy depends on the hypotheses to be tested and often pre-experiments might assist in determining the numbers of samples to be analyzed. Furthermore, the strategy used to sample the rhizosphere influences the data obtained. Typically composite samples from the root system of several plants are analyzed for reporting in a representative manner on the structural and functional diversity in the rhizosphere and on the variation among replicates within the same treatment and between treatments. We have studied the rhizosphere microbial communities of different plant species from various sites and geographic regions. Usually the plants were destructively sampled by uprooting them and vigorously shaking the roots. Different protocols have been used also in our laboratory and the protocols had to be adapted for various reasons. Therefore it is highly recommended for comparison of data from different studies to carefully read the sampling protocols described, as the fractions of the rhizosphere microbial communities analyzed differed—depending on the protocols applied—in the proportion of rhizoplane and bulk soil microorganisms present. Rhizosphere total community (TC-) DNA was extracted from the soil brushed off from the root (Marques et al. 2014). This technique is typically used when long-distance transport of samples is needed and microbes residing on the rhizoplane or in soil particles glued to the roots or from fine roots were likely missing. However, in most studies performed by our group the complete root system, after vigorous shaking, was cut into pieces and mixed. Subsamples placed in plastic bags were treated in the Stomacher® Circulator after adding saline or water. Via paddle movement cells were detached from the root and soil particles and the Stomacher treatment step was repeated three times. To obtain the microbial pellets, the combined supernatants were centrifuged and the TC-DNA was extracted from the complete pellet with of commercial soil DNA extraction kits (Weinert et al. 2009; Schreiter et al. 2014a). When a combination with cultivation-dependent analysis was done, e.g. to determine the potentially antagonistic fraction (Berg et al. 2002) or to monitor inoculant strains (Adesina et al. 2009; Xue et al. 2013; Schreiter et al. 2014a) an aliquot from the combined supernatant was used for plating of serial dilutions. Recently, we had to modify the protocol in a project aiming to compare the effect of three soil types on the rhizosphere communities. An additional root washing was performed in order to remove big clumps of soil adhering to the roots of plants grown in clay rich soils before the Stomacher® protocol (Schreiter et al. 2014b). The TC-DNA obtained from the pellet gained with this protocol was assumed to represent the genetic information of microbes colonizing the rhizoplane and rhizosphere. Although a complete dislodgment of cells adhering to the roots and soil particles

seems to be impossible, it is important that cells bound to soil particles with different degrees of strength are released with similar efficiency. Another crucial step for the recovery of representative DNA that mirrors the genomes of all microbes present in a rhizosphere sample is the efficient lysis of microbial cell walls. This can be achieved by mechanical cell disruption and by enzymatic or chemical disintegration of cell walls, or a combination of these methods. The efficiency of the different methods used might not only influence the yield but also the presence of genomic DNA in cells difficult to lyse. However, obviously the strength of lysis needs to be a trade-off as too rigorous lysing methods might shear DNA released from cells that are easy to lyse. The DNA yield might vary considerably for different DNA extraction kits used for the same rhizosphere soils. Commercial kits for extraction from soil after a harsh lysis with the FastPrep®-24 Instrument were major achievements and allowed a simplification and miniaturization of the method. Extraction kits are less time-consuming and efficiently remove co-extracted humic acids which would disturb PCR-amplification. Finally, it should be stressed that strict precautionary measures need to be taken to prevent contamination of the DNA during the extraction. In particular, when PCR is used to amplify a target gene that occurs less frequently, e.g. antibiotic resistance genes or transgenic DNA, the extraction of DNA, preparation of PCR reactions and analysis of PCR products need to be done in separate rooms.

30.3 Bacterial and Fungal Community Composition in the Rhizosphere

PCR-based amplification of 16S and 18S rRNA gene or ITS fragments from rhizosphere DNA and their subsequent analysis by fingerprinting, cloning and/or sequencing are most frequently used to study the composition of microbes in the rhizosphere and the effects of treatments. The rapidly growing database of ribosomal rRNA gene sequences contains presently more than a million good quality 16S rRNA gene sequence entries deposited in Ribosomal Database. A disadvantage of using ribosomal rRNA gene fragments is that bacteria possess different numbers of ribosomal RNA operons. The numbers of 16S rRNA operons are assumed to reflect different ecological strategies of bacteria (Klappenbach et al. 2000) and sequence heterogeneity of the different operons might occur (Nübel et al. 1996). Costa et al. (2007) proposed the *Pseudomonas*-specific *gacA* gene as an alternative marker for studying their community composition. However, no matter which gene is targeted, one major limitation that remains is that gene fragments of less common populations are often not represented in clone libraries or fingerprints, especially when primers targeting all bacteria, archeae or fungi are used for PCR amplification. Bent and Forney (2008) termed this problem “the tragedy of the uncommon”. The application of group-specific primers targeting the 16S rRNA gene can assist in studying less common populations (Heuer et al. 1997; Heuer et al. 2002; Gomes et al. 2001; Costa et al. 2006a; Costa et al. 2006b; Weinert et al. 2009). The sequence diversity among 16S and 18S rRNA gene or ITS amplicons from TC- DNA can be analyzed

by various techniques such as the terminal restriction fragment length polymorphism (T-RFLP), single strand conformation polymorphism (SSCP) or denaturing gradient gel electrophoresis (DGGE) that were developed in the end of the 1990's. A comparison of these fingerprinting techniques showed that they had similar resolution levels and provided similar results despite the different 16S rRNA gene regions used (Smalla et al. 2007). At that time the great advantage of the fingerprinting techniques was that a sufficient number of replicates could be analyzed in parallel, and when combined with statistical analysis, testing of different biotic and abiotic factors influencing the bacterial and fungal community composition became possible (Kropf et al. 2004). A clear drawback of the molecular fingerprinting techniques was that bands with treatment-dependent intensity had to be excised from the fingerprints, re-amplified, cloned and sequenced. Sequencing of dominant bands with identical electrophoretic mobility detected in the rhizosphere of strawberry and oilseed rape were shown to represent taxonomically different populations (Costa et al. 2006a). On the one hand, 16S rRNA gene fragments from taxonomically distinct populations might have the same electrophoretic mobility due to similar melting behavior while, on the other hand, one population might generate more than one band due to operon sequence heterogeneity. The DGGE fingerprints based on 16S and 18S rRNA gene and ITS fragments were used to study the influence of the following factors on the composition of the bacterial and fungal communities in the rhizosphere: (i) plant species (Smalla et al. 2001; Costa et al. 2006a), (ii) plant growth developmental stage (Smalla et al. 2001; Gomes et al. 2001; Gomes et al. 2003), (iii) the cultivar Weinert et al. 2009), (iv) the site (Costa et al. 2006a, 2006b) (v), the soil type (Schreiter et al. 2014a), and (vi) the effects of inoculants or pathogens (Adesina et al. 2009; Xue et al. 2013).

To obtain information on the taxonomic affiliation of the dominant bacteria in the rhizosphere cloning and sequencing of 16S rRNA gene amplified from TC- DNA of three potato genotypes grown at two sites were used. This approach was rather time and cost intensive and thus typically was not applied for replicates but for pooled samples. The TC-DNA from the potato rhizosphere of replicates of the same samples was also analyzed by PhyloChips (DeSantis et al. 2007). The PhyloChip was hybridized with Biotin-labelled 16S rRNA gene fragments. By means of the PhyloChip a total of 2432 operational taxonomic units (OTUs) were detected in the rhizosphere of potatoes and 864 were detected in all replicates. The major limitation of the PhyloChip approach is that the diversity detected depends on what is on the Chip, and that the hybridization signal intensity cannot be directly related to relative abundance. Nevertheless, PhyloChips are great tools for comparing the relative abundance of particular OTUs within and between treatments (Weinert et al. 2011). Thus OTUs differing in relative abundance in the rhizosphere of the same potato cultivars between sites and, more importantly, between cultivars could be identified. In recent years amplicon sequencing technology became an important tool in rhizosphere microbiology and revolutionized the field. With increasing read length and sequencing depth this technology now allows analyzing multiple replicates as previously done by DGGE to determine the effect of various biotic and abiotic factors on the microbial community composition in the rhizosphere (Marques et al. 2014; Schreiter et al. 2014a). The community composition analysis done by pyro- or illumina sequencing

at a much higher resolution level largely confirmed data obtained by DGGE. The main advantage of amplicon sequencing is that at the same time insights into the taxonomic composition and identification of genera differing in relative abundance depending on the treatment becomes feasible. Although the assignment to species level is only achieved for a fraction of the sequence reads, the situation will improve with increasing read lengths. However, researchers should keep in mind that there is a large diversity beyond the 16S rRNA gene level (Eltlbany et al. 2012). Recent insights come from the determination of the plant microbiome by direct sequencing of DNA (metagenome) or cDNA (metatranscriptome). Presently, a major limitation of the direct sequencing approach is that typically no replicates were sequenced. The enormously large sequence data sets can provide insights into metabolic pathways, plant effectors, and mobile genetic elements (MGE) which can be the basis for generating new hypotheses.

The TC-DNA can be also used to quantify the abundance of beneficial or plant pathogenic bacteria by PCR-Southern blot hybridization (Eltlbany et al. 2012). The presence of antibiotic resistance genes and MGE in TC-DNA can be determined by quantitative real-time PCR (qPCR) and Southern blot hybridization. The latter approach was shown to be more sensitive and specific than qPCR but remained semi-quantitative. Quantitative real-time PCR should be done, if possible, with Taqman probes instead of Evagreen in order to achieve a high specificity.

30.4 Main Findings Obtained by Molecular Analysis of Rhizosphere Plant Species and Growth Stage-Dependent Diversity

DGGE fingerprints of bulk soil and rhizosphere samples from strawberry, oilseed rape and potato plants that were grown in a randomized plot design at the same field site revealed an enrichment of specific bacterial populations in the rhizosphere (rhizosphere effect) and plant species-dependent bacterial community composition (Smalla et al. 2001; see Fig. 30.1). Bulk soil fingerprints were characterized by many equally intense bands indicating a high evenness while in the rhizosphere fingerprints of the several stronger bands were detected, indicating an enrichment of some populations in response to root exudates and a reduced evenness. Some bands showed a plant species-dependent enrichment. Bands that were detected only in the rhizosphere fingerprints of strawberry plants were identified after cloning of the re-amplified PCR products and sequencing indicated an enrichment of *Actinobacteria* in response to the growing strawberry plants. Furthermore, the early studies by Smalla et al. (2001) and Gomes et al. (2001) already showed that different plant developmental stages were characterized by different bacterial community compositions. This finding was also observed for lettuce grown in three soils by means of amplicon sequencing (Schreiter et al. 2014a). When strawberry and oilseed rape plants were grown at different field sites, the rhizosphere fingerprints were influenced by both the site and the plant species. Interestingly, the actinobacterial DGGE fingerprints of

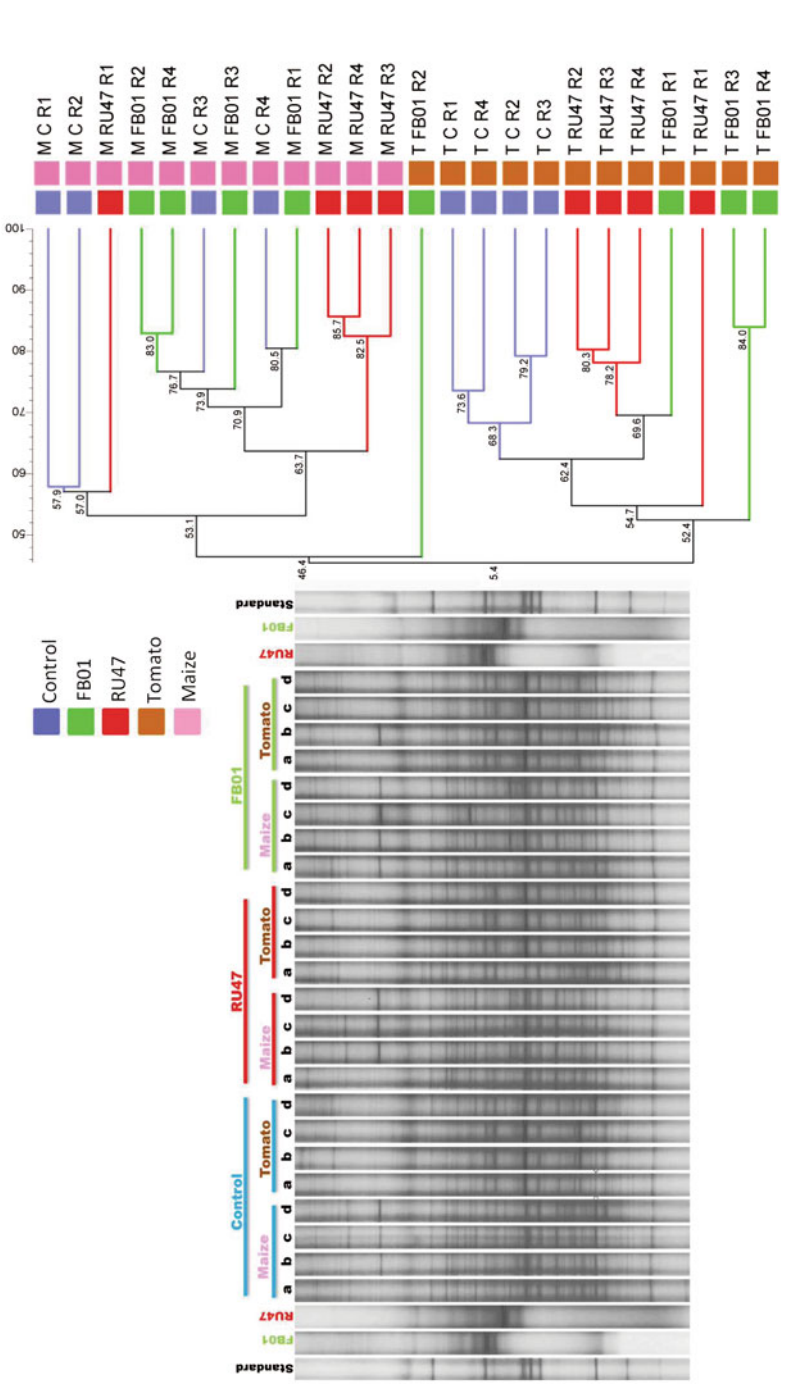

Fig. 30.1 The effect of *Pseudomonas jessenii* RU47 (RU47) and *Bacillus amyloliquefaciens* FZB42 (FB01) on total bacterial community in rhizosphere obtained by DGGE. The unweighted pair group method with arithmetic mean (UPGMA) analysis of this gel revealed a strong effect of the plant species on the composition of the rhizosphere bacterial community

strawberries grown at three sites displayed highly similar actinobacterial community compositions indicating that *Actinobacteria* did strongly respond to the strawberry exudates (Costa et al. 2006a). Similarly, *Pseudomonas* populations were enriched in the rhizosphere of strawberries as revealed by sequencing of bands from the *Pseudomonas* fingerprints from strawberry. The sequences of populations enriched in the strawberry rhizosphere grown at three sites were identical to those from isolates with in vitro antagonistic activity towards *Verticillium dahliae* (Costa et al. 2007).

Plant Genotype-Dependent Diversity In contrast to the effect of the plant species and the site, the influence of the plant genotype on the microbial community composition in the rhizosphere is much more subtle. In order to investigate the effect of transgenic potato plants, five different potato cultivars and two transgenic lines grown at two sites were investigated by DGGE fingerprints (Weinert et al. 2009). Transgenic potatoes were found to be in the normal range of variability among different cultivars. PhyloChip analysis revealed that OTUs differing between cultivars belonged to the *Pseudomonadales*, *Enterobacteriales* and *Actinomycetales* (Weinert et al. 2011). Moreover, the bacterial community compositions in the tuber rhizosphere of three sweet potato genotypes were recently compared by DGGE and amplicon sequencing of 16S rRNA gene fragments. While DGGE fingerprints showed only minor plant genotype-dependent differences at both sampling times, amplicon sequencing allowed identifying plant genotype-specific populations which were linked to low starch content. However, in the tuber rhizosphere of all plant genotypes, *Bacillus* and *Paenibacillus* were significantly enriched compared to bulk soil.

Site- and Soil Type-Dependent Microbial Diversity DGGE and amplicon sequencing analysis was used to analyze the effect of soil types on the microbial community composition in the rhizosphere under field conditions. Earlier studies from our group had already provided insights into the effects of different sites (Heuer et al. 2002; Costa et al. 2006a; Weinert et al. 2009) as it was assumed that the microbial community composition was not only influenced by the soil type but also by cropping history, agricultural management practices and climate. The study by Schreiter et al. (2014a) was the first to show under field conditions that three soils that had been kept in an experimental plot system under identical cropping history and weather conditions at the same field site for more than 10 years still displayed a distinct bacterial community composition in the rhizosphere of lettuce, indicating that the soil properties (mineral and organic composition, pH) are indeed a major factor shaping the microbial community composition in the rhizosphere.

Taxonomic Composition Cloning and sequencing of 16S rRNA gene fragments amplified from TC-DNA of the rhizosphere of three potato varieties grown at two sites showed a similar composition of major phyla and classes in the potato rhizosphere with the phylum *Proteobacteria* being the most abundant, followed by *Acidobacteria*, *Actinobacteria*, *Firmicutes, Bacteroidetes* and *Verrucomicrobia.* Interestingly, despite the low coverage (approx. 150 sequences per site) sequences affiliated to the *Acidobacteria*, *Verrucomicrobia* or phylum TM7 were detected in the rhizosphere of potatoes of all cultivars and from both sites (Weinert et al. 2011).

Cultivation-independent analysis clearly showed that these organisms which are difficult to culture are abundant in the rhizosphere but their role, e.g. in the dialogue with the plant, still needs to be revealed. Amplicon sequencing of 16S rRNA gene fragments from the rhizosphere of lettuce grown under field conditions in three soils revealed that *Proteobacteria* were strongly enriched in the rhizosphere of lettuce in all three soils compared to bulk soil while the relative abundance of *Actinobacteria* decreased. Several genera such as *Sphingomonas*, *Variovorax*, *Pseudomonas*, and *Rhizobium* were enriched in the rhizosphere of lettuce, independent of the soil type. Many dominant OTUs (defined at 97 % sequence identity) in the rhizosphere of lettuce were shared among the three soil types although some were soil type-specific (Schreiter et al. 2014a). Whereas in the tuber rhizosphere of sweet potato in particular the relative abundance of *Firmicutes* (*Bacillus* and *Paenibacillus*) was enriched compared to bulk soil (Marques et al. 2014).

Effects of Inoculants DGGE fingerprints and amplicon sequencing of 16S rRNA gene fragments were also used to investigate the effect of inoculants on the indigenous microbial communities in the rhizosphere (Götz et al. 2006; Adesina et al. 2009; Grosch et al. 2012; Xue et al. 2013). Compared to the effect of the plant species, the soil type or the year-to-year variation, inoculants influenced rhizosphere microbial communities to a lesser extent. Interestingly, the composition of the indigenous microbial community was most strikingly influenced by a mixture of *Trichoderma viridae* and *Serratia plymuthica.*

Detection of Antibiotic Resistance Genes and Mobile Genetic Elements The importance of horizontal gene exchange for short-term bacterial adaptability and successful colonization of new ecological niches has only recently been fully recognized (Heuer and Smalla 2012). The rhizosphere provides a natural hot spot of horizontal gene transfer as nutrient availability, bacterial cell numbers and activity are increased compared to the bulk soil. We used PCR-Southern blot hybridization and qPCR to detect resistance genes and MGE-specific sequences. We could show that the abundance of sulfonamide resistance genes (*sul1*, *sul2*) was unexpectedly lower in the rhizosphere of maize and grass grown in manure-treated soils compared to control. Another interesting observation was the enrichment of class 1 integrons and IncP-1 plasmids in the rhizosphere of lettuce grown in three soils (Jechalke et al. 2014). This increased abundance might be caused by aromatic compounds in the root exudates of lettuce (Neumann et al. 2014).

Conclusion

The analysis of TC-DNA from the rhizosphere became an important component of the tool set available in rhizosphere microbial ecology and provided important insights of practical relevance, e.g. for plant breeding or biocontrol. Conclusions from 16S rRNA gene based analysis should be drawn cautiously as the resolution level is limited and diversity beyond 16S rRNA gene sequences is high and not captured. Therefore, methods analyzing TC-DNA should be combined with microscopy and

cultivation approaches. Likely, new image analysis tools and sensitive chemical detection methods will be more and more integrated to better understand the complex interactions in the rhizosphere.

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