MARSCHNER REVIEW

Back to the basics: The need for ecophysiological insights to enhance our understanding of microbial behaviour in the rhizosphere

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Abstract

Background and Scope Microorganisms exhibit an astonishing diversity and wide genetic variability even within species, in particular with respect to their metabolic pathways and host-interactive capabilities. The mosaic genomes that encode these capacities are accountable for the abilities of environmental microbes to survive and thrive in highly complex systems such as soil and the rhizosphere. Whereas credits are to be given to traditional microbiology studies, e.g. with rhizobia and their interaction with the plant, an explosive enhancement of our understanding of the plant-microorganism interactive system has been recently achieved by the broad application of the molecular toolbox, in particular high-throughput sequencing (HTS) technologies. The latter have allowed to access thousands to millions of microbial phylotypes and functions at relatively low cost and effort. While such techniques have improved the accessibility of the taxonomic and functional diversity of rhizosphere and soil microbial communities, detailed insights into organismal ecology and physiology (reflecting the behaviour of populations of cells) within the community in the natural environment are still required.

Conclusions In this review, we first examine the current 'state-of-the-art' of rhizosphere ecology studies and what HTS strategies have added to our understanding of the system. We posit that our capacity to develop and test refined ecological hypotheses is hindered if we solely depend on deep-sequencing methods. Plant-soil-microorganism systems represent one of the most intriguing 'playgrounds' for assessments of ecological theories, since they offer a myriad of ways to investigate ecological interactions (i.e. intra- and inter-specifically), physiological and behavioural traits. In addition, the evolutionary processes that lead to innovation in the microbiota are likely prominent in the rhizosphere. Thus, there is a perceived need to shift our attention from the HTS studies, that extensively map the local microbiota in an overall fashion, to studies focusing on as-yet-unaddressed fundamental questions about the plant-soil microbiota system. Such a paradigm shift will certainly assist us in the unravelling of the building blocks of rhizosphere (and soil) microbial communities, as well as form a basis for targeted manipulation of these in their natural settings.

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Introduction

Soil represents the major biodiverse ecosystem on Earth, harbouring a plethora of microbial species interwoven in a balanced and intricate way (Vogel et al.

2009). Interestingly, different plant species are able to efficiently "select" - from the total pool of microorganisms in the soil - different suites of microbial species that assemble into communities in the rhizosphere (Berg and Smalla 2009). This process is mainly mediated by the physical extension of the plant root system through the soil matrix, which offers colonization sites in addition to providing mucilage and exudates. The latter may act as nutrient sources as well as signals, attracting microorganisms towards the root surface in a more or less specific sense. The most well-known examples of microbes associating tightly with plant roots are given by *Rhizobium* (triggered by specific flavonoid compounds secreted from roots of leguminous plants) and mycorrhizae. We may surmise that this interactive process can actually more often result in intimate microbe-plant interactions, which possibly also results in the colonization of internal plant tissues to yield endophytes (Hardoim et al. 2008).

The rhizosphere, firstly described by Hiltner (1904), is, in its simplest form, defined as the volume of soil around living plant roots that is influenced by the activity of the roots (Darrah 1993; Hinsinger 1998). Thus, it is the physical occupancy of soil by emerging roots and their sloughed–off cells, in addition to the chemical compounds that are exuded [sugars, polysaccharides, amino acids, flavonoids, peptides, proteins, fatty acids (Bowen and Rovira 1999; Nguyen 2003; Jones et al. 2004)] which define the rhizosphere as a very specific site in soil, in which microorganisms are activated. Moreover, the joint activity of roots and microbes promotes physicochemical heterogeneity, that is, spatial and temporal diversity, in the local soil microhabitat. This emerging heterogeneity is revealed as a gradient of different compounds extending from the root, reflected in shifting parameters such as nutrient and signalling compound concentrations, pH, water and oxygen availability and soil physical structure (Hinsinger 1998). Moreover, root exudates are known to vary in accordance with plant species, phenological status and even cultivars within a species (Kowalchuk et al. 2002; Högberg et al. 2006; Micallef et al. 2009). The penetration of soil by plant roots is mainly mediated by chemical interactions with soil particles and diffusional characteristics (Hinsinger et al. 2005; Watt et al. 2006). As a result, root setting and exudation may affect the local microbiota by inciting a very sophisticated interplay of gradient-wise chemical signalling and nutrition.

These effects result in a range of differential microbial strategies and interactions that allow microorganisms to successfully compete, survive and thrive in the rhizosphere biome. A key factor may be to what extent specific microbes are able to make their way to the root surface, where nutrient levels are highest. Pseudomonads are known to often have the capacity to migrate towards plant roots. One recent example is the finding that benzoxazinoids in exudates from maize roots are able to successfully attract *Pseudomonas putida* (Neal et al. 2012). Conversely, plants are thought to be often quite sensitive to the local microbial activity. For instance, they may experience enhanced performance by microbial contributions to their health and development, via assists in nutrient uptake and via phytohormones and enzymes that are produced. As a case-in-point, nitrogen-fixing bacteria such as rhizobia constitute a well-known functional group that contributes to the global input of nitrogen into natural and agricultural soil systems (reviewed in detail by Herridge et al. 2008). The degree to which diverse nitrogen fixers are successful in their rhizosphere colonisation and actual activity is a key issue for many nitrogen-poor soils. In addition, key nutritional functions, such as phosphorus solubilisation, have been reported to be mainly microbial in nature. Phosphorus solubilisation involves a variety of distinct genetic mechanisms that occur across many members of the soil microbiota, including the secretion of organic acids (for a review see Richardson et al. 2009). Also, the production of phytohormones by rhizosphere bacteria, resulting in an altered (often more vigorous) plant development, has been described (Lee and Song 2007). Finally, given its effect on local ethylene levels, the activity of the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase, a feature widespread in plant-associated bacteria (e.g. *Burkholderia phytofirmans*), has been shown to have important implications for rhizosphere functioning (Hardoim et al. 2008). Thus the rhizosphere microbiota is important for the plant, as evidenced by the fact that a wide range of bacteria isolated from the rhizosphere has been shown to be capable of acting as a plant growth or plant health promoting agent. They often do so either by strengthening plant growth or by warding off plant pathogens via the production of antimicrobial compounds or by eliciting plant defence reactions (Sfalanga et al. 1999; Földes et al. 2000; Flores-Vargas and O'Hara 2006; Romanenko et al. 2007).

While several studies so far have focused on the taxonomic and functional aspects of rhizosphere microbial communities across soil types and plant species (e.g. Andreote et al. 2010; İnceoğlu et al. 2011; Hardoim et al. 2011), relatively little is known about the effects of plant root exudates on the gene expression patterns of rhizosphere-associated bacteria (for a few examples, see Pothier et al. 2007; Neal et al. 2012). This is a key emerging field of research, as only by being able to relate specific functioning of rhizosphere microorganisms to local conditions (and understanding the dynamics of this) will we be able to understand and predict rhizosphere function at this level. Here, key questions are (1) how do root exudate compounds shift the gene expression profiles of specific strains in the soil? and (2) what are the roles exerted by these expression systems in the bacterium-plant association and in the adaptation to rhizosphere conditions? The latter question relates to the fact that we often may find enhanced expression of as-yet-unknown gene functions. Last but not least, the plethora of bacterial forms in the rhizosphere, which may differ even within species, and their specific behavioural response when confronted with the pressures exerted by plant roots (thus, defining the intricate processes underlying the ecological interactions in the rhizosphere) is still underexplored. This poses great challenges for research aimed to elucidate the mechanisms underlying the complexity of the functioning of the system.

The present review first examines recent advances in the application of the molecular toolbox to the rhizosphere. This toolbox has greatly expanded over recent years and previous reviews have examined the methodologies (e.g. Van Elsas and Boersma 2011). After discussing the strengths and caveats of the broad use of the current molecular (e.g. HTS) methodologies, we pinpoint how these can be reliably applied to evaluate the fundamental questions about rhizosphere interactions. Next, it is concluded that boundaries are now reached with the HTS methods in terms of the level of resolution that can be reached. A key issue at hand is the lack of understanding of the ecophysiological behaviour of specific microorganisms in the rhizosphere, as well as soil. We thus argue that, to better access this issue, a (renewed) focus on reduced subsets of this microbiota is required. In this approach, more effort should be spent to address the key as-yet-uncultured microorganisms, especially in terms of their behaviour in the system, in addition to enhancing

our understanding of the behavioural aspects of already-cultured organisms. Such efforts should be placed in the context of the great dynamism in the genetic determinants of microbial behaviour, which can occur and spread on mobile genetic elements in the rhizosphere (Van Elsas et al. 1988), leading to the wealth of diverse organismal types that are selected in this habitat. We will, thus, not only enhance our understanding of microbial interactions and behaviour in the rhizosphere, but also decode the plethora of as-yet-untapped eco-evolutionary strategies in the local microbiota. Such approaches will, collectively, shed light on the physiological traits and metabolic adaptations that make the difference for the microbes that dwell under rhizosphere conditions.

A brief retrospect – how have methodological developments changed our understanding of rhizosphere microbiology?

Most of our understanding of the rhizosphere and soil microbiota has been built on a strongly limited view of just a few species that could, traditionally, be cultured under laboratory conditions (Singh et al. 2004). The aforementioned rhizobia and mycorrhizal fungi represent examples of the microorganisms of which we have started to understand the underlying principles of their ecological behaviour in the rhizosphere. In a broader sense, the well-known diction by Martinus Beijerinck (voiced by his disciple Baas Becking) *"Everything is everywhere and the environment selects"* can be interpreted to indicate that one can find any functionally-defined microorganism from a soil in accordance with the milieu used for enrichment and/or isolation. Indeed, as argued in the above, plant roots can be envisioned to confer specific selective pressures on soil microorganisms. However, what Beijerinck, his co-workers and disciples, did not address was the question to what extent the excerpts from natural systems such as soil they isolated reflected the true extant diversity. A similar argument holds for the rhizosphere. And, as we understand now, the best estimates of the true microbial diversity in natural systems like soil/rhizosphere greatly exceed the diversity that is commonly observed from culturing efforts like platings or enrichments. A first glimpse of the truly astonishing diversity in soils was obtained in the late 1970s and early 1980s, with the increasing application of 16S rRNA gene based diversity assessments using directly extracted nucleic acids. Such

studies allowed us to better elucidate the real diversity and structural composition of microbial communities, for instance by community fingerprintings, clone libraries, sequencing and taxonomic inference (Lane et al. 1985; Muyzer et al. 1993; Duineveld et al. 1998, 2001; Smalla et al. 2001; Costa et al. 2006; Van Overbeek and van Elsas 2008; Dini-Andreote et al. 2010). The use of the 16S rRNA gene as a target has exploded till today (broadly discussed in the upcoming sections), allowing to investigate microbial community structures, and shifts in these, in a broad array of environments. A second breakthrough was provided by the development and improvement of HTS (also called 'next-generation sequencing' - NGS) technologies. This development has started with the advent of Roche 454 pyrosequencing in 2005, allowing a significant advance in the ease of sequencing and lowering of its costs. This capability, to massively retrieve genomic information from the environmental microbiota, in addition to the enhanced capabilities to access the metatranscriptome, the metaproteome and the metabonome, has led us into the so-called meta-'omics' era. Since then, the application of the novel methodologies has greatly assisted researchers in the understanding of the dynamics and functioning of extant microbial communities in soil and the rhizosphere (e.g. Berg and Smalla 2009; Barret et al. 2011; İnceoğlu et al. 2011; Tett et al. 2012; Lundberg et al. 2012; Bulgarelli et al. 2012).

A very recent development is given by two remarkable studies that focus on the description and establishment of a so-called 'core' microbiome for the model plant species *Arabidopsis thaliana* (Bulgarelli et al. 2012; Lundberg et al. 2012). Briefly, it was found that some specific taxa, such as those belonging to Proteobacteria, Bacteroidetes and Actinobacteria, were quite constantly present and deterministically occurred in the samples according to presumed niche sorting (i.e. rhizosphere and root endophyte), while other taxa appeared to be more stochastically spread. The latter is exemplified by taxa observed to belong to the class Betaproteobacteria. To explain this finding, the authors suggested that this bacterial group might be saprotrophic (in addition to its stochastic occurrence) and, as such, it would be naturally found on any plant root or plant debris (Bulgarelli et al. 2012). Given the massive effort, such research represents a major step forward in our broad understanding of the microbial types that are selected by plant roots and whether and to what extent plant genotype and developmental stage influence the selective process (for more detail see Lundberg et al. 2012). At this point, we would like to emphasize that deeper sequence analyses and increased taxonomic resolution would probably allow to enhance our knowledge even further. Nevertheless, such an additional knowledge gain might be minimal, as the patterns of root colonization are already discernible on the basis of the current - very impressive - data sets. On the other hand, studies performed at much smaller spatial scale, in which spatial aspects of colonization as well as individual/group behavioural aspects are included, would represent a giant step forward. Therefore, the focus should now be on the determination of the phenotypic diversity of rhizosphere microorganisms at realistic spatial and temporal scales and on how organisms living together in this system interact. On top of that, an assessment of how such interactions boil down to potential group behaviour (e.g. conferring stabilizing mechanisms for microbial success in such environments) is compellingly needed.

Inspired by the ease and feasibility of obtaining time series with genomic data of microbial communities, recent studies have attempted to establish empirical models to predict community assembly in space and time (Fuhrman and Steele 2008; Steele et al. 2011; Larsen et al. 2012a,b; reviewed in detail by Faust and Raes 2012). The system has been compared to an 'artificial neural network' and the approach is thought to represent a step forward in our capacity to connect microbiological and physicochemical data within predictive models. Using such approaches, it may be possible to establish connections between phylotype dynamism and environmental parameters. However, as a consequence, this would demand *n*-fold more experimental testing of the predictions in order to validate the emerging models. Such an approach may be promising in order to evaluate and test the ecological interactions between organisms in plant-soil systems, especially if assumptions are tested within well-designed and validated experiments. Certainly, such experiments place a great demand on our capability to cultivate and experimentally manipulate and assess individual cell populations, both in situ and under controlled experimental conditions.

Rhizosphere organism-based studies in the age of HTS

In the foregoing, we discussed the great steps forward in our knowledge of the rhizosphere that have been

brought about by the advent of the HTS technologies. The ensuing interpretational approaches may indeed indicate the selection of particular bacterial types and functions, and even the potential co-selection of combinations of types. However, such analyses stop short of telling us how individual microorganisms actually respond and 'work' under the local conditions, at the cellular or population level. In other words, we will remain ignorant of the real life history of the individual building blocks of the local microbial communities. Unfortunately, our inability to cultivate the vast majority of the microorganisms present in nature, the so-called 'great plate count anomaly' (GPCA; Staley and Konopka 1985), has long plagued, and is still plaguing, our progress in understanding soil/rhizosphere systems. In fact, the absence of an ecological unit, i.e. an isolated pure culture, drastically narrows down our ability to fully appreciate and characterize the physiological and ecological responses of such organisms in their habitat, for instance, by educated design, manipulation and experimental testing of ecological hypotheses. In soil and rhizosphere systems, the GPCA accounts for 95 – 99 % of total bacterial phylotypes, which implies that only 1 – 5 % of the total is culturable (Nunes da Rocha et al. 2009; Vartoukian et al. 2010; Stewart 2012). Surprisingly, relatively little effort has as yet been spent to overcome the GPCA, for instance by intensifying the efforts to bring organisms into culture. As an example of recent studies circumventing such limitation, Nunes da Rocha et al. (2010), by applying a combination of cultivation approaches [i.e. oligotrophic agar medium amended or not with plant root exudates and/or catalase (used to reduce oxidative stress)], were able to retrieve to culturability hitherto-uncultured bacteria from the potato rhizosphere belonging to the phylum Verrucomicrobia. These authors described 7-fold higher recoveries of cells by applying such approaches, when compared to the use of a commonly-used medium like R2A. This study may serve as an example as to how 'basic' approaches can help to efficiently overcome the GPCA for rhizosphere inhabitants. Moving forward, the authors investigated the ecophysiological traits of other hitherto-uncultured leek rhizosphere isolates, i.e. strains of the genus Holophaga (Nunes da Rocha 2010). Strikingly, the Holophaga isolates presented high mobility in in vitro tests, which might be linked to their ability to rapidly respond to root exudate signals. Moreover, other traits were also observed, such as the ability of these isolates to produce biofilms as well as to aggregate (in this case by forming chains of cells). In the light of these traits, it was suggested that these strains revealed group behaviour, possibly used to avoid predation by protozoa, as previously discussed by Young (2007).

These examples point us to a key paradigmatic contention about what drives the assembly of the microbiota in the rhizosphere: the most successful members of the local microbial community might be those that contain traits or trait combinations that allow them to have an edge at any point in time or space. The system thus has a 'specific carrying capacity' (SCC) for each of the plethora of rhizosphere inhabitants and this SCC may itself be a dynamic entity that depends on plant and soil characteristics. Under this vision, the local rhizosphere microbiota is made up of organismal types of which the population sizes and activities are each governed by their SCCs. As this contention is built upon the phenology of the local organisms, it is the functional complement of the rhizosphere microbiota that matters, and not so much the phylogenetic make-up of the community. If we shift our thinking just a bit further, stepping out of the fixation that traits and combinations of traits are invariably bound to phylogeny, we may start to discern a picture of the rhizosphere microbiota as being made up of members that represent combinations of traits that make them successful anywhere in the plant growth cycle. The following example may, in a simple way, illustrate this point (for more ecophysiological traits examples see Table 1). When monitoring the population dynamics of the species *Pseudomonas fluorescens* and *Bacillus subtilis* added into the system, to address their response to wheat root development, it was observed that - while *P. fluorescens* presented a slow, steady decline over time - *B. subtilis* populations rapidly decreased and stabilized at low levels (ca. 10^3 CFU per g of soil) (Van Elsas et al. 1986). This finding supports the hypothesis that the population size of each local organism is reigned by the SCC which, at each point in time, is imposed on it by the system. The SCC is ultimately limited by nutritional facets and determines the constraints of the local niche. The collective SCCs, which by nature are dynamic, determine the collective niche for the local microbiota, as influenced by the system. For the microbes themselves, their intrinsic genomic information, as well as their capacities to acquire such information from their surroundings [by horizontal

gene transfer (HGT), for instance by conjugation, as previously described in the rhizosphere (Van Elsas et al. 1988)], plays a key deterministic role in their success, and a core set of traits that confer rhizosphere competence may exist. Thus, a plethora of diverse ecological and functional types, which consist of different gene combinations, may be seen to coexist in the system.

Table 1 depicts some selected traits that are commonly deemed to be important for rhizosphere competence and reveals their presence in current key

rhizosphere inhabitants. It is obvious that different combinations of such traits offer different ecological opportunities to the gene hosts and so different niches that coexist can be filled. The forces of HGT and selection are continuously shaping the local microbial communities. Hence, to really understand the rhizosphere microbial system, a focus on both HGT and behaviour/niche occupancy is required, which is a very desirable research direction. Thus, key questions that pertain to the big question about how microorganisms respond to the dynamic local conditions in their

Table 1 Selected bacterial ecophysiological traits involved in their 'life strategy' to survive and thrive in the rhizosphere/soil system

Class	Ecophysiological trait	Bacterial types (examples)	References
I Interactions with plant metabolism	Plant hormone biosynthesis	Several plant-associated bacteria	Baca and Elmerich 2003
	ACC deaminase	*Burkholderia phytofirmans* PsJN	Sessitsch et al. 2005
		Variovorax paradoxus 5C-2	Dodd et al. 2009
	Biological nitrogen fixation	Rhizobia group (several bacterial types)	Willems 2006
		Acetobacter diazotrophicus PA15 *Azoarcus* sp. BH72	Rosenblueth and Martínez-Romero 2006
		Klebsiella pneumoniae 342	
	Induced systemic resistance	*Bacillus subtilis*	Ongena and Jacques 2008
		Bacillus amyloliquefaciens ES-2	Sun et al. 2006
II Competitiveness	Antibiotic production	*Pseudomonas aureofaciens* 30–84	Morello et al. 2004
		Serratia plymuthica PRI-2C	Garbeva et al. 2012
	Surfactin production	*Bacillus subtilis* 6051	Pal Bais et al. 2004
		Pseudomonas fluorescens SS101	Mazzola et al. 2007
	Cyanogenesis	*Pseudomonas fluorescens* SS101	Gallagher and Manoil 2001
		Pseudomonas aeruginosa PAO1	Rudrappa and Bais 2008
	Methylotrophic metabolism	*Methylobacterium nodulans* ORS2060	Kaparullina et al. 2011
		Methylobacterium spp.	Andreote et al. 2009
III Chemotaxis and quorum sensing	Motility	*Pseudomonas stutzeri* A15	Rediers et al. 2003
		Pseudomonas fluorescens WCS365	de Weert et al. 2002
		Pseudomonas putida PaW8	Turnbull et al. 2001
		Azospirillum spp.	Broek and Vanderleyden 1995
	Passive dispersal facilitation	*Paenibacillus vortex*	Ingham et al. 2011
IV Persistence in the environment	Endospore formation	*Bacillus megaterium* *Bacillus cereus*	Timmusk et al. 2011
		Bacillus pumilus	
		Paenibacillus polymyxa	
V Aggregation	Biofilm formation	*Pseudomonas putida*	Espinosa-Urgel et al. 2002
		Bacillus subtilis 6051	Pal Bais et al. 2004
		Methylobacterium mesophilicum SR 1.6/6 *Methylobacterium extorquens* AR1.6/2	Rosseto et al. 2011
	Cell aggregation	*Azospirillum brasilense*	Burdman et al. 2000

This overview excludes pathogen types that contain dedicated systems to colonize, invade and to be pathogenic to host plants

ACC 1-aminocyclopropane-1-carboxylate deaminase

rhizosphere environment can only be answered at the level of the individual cells/populations. We suggest that the molecular tools that are available are now tuned towards the assessment of what are the key assets of a bacterium that determine its successful behaviour in the rhizosphere.

Monitoring the in situ dynamics of plant root-associated microorganisms

The key questions

As indicated in the foregoing, there are still a number of unanswered key questions about the microbial populations in the rhizosphere that need to be tackled at the organismal level. First, although we now can broadly map the composition of the rhizosphere microbiota, both at the taxonomic and at the functional level, we ignore most aspects of individual behaviour, i.e. at the cell population level. Thus, cell-to-cell interactions, and spatial and temporal aspects of these, remain unexplored by the HTS holistic approaches. Very importantly, we do not yet fully understand what makes an organism a good rhizosphere colonizer, what drives rhizosphere colonization and how many niches there are, or can be, in the rhizosphere. Finally, we constantly underestimate the enormous dynamism, in eco-evolutionary terms, of the organisms that make part of the rhizosphere community.

The methodological developments

On the positive side, there are important developments in the technologies available to address more focused questions about the microbiota in the rhizosphere. As posited in the foregoing, such recent advances in the molecular toolbox, e.g. HTS, have enabled to circumvent the limitations imposed by the GPCA. As a large amount of techniques is currently available, which cannot be extensively addressed individually, we summarize our overview of such tools and their usefulness to monitor and study of single microbial populations in the plant-soil system in Fig. 1. Hereunder, we discuss what the use of such tools has brought us in terms of scientific novelty.

First, important advances in fluorescence in situ hybridization (FISH) have enabled the direct detection and identification of single cells or microcolonies using their signatures in rRNA molecules in the rhizosphere

(Mogge et al. 2000; Amann et al. 2001; Bertaux et al. 2007; Wagner 2009). Key facets of microbial localization and interaction have thus been elucidated (e.g. Gantner et al. 2006). When integrated with the use of a suite of other techniques (i.e. microautoradiography, Raman microspectroscopy or secondary ion mass spectrometry -SIMS), FISH can be applied to provide additional information on:

(i)   the presence of specific target genes or even mRNA molecules in cells,
(ii) specific metabolic activity and
(iii) the presence of chemical compounds in the vicinity of the detected cell (reviewed in detail by Wagner and Haider 2012; also see Watrous et al. 2012).

Conceptually, in the physicochemically heterogeneous rhizosphere system, the application of such tools will allow to investigate and monitor plant-microbe interactions, as significant sample disruption is avoided (in the case of Raman spectroscopy). However, despite the elegance of FISH-based methods, such techniques still pose great methodological impairments, mainly those related to the high background of fluorescent particles within soil samples and difficulties to sort and perform DNA extraction and amplification from fixed cells (Wagner and Haider 2012). Suitable methods to be applied to rhizosphere environments are micromanipulation (Kvist et al. 2007) followed by fluorescence activated cell sorting (FACS; Yilmaz et al. 2010). These methods may produce specific cell fractions representative of rhizosphere situations. Moreover, optical tweezers (Blainey et al. 2011) and laser microdissection (Klitgaard et al. 2005) are methods well suitable for phylogenetically directed cell separation (Wagner and Haider 2012). This represents a major development that may open up rhizosphere (single-cell) genomics, since, once efficiently detected and sorted, retrieved cells can be analysed by genome sequencing following multiple displacement amplification. At this point we argue that, as genomics tools develop rapidly, more effort should be spent on identifying the putative genes that are related to microbial unculturability (e.g. nutritional limitation, microbial dormancy). Ultimately, this will enable us to address the basics of culturability of these organisms to further investigate their ecophysiological properties at an individual/population basis.

Fig. 1 Flow chart describing different approaches to achieve insights in microbial behaviour in the rhizosphere
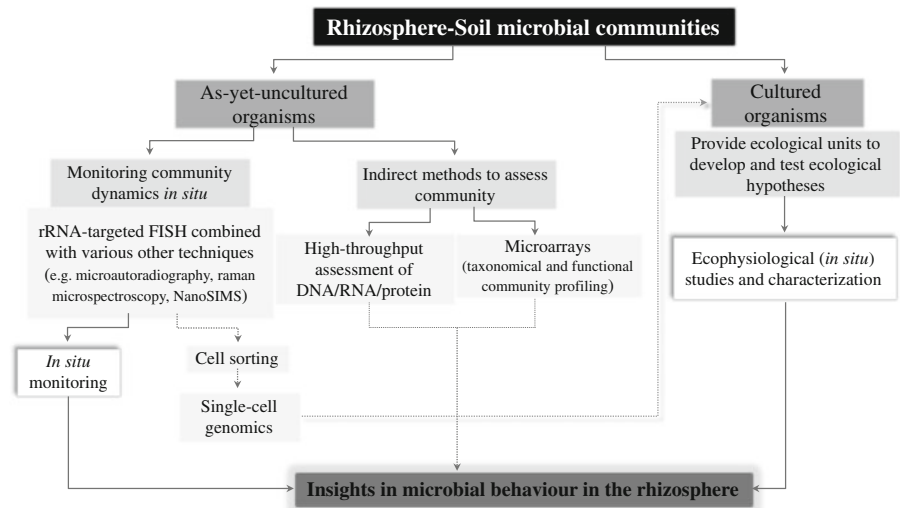
Bacterial behaviour in the rhizosphere

Despite the fact that it was historically thought to represent a cognitive process, which therefore would not be applicable to 'non-thinking' microorganisms, behaviour is an area in which theory and experimental microbial ecology are closely linked (Prosser et al. 2007). Indeed, bacterial inhabitants of the rhizosphere need to deal with a myriad of environmental factors in a spatially and temporally quite 'erratic' manner. Hence, it is the exact nature of the dynamism under such conditions that determines the specific, and also overall, behaviour of the local cell populations, which is primarily regulated at an individual signal threshold basis (Fig. 2). Given the complexity and heterogeneity of the habitat in space and time, it is very difficult to point to simple rules that govern the behaviour of microorganisms in the rhizosphere. Also, a plethora of life and survival strategies is known to occur among rhizosphere inhabitants. For instance, some bacteria, e.g. *Bacillus* types, have the capacity to enter the sporeform and thus survive the harsh conditions that often prevail in the rhizosphere. It is well known that a particular bacillary type, *Bacillus subtilis*, shows a differentiation process in the laboratory, yielding a small suite of physiologically-adapted forms, including sporulating, protease-secreting, polysaccharide matrix building and lysing cells (Bassler and Losick 2006; Shank et al. 2011; Chastanet and Losick 2011; McLoon et al. 2011). In a sense, the organism appears to show some kind of orchestrated group behaviour. Collectively, such bacilli thus may make up a

cooperating population. In fact, in laboratory studies, the organism has been indicated to enter a so-called bi-stable condition (Veening et al. 2008). However, we basically ignore to what extent this species (as well as other bacterial species out of the thousands present in one gram of rhizosphere soil) will follow similar or other patterns of differentiation in the rhizosphere. Well-know rhizosphere inhabitants, such as fluorescent pseudomonads, are not known to exhibit a differentiation process to the magnitude of that observed in *B. subtilis*, yet they do reveal intricate colony morphology changes in in vitro conditions (Spiers et al. 2002; Achouak et al. 2004; Bantinaki et al. 2007; McDonald et al. 2009; Lalaouna et al. 2012). These changes point to modifications at the cell surface as well as in major physiological processes. It is likely that such morphological/behavioural diversities in the two organismal groups are locally-determined and, given the great heterogeneity within the rhizosphere, one can envisage a plethora of diverse physiological responses of the same population at spatially different sites in the rhizosphere. Not to speak of the possibility of temporal differences, in accordance with shifting plant physiologies, root exudation and growth/death patterns.

Whatever group of rhizosphere bacteria we are studying, we in most cases do not understand the nature and functioning of the numerous signalling compounds that are often swapped within and across the microbial populations in the rhizosphere, nor do we understand the genetic regulatory mechanisms behind it. Such compounds are known to direct the
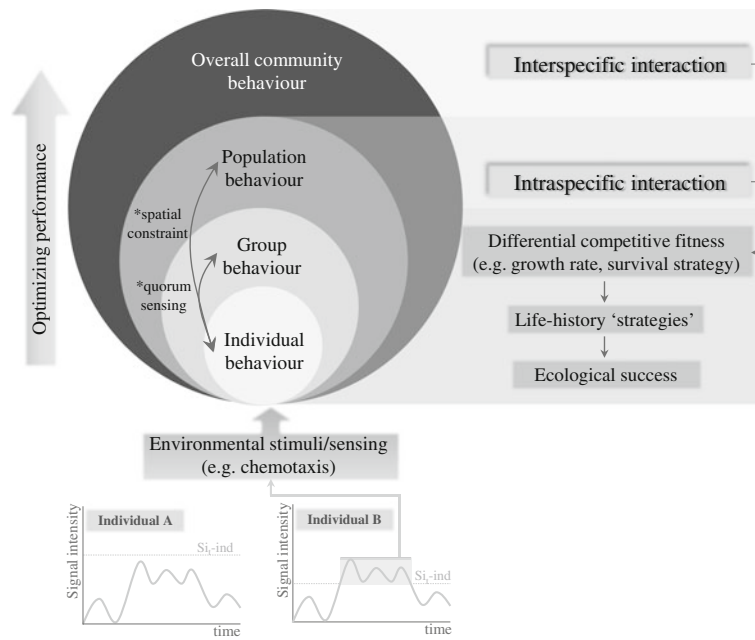
Fig. 2 Schematic representation of a microbial behavioural process, ranging from individual ecological units to overall microbial communities. Microorganisms possess a vast array of regulatory systems enabling them to sense and respond to many simultaneous environmental signals (e.g. from root exudates) at a determined threshold value, to optimize their performance. This unique capacity to monitor their surroundings is ultimately responsible for the fate of each individual population in the rhizosphere. Trade-offs in investment in cell growth and differentiation are responsible for the life history of a cell, leading to ecological success. Here, 'group' is defined as an aggregate of cells belonging to the same species. Abbreviation: Si_t-ind: signal intensity threshold at individual level

physiological responses of the rhizosphere inhabitants and thus have a great bearing on the community make-up and functioning. Moreover, there are cases of cross-talk and overhearing, in which neighbouring organisms perceive the presence of each other and respond, in a physiological sense, accordingly (Garbeva et al. 2011). Such processes are fundamental in the web of interactions within the rhizosphere, yet will remain unnoticed in all taxonomically-based assessments. Hence, we argue that a much stronger focus needs to be placed on studies in which interspecies interaction mechanisms are addressed, in order to elucidate how microorganisms perceive each other and what their physiological responses are when facing different organisms and signalling molecules. Such an effort will ultimately lead us to understand how physiological changes enhance (or not) the fitness of a group, leading to successful niche occupancy.

Multi-species interactions - sensing your neighbour

It is known that, in natural communities, microorganisms explore their biotic and abiotic environments by perceiving and responding to a wide range of chemical stimuli (Taga and Bassler 2003; Ryan et al. 2008; Shank and Kolter 2009; Straight and Kolter 2009). Indeed, cell surface localized sensing systems that microbes have evolved likely provide these with information on the identity of the neighbouring (micro- or macro-) organisms. Moreover, sensing systems have evolved that allow perceiving the surrounding chemical conditions (such as nutrient availability), indicating the possibilities for successful chemotaxis. The ability to continuously perceive signals from the environment, biotic or abiotic, may enable a microorganism, for instance in soil and rhizosphere, to differentiate between a 'good' and a 'bad' local condition, which might directly impinge on its survival strategy.

Recent behavioural studies with bacteria have revealed that some of these can alter their gene expression profiles when confronted, for instance, with other bacteria or fungi (Garbeva and de Boer 2009; Tai et al. 2009; Garbeva et al. 2011; Nazir 2012). As an example, Nazir recently showed that *Burkholderia terrae* strain BS001, in its interaction with the saprotrophic fungus *Lyophyllum* sp strain Karsten, upregulated a

selected set of genetic systems that were hypothesized to be important in the interaction (Nazir 2012). These included genetic systems that allow a close interaction with the fungus, like genes related to the type three secretion system, chemotaxis and biofilm formation. Furthermore, most characterized bacterial species harbour multiple two-component signal transduction systems that enable the coupling of a diverse array of adaptive responses to neighbouring macro- and micro-organisms and to abiotic environmental changes (Gao et al. 2007). For instance, *Pseudomonas* species, which are frequently isolated from the rhizosphere (as well as soil), possess a range of two-component signal transduction proteins. Totals of 212 such systems were found in *Pseudomonas fluorescens* Pf0-1, 223 in *P. fluorescens* Pf-5, 179 in *P. putida* KT2440 and 203 in *P. syringae* DC3000 (Garbeva et al. 2011). Such signal transduction systems have been described as sensing devices that allow rapid responses by the organisms to surrounding environmental influences and changes therein (Heeb and Haas 2001; Dubuis et al. 2007; Humair et al. 2010).

Different genes involved in metabolic strategies thus allow microbial species in soil and rhizosphere to respond to environmental cues and thrive (Table 1, class II). Overall, genes that are upregulated in cases of nutrient availability are involved in nutrient sensing, capture and metabolism. Those active during interspecific bacterial interactions in soil and the rhizosphere are often associated with the production of antibiotics, which might act as either a growth inhibitor or a chemical signal in cell-to-cell communication (Fong et al. 2001; Goh et al. 2002; Harrison et al. 2008; Garbeva and de Boer 2009; Wecke and Mascher 2011). This suggests that antibiosis is a key trait in common defence or offensive strategies in microbial interactions. In addition, genes conferring resistance against toxins were also found to be upregulated during competitive interactions (Dantas et al. 2008; Martinez 2008, 2009), suggesting possible protection against self-intoxication and/or intoxication from toxins produced by other member(s) of the community. Remarkably, recent studies have noted that several genes that are observed to be differentially expressed during interspecific interaction experiments matched as-yet-unknown functions (Garbeva and de Boer 2009; Garbeva et al. 2011). Thus, at present our understanding of species interactions and microbial behaviour in the rhizosphere may still be hidden in,

so far, cryptic gene regions. An overview of currently-available annotated bacterial genomes teaches us that, roughly, between 25 and 40 % of the coding regions of the genomes of soil bacteria refers to unknown functions. It is well possible that such functions play major roles in the establishment of bacteria in soil and rhizosphere and in their interactions with their biotic and abiotic environments.

In summary, the rhizosphere, much like soil systems, very often offers a carbon-limiting environment to the microbial inhabitants (Demoling et al. 2007; Rousk and Baath 2007). To better understand the interactions between the organisms in such systems, experiments must take into account the conditions that reign locally. Thus, in-depth studies focusing on the analysis of fundamental mechanisms and expression of genes underlying the local microbial interactions are urgently needed. For instance, competitive success of a strain can be assumed on the basis of the resistance of a strain to a competitor's toxin. This would reflect the ability of the strain to compete and establish a strategy like production of a counter antimicrobial compound. Besides, modulation of the expression of other genes related to essential cellular processes may also be required to sustain a microbial population and bring it in a required dynamic state. At this point, experimental tests allowing the evaluation of metabolic and physiological traits of mixed populations might offer great potential to reveal the relationships and behaviour of isolated strains. Ultimately, this will also guide us to a better understanding of the basic mechanisms that rule microbial community assembly and dynamics in complex systems, such as soil and the rhizosphere.

Outlook

On the basis of the foregoing arguments, it appears that a better focus on microbial behavioural studies, with organisms that can be studied in an ecophysiological manner, is required to achieve progress in our understanding of the rules that underlie the dynamics of the rhizosphere microbiota. Thus, fundamental questions regarding basic ecological principles on microbial community structuring, rather than holistic ones, can be answered. For instance, how do key rhizosphere inhabitants perceive their local environments and how does this change in time? What

organismal neighbours 'matter' and which ones do not? What type of physicochemical environment is favourable, and how do dynamic changes govern the establishment and survival of the organism? How does a rhizosphere organism obtain its nutrients and how do nutrient gradients evolve? What is the role of specific cooperation between microorganisms, for instance conferring strong stabilizing mechanisms acting on community make-up? How do horizontal gene transfer processes shape the key members of the rhizosphere microbiota and how dynamic is the genetic make-up of the latter? It is clear that one would need a deep understanding of the key organisms in the rhizosphere in terms of their ecophysiological responses to key triggers in the system. Thus, molecularly-based assessments, e.g. via direct mRNA (metatranscriptomics) or protein (metaproteomics) studies, might be one important way to make further progress. Such assessments could be performed even without the need to culture the organism. Conversely, we advocate that studies with isolated strains with respect to their behaviour in the rhizosphere, at the cell/population levels, would offer great benefits, improving our understanding of their ecophysiology and performance. For instance, the behaviour of the aforementioned *B. subtilis*, in terms of its potential differentiation in the rhizosphere, has remained totally unexplored. Such studies open up an avenue of possibilities to explore and test ecological theories, with a firm basis in microbiology, with respect to the rules that govern the colonization of the rhizosphere by local microorganisms. In the studies, the degree of diversity, richness and evenness of the community can be manipulated.

Unfortunately, our inability to cultivate the vast majority of the microorganisms in soil and rhizosphere has long been a factor limiting progress in this area, and the GPCA is still limiting progress. There is indeed a perceived need for studies that successfully isolate as-yet-uncultured strains from the rhizosphere, including more strains of the same species. This will allow us to obtain a broad view of what microbial types are successful in the rhizosphere. It will also enable the conduction of multi-species studies that pave the way towards an understanding of what drives the ecology and evolution of successful organisms (on the basis of their genomic contents) in the rhizosphere.

Clearly, the rapid emergence of ever more sophisticated DNA sequencing tools has yielded considerable progress in our understanding of the rhizosphere.

The resulting massive HTS studies of microbial communities in the rhizosphere will likely lead to an enormous database/inventory of microbial types that abound in the system. Such research efforts might seem to be driven merely because they are possible (Prosser 2012), but, in reality, are not without merit. As argued, applying the tools in time courses yields valuable information about the rise and fall of bacterial empires in the rhizosphere, and thus – if linked to so-called metadata – pinpoints the ecological conditions that favour each one of such populations. However, there is still an unavoidable inadequacy in the measurements of phylogenetic diversity, which hampers the attempts to correlate the data obtained and their usefulness to predict and model community structure and functionality. Such inadequacy lies in the inherent inability of such methods to deeply assess the behaviour of the individual components of the local microbiota that is assessed. Thus, additional small-scale studies are required which focus on those organisms that are suspected to be of key relevance in the rhizosphere and/or soil. Such studies can pinpoint the major drivers of rhizosphere community structure, leading to a better understanding of niche sorting and its dynamics in the rhizosphere. Ultimately, this will enhance our ability to predict the composition and ecophysiological behaviour of members of some of the key rhizosphere populations, with potentially great implications for successful application of microorganisms to the rhizosphere.

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