Assemblage and functioning of bacterial communities in soil and rhizosphere

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Chapter 6

General discussion

The main goal of the research described in this thesis was to obtain a better understanding of the assemblage and diversity of bacterial communities in soil and rhizosphere as well as of their functionality. To reach this, I manipulated the community diversity by use of the so-called dilution approach focusing on both the effects of soil and plant on the community assemblage. In order to study the microbial community diversity and functionality, I applied a combined approach of next generation amplicon and shotgun metagenome sequencing followed by advanced bioinformatics and statistical analyses. Here, I will first discuss the methodology of studying microbial diversity in soil, which could be used as a general approach in further studies to analyze microbial diversity experimentally. Secondly, I will discuss the importance of the impact of soil and the relevant physicochemical soil characteristics on the structuring of microbial communities in soil. Thirdly, I will concentrate on the microbial community assemblage processes operating in soil and rhizosphere at both taxonomic and functional levels. In the fourth section regarding plant-microbe interactions, I will discuss the feedback of soil-borne bacteria and plants, and the functional traits that determinate the relationship between the bacterial community and plant growth. Finally, I will discuss ideas and directions for future research on soil microbial diversity.

6.1 Methodology: assessing the microbial community diversity in terrestrial ecosystems

Previously, many studies on the creation and functionality of biodiversity have focused on macro-organisms and much less on microorganisms (Bever 1994, Shanmugam et al 2011, Tilman et al 1997) despite the increasingly recognized importance of microbial diversity in terrestrial and other natural ecosystems (Thiele-Bruhn et al 2012, Wall et al 2015). One of the major hurdles in these studies is the lack of sound approaches to manipulate experimentally microbial biodiversity. One of the main approaches applied to microbial biodiversity and assemblage studies is the so-called dilution approach, which is used here. Until now, the studies performed to assess microbial biodiversity based on this approach have often been restricted by low-resolution based analytical methodologies (Griffiths et al 2001, Mandeel et al 2005, Nielsen et al 2015, Prakamhang et al 2015, Wall and Six 2015). So, they failed to comprise the total microbial community profiles, while there are sufficient arguments that it is of utmost importance to study the diversity and functionality of the total microbiome in terrestrial ecosystems (Berendsen et al 2012, Chaparro et al 2014).

The estimates of bacterial diversity based on the results obtained with the dilution approach revealed that the bacterial community diversity was reduced significantly at species or OTU level by dilution of a soil suspension (Chapters 2 and 3). Previous studies claimed that by dilution particularly rare species were removed from soil suspensions and that therefore the abundant ones would dominate the microbial community formed after incubation of the diluted suspensions in soil (Franklin and Mills 2006, Garland and Lehman 1999). As the role of rare species in ecosystem functioning is a hot topic in ecology (Gaston 2012, Pedros-Alio 2012), I was especially interested in the possibilities provided by the dilution approach to, indeed, separate abundant and rare species. The results of my studies, however, showed that unique species were present in all dilutions including the most diluted suspensions. Probably certain species are suppressed for the sequencing assessments in the less diluted suspensions and only showed up in the more diluted, less diverse, suspensions. Thus, the conclusion can be drawn that the common presumption underlying the dilution approach that rare species would be out diluted, is not correct. Thus, the dilution approach does not allow for the separations of rare and

abundant species, and, so, is not the appropriate approach to study the importance of rare and/or abundant microorganisms in ecosystems.

It should be pointed out however, that it is hard to formulate a clear-cut definition of 'abundant' and 'rare' organisms (Fuhrman 2009). Rare species are often described as organisms occurring in the relative abundance range of approximately 0.1% (Postma-Blaauw et al 2005) to 0.01% (Qin et al 2010) of the total community. However, organisms that do occur in one environment in that abundance range may become common, even dominant, when the environment changes (Kulmatiski et al 2008). So, they may be regarded as a seed bank of diversity and functionality when local conditions change through natural or anthropogenic causes (Fuhrman 2009). Yet, certain studies indicated that it would be the abundant species that mainly perform most of the functions in marine ecosystems (Cottrell and Kirchman 2003). Similarly, studies based on advanced sequencing approaches indicated that the abundant members of the community are primarily responsible for most major biogeochemical processes such as the nutrient cycling (Pedros-Alio 2006). So, in conclusion: our understanding of the functional importance of different groups, *i.e.* rare/abundant species, in natural ecosystems is limited mainly by limitations to the possibilities provided by the currently available methodological approaches to provide a comprehensive assessment of the microbial community diversity at the phylum (van de Voorde et al 2012) and/or the OTU level (Bulgarelli et al 2015).

Recently, new low-cost, high-throughput sequencing approaches have greatly improved the understanding of the huge diversity of microbial communities in ecosystems (Bulgarelli et al 2015, Franzosa et al 2015, Lebeis et al 2015, Rodrigues et al 2013, van de Voorde et al 2011, van de Voorde et al 2012). High-resolution sequencing approaches have the potential to allow for the detection of the entire microbial community structure including the most dominant and the rarest species (Lynch and Neufeld 2015, Pester et al 2010). I applied these approaches in the study described here. Continuing advances in sequencing technology have allowed for studies on diverse microbiomes, ranging from natural environments (Mendes et al 2014) to the human body (Tremaroli and Backhed 2012). Although these approaches have been proven highly effective, there are still limitations based on the current DNA sequence-based methods. For example, upon application of these approaches, a clear

definition of a microbial species is still lacking (Nielsen et al 2015). Usually we rely on the sequence similarities of taxa-specific DNA subunits to distinguish microorganisms, using the term "Operational Taxonomic Unit, or OTU" rather than species. Most sequencing approaches provide, at best, species-level taxonomic resolution, but many important phenomena may be present at the strain level. Furthermore, uncultured microorganisms represent the majority of microbial diversity, and, as their functional potential is largely unknown, the databases used for annotation of the functional genes underrepresent dramatically the overall microbial functional potential. In addition, a fundamental limitation of metagenome sequencing is that the presence of a functional gene does not necessarily represent its activity, as host organisms may be dormant, inactive or only active in certain condition. Thus, additional integrated approaches, such as RNA (transcriptomics) and proteins (proteomics), are required to fully describe a microbial community and it's functioning.

The network analysis described in Chapter 4 showed a more tighter and complex network of rhizosphere communities than that of bulk soil communities, including more keynote species mainly belonging to different genera. These key members of the rhizosphere microbial communities may also be the key intermediaries in plant-microbe associations. Indeed, in Chapter 5, two groups, *i.e. Arthrobacter* and *Planctomycetaceae*, which were identified as strongly enriched families in the rhizosphere and important intermediates in the networks in the rhizosphere (Chapter 4), were also identified as potential candidates to explain best the differences in plant biomass production after incubation of the undiluted 10^{-1} suspension using unsupervised multivariate analysis. Further partial correlation revealed that *Arthrobacter* was the taxonomical group most related to plant growth. However, *Arthrobacter* had a lower betweenness centrality, *i.e.* the extent of network interactions, than *Planctomycetaceae* in the 10^{-1} rhizosphere community (317 for *Planctomycetaceae* and 163 for *Arthrobacter*, respectively). This suggests that *Arthrobacter* might have been more important for plant-microbe interactions, while *Planctomycetaceae* mediated more network associations. The role of the other key intermediate groups of the rhizosphere and soil networks was not further assessed and, at least, their impact on plant biomass production was negligible as compared to that of *Arthrobacter* and *Planctomycetaceae.* This

points to both the power and the limitations of network analyses for detecting species associations in plant-soil systems.

6.2 The impact of soil on the structuring of soil bacterial communities

Previous studies have found that soil is one of the most important factors structuring microbial communities (Berg and Smalla 2009, Garbeva et al 2004, Kuramae et al 2012). Results from my study indicated (Chapters 2 and 3), indeed, that different soils had a strong steering, selective, effect on shaping bacterial communities.

As described in previous studies, soil type has been ranked as the most important factor determining the structure of microbial communities, followed by time, specific farming operations, management systems and spatial variation (Bossio et al 1998). The factors in soils that may potentially affect microbial communities and thus may explain differences and shifts in community structure are pH (Lauber et al 2009), phosphate availability (Faoro et al 2010), and organic matter content (Verbruggen et al 2010). The soils I used in the cross-dilution experiment differed in these factors. The Utrecht soil was characterized by low pH, the Clue soil was characterized by high phosphate content, and high organic matter while the Meijendel was characterized by a relatively high pH. All these soils contained a characteristic microbial community and the factors mentioned are likely the driving variables shaping the bacterial communities in these soils (Chapter 3).

Previously, studies have focused on the importance of single (a)biotic factors and much less on the integrated soil characteristics when evaluating the effects of soil on microbial community structure and function (Murty et al 2002, Torsvik and Ovreas 2002), despite the increasing recognition of the importance of the overall environment on the structuring of microbial communities in soil and their biodiversity (Fierer and Jackson 2006, Hogberg et al 2007). I showed that the structure of the bacterial community was changed dramatically after incubation in soil as compared to the structure of the community in the suspension (Chapters 2 and 3). This strongly points to the overriding impact of soil, as an important, decisive, factor in the assemblage of bacterial communities in soil, which is likely due to the integrated physical and chemical

characteristics of the soils. Therefore, I suggest that a combination of abiotic factors, and not only pH or another single factor determines the structure of soil bacterial communities.

6.3 Bacterial community assemblage in soil and rhizosphere

Plants are known to significantly select for specific microorganisms in the rhizosphere (Haichar et al 2008, Mendes et al 2014). This is called the 'rhizosphere effect'. It is known that plant species have rather specific effects on the structure of the rhizosphere microbial communities, even at the genotype level, (Berg and Smalla 2009, Duineveld et al 2001, Haichar et al 2008). I also observed a considerable effect of the presence of plants on the bacterial communities in the rhizosphere at both taxonomic and functional levels (Chapter 4).

Earlier studies indicated that plants influence the composition and activity of the rhizosphere microbiota by selecting specific microbial populations from the soil-borne microbial reservoirs (Berg 2009, van Overbeek and van Elsas 2008), and, thus, the microbial community in the rhizosphere is a subset of the bulk soil (Duineveld et al 2001). Results presented in this thesis clearly indicate that the composition of the rhizosphere communities was dramatically different from that of the soil communities in terms of the dominant species. That does not hold for the abundant phyla of *Proteobacteria*, which showed to be highly diverse both in soil (Chapters 2 and 3) and rhizosphere (Chapter 4), which is consistent with the common concepts on the lifestyle of *Proteobacteria* (Fierer et al 2007). In agreement with earlier observations, within the phylum of the *Preoteobacteria*, bacteria from the families of *Pseudomonadaceae* (DeAngelis et al 2009) or of *Burkholderiaceae* (Pastorelli et al 2011, Uroz et al 2010) are among the most abundant members of the rhizosphere communities. It is, therefore, remarkable that 'transporters' genes that could be assigned to *Pseudomonaceae* were overrepresented in the bulk soil and not in the rhizosphere. As the occurrence of 'transporters' genes was found to be a determinative factor explaining plant biomass production (Chapter 5), this questions the significance of this group of bacteria as plant growth promoting organisms. Also, the relative abundance of *Actinobacteria* was found to be significantly larger in the rhizosphere of *Senecio* plant than in

the bulk soil while the Shannon diversity index for this phylum was significantly lower in the rhizosphere, which could be explained by the large relative abundance in the rhizosphere samples of one family, *i.e. Micrococcaceae* to which *Arthrobacter* belongs (Chapter 4). Interestingly, in line with these observations we clearly showed in Chapter 5 that *Arthrobacter* was significantly positively correlated to plant biomass more than any other group of bacteria (Chapter 5).

Based on the possibilities provided by the advanced sequencing approaches available, the concept of 'rhizosphere effect' should not only be limited to species but should be extended to the selection of functional genes in the soil microbiome (Mendes et al 2014, Ofek-Lalzar et al 2014). One of main goals of metagenomics has always been to link functional genes to particular organisms (DeLong 2009). The results presented in chapter 4 clearly illustrate the process of rhizosphere selection both at the community composition and functioning levels. We showed that the enrichment processes in the rhizosphere selects for microorganisms with specific functional traits including 'transporters', 'Embden Meyerhof Parnas' (EMP) and 'hydrogen metabolism'. The genes related to 'transporters' have been described in earlier observations by Mark et al (2006) and Mendes et al (2014) who showed that transporter systems are frequently enriched in the rhizosphere. The 'transporters' genes were positively related to plant growth. Because they were enriched in the rhizosphere compared to the soil we can conclude that plants positively affect bacterial species with such genes and this may in part explain the positive correlation with plant growth. At the same time bacterial species with these 'transporters' genes may stimulate plant growth, which would contribute to the positive correlation. Whether either one of the two or both explanations are true cannot be concluded from our experiments with certainty. Another over-represented group of functions in the rhizosphere is linked to EMP cycling. The EMP pathway is the most common bacterial glycolytic pathway for cellular energy production (Flamholz et al 2013). Considering that plants provide a wider and more complex range of substrate in the rhizosphere than is available in the soil, and thus provide better conditions for bacterial growth and activity we could expect, indeed, that the genes related to energy production will be over-represented in the rhizosphere metagenome as compared to the soil metagenome. Similarly, 'hydrogen metabolisms' also involve genes related to energy-generating mechanisms of specific microbial species such as nitrogen-

fixing bacteria (Eisbrenner and Evans 1983). Therefore, the group of genes related to 'hydrogen metabolism' might also be over-represented in the rhizosphere than in the soil, as we discussed earlier.

6.4 Impact of the rhizosphere microbiome on plant growth

One of the main results of the metagenomics analysis was the identification of particular functional genes activated in the rhizosphere, which determines plant microbe interactions. As mentioned above the results described in Chapter 4 demonstrated that selection of functions took place in the rhizosphere resulting in over-representation of particular functional genes in the rhizosphere compared to bulk soil. Although earlier studies have identified particular functions beneficial to plant growth, including nitrogen fixation or disease suppression (Quecine et al 2012, Tittabutr et al 2013), generally, the microbial functional traits that contribute to plant fitness have been largely unknown. In Chapter 5 I identified both the species and functional genes that potentially had most influence on plant growth by unsupervised multivariate analysis. As mentioned earlier based on unsupervised multivariate analysis, *Arthrobacter* and *Planctomycetaceae* were selected as potential candidates to explain the differences in plant biomass production, with *Arthrobacter* having the strongest impact. Tahir et al (2015) showed, after analysis of the wheat rhizosphere using 16S rRNA gene sequencing, that *Arthrobacter* belonged to the plant health promoting rhizobacteria. Several species of *Arthrobacter* have been described as plant growth promoting rhizobacterium (Gusain et al 2015, Ullah and Bano 2015). *Planctomycetales* is also a rhizosphere species (Tahir et al 2015), but its functionality is until now largely unknown.

The results presented in Chapter 5 also illustrated the importance of particular functional gene category for regulating plant growth. Interestingly, the functional genes of 'transporters' in the rhizosphere, which I already observed as being positively selected in the rhizosphere in Chapter 4, also appeared to be positively correlated to plant growth (Chapter 5). This provides evidence that plants may select for particular functional genes that promote their own growth. Interestingly, the frequency of 'transporters' genes was higher in *Arthrobacter* than in most other components of the bacterial community. By using partial correlation analysis, I proved that *Arthrobacter*

was not significantly correlated to plant biomass when taking 'transporters' genes into account, which suggests, that the functional genes explained better the plant-bacteria interactions than the community composition. Specifically, the 'monosaccharide transporters' genes were significantly positively correlated to plant biomass when all three dilutions samples were taken together, and this group of genes increased significantly upon dilutions in the rhizosphere. So, it is not enough to know who is there, but more importantly is to know what are they doing (Xu et al 2014).

However, we should exercise caution with the assertion on the importance of certain functional traits because it is extremely unlikely that a single function determines the differences in plant biomass production. Indeed, we also observed that 'nucleic acid metabolism' genes were also positively correlated to plant biomass production. The nature of this particular relationship is still unclear to us, but this may be related to cellular growth processes, which indicates a higher bacterial abundance/activity in the rhizosphere than in the soil, as also shown in Chapter 5. Consequently, this group of genes may point to a positive relationship between bacterial and plant growth.

We also observed functional genes including 'cellular response to stress' and 'saccharide metabolisms' that were negatively correlated to plant biomass production. As was described above, these functional genes were under-represented in the rhizosphere as compared to their abundance in the soil, suggesting that plant selected against such genes in the rhizosphere. One of the explanations could be that plants create a less hostile environment for microbial community in the rhizosphere by the rhizodeposition processes. As a result, this may lead to a negative correlation between 'cellular response to stress' genes and plant growth. Similarly, if plants produce more saccharides that become available for the rhizosphere community, microbial genes related to their biosynthetic pathway might be suppressed in the rhizosphere. However, in that case, one would expect that the 'saccharide metabolisms' genes were over-represented in the bulk soil, which they were not.

6.5 Final conclusions and future perspectives

A few points to consider in future studies concern the theoretical concept on assemblage of microbial communities, the separation of rare versus abundant species, the plant soil feedback effects and the selection processes operating in the rhizosphere.

Although I did not include this in this thesis, I did assess the rules leading to the assemblage of microbial communities in soil and rhizosphere using the theories niche-based and neutral mechanisms. These theories are based upon macro-ecological concepts, but have been used frequently in microbial ecology to describe microbial community assembly processes. The niche-based assemblage concept predicts that the assemblage of a community is based on niche partitioning of the limited resources between competitive species or the differentiation of niche space within a community and have been used to explain microbial community assemblage processes in, for instance, lakes (Van der Gucht et al 2007), soil (Fierer and Jackson 2006, Lozupone and Knight 2007), rhizosphere (Mendes et al 2014) and human gut (Lu et al 2014). The neutral theory is based on the assumption that the differences between members of an ecological community of similar species or species from the same trophic level are "neutral," or irrelevant to their success. In the light of my results on the importance of functionality rather than of taxonomic composition for the functioning of microbial communities in terrestrial ecosystems, it is recommendable to extend these concepts focusing on species functionality in order to be able to better understand how microbial communities are shaped in soil and rhizosphere. It should be taken into account that these models for microbial communities are mostly applied to the entire microbial community. This can lead to a strong underestimation of the selection effects. Selection is most likely occurring within groups of organisms, such as pollinators, insect herbivores etc. that share important ecological features. However, scale is a significant problem in microbial ecology. The entire microbial community encompasses a very diverse set of such ecological features and by pooling all microbes into one group selection may appear neutral while in fact it is not. Finding the “pollinators” within microbial communities is one of the challenges for modern microbial ecology.

The existing assumption linked to the dilution approach is that the approach allows for the separation of rare from abundant species. However, I

showed that unique species were detected even in the most diluted suspension and that rare/less abundant species could become abundant in another environment. This may be effectuated by the dilution procedure in which a less competitive environment may be created for rare species so to flourish more than in a more competitive environment of a less diluted inoculum. Thus, it is impossible to investigate their importance in natural ecosystems by use of the dilution approach. As the long tail of less abundant/rare species is a typical characteristic of highly diverse natural microbial communities, there is still an urgent need to develop appropriate methodologies to separate less and more abundant microbes that allow for specific investigations of their behavior and activities.

Future studies also need to compare plant-soil feedback processes across ecosystems and across successional stages within these ecosystems. One of the most reported findings regarding plant-soil feedback effects is that these effects are negative regarding plant growth (Bever 2003, Lankau et al 2011). So, these issues need to be further consideration under controlled conditions and time scales in order to enable the determination of the potential factors explaining the feedback processes. In these studies functional traits rather than taxonomy should be the target of fundamental research.

Finally, further studies should address how microbial communities are structured and selected at both the taxonomic and functional levels, in distinct soil types and in the presence of specific plant species. Metagenomics analysis has provided information about which microorganisms are present and what they are capable of doing. However, the detection of functional genes is not evidence of their activity. Further functional gene expression analysis including metatranscriptome analysis can provide information about what microorganisms are actually doing. Therefore, integrated experimental, including sequencing approaches, together with computational analysis, are needed to improve our understanding of microbial functionality in specific niche and plant- microbe interactions.

6.6. References

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