Chapter 9
Recent Advances in Functional Genomics and Proteomics of Plant Associated Microbes

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9.1 Introduction

Research in soil microbiology has concerned the determination of the presence of gene sequences so as to assess microbial diversity rather than the determination of gene expression. Generally these molecular techniques are based on the specific amplification of the target nucleic acid by polymerase chain reaction (PCR) with either restriction analysis or separation by denaturing or conformational properties of the resulting amplicons (Lynch et al. 2004). On the other hand microbial activities in soil have been measured by classical techniques such as those for determining soil respiration, enzyme activities, N mineralization, adenylate energy charge, leucine and thymidine incorporation, etc., with no idea of gene expression. The rhizosphere effects on microbial diversity and activity are discussed in Chap. 14 of this book.

Rapid progress in genomics has led to the availability of full genome sequences of hundreds of microorganisms, mostly bacteria (DeLong 2002). Combinations of new molecular methodology and genomics have been used successfully to link microbial phylogeny with function in several ecological studies and the same approach could provide significant insights into plant–microbe interactions in the rhizosphere. The functional genomics is based on a holistic or systemic approach with studying information flow within a cell and this requires the application of high throughput methods using automated technologies, which allow functional analysis of genome, proteome and metabolome of an organism (Wren 2000). In this way it is possible to get an insight on interplay of a large number of gene products and the relative consequences of this communication to the physiology of a cell. In contrast, molecular biologists have followed the reductionistic approach by studying single genes, and the individual actions of genes with a step-by-step characterization of metabolic pathways. Thus, an efficient DNA extraction with

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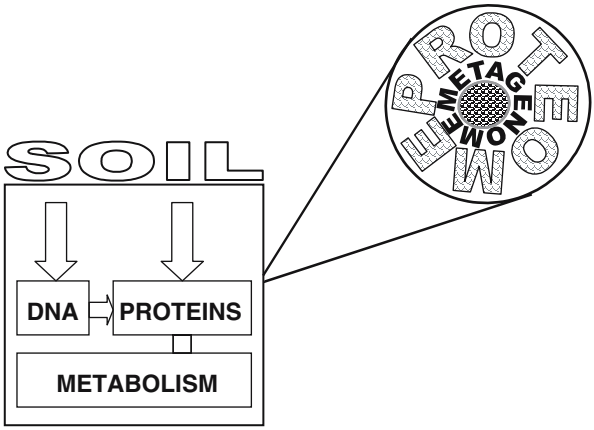

Fig. 9.1 Holistic view of genomics and proteomics of rhizosphere

a high quality DNA sequence is followed by the analysis of the synthesised proteins.

It is possible to apply the functional genomics to the rhizosphere if we assume that rhizosphere soil can be seen as an organism and the genome of this organism includes genomes of all organisms inhabiting the rhizosphere soil (metagenome), the proteome includes all proteins associated with these organisms and the metabolism includes all metabolic reactions of these organisms (Fig. 9.1). However, this holistic view must consider that:

1. Both abiotic reactions and reactions catalysed by extracellular enzymes adsorbed by soil colloids can occur in soil and they are not under the control of any gene (Nannipieri et al. 2003).
2. Several methodological problems exist due to the complexity of soil. For example, as will be discussed later, only recently techniques for determining mRNA has been set up for soil due to problems in extracting and manipulating mRNA molecules, which are less stable than DNA molecules (Nannipieri et al. 2003). Methodological problems also exist in analysing the final products of gene expression, the proteins (proteomic approach), in soil (Nannipieri 2006; Ogunseitan 2006).

The aim of this chapter is to discuss the different approaches and relative techniques for studying the functional genomics of the rhizosphere soil. Gene expression at both transcription and translational levels will be discussed. Since the matter is relatively new and the relative bibliography is scarce, the discussion will mainly concern the potential applications by underlying the potential advantages and drawbacks of the various methods. Studies of gene expression in bulk soil will be also reviewed by considering both advantages and disadvantages of the employed assays and discussing the meaning of the relative results with the aim to evaluate the potential insights that can be got by carrying out these studies in the rhizosphere soil.

9.2 Plant and Genetic Diversity in the Rhizosphere Soil

Plants can influence the genetic diversity of microorganisms inhabiting the rhizosphere; for example, plants select for a higher diversity of ammonia-oxidizing and nitrogen-fixing bacteria in the rhizosphere, when compared with bulk soil (Briones et al. 2003; Cocking 2003); on the other hand soil microorganisms may affect plant diversity through providing competing advantages to specific plants (Bever 2003). It is not known if the higher diversity of functional genes depends on a higher diversity and number of microorganisms in the root regions as a consequence of the accessibility of C source or the results of a natural selection, whereby plant supplies C and in turn the microbes provide N, P, other nutrients and protection against pathogens and herbivores. Obviously these hypotheses cannot be verified by research only based on fingerprinting techniques but it is important to study the utilization of plant C by microbial species with a particular functional role by using advanced techniques, which allow correlating a specific metabolic activity with phylogenetically identifiable units in natural environments (Gray and Head 2001; Wagner 2004).

Bacteria frequently exchange genetic material and the acquired DNA can be integrated in the recipient cell genome by recombination catalyzed by bacterial integrases, and the selective advantages acquired by the new genetic material may be maintained or lost depending on the characteristics of the environment. Particular genes required for effective root colonization by bacteria can be subjected to phase variation, a DNA rearrangement process, regulated by site-specific recombinase (Dekkers et al. 1998a). A rhizosphere-incompetent *Pseudomonas* strain was capable of colonising root tips after transfer of the site-specific recombinase from a rhizosphere-competent *Pseudomonas fluorescens* (Dekkers et al. 1998b). The presence of plasmids with novel gene clusters containing open reading frames (ORFs) encoding small proteins are advantageous for *Pseudomonas* spp. to colonise roots during particular periods of plant growth (Liley and Bailey 1997). The encoded proteins are assumed to play a role in the cell-to-cell contact mechanism leading to horizontal gene transfer in the natural habitat.

Studies on genetic traits of *P. fluorescens* and *P. putida* responsible for successful colonization of roots include those encoding molecules involved in iron uptake, NADH dehydrogenase, extracellular proteins, surface proteins, lipopolysaccharides, and constituents of flagella (Espinosa-Urgel 2004). Some of these factors and the cell-cell communication through quorum sensing are responsible for the formation of biofilms, which seems essential for the root colonization.

Horizontal gene transfer in nature can occur between same or different species which are similar taxonomically (Veal et al. 1992; Nielsen et al. 1998; Jain et al. 1999) and it is not limited to bacteria but may concern fungi (group I introns located in mitochondrial genome and nuclear ribosomal RNA genes), which can be found in different plants (Cho et al. 1998). Plant surfaces, including rhizoplane, are considered hot spot for conjugation probably because the close proximity of bacterial cells in biofilms facilitates the exchange of genetic material (van Elsas et al. 2003; Espinosa-Urgel 2004). Transfer by conjugation does not seem related to the micro-

bial activity, determined by incorporation of ^{3}H-leucine into cells, since, in spite of the fact that this activity was similar in the rhizosphere of barley, wheat and pea, the conjugal transfer of plasmid between *P. fluorescens* and *S. plymuthica* was the highest in the pea rhizosphere (Schwaner and Kroer 2001).

Of the three horizontal gene processes (conjugation, transduction and transformation) occurring in bacteria, transformation is the only one involving extracellular DNA. Under natural soil conditions, transformation is likely to be due to illegitimate recombination (Mercier et al. 2006). It has been calculated that natural transformation of bacteria in soil is an extremely rare event and no specific reports on the frequency of transformation in the rhizosphere have been reported; thus there is no conclusive evidence of enhanced competence or more availability of extracellular DNA in the rhizosphere than in the bulk soil (Mercier et al. 2006). It is not clear whether under peculiar rhizosphere conditions, such as reduced pH and Eh, enhanced microbial activity, the extracellular DNA may be more or less available to soil microorganisms. The extracellular DNA is thought to be rapidly degraded in soil environment (Blum et al. 1997) unless it is stabilized through adsorption by soil colloids (Paget et al. 1992); DNA adsorbed by soil colloids can persist for a long time (Nielsen et al. 1997; Gebhard and Smalla 1999), maintaining its transforming capacity (Pietramellara et al. 1997; Nielsen et al. 2000; Ceccherini et al. 2003).

9.3 Gene Expression in the Rhizosphere Soil

9.3.1 Reporter Gene

Both rDNA and rRNA techniques do not allow determining the phenotypic expression in the rhizosphere soil of the specific genes and this shortcoming can be overcome by a reporter gene, expressed from a constitutive or inducible promoter resulting in a detectable phenotype. It is important to select a representative soil microorganism or a specific microorganism for the specific purpose. There are several available reporter genes such as those encoding substances responsible for the production of bioluminescence (*lux*), green fluorescent protein (*gfp*), β-galactosidase (*lac*Z) and ice nucleation promoting proteins (*ina*Z) (Sørensen and Nybroe 2006; Burmølle et al. 2006). The proper selection depends on: i) the type of detection used to quantify the reporter gene expression; ii) the sensitivity of the used reporter; iii) the type of sensing promoter (Saleh-Lakha et al. 2005).

Whole-cell bacterial biosensors have been used for monitoring the availability of C, N and P in the rhizosphere soil. This information can be obtained using *lux*-marked *Pseudomonas fluorescens* strains in which bioluminescence is regulated by the cellular levels of $FMNH_2$, in the presence of sufficient aldehyde and oxygen (Amin-Hanjani et al. 1993; Kragelund et al. 1995, 1997; Yeomans et al. 1999). Such strains are bioluminescent under starving conditions whereas assimilation of nutrients reduces the bioluminescence (Yeomans et al. 1999;

Roca and Olson 2001). Using the whole cell biosensor *Pseudomonas fluorescens* 10586 pUCD607 responding to a range of compounds present in the root exudate, Darwent et al. (2003) reported that a reduced supply of NO_3^- to winter barley induced a greater root exudation. DeAngelis et al. (2005) found a significantly lower NO_3^- availability in the rhizosphere of wild oat (*Avena fatua*) than in bulk soil; in addition, competition for NO_3^- between roots and the whole-cell bioreporters could be removed by soil amendment with NO_3^-; they used two bacterial biosensors set up by fusion of promoterless ice nucleation *ina*Z from *P. syringae* and *gfp* with the nitrate-regulated promoter of *nar*G in *E. cloacae* EcCT501R.

A simultaneous measurement of C, N and P in the rhizosphere soil solution has been conducted by Standing et al. (2003) by using a tripartite reporter gene system. However, responses of bacterial biosensors to N and P limitations in rhizosphere may depend on both the concentrations and forms of nutrients; for example, the *Pseudomonas* N-reporter strains reacted towards limitation by both NH_4^+ and common amino acids (e.g. glutamate). However, it is important to know the reaction of the reporter to other N-containing compounds of root exudates. A partial answer has been obtained by using reporter bacteria responding specifically to individual amino acids. The induction of a lysine-responsive *P. putida* reporter was demonstrated in rhizosphere of corn, but not in the bulk soil (Espinosa-Urgel and Ramos 2001) and a tryptophan-reporter strain showed significant induction in older root segments of Avena grass, but not at the root tip (Jaeger et al. 1999). Kuiper et al. (2001) demonstrated that uptake regulation of putrescine, commonly released in root exudates of tomato, was important for growth and root colonization of rhizobacteria using a *P. fluorescens* reporter strain. By using a whole cell biosensor constructed in *Pseudomonas* spp. by fusing *lux*-reporter genes to the promoters of operons active in the synthesis of ribosomal RNA, Marschner and Crowley (1996) demonstrated that rDNA genes were highly expressed and thus growth of *P. fluorescens* was higher in the rhizosphere of pepper than in the bulk soil. Ramos et al. (2000), using a ribosomal promoter inserted with a *gfp*-reporter encoding for an unstable GFP variant, demonstrated that the growth of a *P. putida* strain was greater at the root tip than in mature or senescent root areas.

The iron stress response of pseudomonads has been monitored by using an iron-regulated, ice nucleation gene reporter (*ina*Z) (Loper and Henkels 1997; Marschner and Crowley 1997, 1998). It has been shown that iron stress was greater in the zone behind root tips than in older lupine (*Lupinus albus*) and barley (*Hordeum vulgare* L.) root zones and bulk soil (Loper and Henkels 1997). In addition, the iron stress response of *P. putida* Pf-5 could be shut down by the production of phytosiderophores after induction of the iron stress response in plant roots of rice and barley (Marschner and Crowley 1998). Ice nucleation activity expressed by rhizosphere populations of *Pseudomonas fluorescens* Pf-5 decreased from one to two days after the bacterium was inoculated onto root surfaces, suggesting that iron became more available to rhizosphere populations of Pf-5 once they were established in the rhizosphere.

Whole cell (usually enteric bacteria such as *E. coli* and *S. typhimurium*) biosensors, whose response is controlled by the expression of global regulatory genes (regulons), have been also used to monitor microbial activity as affected by fluctuations of temperature, osmolarity, moisture, radicals concentrations, etc. in the rhizosphere soil due to plant activity (van Dyk et al. 1995; Ptitsyn et al. 1997; Vollmer et al. 1997; Bechor et al. 2002). Other whole cell biosensors have been used to detect antibiotics (Bahl et al. 2004), endocrine disruptors (Desbrow et al. 1998) and quorum sensing molecules (Andersen et al. 2001). Using a *Rhizobium* strain carrying a *nod*C-*lac*Z fusion, Bolanos Vasquez and Warner (1997) studied the activation of the *nod* gene by different flavonoids from bean plants. Since this pioneer observation, a large number of bioreporters for the study of chemical colloquia involved in bacterial growth and bacterial activity in rhizosphere and bulk soil have been constructed (Rainey 1999; Timms-Wilson et al. 2000; Allaway et al. 2001; Marco et al. 2003). For example, whole-cell bacterial biosensors, based on constructs with *gfp* as a reporter system, have been set up for detection of *N*-acyl homoserine lactones (AHLs) signals involved in quorum sensing phenomena (Steidle et al. 2001). It is believed that these signals are common among rhizosphere bacteria colonising roots (Zhang and Pierson 2001). Identification of promoters activated upon the exposure of bacteria such as *Rhizobium* or *Pseudomonas* species to the rhizosphere environment can be carried out by inserting a promoterless reporter system into a host strain, with the following recovery of the activated cells to identify the activated promoter by gene sequencing at the site of reporter insertion. Thus, the use of a *Rhizobium* strain with a promoterless *gfp* allowed one to identify rhizosphere-activated promoters controlling the synthesis of thiamine and cyclic glucan, or surface growth-activated promoters controlling methionine synthesis or putrescine uptake (Allaway et al. 2001).

The use of whole cell biosensors has increased our knowledge on the interactions between plants and plant pathogens and on the relative biological control strategies. For example, Smith et al. (1999), using the biosensor *P. fluorescens* F113, found that the fungus *Pythium ultimum* releases molecules which down-regulate the *rrn* promoter of ribosomal RNA synthesis in the bioreporter and Lee and Cooksey (2000) reported that *P. putida* activates promoters controlling the synthesis of ATP binding cassette (ABC) transporter proteins in the presence of the fungus *Phytophthora parasitica*. These findings suggest that in some cases the pathogenic attack of a plant root is also connected to a reduced activity of beneficial rhizobacteria.

9.3.2 Extraction and Characterization of mRNA

The relationship between the amount of rRNA and cell activity is not valid for soil microorganisms because most of them under dormancy show high ribosome level so as to be ready to respond to suitable environmental conditions, such as the appearance of substrate (Kowalchuk et al. 2006). In order to determine the expression of a gene it is necessary to monitor its transcript; this can be done by determining

the relative mRNA or indirectly through the detection of the signal of a reporter gene fused to the target gene, as discussed above (Krsek et al. 2006). Given the rapid degradation of non-functional mRNA in the environment, the analysis of the target mRNA represents one of the best opportunities to analyse the activity of the target gene. However, as it is discussed below, a complete functional assessment of microbial communities in soil should also involve the relative encoded proteins.

Successful extraction and characterization of mRNA from soil has been delayed with respect to that of DNA, in spite of the fact that extraction procedures of RNA and DNA from soil are similar in principle. Unfortunately, RNA extraction requires several precautions such as the inhibition of RNase activity, using, for example, diethylpyrocarbonate in the RNA extraction solution (Bakken and Frostegård 2006). Since both skin and many microorganisms can be sources of RNases, care should be taken in keeping solutions and equipments free from these enzymes. The fast turnover rate of mRNA in prokaryotic cells is another problem; indeed, the half-life of specific *E. coli* mRNA ranges from 0.5 to 20 min (Nierlich and Murakawa 1996). Fortunately, slower growing microorganisms, the majority of microorganisms inhabiting soil, have a longer mRNA half-life (Krsek et al. 2006). The situation is completely different for mRNA of eukaryotic cells where transcription and translation are physically separated by nucleus membrane with temporal separation of the two processes. In addition, eukaryotic mRNA often undergoes post-transcriptional modifications, such as splicing of intervening sequences or methylation before being translated, and for this reason the half-life of this mRNA can be prolonged to several hours (Sarkar 1997).

Several methods exist to characterize mRNA so as to assess differences in gene expression (Krsek et al. 2006). Subtractive hybridization involves synthesis of cDNA from total RNA extracted from a wild type of mutant cultivated under certain conditions and then this cDNA is hybridised with RNA from the respective strain or mutant cultivated under different conditions. After elimination of double-stranded cDNA-mRNA hybrids, the composition of single-stranded cDNA is analysed (Utt et al. 1995). Subtractive hybridisation has been used to compare the activity of genes responsible for the nodulation of two strains of *Bradyrhizobium japonicum* in culture with or without water extract of a sandy loam soil or root exudates from aseptically grown soybean seedlings (Bhagwat and Keister 1992).

Another assay involves the formation of an hybrid between RNA and a labelled RNA probe, complementary to the gene of interest and prepared by a transcription anti-sense. The double-stranded RNA hybrid is analysed after removal of the non-hybridised RNA by degradation by ribonuclease (Fleming et al. 1993). This technique was used to quantify transcript levels of NAH7 naphthalene dioxygenase (*nah*A) gene with mRNA extracted by a modified hot phenol method from a polycyclic aromatic hydrocarbons (PAHs) contaminated soil. The level of transcripts correlated significantly with [^{13}C] naphthalene mineralization rate and soil naphthalene concentration; the use of a naphthalene-lux reporter system showed that the hydrocarbon was available to microorganisms (Sanseverino et al. 1993–1994).

A differential display method is based on the fact that 100% of eukaryotic mRNA and 2–50% of prokaryotic mRNA present polyadenylate tails (Krsek et al.

2006). In contrast to other methods, this assay does not require one to know gene sequence to design the primers. Indeed, the 3′ primer used in the reverse transcription is made of a poly(T) region with two additional bases recognising a minor part of the target mRNA and the 5′ primer is short and should anneal with 500 bp of the 3′ primer. Then the PCR products are characterised by techniques generating a fingerprint, and single cDNA bands can be excised, sequenced or cloned (Krsek et al. 2006).

Genes involved in production and resistance of streptothricin (ST), an antibiotic from *Streptomyces rochei* F20, have been studied in liquid culture, in soil and in the rhizosphere of spring wheat (Anukool et al. 2004). RNA was extracted and amplified by RT-PCR with primers specific for either ST resistance gene (*sst*R) or ST biosynthesis gene (*sst*A) coding for peptide synthetase. The *sst*R mRNA was detected in both sterile and non-sterile rhizosphere whereas both transcripts were not detected in the rhizoplane.

Transcripts of ligninolytic enzymes (*lip*) of *Phanerochaete chrysosporium* involved in the degradation of wood have been monitored by extracting poly (A) RNA by magnetic capture (Janse et al. 1998). Transcription patterns of all ten *lip* genes and three manganese peroxidase (*mnp*) genes, both quantified by competitive RT-PCR with full-length genomic subclones, were markedly different in wood when compared with those got with defined media or from soil cultures. It was suggested that this depended on the specialisation of different enzymes to different environmental conditions. Indeed transcription of *lip* gene in *Phanerochaete chrysosporium* is regulated by the availability of C and N sources as determined by competitive RT-PCR (Stewart and Cullen 1999). In addition, iron has been found to regulate the transcription of 97 differentially expressed cDNA fragments in *Phanerochaete chrysosporium*, most of them encoding proteins involved in iron uptake (Assmann et al. 2003). Obviously the regulation of genes in *Phanerochaete chrysosporium* in the rhizosphere depends on the competition between plants and microbes for nutrients and in the case of iron availability, it can play a role in the biological control (Crowley 2001).

9.3.3 Linking Enzyme Activity to Gene Expression

Measurements of enzyme activity represent one of the classical determinations carried out in bulk and rhizosphere soil (Nannipieri et al. 2003). As discussed in Chap. 14, the interpretation of these measurements is not simple due to the many locations of enzymes contributing to the overall measured enzyme activity. Only a few studies have been carried out to link enzyme activity with expression of genes encoding the protein molecules responsible for the target enzyme activity (Fig. 9.2). The reverse transcription has been used to monitor the expression of three manganese peroxidase (*mn*P) genes during removal of PAHs by *Phanerochaete chrysosporium* grown in presterilized soil (Bogan et al. 1996a). The maximum level of transcripts preceded for one to two days the highest manganese peroxidase extracted from soil

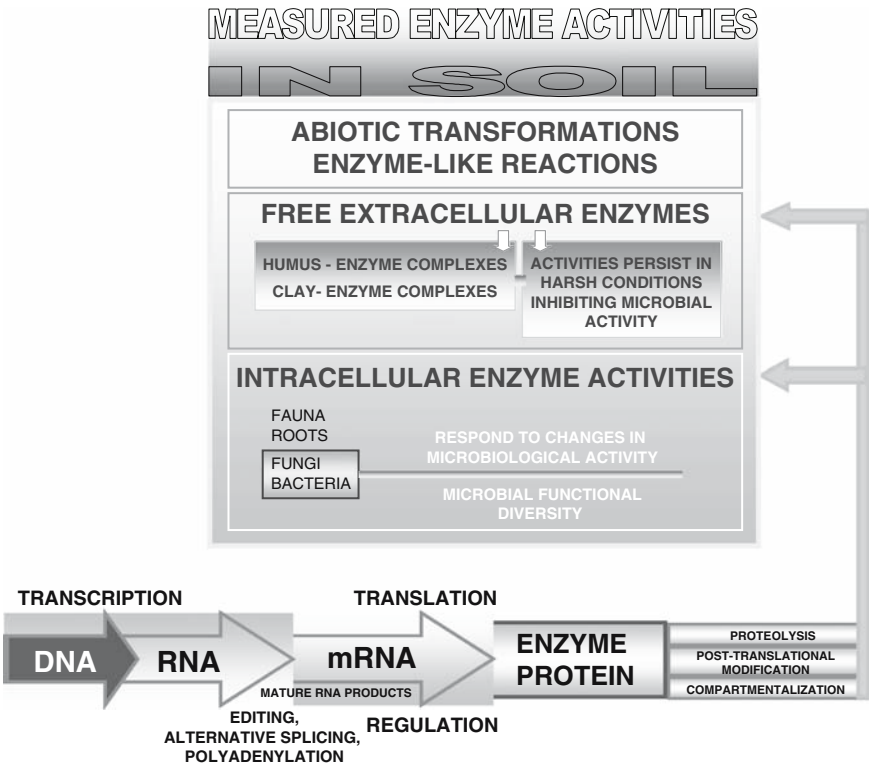

Fig. 9.2 Linking enzyme activity with expression of genes

by the method of Bollag et al. (1987) and both peaks occurred during the maximum rate of two PAHs, fluorene and chrysene, degradation. Bogan et al. (1996b) quantified transcripts of all ten known lignin peroxidase genes (*lip*A-*lip*J) of *Phanerochaete chrysosporium* by competitive RT-PCR, after extraction of mRNA from soil microcosms amended with anthracene. Lip proteins were also extracted from soil microcosms, purified and applied to a nitrocellulose membrane before their quantification by Western blotting with specific monoclonal antibodies. Levels of transcripts were significantly correlated with levels of proteins, even if there was a two-day delay in the peak of the latter respect to the peak of the former. Transcripts of nine *lip* were detected in a soil contaminated by a waste. Studies on both manganese and lignin peroxidase are important not only for the implications on bioremediation of polluted soils but also for a better understanding of the degradation of litter since the importance of these enzymes in the degradation of the lignocellulose complex, which is the main component of the plant residues.

However, the best study covering all events starting from gene presence, through gene expression and up to the detection of target enzyme in soil, has been carried so far by Metcalfe et al. (2002). Chitinase activity was measured by loss

of chitin in buried litter-bags and by a luminescence assay by using as a substrate 4-methylambelliferyl-$(GlcNAc)_2$, whereas the composition of community was evaluated by DGGE. In addition, samples from community DNA was amplified with primers targeted to a gene fragment from family 18 group A chitinases. The addition of sludge to the pasture soil increased the chitinase activity and the number of actinobacteria with prevalence of actinobacterium-like chitinase sequences. Unfortunately extraction of transcripts was unsuccessful probably due to the adsorption of mRNA by soil colloids and the target enzyme proteins were not monitored.

9.3.4 Proteomic Approach

The proteomic approach involves the study of all proteins of a cell so as to get an integrated view of the cell itself. It is well established that more protein isoforms can be synthesised by a single gene because mRNA molecules can be subjected to post-transcriptional control such as alternative splicing, polyadenylation and mRNA editing (Graves and Haystead 2002). The recent availability of extensive metagenomic sequences from various environmental microbial communities has extended the postgenomic era to the field of environmental microbiology. Although still restricted to a small number of studies, metaproteomic investigations have revealed interesting aspects of functional gene expression within microbial habitats that contain limited microbial diversity. These studies highlight the potential of proteomics for the study of microbial consortia. However, the application of proteomic investigations to complex microbial assemblages such as seawater and soil still presents considerable challenges. Nonetheless, metaproteomics will enhance the understanding of the microbial world and link microbial community composition to function (Wilmes and Bond 2006). Although the number of proteins that have been identified and separated using available proteomic analysis methods is encouraging, the diversity of other microbial ecosystems still poses enormous challenges.

Recently it has been said that we need to study microbial proteomics in soil and thus to go beyond DNA and mRNA characterization in order to have a better understanding of soil functionality (Nannipieri 2006; Ogunseitan 2006). Unfortunately, soil proteomics is still in its infancy. According to Nannipieri (2006), one of the main methodological problems is to extract specifically intracellular proteins in the presence of a large background of extracellular proteins; these extracellular proteins are stabilised, that is protected against proteolysis, by their association with soil colloids. On average only 4% of the total organic N is present as microbial N whereas amino acid N released after acid hydrolysis can account for 30–45% of the total organic N in soil mostly as extracellular protein N or peptides N (Stevenson 1986). By considering the protein N distribution in soil it has been hypothesised that soil proteomics might be subdivided in *functional proteomics* and *structural proteomics* (Nannipieri 2006). The latter concerns the characterization of the extracellular protein N stabilised by soil colloids so as to improve our understanding of the mechanisms

responsible for such a stabilization and gives insight on the dominant extracellular proteins before soil sampling. The combination of this proteomic approach with carbon dating might also reveal the period in which these extracellular proteins were dominant. On the contrary *functional proteomics* concerns proteins synthesised by microbial cells involved in biochemical processes occurring in soil in lab experiments or in the field at the moment of soil sampling. The study of functional proteomic can improve our understanding of degradation of organic pollutants and organic debris, nutrient cycling, blockage of inorganic pollutants, molecular colloquia between microorganisms, between plant roots and microorganisms and between plant roots. As is done for the extraction of nucleic acids from soils, two different approaches have been proposed to extract proteins from soil: i) separation of microbial cells from soil particles and successive cell lysis with release of proteins; ii) cell lysis in situ with extraction of proteins (Nannipieri 2006).

A pioneeristic proteomic approach in the rhizosphere was followed by Lambert et al. (1987) with characterization of rhizobacteria, isolated by cultivation from the rhizosphere soil of maize, by dodecylsulfate-polyacrylamide gel electrophoresis of total cell proteins These proteins were extracted by treating cultivated bacterial cells with lysozyme, followed by boiling with a buffer (a mixture of sodium dodecyl sulfate, β-mercaptoethanol, Tris, EDTA, sucrose, bromophenol blue). A similar approach was also carried out by Lambert et al. (1990) to characterize fast-growing, aerobic and heterotrophic bacteria isolated from the root surface of young sugar beet; in this case proteins were extracted from bacterial cells as mentioned above after sonication. The main bacterial species were *Pseudomonas fluorescens, Xanthomonas maltophilia, Pseudomonas paucimobilis,* and *Phyllobacterium sp*.

Recently the proteomic approach has been applied to characterize cell surface proteins of *Pseudomonas putida*, a plant-growth promoting rhizobacteria active against pathogens, capable of degrading pollutants and able to form biofilms (Arevalo-Ferro et al. 2005). The latter property and the cell-to-cell communication system (quorum sensing, QS), that enables the bacteria to co-ordinate the expression of special phenotypes in a cell density manner, seem to be involved in bacterial rhizosphere competence. Mature biofilms, complex three-dimensional structures where cells are embedded in a thick matrix of extracellular polymeric substances (EPS), show channels allowing the passage of nutrients to the interior parts and the transport of wastes to the external side, both moved by fluids (O'Toole et al. 2000; Hall-Stoodley et al. 2004). Initial steps in biofilm formation involve attachment to a surface, followed by formation of microcolonies and maturation of microcolonies in EPS-encased three-dimensional biofilms. Arevalo-Ferro et al. (2005) compared two-dimensional gel electrophoresis (2-DE) protein profiles of a strain and the QS-deficient mutant grown either in planktonic cultures or in 60 h old mature biofilms. The spots of differentially expressed proteins were excised from the 2-DE gel and the relative proteins were identified by peptide mass fingerprinting and database search. Almost 40% of the surface proteins were affected by the sessile life suggesting that an important fraction of the bacterial genome was involved in biofilm physiology; in addition, almost 10% of the protein spots were controlled by the QS system and differed if the cells were grown in surface or in suspension.

The state-of-the-art of soil proteomic has been recently reviewed by Nannipieri (2006) and Ogunseitan (2006). Schulze et al. (2005) have used the mass spectrometry based proteomics to characterize proteins of leachate from some soils with the aim to determine the phylogenetic origin and the potential catalytic functions of proteins. It was observed that the number of bacterial proteins in the forest soil was greater in winter than in summer; in addition, enzymes, such as peroxidase, involved in the degradation of complex molecules were present in water from the forest soil whereas enzymes such as transferases, which might be involved in methane production, were present in waters from peat bog.

9.4 Linking Gene Expression to Functions: The Use of Stable Isotope Probes (SIP)

A major advance in linking functional activity to community structure came with the development of stable isotope probing (SIP) (Radajewski et al. 2000), which involves tracking of a stable isotope atom from a particular substrate into components of microbial cells that provide phylogenetic and functional information, such as lipid, DNA or RNA. Indeed the major advantage of the SIP technique is that ^{13}C-enriched DNA will contain the entire genome of each functionally active microbe of the community. Detailed methodology, potential and future improvements needed for the SIP technique have been already reviewed (Radajewski et al. 2003; Wellington et al. 2003; Manefield et al. 2006). Successful applications of this technique are restricted to reactions of microbial anabolism, because SIP is based on assimilatory processes (Manefield et al. 2006). Thus non-assimilatory chemical transformations, which also occur in soil, fall outside the applicability of SIP. Even if the SIP technique can theoretically be applied to trace the assimilation of any element of biological importance that has a stable isotope, it has almost exclusively been restricted to the use of ^{13}C. The labelling and detection of DNA using $H_2{}^{18}0$ can allow characterising active community members by using a substrate-independent technique.

The SIP was first applied to monitor microorganisms responsible for the assimilation of C from the greenhouse gas methane in a freshwater sediment environment (Boschker et al. 1998). Stable isotope labelled methane ($^{13}CH_4$) was pulsed into the microbial community of the sediment and labelled polar lipid derived fatty acids (PLFAs) from methane assimilating organisms were separated from extracted PLFAs and analysed for the ^{13}C enrichment by isotope ratio mass spectrometry (IRMS). Species belonging to the *Methylobacter* and *Methylomicrobium* genera, were the organisms responsible of methane oxidation.

Radajewski et al. (2000, 2002) were the first to use the ^{13}C labelled substrates (methane and methanol) to label nucleic acids of soil. Equilibrium density centrifugation in CsCl gradients was used to separate ‘heavy’ (labelled) from ‘natural’ (unlabelled) DNA and 16 S rRNA gene clone libraries constructed from ‘heavy’

DNA were sequenced to obtain the identity of organisms assimilating the used substrates. By amplifying functional genes involved in the oxidation of one carbon compound it was found that not only were methanol dehydrogenase and methane monooxygenase genes involved in methanol and methane assimilation, but also species encoding ammonia monooxygenase had assimilated ^{13}C as $^{13}CO_2$ generated by the methylotrophs (Radajewski et al. 2002). Indeed a serious drawback of the SIP approach is the possibility of secondary feeding on breakdown products of the primary substrate. Another drawback of this technique is the presence of unlabelled substrates native to the system that will compete for assimilation. This has led researchers to apply artificially high concentrations of labelled substrates into soil microcosms for extended periods of time (Manefield et al. 2006) but the relevance of this approach at the real situation in situ has been questioned and the use of pulse ^{13}C labelled compounds has been suggested (Jeon et al. 2003; Padmanabhan et al. 2003). Another important issue of any SIP methodology concerns the degree of labelling, which depends on number of organisms using the established substrate (Manefield et al. 2006). When the substrate is consumed by a broad diversity of organisms, the degree of labelling in any substrate-using taxa will be low, making separation by density problematic, whereas if the substrate is consumed by a small number of taxa then the degree of labelling in the specific taxa will be high, facilitating isolation by density (Manefield et al. 2006).

There are differences in using fatty acids or nucleic acids as biomarkers in the SIP technique; PLFAs are more rapidly labelled and give more quantitative information when analysed with IRMS than nucleic acids (Manefield et al. 2006). However, extraction of PLFAs from soil is more laborious that nucleic acid extraction. In addition, the PLFA based SIP gives an inferior phylogenetic resolution than that offered by nucleic acid based biomarkers and signature PLFAs have to be identified from close culturable relatives (Manefield et al. 2006).

Accurate results can be obtained by applying SIP to soil if the delivery of a pulse is carefully planned by considering the ability of soil colloids to adsorb biological molecules, and the solubility and volatility of the used substrates. In addition, since cell replication in soil is slow there are limitations to the isotopic enrichment of DNA with pulse labelling unless the duration of a pulse is extended. To solve this problem, 16 S rRNA based SIP methodologies have been developed for studying phenol degradation in the activated sludge community of an industrial waste water treatment plant since rates of RNA synthesis are always higher than those of DNA due to the fact that RNA is turned over in bacteria independently of replication (Manefield et al. 2002a,b). The RNA-SIP technique has also been applied to soil to identify methylotrophs responsible for methanol assimilation in a rice field soil (Lueders et al. 2004a) and bacteria and archaea responsible for syntrophic propionate degradation in flooded soil (Lueders et al. 2004b). In spite of the fact that the use of RNA as a biomarker and the precise quantitative examination of gradient profiles has enhanced the sensitivity of NA-SIP, the application of the technique to rhizosphere and bulk soil still presents some drawbacks such as the ability to extract clean and intact DNA or RNA from the soil or to sufficiently label nucleic acids of

microorganisms involved in the metabolism of plant root exudates (Manefield et al. 2006). Grassaland monoliths (400 mm diameter × 200 mm deep) were pulsed with $^{13}CO_2$ to promote the release of ^{13}C labelled root exudates into soil (Ostle et al. 2003) but the analysis of 16 S rRNA from root associated soil by IRMS and equilibrium density centrifugation showed that the degree of labelling was too low to get meaningful results (Griffiths 2003; Manefield et al. 2006). On the other hand the PLFA-SIP analysis of soil samples derived from a $^{13}CO_2$ plant pulse showed the assimilation of root exudates by Gram-negative bacteria and fungi, but the phylogenetic resolution was low (Treonis et al. 2004). Therefore, it is problematic to use $^{13}CO_2$ to label soil microbes via root exudates because a broad range of labelled organic compounds are released as root exudates and several microbial species can use the labelled root exudates. According to Manefield et al. (2006) it can be more rewarding to pulse directly soil with labelled root exudate compounds and monitoring microorganisms of rhizosphere soil involved in the assimilation of the target compound by the use of any SIP technique. This can be done in experimental systems simulating the delivery of root exudates into soil (Badalucco and Kuikman 2001; Falchini et al. 2003).

9.5 Other Techniques Used to Link Activity to Phylogenetic Information

Radioactive isotope based methods can be used to study the link between the function and the presence of any taxa. The fluorescence in situ hybridisation (FISH) allows the phylogenetic identification of uncultured bacteria in natural environments using fluorescent group specific phylogenetic probes (targeting rRNA) and fluorescence microscopy. FISH targeting intracellular rRNA has been widely used over the past decade as a type of 16 S rRNA targeting method to identify and quantify certain bacteria in soils, sediments, and activated sludge. Although FISH is useful, difficulties are often encountered when it is used on complex community samples. Problems encountered include variable probe binding to different rRNA target sites and highly autofluorescent samples or cell backgrounds. Another shortcoming of this approach is that cells must be counted visually under a microscope, which is laborious. Quantitative membrane hybridization of labeled DNA probes with bacterial community RNA can avoid these problems. By combining FISH with microautoradiography (MAR), it is possible to identify individual cells, to determine the three-dimensional position of a cell and quantify the active population utilizing a specific substrate. The combination of FISH–MAR has been used successfully to monitor the substrate utilization by bacterial groups of an activated sludge system (Lee et al. 1999**;** Gray and Head 2001) and in various natural environments (Gray et al. 2000). This technique might be used to study plant-microbial interactions, even if it may be difficult to apply this technique to soil. Indeed, it is necessary to extract cells from soil in order to eliminate interferences by soil particles and increase the sensitivity of the technique (Krsek et al. 2006).

Both chemical and physical methods have been used and they present drawbacks such as incomplete recovery, rupture of cells and changes in cell physiology during the separation of cells from soil particles. In addition, the FISH-MAH is a time-consuming and labor intensive technique, and because of the vast diversity of microorganisms in soil, it is almost impossible to design probes and analyse data to resolve all individual species. An advancement in the simultaneous monitoring of the diversity and substrate incorporation by complex microbial communities has been obtained by combining FISH-microautoradiography with an isotope microarray (Adamczyk et al. 2003). The RNA, extracted from ammonia-oxidizing bacteria in pure culture or in the activated-sludge samples both grown in the presence of ^{14}C bicarbonate, was fluorescence labeled and microarray hybridized. It was shown that incorporation of ^{14}C into rRNA could be detected by scanning all probe spots for fluorescence and radioactivity. Therefore, the isotope array enables the application of many probes in parallel, whereas this cannot be done with FISH-MAH, and to measure directly the substrate incorporation into target nucleic acids in contrast to DNA or RNA-SIP.

Another technique allowing detection of active microbial species in environmental samples involves incubating these samples with bromodeoxy uridine (BrdU); the BrdU in the DNA of active microorganisms can be separated from unlabelled DNA by immunocapturing. Then the labelled DNA can be characterised by profiling or cloning and sequenced (Borneman 1999; Yin et al. 2000). Unfortunately not all microbial cells are capable of taking up BrdU, and with FISH-MAH and SIP techniques the conditions of the assay can be different respect to those occurring in situ.

9.6 The Metagenome

The total number of prokaryotic cells on earth has been estimated at $4–6\times10^{30}$, thought to comprise between 10^6 and 10^8 separate genospecies (distinct taxonomic groups based on gene sequence analysis). It is widely accepted that this diversity presents an enormous (and largely untapped) genetic and biological pool that can be exploited for the recovery of novel genes, entire metabolic pathways and their products. Observations showing that culturing yields a fraction of the microbial diversity evident from microscopic analysis have been consistently supported by the results of phylotypic analyses on community DNA preparations, leading to the concept of ‘unculturables’. The apparent underestimation of true microbial diversity derives largely from a reliance on culture based enumeration methods. There is a growing belief that the term ‘unculturable’ is inappropriate and that in reality we rather have yet to discover the correct culture conditions (Cowan et al. 2005). The development of metagenomic technologies over the past 10 years tries to overcome this bottleneck by developing and using culture-independent approaches.

One of the metagenomic approaches which generates a massive amount of data on microbial ecology is the reconstruction of the metagenome of an ecosystem

using random shotgun sequencing. The basic steps of DNA library construction (generation of suitably sized DNA fragments, cloning of fragments into an appropriate vector and screening for the gene of interest) have been extensively and successfully used for over three decades. As there are no obvious limitations in translating the technologies of genomic library construction and screening to metagenomic libraries, it is perhaps surprising that metagenomics only developed in the mid-1990s with the successful application of library construction to marine metagenomes. The metagenomic approach is based on cloning the total microbial genome (the metagenome) extracted from natural environments in culturable bacteria such as *Eschericha coli* (Handelsman et al. 1998; Rondon et al. 2000). The recent development of technologies designed to access this wealth of genetic information through environmental nucleic acid extraction has provided a means of avoiding the limitations of culture-dependent genetic exploitation. Since most of bacteria living in natural environments such as soil are unculturable, the metagenomic approach can allow characterizing the unknown genome of these unculturable bacteria with the probability of finding novel microbial products such as antibiotics and enzymes and assessing specific metabolic and ecological functions (Schloss and Handelsman 2003). By virtue of their low copy number, bacterial artificial chromosome (BAC) vectors can be used to propagate large DNA fragments (around 100 kb), which would otherwise be unstable (Wellington et al. 2003). The major criticism of this technique is that it might miss members of the microbial community which are low in number but perform essential processes.

Microbial DNA extracted directly from soil has been used successfully to construct a BAC library with an average insert size of between 37 kb and 150 kb (Béjà et al. 2000). It is probably too early to state that metagenomic gene discovery is a technology that has 'come of age'. New approaches and technological innovations are reported on a regular basis and many of the technical difficulties have yet to be fully resolved. However, there can be little doubt that the field of metagenomic gene discovery offers enormous scope and potential for both fundamental microbiology and biotechnological development (Cowan et al. 2005). Approaches that enrich for a portion of the microbial community or for a collection of metagenomic clones will enhance the power of metagenomic analysis to address targeted questions in microbial ecology and to discover new biotechnological applications. Metagenomics in plant–microbial interactions has not yet been attempted, but this approach holds great promise to link phylogeny of rhizosphere microbes to function, especially when a functional gene is known.

9.7 Microarray

DNA microarrays have recently been developed for quantifying and monitoring bacteria members of communities in various environments. Sequence analysis of large insert libraries with environmental DNA combined with genetic and functional analysis has the potential to provide significant insight into the genomic

potential and ecological roles of cultured and uncultured microbes. Microarrays (or microchips) technique is a powerful tool that is becoming increasingly used to monitor gene expression under different growth conditions, to detect specific mutations and to characterize microorganisms in environmental samples (Zhou 2003). Microarrays thus represent a powerful high-throughput system for analysis of genes. They are typically used to monitor differential gene expression, to quantify the environmental bacterial diversity and to catalogue genes involved in key processes. This approach identifies only those genes transcribed in the analysed community and is therefore very valuable for the identification of functional genes with relevance to the ecosystem. Compared with traditional nucleic acid hybridisations on porous membranes, glass slide based microarrays offer the additional advantage of high density, high sensitivity, rapid (real-time) detection, lower costs, automation and low background levels. The methodology, advantages and challenges in applying microarrays to environmental studies have been reviewed by Zhou and Thompson (2002). Microarrays can be classified, by considering the type of probe arrayed and the potential application, in: i) Phylogenetic Oligonucleotide Arrays (POAs), used for assessing microbial diversity and based on probes from rRNA genes; ii) Functional Gene Arrays (FGAs), used to evaluate microbial activities and based on the use of probes for gene coding proteins involved in specific functions; iii) Community Genome Arrays (CGA) containing probes of large fragments of DNA and used for detecting target genes and organisms (Schadt and Zhou 2006). The probes designed for POAs are shorter than those designed for FGAs because the latter should target protein-coding genes. In order to detect the presence of functional genes, total RNA is extracted from environmental samples, reverse transcribed and probed against microarrays (Saleh-Lakha et al. 2005; Schadt and Zhou 2006). However, the RNA extraction efficiency from environmental samples is much lower than that from pure culture of bacteria and thus signals cannot often be detected on microarrays. Microarrays have been used successfully to differentiate microbial community composition in environmental samples (Peplies et al. 2003) and to detect and quantify the functional diversity of genes involved in nitrogen cycling, such as *amo*A, *nif*H, *nir*K, *nir*S (Taroncher-Oldenburg et al. 2003) and *nir*S (Cho and Tiedje 2002) and methane oxidation (*pmo*A) (Stralis-Pavese et al. 2004). However, the application of this technique to soil microbiology is still potential.

In addition to low mRNA extraction efficiencies from environmental samples, the application of microarrays to environmental samples presents the following shortcomings: i) presence of background noise in environmental samples and false positive hybridisation signals, with problems in quantifying microbial populations from environmental samples; ii) even though this is a high throughput technology with one chip being used for a thousand probes, the current capacity of microarrays is limited with respect to the number of genes needed for monitoring the enormous diversity of the soil microbial community; iii) the hybridization of probes with target nucleic acids can be difficult due to stable secondary structure of DNA or RNA; iv) in spite of the fact that it is assumed that target sequences containing single base mismatches can be differentiated by microarray hybridization, generally discrimination can occur in the case of more than one single base mismatch; v) both humic

and clay substances can interfere with hybridization; vi) the functional analysis is based on genes and pathways that have been revealed through isolates studied in the laboratory (Saleh-Lakha et al. 2005; Schadt and Zhou 2006).

Though several challenges still remain to be overcome to apply microarray technology most effectively to monitoring gene expression in complex microbial ecosystems (e.g. increasing the sensitivity), the potential and capability of this promising in situ technology appears to be highly attractive. In spite of these shortcomings, microarrays have been used to monitor functions of sulfate reducing and nitrate-fixing bacteria from soil (Loy et al. 2004; Stewart et al. 2004), for detecting genes involved in the biodegradation processes (Rhee et al. 2004) and methanotrophs in the environment (Bodrossy et al. 2006).

Avarre et al. (2007) have recently reviewed the use of DNA microarrays for detection and identification of bacteria and genes of interest from various environments. So far, most of the genomic methods that have been described rely on the use of taxonomic markers (such as 16 S rRNA) that can easily be amplified by PCR prior to hybridization on microarrays. However, taxonomical markers are not always informative on the functions present in these bacteria. Moreover, genes for which sequence database is limited or that lack any conserved regions will be difficult to amplify and thus to detect in unknown samples. Furthermore, PCR amplification often introduces biases that lead to inaccurate analysis of microbial communities. An alternative solution to overcome these strong limitations is to use genomic DNA (gDNA) as target for hybridisation, without prior PCR amplification (Avarre et al. 2007). Though hybridization of gDNA is already used for comparative genome hybridization or sequencing by hybridization, in addition to the high cost of tiling strategies and important data filtering, its adaptation for use in environmental research poses great challenges in terms of specificity, sensitivity and reproducibility of hybridization. This specificity problem is inevitable as long as many probes are hybridized to targets at the same time.

In view of these problems, Hoshino et al. (2007) have recently developed an RNA microarray in which total RNA from a microbial community is attached to a glass slide, and specific rRNAs are detected by fluorescently labeled oligonucleotide probes. In this RNA array method, hundreds of RNA samples can be analysed at the same time, and direct attachment of RNA onto the slide glass can omit PCR bias, while it cannot detect a low amount of target gene as functional gene. Moreover, any specificity problems of hybridization cannot arise because only a few probes are used for hybridization. The RNA microarray requires only 4 h for hybridization and enables double staining and estimating relative abundance of rRNA.

9.8 Conclusions

The study of gene expression in the rhizosphere soil is only just starting because of the limitations in the use of most of the relative techniques, including those for characterising synthesised proteins in the rhizosphere. For this reason the link

between the composition of the microbial community and the biochemical processes occurring in the rhizosphere is still vague. New cultivation methods continue to be developed to improve our ability to capture a greater diversity of microorganisms within the environment. However, once these techniques are fully developed for studying soil samples, it will be possible to assess simultaneously in a single assay microbial diversity and most of the processes occurring in the rhizosphere by using, for example, functional microarray techniques. This will permit a better understanding of processes such as biological control by rhizobacteria, stimulation of microbial activity by root exudates, competition between microorganisms and plant roots for nutrients, molecular colloquia between microorganisms and between plant root and microorganisms. Development of soil functional genomics will also clarify whether the higher diversity of functional genes (and microbial species) of the rhizosphere than bulk soil is solely linked to the greater C availability, or is the result of a selection mechanism where plant actively select species capable of supplying nutrients.

Although microarrays can provide rapid generation of large functional datasets, this technique has several shortcomings including the fact that the functional analysis described is based on genes revealed in isolates studied in the laboratory with exclusion of genes of unculturable microorganisms.

The use of techniques such as SIP have permitted a better understanding of the link between the function and microbial cells involved in the specific function. However, these techniques not only need to be methodologically improved but also applied in a suitable way to the rhizosphere soil. According to Manefield et al. (2006) it can be more rewarding to use labelled root exudate compounds and monitoring microorganisms of rhizosphere soil involved in the assimilation of the target compound by the use of any SIP technique than to pulse the whole seedlings and then monitoring labelled nucleic acids or PLFA of microorganisms of rhizosphere metabolising the labelled root exudates.

Reporter technology has been used to assess several functions in the rhizosphere soil including gene expression even at the single cell level The ever increasing knowledge of the promoter and regulator gene along with the refinement of reporter gene insertion techniques will allow use of the reporter gene technique for monitoring induction, expression and regulation of virtually any gene in the rhizosphere. In addition, in this case the methodological improvement of the technique will also allow the design of new reporter bacteria to respond to specific root exudates, as it already occurs for specific signals involved in molecular colloquia (Sørensen and Nybroe 2006).

Nucleic acid based methods present some limitations for getting information on functions expressed by microbial communities in situ. In this context, the large scale study of proteins expressed by indigenous microbial communities (metaproteome) should provide information to gain insights into the functioning of the microbial component in ecosystems. Characterization of the metaproteome is expected to provide data linking genetic and functional diversity of microbial communities. Studies on the metaproteome together with those on the metagenome and the metatranscriptome will contribute to progress in our knowledge of microbial

communities and their contribution in ecosystem functioning (Maron et al. 2007). Looking to increase our understanding of the role that members of a microbial community play in ecological processes, several techniques have been developed that are enabling greater in depth analysis of environmental metagenomes.

Microarrays for investigating the content of microbial communities in environmental samples are still in their infancy. One of the main reasons is the technical difficulty inherent in the hybridization of gDNA. Avarre et al. (2007) have proposed few strategies that may contribute to improve detection of gDNA with microarrays based on a simple and low-cost design, like isothermal probes, sensitivity enhancement, competitive hybridization or the use of the promising gold nanoparticles technology. Once this limitation is overcome, we can predict an explosion of microarray based investigations of microbial communities, which will eventually lead to a dramatic increase in our knowledge of the microbial genetic structure and composition of samples of high environmental and ecological value.

RNA microarray protocol developed by Hoshino et al. (2007) for determining the abundances of individual bacterial members in complex microbial communities in terms of requirement of much less RNA than conventional membrane hybridization methods is quite useful. Because such a small amount of RNA is required, sufficient signal could be obtained beginning just 1 h after hybridization, whereas overnight hybridization is required to obtain sufficient signal in membrane based methods. This RNA microarray enables hundreds of samples to be easily and rapidly processed to determine the relative abundances of individual members in complex bacteria communities. This protocol will be useful in determining the abundances of bacteria in, for example, wastewater treatment processes and bioremediation of soils.

Proteomics thus still has a great potential for the functional analysis of microbial communities of rhizosphere soil because protein expression is linked to specific microbial activities in a given ecosystem. Unfortunately this technique also has several shortcomings that prevent its successful application to soil, as unlike other cellular macromolecules, proteins exist in many different biological and physical conformations. Consequently, there is no universal extraction protocol that can be followed up to now. Despite the limited number of environmental proteomic investigations that has been carried out, it is clear that metaproteomics has huge potential in the field of environmental microbiology. It is necessary to set up experimental protocols allowing extraction of intracellular proteins without extracting extracellular proteins, the latter largely prevailing over the former in soil. Although it remains a daunting task to elucidate all of the functional proteins that are contained within an environmental sample, metaproteomics will find immediate use in studies focusing only on parts of expressed environmental proteomes. Investigations that focus on limited numbers of highly expressed proteins can have immediate impacts on developments in the field (Wilmes and Bond 2006). However, because protein expression is a reflection of specific microbial activities in a given ecosystem, proteomics has great potential for the functional analysis of microbial communities. We believe that the elucidation of metaproteomic expression will be central to functional studies of microbial consortia. Metaproteomics will gain momentum

with the advent of further environmental sequencing projects and it will provide a useful tool with which to focus research direction.

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