Functional Metagenomics for Rhizospheric Soil in Agricultural Systems

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Abstract

The study of plant microbiota has been stimulated by recognizing the fundamental role that the genetic capacity of the associated microbial communities has to modulate the phenotypic expression of plants, which is crucial for its health, physiology, and productivity. All genes in a metagenome can be described by whole-metagenome shotgun sequencing, but it is time-consuming, and a high level of experience is required. Alternatively, the amplification and high-throughput sequencing of the16S-rRNA gene allow describing the microbial composition. Then functional activities can be inferred by listing the abundance of each gene. Also, the identification and the quantification of microbiome transcripts are now accessible to determine the profile and changes in gene expression occurring in a microbial community in response to environmental or experimental variations. Considering the beneficial role of microbial communities in soil environments, it is important increasing the understanding of plant-microbe relationships to provide biotechnological information for control and management with sustainable practices.

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8.1 Introduction

Agriculture constitutes a cornerstone activity to produce food at large-scale and also biomaterials for biotechnological purposes (Béné et al. 2016; Jones and Ejeta 2016). However, the increasing food demand together with the environmental impacts caused by agro-industries represents a challenge that requires more intensive yet environmentally safe production, as well as the sustainability of the resources involved in this activity (Gebbers and Adamchuk 2010).

Microbial communities play essential roles for the excellent performance of agriculture through the decomposition, solubilization, and recycling of nutrients and toxic compounds, competing against pathogens and producing desirable physicochemical and biological conditions for the cultured species. Plants are colonized by a number of microbes that together can reach higher cell loads than the plant itself. Most of these microbes thriving in the rhizosphere and nearby are directly or indirectly associated to the plant (Mendes et al. 2013), which can be considered as a harboring host and neighbor mutualist body for those microbes that are not in contact with the plant but interacting with it. Beneficial rhizospheric microbes can alter the morphology of plants while enhancing their growth and increasing mineral availability (Lakshmanan et al. 2014). Microbiome soil in plants is also fundamental because it can secrete growth hormones while inducing the immune system. Diseases in crops or other food-producing environments are sometimes strongly linked with the changes in the environmental microbiome.

The study and characterization of the microbiota associated with plants have recently considered the genetic stock of microorganisms as endophytes and epiphytes (in and on plants, respectively), as an extension of the host genome and with a fundamental role in its phenotype. The sum of all genetic information or hologenome (Theis et al. 2016) enables the adaptation process to new or changing environmental conditions and the ability to resist pathogens. However, some circumstances could affect these microbes.

Anthropogenic activities causing changes in soil affect both the soil microbiome and the organisms living in and on them. These changes can consequently alter the functional profile of the microbiome, affecting the occurrence and abundance of essential metabolic pathways that maintain a balance within the systems. Herein, there are three groups of microbes present in the rhizosphere, including commensal, beneficial, and pathogenic (Berendsen et al. 2012). Microbiomes can activate complex signaling pathways in response to biotic or abiotic stress, leading to localized and systemic defenses (Lakshmanan et al. 2014), but these functions may be impaired by the occurrence of pathogenic blooms. Therefore, understanding the taxonomic and functional microbiome profiles caused by the occurrence of microbial pathogens in food production systems may provide valuable information to understand the negative effects of these phenomena and devise strategies to alleviate the effects or eradicate the pathogens; in addition, crops can be engineered through their microbiota favoring desirable responses. Genomic disciplines, but particularly functional metagenomics, can provide adequate insights of microbiomes.

8.2 From Functional Metagenomics to Food Production

Microbes associated to plants are essential for their adequate development; in this regard, plant microbiome is a key determinant of their health, physiology, and productivity (Mendes et al. 2013; Berendsen et al. 2012), and at the same time, much of these microbes participate in biogeochemical cycles. Particularly, these microbes can influence seed germination, seedling vigor, plant growth and development, nutrition, diseases, and development (Qin et al. 2011; Mendes et al. 2013). At some extent, the functions of this microbiota can be extrapolated to that of animals, where these microbes are considered as an annexed organ or a host's genome extension, and therefore the entire comprehension of the biology of a plant also depends on the knowledge gathered about its microbiota. Microbes including bacteria, archaea, fungi, protozoa, and algae are usually part of these microbial consortia.

This is a symbiotic relationship where plants depend on specific functions of the microbes while producing photosynthetically fixed carbon and other exudate components for the microbes thriving in the spermosphere, phyllosphere, rhizosphere, and mycorrhizosphere (Mendes et al. 2013). Despite a considerable number of studies having demonstrated these associations, the diversity and complexity of these diversity and interaction networks suggest that our current knowledge about this subject is still limited. Understanding the plant microbiome is an unavoidable cornerstone not only to comprehend the biology of plants but also to identify microorganisms that can be exploited for biotechnological purposes (e.g., improving plant growth and health) or regulated to avoid undesirable scenarios (disease).

Advances in genomics science shed light to some biological processes as virulence or resistance to antibiotics of pathogenic microorganisms, which play important roles in agricultural system industries (Lazarevic and François 2013). Current technologies not only provide better pictures about the taxonomic structure of microbial communities but also show accurate predictions about the functional capabilities and even real-time activities of microbes.

The study of the microbiome from this perspective is biotechnologically relevant because the information associates some of the translational applications with basic biology. Despite metabolic and regulatory networks being difficult-to-approach subjects for microbiologists, this kind of information may serve to identify targets for molecular therapy, particularly microbiomes (Vargas-Albores et al. 2018). For example, crops affected by pathogenic bacteria may require alleviating strategies including the use of antibiotics. However, functional metagenomics can provide information about potential resistance to particular kinds of antibiotics and suggest a more targeted and efficient strategy (dos Santos et al. 2017), allowing the use of less antibiotic while alleviating the collateral damage to the environment. Nevertheless, achieving this level of understanding about particular features of a microbiome requires highly specialized sets of tools. Another studies have revealed that manipulating the soil microbiome may result in improved soil health and increased plant fertility (Chaparro et al. 2012); however, knowing the taxonomic profile and functional capabilities of soil and rhizospheric microbiota will provide greater certainty about the parameters or conditions to be manipulated.

In addition, rhizospheric soils may also be reservoirs of opportunistic pathogens; these can disrupt in host microbiota by competition for resources, release of antimicrobial compounds, or antagonism against beneficial microorganisms. Understanding pathogen functionality, health, and productivity of important agriculture species can be improved by elucidating the taxonomic diversity and functional potential of their respective microbiota. Pathogen outbreaks usually occur when the conditions allow their proliferation; this means that pathogens can either be introduced into the systems or can be part of the environmental microbiota in a dormant or fully active state but not being virulent (Mendes et al. 2013). Conditions in the outside-host environment are prone to fluctuate over time affecting the microbiota and the activity of some pathogens.

One of the main fluctuating conditions affecting the microbiota is the soil nutrient profile, which is not for the plant but rather for the microbiota; for example, N-fertilization is commonly used in several crops particularly for modern varieties; however, this fertilization reduces microbial biomass and diversity, and consequently some functional capabilities may be lost (Ramirez et al. 2010). These kinds of strategies may have short-time beneficial effect on production but may have consequences in the long term because the soil microbiota may lack adequate functional profile to stimulate the responses in the plants of future crops or inclusively may fail to suppress pathogens. In addition, these modern plant varieties may have lost their ability to support microbiomes that degrade organic nitrogen and solubilize mineral nutrients such as phosphorus (Wallenstein 2017). In this regard, taxonomic and functional metagenomics insights could offer clues for performing strategies minimizing this collateral damage to the rhizospheric microbiota. For example, Wallenstein (2017) suggests engineering rhizospheres through inoculants that form connections with the native microbiome or soil amendments that stimulate microbial activity (Fig. 8.1).

Rhizospheres are highly structured, and the microbiome-plant interconnections through different signaling pathways as well as nutrient interchange (including root exudations) constitute a common process during the life cycle of a plant. As crops have been historically selected considering traits for intensive management, these connections were decreased. However, Wallenstein (2017) argued: "in the future, a systems approach to rhizosphere engineering could restore some features of natural rhizospheres through soil amendments, inoculants and plant traits that support beneficial microbiomes" (Fig. 8.1).

The improvement and increased use of high-throughput sequencing methods and the availability of generating genome information have significantly contributed to achieving deep approaches in functional metagenomics (Loman and Pallen 2015). Functional metagenomics begins with the isolation of DNA from microbial samples of environmental systems (Lam et al. 2015). In this context, there are two pathways to understand how these genes are involved with the environment; the first one is performing a molecular shotgun sequencing or also denominated whole-metagenome shotgun sequencing (WMS) from genomic DNA fragments of a metagenome community. The second one requires the amplification and high-throughput sequencing of a taxonomic biomarker gene, for example, the16S-

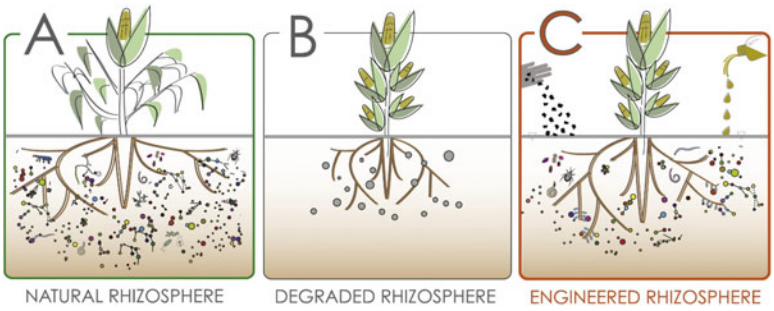

Fig. 8.1 Hypothesized conceptual models of rhizosphere systems in (**a**) natural ecosystems, (**b**) degraded systems such as those under intensive management and high fertilization, and (**c**) rhizospheres that have been engineered through inoculants that form connections with the native microbiome or soil amendments that stimulate microbial activity. (Figure obtained from Wallenstein 2017 (13). Rhizosphere 3(2):230–232 [w/ editorial & author permissions])

rRNA gene, avoiding the need of shotgun metagenomics while allowing, if required, the continuation of the metatranscriptomic and metaproteomic studies. Bioinformatics tools are absolutely required for any of these two approaches to process all of the recovered information (Morgan and Huttenhower 2012; Ortiz-Estrada et al. 2019).

8.3 Describing Microbial Communities Through the 16S rRNA Gene

Because the 16S rRNA gene is the most ubiquitous gene in the prokaryotic world and contains a combination of conserved and variable regions, it has been used for the classification of ribosomal RNA (rRNA) sequences and become a common approach for taxonomic identification of bacteria-forming complex communities (Glöckner et al. 2017). The 16S approach is mainly focused to elucidate the bacterial diversity, structure, and organization of microbiota as a community (Morgan and Huttenhower 2012). Nowadays, shotgun metagenomics is a very reliable technique involving the reconstruction of gene sets or genomes requiring a high level of experience and is time-consuming. For these and other reasons, studying microbial communities through 16S rRNA-targeted sequencing remains a valid approach.

Despite the many packages available on the market containing bioinformatics pipelines to analyze 16S rRNA gene amplicons, the most used open-code software tools are *Mothur* and Quantitative Insights Into Microbial Ecology (*QIIME*). These are based on a clustering-first approach, and the most recent pipelines including Kraken, CLARK, and One Codex use an assignment-first approach (Siegwald et al. 2017). These tools called pipelines are used to process the sequences by demultiplexing and quality filtering, classifying, aligning, and assigning sequences to operational taxonomic units (OTU) followed by the capability to process the information by microbial or ecological analyses like α- and β-diversity based on 16S rRNA gene (Glöckner et al. 2017; Almeida et al. 2018). The tools mentioned above

use SILVA as first-option reference datasets for taxonomic classification, but there are also other databases including the Greengenes rDNA, the NCBI Taxonomy, and the Ribosomal RNA Database project (RDP) (Balvočiūtė and Huson 2017).

Nonetheless, this is an approach to study bacteria and archaea but does not target eukaryotes. For targeting these last microbes, the 18S rRNA gene is required. This taxonomic biomarker gene is part of the structural RNA for the small component of eukaryotic cytoplasmic ribosomes. Unfortunately, the tools and protocols to study and valuate this biomarker are not as developed and extended as those for the 16S rRNA; however, there are some recommended software and useful pipelines to address this topic, particularly for amplification, sequencing, and taxonomic classification (Yang et al. 2013; Wang et al. 2014; Popovic and Parkinson 2018), whereas the rest of the statistical analyses have pretty much the same basis.

8.4 Drawing the Metabolic Potential

8.4.1 Targeted Metagenomics

A decade ago, providing a picture of the taxonomic profile and structure through 16S rRNA sequencing was considered as the limit to where this technique could lead; however, the feasibility of connecting databases containing information from simple biomarkers to those containing information on complete genomes made possible to infer the functional capabilities of the microbial community based in 16S rRNA sequencing. The correlation between taxonomy and metabolic function allows carrying out work focused on the identification of community structure and composition through analysis of taxonomic biomarker genes.

There are bioinformatics tools that construct an approach of functional metagenomics of a microbiome (Langille et al. 2013) (Fig. 8.2). These use the information from databases about the microbes constituting any niche and infer the metagenome of such microbial community and constructing a gene catalog with the abundance of different gene families. For example, tools like Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) are used for this purpose. In this kind of approach, the efficiency of functional predictions depends on the taxonomic classification accuracy, the genomes used as reference, and the information collected in databases from the closest ancestors. The databases of genes used for the metagenome construction are the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Clusters of Orthologous Groups of proteins (COG) (Kanehisa et al. 2008).

This, of course, is an imperfect association but can provide valuable information to evaluate factors influencing the functionality of microbial communities. One of the disadvantages of this approach is that this software uses the Greengenes database, which has not received actualizations since several years ago. However, there are other options such as Tax4fun, an R package using the SILVA database as

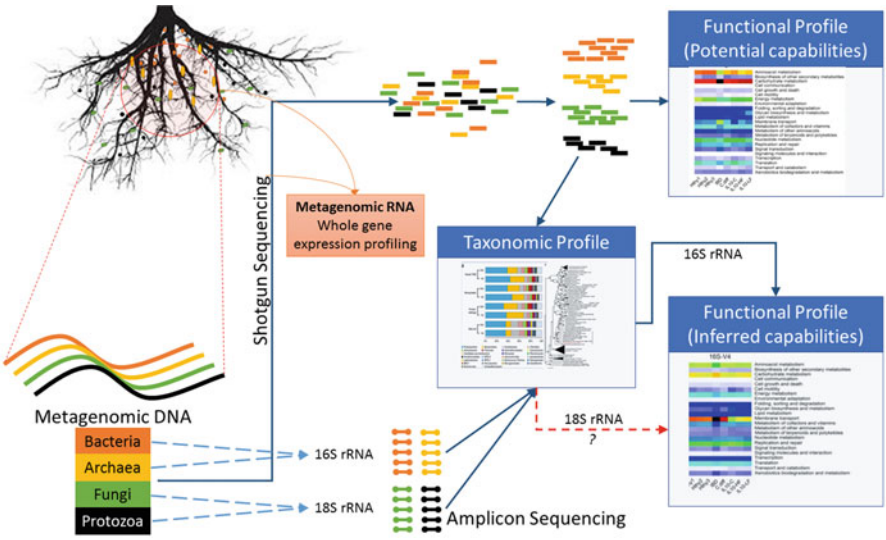

Fig. 8.2 General scheme for obtaining information about the taxonomic and functional profiles of microbiomes through metagenomics (shotgun sequencing) and targeted metagenomics (amplicon sequencing) approaches. To date, there are no bioinformatics tools for inferring functional profiles of eukaryotes using 18S rRNA; however, it is theoretically possible

reference and reported to be more accurate for some microbial communities (Aßhauer et al. 2015); however, neither PICRUSt nor Tax4fun has extensions to consider 18S rRNA data, and therefore the study of the microbiome may be incomplete.

Another issue to consider is that despite the easy generation of biomarker libraries, the manual exploration of the metabolisms associated with all observed OTUs is impractical; therefore, the researcher has no other option but to trust the gene family ordering process performed by the software.

On the other hand, there is evidence suggesting that in spite of the bias introduced by this imperfect correlation, shotgun metagenomics has validated the results obtained through targeted metagenomics using taxonomic biomarkers (Jovel et al. 2016).

Studies evaluating the rhizosphere have used these tools. Herein, Zhu et al. (2016) not only evaluated the effect of urea-based fertilizer rate on maize root exudation, the associated rhizosphere microbial community, and nitrogen-use-efficiency but also explored via PICRUSt the metagenomics contribution of bacterial OTUs obtained from 16S rRNA sequencing. Briefly, results revealed that some nitrifying and denitrifying genes were significantly influenced by the N rate, whereas some nitrogen-fixing and urease genes were not. However, on a total abundance basis, all N-cycle genes increased significantly as the N rate increased.

8.4.2 Metagenomics

The genomics discipline studying the genetic material recovered directly from environmental (or experimental) samples is known as metagenomics. To detect all of the genes contained in a microbial community, a shotgun metagenomics sequencing is performed, followed by annotations providing detailed output sets of metabolic and functional profiles (Vargas-Albores et al. 2018) (Fig. 8.2). Despite bioinformatics backgrounds being required to perform mandatory pipelines including quality control, assemblage, and annotation, there are user-friendly computing (public and private) software allowing the analysis of these data by most biologists. For instance, MG-RAST and CLC Genomics Workbench (Microbial Metagenomics Module) and other programs involve user-friendly end-user systems to perform complete mandatory pipeline processes. HUMAnN is another computational pipeline developed to determine the relative abundance of gene families and metabolic pathways from short-read sequence datasets (Aßhauer and Meinicke 2013), while STAMP is a friendly software to perform statistics of data obtained from targeted or shotgun metagenomics (Parks et al. 2014). There are plenty of free tools available; however, the sets of tools to use to establish a valid pipeline depend on the objectives of the study and the computing skills of the biologist.

Even though targeted metagenomics (16S rRNA) is an approach for the metagenome, shotgun sequencing offers not only more complete and precise results but also allows digging more into the functional potential of any microbial community, obtaining the genomic information from different taxa, and inferring metaproteomes from raw metagenomics sequences (Rooijers et al. 2011).

Studies related to agricultural sciences are still few but rapidly growing since microbiologists working in these disciplines are adopting these tools in an accelerated manner. Mendes et al. (2014) performed shotgun metagenomics to investigate the taxonomic and functional profiles of microbial communities in the bulk soil and in the rhizosphere of soybean plants and tested the validity of neutral and niche theories to explain the rhizosphere community assembly processes. This approach demonstrated that the assembly of the microbial community in the rhizosphere is based on niche-based processes as a result of a selective pressure exerted by the plant itself and other environmental factors.

Unlike targeted metagenomics, this approach can collect information about all of the microbial taxa thriving in the rhizosphere but requires additional processes for cleaning, ordering, and annotating sequences (Quince et al. 2017).

8.4.3 Metatranscriptomics

Transcription is the first phase of gene expression in which a particular segment of DNA is duplicated/transcribed into RNA language (especially mRNA). All of the living microbes thriving in any niche that is metabolically active are contributing to the activities of the microbiome. These functions can be estimated and tracked through metatranscriptomics.

The identification and quantification of microbiome transcripts are now possible. Metatranscriptomics is an "omics" discipline enabling researchers to determine the profile and changes in gene expression occurring in a microbial community in response to environmental or experimental variations. Metatranscriptomics estimates the function and activity of complete sets of transcripts (RNA-seq) obtained from any sample. Therefore, the "metatranscriptome" (messenger and noncoding RNAs) offers a vision framework about the regulatory networks and gene expression occurring at the time of sampling. This is a non-genomics discipline but that can complement and strengthen the results obtained through metagenomics.

Relevant biological processes depending on small RNAs such as virulence, quorum sensing, and stress/immune responses can be accessed through this approach (Bejerano-Sagie and Xavier 2007). This approach allows understanding some of the biomolecular networks dictating emergent phenotypes in the microbiome and their roles in the agricultural systems. In addition, because of microorganisms and mechanisms involved in relevant functions including disease suppression in soil and microbiome-plant interaction are still poorly known, these kinds of disciplines with their respective techniques may unveil the role of genes responsible for these mechanisms (Kothari et al. 2017).

For example, Hayden et al. (2018) performed a comparative metatranscriptomic approach assessing the taxonomic and functional characteristics of the rhizosphere microbiome of wheat plants grown in adjacent fields previously known as suppressive and nonsuppressive to the pathogen *Rhizoctonia solani* AG8 (major pathogen of grain crops). The authors reported that suppressive samples showed greater expression of a polyketide cyclase, a terpenoid biosynthesis backbone gene, and several cold shock proteins, whereas nonsuppressive samples showed higher expression of antibiotic genes.

Furthermore, the combination of data obtained from metagenomics and metatranscriptomics can provide information about of the changes in gene expression that are accompanied by the changes in the microbiome structure, enabling to know what species are present in a given niche, their functional capabilities, and their real-time activities under particular scenarios.

8.5 Future Perspectives

Microbial communities play an important role for agriculture progress. Alterations of these types of ecosystems allow the entry of pathogens resulting in diseases that disrupt the industry development. In recent years, interest in understanding the activities of these microbial communities has increased. However, one of the main challenges in this area is discriminating and/or interpreting this massive amount of information. Bioinformatics tools have become indispensable in metagenomics research, allowing to know the structure and metabolic potential of environmental microbes. Improving the accuracy of the results is encouraged. One option is the single-molecule real-time sequencing (SMRT) developed by Pacific Biosciences (PacBio), which produces long readings and provide large scaffolds (Rhoads and

Au 2015). Despite having closely similar error rate to Illumina Miseq platform and Roche 454, it is expected to increase its efficiency and eventually displace small fragment sequencing by synthesis (Wagner et al. 2016).

A few metagenome studies by sequence-based metagenomics approaches have been performed for plants and associated microorganisms. Busby et al. (2017) mentioned the study of the interaction of plant-microbes in a global context could lead to find a natural mechanism facilitated by microorganisms controlling diseases by their ecological process. For example, pathogens may develop resistance mechanism that complicates their elimination in soil systems. Functional metagenomics has facilitated the detection of new antibiotic resistance mechanisms and find out the source of antibiotic resistance from total microbiome. Understanding these mechanisms will provide strategies in antibiotic improvement and alternatives in antibiotic resistance (Pehrsson et al. 2013). In addition, a total comprehension of microbial effects in natural alliance at culture systems will improve productions beyond genetic alteration (Lakshmanan et al. 2014).

Considering the beneficial role of microbial communities in soil environments, it is important to increase the understanding of microbial pathogens and provide biotechnological information for their control and management with sustainable practices.

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