

Current overview on the study of bacteria in the rhizosphere by modern molecular techniques: a mini–review

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Abstract

The rhizosphere (soil zone influenced by roots) is a complex environment that harbors diverse bacterial populations, which have an important role in biogeochemical cycling of organic matter and mineral nutrients. Nevertheless, our knowledge of the ecology and role of these bacteria in the rhizosphere is very limited, particularly regarding how indigenous bacteria are able to communicate, colonize root environments, and compete along the rhizosphere microsites. In recent decades, the development and improvement of molecular techniques have provided more accurate knowledge of bacteria in their natural environment, refining microbial ecology and generating new questions about the roles and functions of bacteria in the rhizosphere. Recently, advances in soil post–genomic techniques (metagenomics, metaproteomics and metatranscriptomics) are being applied to improve our understanding of the microbial communities at a higher resolution. Moreover, advantages and limitations of classical and post–genomic techniques must be considered when studying bacteria in the rhizosphere. This review provides an overview of the current knowledge on the study of bacterial community in the rhizosphere by using modern molecular techniques, describing the bias of classical molecular techniques, next generation sequencing platforms and post–genomics techniques.

Keywords: Microbial ecology, molecular techniques, rhizosphere, next–generation sequencing

1. Introduction

The rhizosphere, is defined as the soil zone under the influence of plant roots, a site of high microbial activity, characterized by a great array of complex and dynamic physical, chemical and biological interactions. In the rhizosphere, microorganisms have an important role in the organic matter transformations and biogeochemical cycles of plant nutrients. In this context, a substantial number of bacterial species interact with their host plants and may exert beneficial effects on plant growth, plant nutrition and disease suppression (Avis *et al.*, 2008, Pii *et al.*, 2015). Thus, numerous studies have been devoted to isolate and describe the activities of diverse plant growth-promoting rhizobacteria (PGPR). Among the main functions attributed to PGPR are: biological N₂ fixation, phytopathogen biocontrol, phosphate solubilization, production of phytohormones and enzymes, among others. Nowadays, PGPR represent an attractive alternative for chemical fertilizers as biofertilizers, phytostimulators, rhizoremediators and phytopathogen biocontrol agents (Lugtenberg and Kamilova, 2009). Despite the perception that PGPR are considered as not dangerous to the environment, their mass application in agriculture have been limited due to their low efficiency in field-level applications (Babalola, 2010). There is still a lack of information on PGPR ecology (diversity, competence, distribution, communication, etc.) in the rhizosphere (Compant *et al.*, 2005; Lambers *et al.*, 2009; Singh *et al.*, 2011); however, a considerable interest has been generated in their study and potential use in crop production around the world (Morrisey *et al.*, 2004).

Currently, it is recognized that the rhizosphere microbiome harbors thousands of different bacterial, archaeal, viruses, fungal and other eukaryotic taxa. A gram of rhizosphere soil might contain around 10⁹ microbial units and 10⁶ distinct taxa (Torsvik *et al.*, 2002; Curtis and Sloan, 2005). The use of molecular techniques have allowed the characterization of unculturable organisms at a level never seen before, since only 1% of soil microorganism have actually been cultured (Ver Berkmoes *et al.*, 2009; van Elsas and Boersma, 2011). Molecular techniques based on DNA analyses provide important tools to evaluate bacterial community composition, identifying the abundance of genes that could be involved in rhizosphere processes. With the advent of next generation sequencing technologies (such as Roche 454 and Illumina platforms), it has become possible to characterize the composition and activity of bacteria at much higher resolution than before. Metagenomics (Handelsman *et al.*, 1998; Myrol and Nannipieri, 2014) approaches can allow characterization of the unknown genomes of unculturable bacteria with the probability of finding novel microbial products (antibiotics, enzymes, anti-carcinogenics, etc.) (Nannipieri *et al.*, 2008). These high resolution analyses of the taxonomic composition of rhizosphere soil provide baseline information on the specific microbiome members living in rhizosphere environments. In addition, we can thereafter infer the community composition at specific root zones, and their functional relationships with plant growth, nutrient uptake, and plant health. Moreover, both RNA and proteins (proteomics) can provide some information on

gene activity, such as metatranscriptomics (total RNA from all bacterial communities of rhizosphere soils) inferring soil–microbial functionality (Bastida *et al.*, 2009).

This review provides an overview of the current knowledge on bacterial community in the rhizosphere by using modern molecular techniques, describing the bias of classical molecular techniques, next generation sequencing platforms and post–genomics techniques.

2. Ecology of bacteria in the rhizosphere

2.1. Abundance and diversity

The rhizosphere harbors a wide variety of bacteria species, and the compositions of bacterial communities differ according to root zone, plant species, plant phenological phase, stress and disease events (Rovira, 1965; Hinsinger *et al.*, 2009; Marschner *et al.*, 2011). Dominant bacterial phyla are members of the classes Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes and Acidobacteria in the rhizosphere of sugarcane, pea native hardwood forest and conifer plantations (Mendes *et al.*, 2011; Weinert *et al.*, 2011; Yang *et al.*, 2012). Among them, Proteobacteria are considered to be the most abundant bacterial group in the rhizosphere due to their ability to respond to labile C sources, showing fast growth and adaptation to the diverse plant rhizospheres (Bulgarelli *et al.*, 2013; Chaparro *et al.*, 2014; Peiffer *et al.*, 2013). Proteobacteria is followed by Acidobacteria, which have been attributed an important role in the C cycle in soils due to their ability to degrade cellulose and lignin (Ward *et al.* 2009). In relation to Actinobacteria, they have been associated with disease suppressive soils and their ability to increase root nodulation

and promote the growth of plants (Mendes *et al.*, 2011; Tokala *et al.*, 2002).

An important bacterial group in the rhizosphere is defined as PGPR, which are able to promote the growth, nutrient uptake and pathogen biocontrol in plants (Avis *et al.*, 2008; Mendes *et al.*, 2011). According to the current GenBank database from National Center for Biotechnology Information (NCBI), the most abundant groups of bacteria which potentially can promote plant growth are Actinobacteria, Firmicutes, Proteobacteria (Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria) and Bacteroidetes (Figure 1), and particularly the genera *Rhizobium*, *Azospirillum*, *Burkholderia*, *Pseudomonas* and *Enterobacter*. Other bacterial groups reported in the rhizosphere are Verrucomicrobia, Sphingobacteria, Flavobacteria, Deinococcus, Fusobacteria, and Epsilonproteobacteria. These bacterial groups are not commonly observed as dominant groups and/or PGPR in the rhizosphere, although this may be due to our limited knowledge of their taxonomy and functional gene sequences. It is also possible the presence of low abundance bacterial group with specific metabolic capabilities and/or adapted to the characteristic of specific plant species rhizosphere, which make that process of soil DNA extraction to be insufficient to be detected. In addition, studies have described the influence of agriculture practices and soil pH on bacterial community composition (Cheng *et al.*, 2010; Jorquera *et al.*, 2014; Pisa *et al.*, 2011; Malik *et al.*, 2013; Chen *et al.*, 2013; Nguyen *et al.*, 2013), as well as the occurrence of bacteria species having dependence on other rhizosphere microorganisms for their own growth (Hirsch *et al.*, 2010).

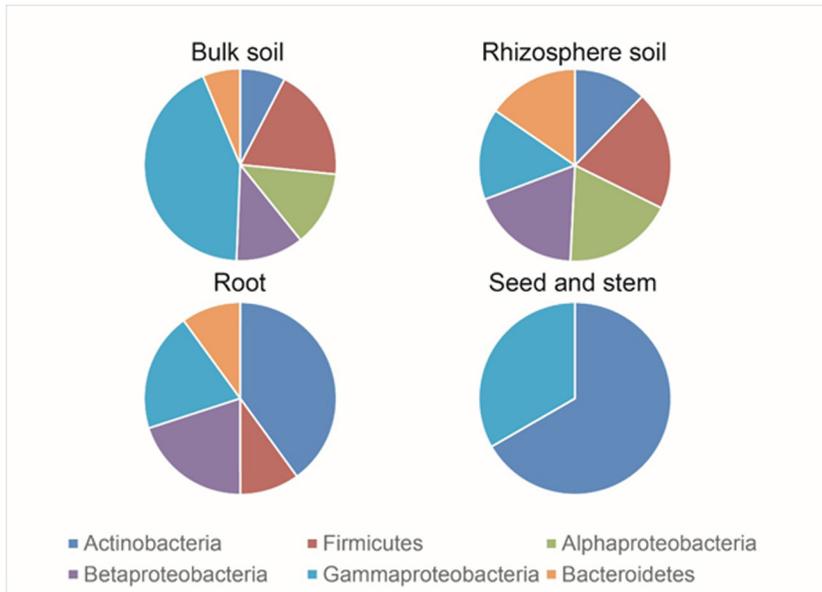


Figure 1. Taxonomic affiliation of 157 entries of plant growth-promoting rhizobacteria (PGPR) in GenBank database in relation to soils (bulk and rhizosphere) and plant tissues (root, seed and stem).

2.2. Colonization and competence

It is known that root exudates released by plants into the rhizosphere are used by bacteria as nutrient sources regulating their activities and growth. Root exudates include the releasing of ions, oxygen, water, and organic compounds, such as sugars, organic acids, amino acids, enzymes, growth factors and others (Marschner *et al.*, 2004; Bais *et al.*, 2004; Bertin *et al.*, 2003; Uren, 2000). Lugtenberg *et al.* (1999; 2001) demonstrated that sugars and organic acids are important for tomato root colonization by bacteria and fungi. In contrast, Karlsson *et al.* (2012) showed that archaeal abundance was decreased by root exudates, probably as result of their lower growth rates and competitiveness compared with bacteria and fungi. The colonization of rhi-

zosphere by bacteria also depend on their motility (Martínez-Granero *et al.*, 2006; Raaijmakers *et al.*, 2009; Capdevila *et al.*, 2004), availability of inorganic compounds (P and Fe) (Raaijmaker *et al.*, 1995; Yang and Crowley, 2000; Marschner *et al.*, 2004; 2011) and production of antibiotics (Raaijmaker *et al.*, 1995; Doornbos *et al.*, 2012). In this context, *Pseudomonas fluorescens* strain F113 has been used as model microorganisms for studying the root colonization, motility and competence of the indigenous microorganisms of the rhizosphere. Thus, Capdevila *et al.* (2004) revealed that the motility is essential for root colonization, and that non-motile mutant strains were displaced from the root tip of alfalfa when competed with the wild-type strain. Hyper-motile mutant strains showed a higher colonization in distal parts of the alfalfa root. Plants also can

influence microbial competition by root exudation. For example, white lupins (*Lupinus albus* L.) acidifies the rhizosphere by releasing protons from roots, and thus reducing bacteria growth, or they may excrete isoflavonoids, chitinase and glucanases against pathogenic fungi (Weisskopf *et al.*, 2006).

2.3. Distribution along the rhizosphere microsites

The abundances, diversities and activities of microorganisms differ along the rhizosphere microsites, depending on depletion/availability of nutrients caused by root exudation and competition between microorganisms. It has been described that greater numbers of bacteria occur around the root tip zone (where the highest sucrose or tryptophan exudation occurs), whereas the lower bacterial abundance occurs around the mature root zone (Jaeger *et al.*, 1999). Cellular death with subsequent cell lysis also releases debris which can be used as energy sources for microorganism to survive in the rhizosphere (Watt *et al.*, 2006). Bacterial compositions also depend on soil type, plant species, plant growth seasons and climate conditions. Models of plant–bacteria interactions in different root zones have been proposed (Jaeger *et al.*, 1999; Marschner *et al.*, 2011; Yang and Crowley, 2000). These models support that root exudates are released in a differentiated manner along the root, resulting in a heterogeneous distribution of bacterial communities along root zones (Figure 2). In root tips and the proximal elongation root zone, the exudation rates are high and colonizers use easily metabolizable sugars and organic acids. In contrast, exudation rates and microbial densities are lower in root hair zones. In the mature root

zones, the bacterial growth is lower due to low metabolizable C input (Marschner *et al.*, 2011). In addition, we also may suppose that some bacterial populations are unique and have adapted to the rhizosphere by using specific compounds secreted from roots, and that the plant itself interferes with the associated bacterial community independent from soil type (deCampos *et al.*, 2013).

2.4. Cell–to–cell communication

The cell–to–cell communication between bacteria in the environment is relevant due to its role in regulating vital functions, biofilm formation, virulence, symbiosis, extracellular enzyme production, antibiotic production, DNA transfer, etc. (Elasri *et al.*, 2001; Raina *et al.*, 2009; DeAngelis *et al.*, 2007). The modes of signal transduction in prokaryotes linking environmental signals to cellular responses are mainly attributed to one–component and two–component systems (Ulrich *et al.*, 2005). In general, soil bacteria can sense the presence of complex molecular exudates through a membrane–bound sensor histidine protein kinase (Fauren *et al.*, 2009). For example, in many Gram–negative bacteria, the beneficial or pathogenic interactions with their host plant are influenced by a GacS sensor kinase and GacA transcription factor (GacS/GacA). This two–component system recognizes environmental signals involved in pathogenicity to plants (Heeb and Haas, 2001). On the other hand, a one–component system implicated in communication of the plant is NodD proteins in *Sinorhizobium meliloti* that perceives flavonoids and activates expression of nodulation (nod) genes, which are required for symbiotic development in alfalfa (Peck *et al.*, 2006).

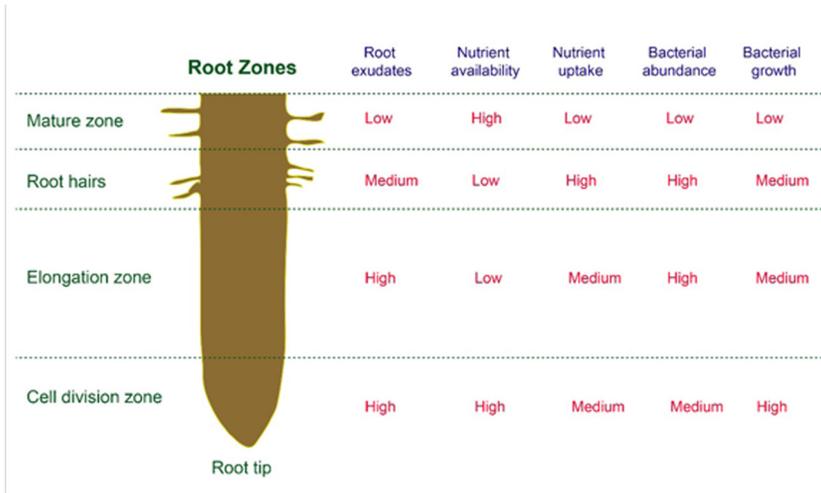


Figure 2. Plant and bacteria properties in different root zones according to models proposed (Jaeger *et al.*, 1999; Marschner *et al.*, 2011; Yang and Crowley, 2000)

The cell-to-cell communication in bacteria may involve quorum sensing (QS), where acyl-homoserine lactone (AHL) and compounds N-acylated derivatives of L-homoserine lactone (acyl-HSL) are the main signaling molecules produced by Gram-negative bacteria, and are released to the environment in order to reflect their population density. Cha *et al.* (1998) showed that QS systems mediated by AHLs were 10-fold higher in the rhizosphere than bulk soil, explained by high abundance of bacterial populations in the rhizosphere. Another study revealed that *Burkholderia graminis* strains M12 and M14, plant growth-promoting rhizobacteria, can produce AHLs that induce protection against salt stress in tomato plants (Barriuso *et al.*, 2008). In Gram-positive bacteria, cell-to-cell communication is controlled by a two-component signal-transduction mechanisms (histidine kinase), releasing oligopeptides in response to changes in cell density (Raina *et al.*, 2009).

Finally, it was postulated that our knowledge of cell-to-cell communication in bacteria could allow us to decipher how stable communities of PGPR can be promoted in the plant rhizosphere (Hirsch *et al.*, 2003), involving further investigations focused on identification of AHLs produced by PGPR (Barriuso *et al.*, 2008) and used as biosensors to understand how these molecules are involved in the diverse rhizosphere processes.

3. Classical molecular techniques in the study of bacteria in the rhizosphere

During the last few decades, a wide variety of molecular techniques have been developed and used as valuable tools for the study of diversity and function of bacteria in the rhizosphere (Hill *et al.*, 2000). However, the biases of each molecular technique must be considered and evaluated during their application in the study of bacteria in the rhizosphere.

3.1. Fingerprinting techniques

PCR-based fingerprinting techniques, such as terminal restriction fragment length polymorphism (T-RFLP), denaturing gradient gel electrophoresis (DGGE) and single strand conformation polymorphism (SSCP), have been widely used in the study of bacterial communities in the rhizosphere (Berlec, 2012; Ascher *et al.*, 2011). However, they determine only dominant bacterial populations within the communities, and may consider both active and inactive bacterial populations (van Elsas and Boersma, 2011). Smalla *et al.* (2007) compared bacterial diversities from several soils and found that T-RFLP was less efficient in assessing bacterial diversity in soil than DGGE, but T-RFLP yielded more reproducible results for comparing large numbers of samples. In general, fingerprinting techniques rely on the amplification of 16S rRNA gene fragments by PCR, but 16S rRNA gene copy number per genome vary from 1 up to 15 or more copies depending on the bacterial species. The numbers of rRNA gene copies are related to the life strategy of bacteria; taxa with low copy numbers and inhabit low nutrient environment (oligotrophic) (Větrovský and Baldrian, 2013; Kang *et al.*, 2010).

3.2. Quantitative PCR (qPCR) and gene expression

The qPCR is a molecular technique widely used for detection and quantification of specific genes and their expression from DNA and RNA samples from various environments (Deepak *et al.*, 2007). It is a sensitive technique allowing detection of the signal produced by DNA fragment amplification in real time during each cycle of the PCR reaction. The limitations of real-time

PCR are related to primer specificity, amplification efficiency, and the concentration of RNA in rhizosphere sample, which may be too low for accurate detection (Marschner *et al.*, 2011). However, this technique can provide important insight into the distribution of specific bacterial genes in the rhizosphere and bulk soils (van Elsas and Boersma, 2011, Sørensen *et al.*, 2009), because it is not only used to detect and quantify 16S rRNA genes but also functional genes involved in relevant processes in the rhizosphere, such as nutrient cycling and phytopathogen bio-control.

The study of gene expression in the rhizosphere soil is still limited (Nannipieri *et al.*, 2008). The majority of previous studies focused on gene expression of *Pseudomonas* species used as a model to study plant-bacteria interaction in the rhizosphere. Barret *et al.* (2011) developed a list of 13 bacterial genes of *Pseudomonas* spp. induced in the rhizosphere in response to root exudates. These genes have functions in metabolism, motility, signal transduction, and unknown functions. Few studies have evaluated the gene expression in the rhizosphere soil. Some examples are: hydrogen cyanide (HCN) induces gene expression in the rhizosphere strawberry of a plant pathogen *Verticillium dahlia* (Decoste *et al.*, 2010), gene expression of *Metarhizium anisopliae* that are induced by bean root exudates (Pava-Ripoll *et al.*, 2011), expression of *nifH* genes by diazotrophic bacteria in the *Spartina alterniflora* rhizosphere (Brown *et al.*, 2003), *beta-propeller* phytase gene expression in *Bacillus* sp. strain MQH15 in ryegrass rhizosphere (Jorquera *et al.*, 2013), and quantification and characterization of genotypes of *Pseudomonas fluorescens* in rhizosphere soil based on 2,4-diacetylphloroglucinol (2,4-DAPG) genes (Mavrodi *et al.*, 2007).

3.3. Microarray and transcriptome

The analysis of transcriptome profiles of rhizosphere strains by microarrays can give information on gene expression involved in the synthesis of several signals to control the bacterial activity in the rhizosphere (Wu *et al.*, 2011). Tremblay and Déziel (2010) analyzed transcript profiles related to motility in *Pseudomonas aeruginosa*, a ubiquitous Gram-negative bacterium, by GeneChip® microarray. The results showed that swarming colonies displayed general down-regulation of genes associated with virulence and up-regulation of genes involved in energy metabolism. Van Puyvelde *et al.* (2011) studied the transcriptome of *Azospirillum brasilense* demonstrating that auxin indole-3-acetic acid is a signal molecule affecting its arsenal of transport proteins and cell surface proteins. However, microarray analysis relies on known genes from bacterial species; however, unknown genes are not detected. Therefore, this technique cannot provide information on the distribution and activities of unknown bacterial genes in the environment (van Elsas and Boersma, 2011).

In this context, Shidore *et al.* (2012) analyzed the transcript profiles of *Azoarcus* sp. strain BH72, an endohpytic strain, exposed to root exudates released by *Oryza sativa*. The microarray analysis showed 2.4% and 2.0% of genes up-regulated and down-regulated, respectively; however, modulated gene expression included a few whose involvement in plant-microorganisms interaction had already been established, whereas a large fraction comprised of genes encoding proteins with putative or unknown functions.

3.4. Biosensors

Biosensors are defined as bacterial cells harboring a reporter gene, which is usually a fluorescence marker such as a green fluorescent protein (GFP) cassette expression (Sørensen *et al.*, 2009). This system allows detection of activity and colonization of bacteria at the single cell level in rhizosphere microsites by epifluorescent and confocal microscopy. Germaine *et al.* (2004) and Götz *et al.* (2006) successfully introduced GFP-tagged plasmids to localize and monitor the rhizosphere colonization of *Pseudomonas putida* strain PRD16, *Enterobacter cowanii* strain PRF116 and endophytic bacterial strains. Recently, Weyens *et al.* (2012) investigated the colonization and its capacity to promote plant growth by endophytic *P. putida* strain W619 with insertion of GFP-labelled, concluding that it did not promote growth. However, a limited number of reporter genes are available, high background fluorescence can limit detection, and the performance of biosensors can be variable depending on manipulation and sample preparation (Marschner *et al.*, 2011).

3.5. Proteomics

Proteomics, defined as the entire protein complement expressed by a genome or by a cell, can be used for evaluating expression and localization of proteins, as well as for analysis of post-translational modifications (Wilkins *et al.*, 1995). However, the extraction of intracellular proteins from soil is a methodological challenge, due to the stability of proteins (protected against proteolysis) and that they may be strongly adsorbed onto soil minerals or co-purify with humic acids

or soil colloids that will interfere with analysis (Nannipieri, 2006, Arenella *et al.*, 2014). According to Nannipieri (2006), intracellular protein–N that is the product of microbial activity accounts for an average of only 4% of the soil total N, whereas a value ranging from 30 to 50% is due to protein–N stabilized by surface–reactive particles. Therefore, the characterization of intracellular proteins should give insight into microbial functioning of the rhizosphere soil at the sampling time, whereas the characterization of the extracellular stabilized proteins should give indication on past microbial events. Keiblinger *et al.* (2012) compared four proteins extraction methods in two soils, the method with sodium dodecyl sulfate–phenol gave the highest yield. Recent studies have indicated that proteins associated with metabolic functions such as carbohydrate catabolism and energy production, lipid and amino acids biosynthesis, signal transduction, and membrane transport are the most abundant in rhizospheres and agricultural, potting, and forest soils (Damon *et al.*, 2012; Wu *et al.*, 2011; Lin *et al.*, 2013). Usually, bacterial proteomics analyses are conducted by 2–dimensional polyacrylamide gel electrophoresis (2D–PAGE), 2D–difference gel electrophoresis (DIGE), and mass spectrometry (MS) for investigating expression gene detection involved between plant–pathogen, nitrogen–fixing bacteria in legumes and endosymbiotic interactions (Bestel–Corre *et al.*, 2004; Cheng *et al.*, 2010; VerBerkmoes *et al.*, 2009). Another technique for the study of the ecology in rhizosphere bacteria is SIP (stable isotope probing)–protein technique with ^{13}C or ^{15}N that can be used for identifying bacterial populations involved in metabolic or biogeochemical processes in the bulk and rhizosphere soils (Bastida *et al.*, 2009).

4. Post–genomic techniques in the study of bacteria in the rhizosphere

Currently, it is assumed that the development and improvement of techniques such as metagenomics, metaproteomics, and metatranscriptomics, will provide more accurate evaluation of the activities and compositions of microbial communities in rhizospheres than classical molecular techniques, generating new questions about the roles and functions of these microbial communities (Hirsch *et al.*, 2010; Sørensen *et al.*, 2009).

4.1. Metagenomics

In order to study and evaluate the richness, distribution and activity of microbial communities in bulk and rhizosphere soils, it is important to understand the ecological functions of each species. Modern molecular techniques have shown that bacterial diversity of bulk and rhizosphere soil is much greater than was predicted. Soil metagenome study of 16S rRNA gene and ITS1 region using next generation sequencing or second–generation sequencing technologies (Niedringhaus *et al.*, 2011) have revealed that 1 g soil sample may contain 33,346 bacterial and archaeal OTUs (Mendes *et al.*, 2011), 3,320 fungal OTUs (Schmidt *et al.*, 2013), 145 to 200 of fungal OTUs (Xu *et al.*, 2012), and 300 archaea OTUs (Pires *et al.*, 2012). Both Roche 454 and Illumina platforms have been used to address the bulk and rhizosphere soils (Table 1). Roche 454 pyrosequencing platform produces long read length (<450 bp) and high consensus accuracy more than Illumina platform (Unno, 2014). Uroz *et al.* (2010) by 454 pyrosequencing compared the bacterial diversity of oak rhizosphere and bulk soil, finding that Proteobacteria, Acido-

bacteria, Actinobacteria, Bacteroidetes were the dominant taxa. Recently, Lagos *et al.* (2014) characterized the composition of bacterial communities of rhizosphere microsites (root tips and mature root zones) of *Lolium perenne* and found that Proteobacteria, Actinobacteria and Acido-

bacteria were the dominant phyla. Similarly, Sun *et al.* (2014) characterized the diversity of bacterial communities of apple rhizosphere by Illumina sequencer and found that Proteobacteria, Acidobacteria, Bacteroidetes, Gematimonadates and Actinobacteria were the dominant groups.

Table 1. A summary of next generation sequencing platforms used in bacterial ecology studies

Company/ Platforms	Read length (bp)	Run time	Sequencing principle	Library amplification	Signal detection	System overview
<i>Roche</i>						
454 GS FLX Titanium XL+	~1000	23 h	Pyrosequencing by synthesis	Emulsion PCR on microbeads	Optical detection of light from pyrophosphate release on nucleotide incorporation	Single gene; genome and transcriptome sequencing
454 GS FLX Titanium	~600	10 h				
454 GS Junior	~400	10 h				
454 GS Junior+	~700	18 h				
<i>Illumina</i>						
Miseq	~300	~5–55 h	Reversible terminator sequencing by synthesis	Bridge PCR on flow cell surface	Optical detection of fluorescent emission from nucleotide incorporation	Single gene; genome sequencing
Hiseq 2500	~125–250	~7–60 h				
Hiseq 3000	~150	1–3 days				
Hiseq 4000	~150	1–3 days				
<i>Pacific Bioscience</i>						
PacBio RS SMRT	20,000	0.5–4 h	Single molecule real time sequencing by synthesis	Not required	Optical detection of fluorescent emission from labelled nucleotide incorporation	Single gene; genome and transcriptome sequencing

4.2. Metaproteomics

Metaproteomics provides a direct measure of proteins present in an environmental sample such as soil, offering information about the functional roles of soil microorganism, such as biogeochemical processes, degradation, or bioremediation processes (Bastida *et al.*, 2012,

Chourey *et al.*, 2010). VerBerkmoes *et al.* (2009) proposed proteogenomics as an important tool for studying the physiology, ecology and evolution of microbial populations, communities and consortia in several environments as a means to connect a microbial species with its function. This approach is important since it combines metagenomics and proteomics and provides for

the verification of metagenomics studies by protein data. However, it is necessary to consider that the databases for soil protein identification are still incomplete. Nevertheless, numerous metaproteomics studies have revealed the diversity of proteins that are expressed by the interactions between plants and soil microbial communities. Recently, Lin *et al.* (2013) compared a metaproteomics profile of rhizosphere of ratoon sugarcane and plant sugarcane. The results revealed that ratoon sugarcane induced significant changes in the soil enzyme activities, the catabolic diversity of microbial community, and the expression level of soil proteins originated from the plants, microbes and fauna. This study also reported that 24.77% of soil proteins are derived from bacteria and most of the up-regulated expression of microbial proteins were involved to membrane transport and signal transduction. Similarly, Wu *et al.* (2011) realized a comparative metaproteomics analysis for determining the changes on soil protein abundance under consecutive monoculture of herb *Rehmannia glutinosa*. The results shown the identification proteins of plants, bacteria and fungi mainly involved in the carbohydrate and energy metabolism, aminoacids metabolism, stress/defense response. However, compared with Lin *et al.* (2013) study, lower percentages (11.65%) of proteins derived from bacteria were found, mainly involved in signal transduction. Moreover, Moretti *et al.* (2012) demonstrated that the rhizosphere of lettuce (*Lactuca sativa*) produced higher amount of proteins related with virulence determinants, energy metabolism, stress/defense response in presence of pathogenic strain of *Fusarium oxysporum* which could be related with the interaction the microbial consortium associated to this fungus.

4.3. Metatranscriptomics

Metatranscriptomics involves the characterization of a set of messenger RNA (mRNA) (transcripts) produced in all cells, which would provide insight into the metabolic processes of a microbial community (Simon and Daniel, 2011; de Menezes *et al.*, 2012). Consequently, metatranscriptomics analysis has the potential to discover novel genes and functions, allowing identification of active community members in both bulk and rhizosphere soils, and to correlate them with their metabolic activities (Kim *et al.*, 2014). However, metatranscriptomic approaches have not been widely used in the rhizosphere, probably due to the instability of mRNAs and difficulties in their extraction from complex ecosystems. Among the more notable methodological challenges are their short half-lives, difficulties in the separation of mRNA from other RNA types (i.e. tRNA, rRNA, miRNA) and interference from humic compounds that co-extract with nucleic acids from soil (Simon and Daniel, 2011). In addition, few studies have applied metagenomics, metaproteomics and metatranscriptomics due also to the difficulties and expense related to simultaneous extraction of nucleic acids and proteins from soil samples (Table 2). Moreover, the majority of studies in bulk and rhizosphere soils are mainly focused on bacteria, demonstrating the need for further studies to obtain further insights into the molecular ecology of other microorganisms present in rhizosphere microbiome, such as fungi, archaea, microalgae, protozoa, etc.

In order to better understand microbial genomes and characterize bacterial phylogeny, the Genomic Encyclopedia of Bacteria and Archaea Project (GEBA Project) was initiated in 2007.

This project is lead by the US Department of Energy (USDOE) Joint Genome Institute, Institute of California Davis, USA, and German Collection of Microorganisms and Cell Cultures, Germany (DSMZ) (Wu *et al.*, 2009). Currently, 200 bacterial genomes have been sequenced. The principal objectives of this project are to improve identification of proteins families, and to identify novel genes and undescribed organisms (<http://goo.gl/FNpdwv> revised in January, 2015). GEBA Project have other cooperative projects, such as GEBA-type strain, GEBA-RNB (Root Nodulating Bacteria), and GEBA-MDM (Microbial Dark Matter). The main objective of GEBA-type strain project is to find novel functions of protein families, and thus, discovery of natural products. On the other hand, GEBA-RNB project is based in sequencing 100 symbiotic bacterial strains isolated from different soils around the world. This project can improve our understanding of the endosymbiotic relations between bacteria and roots,

and nitrogen fixation. The GEBA-MDM project objective is to use single-cell genomics to explore uncultured bacteria and archaea that GEBA project does not include. Hence, the discovery of novel genomes by this project will improve phylogeny and the evolution of bacterial and archaeal domains (Rinke *et al.*, 2013).

Finally, the Pacific Biosciences single-molecule real-time (PacBio RS SMRT) sequencer is a third-generation sequencing technology (Niedringhaus *et al.*, 2011). It is noteworthy that the PacBio RS SMRT has been applied to genome sequencing of *Streptomyces* sp. strain Mg1, a soil bacterium can cause lysis and degradation of *Bacillus subtilis* (Hoefler *et al.*, 2013). Moreover, it may be a suitable platform for longer sequencing reads of 16S rRNA genes from environmental samples; according to Mosher *et al.* (2013), this approach could provide more information about microbial taxonomy and the phylogeny of microbe inhabitants of the rhizosphere.

Table 2. Applications and limitations of post-genomic techniques currently used in soil Microbiology

Technique	Target	Application	Limitations	References
<i>Metagenomics</i>	DNA	Biodiversity and gene quantification	<ul style="list-style-type: none"> – DNA is extracted from all cells (active and non-actives) – Limited to the dominant members of the microbial community of soils 	Bastida <i>et al.</i> , 2009; Simon and Daniel, 2011; van Elsas and Boersma, 2011
<i>Metatranscriptomics</i>	RNA	Biodiversity and gene expression	<ul style="list-style-type: none"> – Liability of RNA – Interference by soil humic compounds 	Simon and Daniel, 2011; Bastida <i>et al.</i> , 2009
<i>Metaproteomics</i>	PROTEINS	Gene functions, activities and metabolic functions	<ul style="list-style-type: none"> – Intracellular proteins–N only 4% of total N – Prevalence of extracellular stabilized proteins–N not involved in the microbial activity at sampling but expression of past event – Strongly adsorbed onto soil minerals or humic colloids 	Simon and Daniel, 2011; Bastida <i>et al.</i> , 2009; Nannipieri, 2006

5. Conclusions and perspectives

Rhizospheres are complex and dynamic habitats characterized by high microbial activities. The diversities in different zones in the rhizosphere can be influenced by plant–bacteria and bacteria–bacteria interactions regulated by communication, competition along root zones, plant growth stage, and plant species. The advances in soil molecular and post–genomic techniques will continue to improve our understanding of the compositions and activities of soil microbial communities, to target the rare or low abundance bacterial populations in the rhizosphere, and to predict *in situ* responses, activities, and growth of bacterial communities. Until very recently, few studies of soil and rhizosphere soils have been performed using metagenomics, metaproteomics and metatranscriptomics; these approaches may be limited by difficulties of extractions and purification of nucleic acids and proteins from complex environmental samples. Advances in nucleotide and protein sequencing techniques might allow lower costs, making them more accessible, expanding libraries DNA or database to continue uncovering more genomes and functions of microbiota of bulk and rhizosphere soils. Being an interesting approach for improving strategies to select indigenous potentially beneficial bacterial strains that may function as biofertilizers or bioprotectors, to understand the importance of the rhizosphere microbiome in promoting plant health and, to study the mechanisms of plant impacts (exudates) on the rhizosphere soil microbiome (Bakker *et al.*, 2013). Therefore, collaborative efforts are needed for future biotechnological developments allowing the establishment of adequate and efficient strate-

gies of rhizosphere management, benefitting the yield and sustainability of agricultural systems.

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