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Nazir, R.; Warmink, J.A.; Boersma, H.; van Elsas, J.D.

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Mechanisms that promote bacterial fitness in fungal-affected soil microhabitats

Rashid Nazir, Jan A. Warmink, Hidde Boersma & Jan Dirk van Elsas

Department of Microbial Ecology, Centre for Ecological and Evolutionary Studies (CEES), University of Groningen, Haren, The Netherlands

Correspondence: Jan Dirk van Elsas, Department of Microbial Ecology, Centre for Ecological and Evolutionary Studies (CEES), University of Groningen, Kerklaan 30, 9750RA Haren, The Netherland. Tel.: +31 5 0363 2151; fax: +31 5 0363 2154; e-mail: j.d.van.Elsas@rug.nl

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Abstract

Soil represents a very heterogeneous environment for its microbiota. Among the soil inhabitants, bacteria and fungi are important organisms as they are involved in key biogeochemical cycling processes. A main energy source driving the system is formed by plants through the provision of plant-fixed (reduced) carbon to the soil, whereas soil nitrogen and phosphorus may move from the soil back to the plant. The carbonaceous compounds released form the key energy and nutrient sources for the soil microbiota. In the grossly carbon-limited soil, the emergence of plant roots and the formation of their associated mycorrhizae thus create nutritional hot spots for soil-dwelling bacteria. As there is natural (fitness) selection on bacteria in the soil, those bacteria that are best able to benefit from the hot spots have probably been selected. The purpose of this review is to examine the interactions of bacteria with soil fungi in these hot spots and to highlight the key mechanisms involved in the selection of fungal-responsive bacteria. Salient bacterial mechanisms that are involved in these interactions have emerged from this examination. Thus, the efficient acquisition for specific released nutrients, the presence of type-III secretion systems and the capacity of flagellar movement and to form a biofilm are pinpointed as key aspects of bacterial life in the mycosphere. The possible involvement of functions present on plasmid-borne genes is also interrogated.

Introduction

The natural 'loose' cover of the earth's surface, known as soil, contains a large and complex community of living organisms (collectively coined the Living Soil). The soil biota as a whole plays an important role in the decomposition of soil organic matter and in nutrient cycling (Coleman *et al.*, 2004; Wardle *et al.*, 2004), which are key processes that determine soil fertility, productivity and global biogeochemical cycling. Next to bacteria, archaea and fungi, the living soil contains organisms such as protozoans, nematodes and higher organisms. Collectively, these organisms form a foodweb, in which organic material and energy are cycled. By their interaction with plant roots, some fungi – called mycorrhizae – act as providers of carbon and energy sources to the soil, whereas, on the other hand, other fungi (as well as bacteria) in soil are often involved in decomposition and mineralization processes. Thus, relevant carbonaceous compounds are continuously introduced into soil, cycled and plant nutrients are regenerated.

The biological diversity of the living soil is truly daunting, exceeding that found in most other habitats (Dance, 2008). In particular, the abundance and diversity of bacteria are high. Torsvik *et al.* (2002) calculated a prokaryotic, mainly bacterial, abundance of 4.8×10^9–2.1×10^{10} cells cm^{-3}, representing up to 8800 different species genomes, depending on the type of soil. Next to the bacteria, soil fungi are abundant and diverse in soil. Soil heterogeneity is clearly a main factor driving the enormous diversity of soil microbial life (Standing & Killham, 2007), and a range of microhabitats exist in soil that differentially select bacterial (or fungal) types. Figure 1 presents a schematic depiction of the conceptual microhabitats of importance for this review, i.e. the rhizosphere (narrow zone of influence of plant roots), the mycorrhizosphere (zone in soil that surrounds plant roots and fungal hyphae associated with these; Rambelli, 1973), the mycosphere (microhabitat that surrounds the dense fungal hyphae in soil that give rise to fungal fruiting bodies (definition used by Warmink & van Elsas (2008) and the bulk soil. In these microhabitats, key factors such as soil type and chemical

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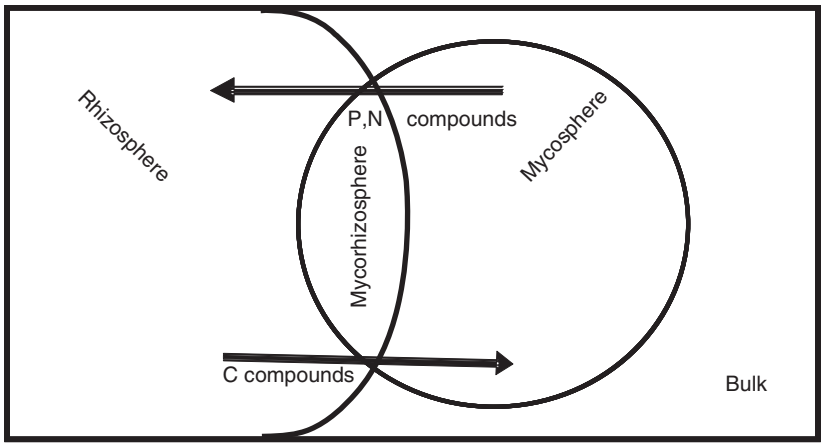

Fig. 1. Schematic description of microbial habitats in heterogeneous soil, and nutrient flow.

status, amount and type of nutrients, pH, moisture and plant or fungal factors such as species/type and age may affect the abundance, community composition and activity of the soil microbiota (Grayston *et al.*, 1998; Garbeva *et al.*, 2004).

We still understand very little of the specifics of the interactions between bacteria and fungi in soil, although a few excellent reviews have gathered the somewhat older information in the area (Johansson *et al.*, 2004; de Boer *et al.*, 2005). Since the publication of these reviews, a number of new observations have been made with respect to the mechanisms of bacterial–fungal interactions in soil. In this review, we examine the current knowledge of the interactions of soil bacteria with soil fungi, in particular mycorrhizal ones, concerning the mechanisms and ecological roles involved. Given the overwhelming role of plants in primary production and their connection to the mycorrhizae, we first briefly discuss the role of plants as major catalysts of the functioning of the living soil.

Plants as drivers of the soil microbiota and the role of mycorrhizae

In the period from the late Ordovician (460 million years ago) to the early Devonian (416 million years ago), land was increasingly colonized by plants (Redecker *et al.*, 2000; Gensel, 2008). Given the primary production by plants (del Giorgio & Cole, 1998) and the known release of carbonaceous compounds by plant roots, members of the soil microbiota would have 'learned' early on how to benefit from the carbon and energy sources that were becoming available in soil. We now know that the rhizosphere, i.e. the narrow zone of soil around plant roots, serves as a hot spot for microbial growth and activity, as it is where plant photosynthates become available for the soil microbiota in the form of root exudate compounds. Up to 30% of the total photosynthate produced by plants can be used by soil microorganisms for growth and cell maintenance (Walker *et al.*, 2003). The composition and quantity of carbonaceous substrates in root exudates may differ depending on the plant species, rhizosphere microsite location and plant growth stage (van Overbeek & van Elsas, 2008) and also on the nutritional requirements of plants themselves. This spatial and temporal variation in carbon availability considerably influences the structure and functioning of the rhizosphere-inhabiting microbial communities. Moreover, these are also affected by the genetic variation within a plant species (Rengel *et al.*, 1996).

A highly evolved strategy of soil microorganisms to directly capture plant-fixed carbon is the direct interaction with plant tissue. The complex structures of plant envelopes, composed of cellulose fibrils embedded in lignin matrices, can be successfully penetrated by the hyphal structures of, in particular, mycorrhizal fungi (Taylor & Osborn, 1996; de Boer *et al.*, 2005). In addition, bacteria that use the action of a specific type-IV and/or other (type-III) secretion system can intimately associate with plant tissue. The resulting interactive processes (including mycorrhizal symbioses and bacterial pathogenesis) have a commonality, i.e. the provision by plants of carbon compounds, such as sugars, to the microorganisms. The mycorrhizal fungi that are associated with plants can also modify plant root functions, for example by tinkering with root exudation (Marschner & Crowley, 1996). They may thus affect the carbohydrate metabolism of the plant (Shachar-Hill *et al.*, 1995) and also influence bacterial populations in the rhizosphere (Azaizeh *et al.*, 1995; Andrade *et al.*, 1998). Overall, mycorrhizal fungi function as 'extenders' of plant roots in the soil, allowing locally enhanced provision of carbonaceous nutrients.

Roles of bacteria and fungi in soil and their interactions

Roles

Given their involvement in key soil nutrient cycling processes, large numbers of specific fungi and bacteria are irreplaceably important for the growth and development of

plants (Poole *et al*., 2001; Johansson *et al*., 2004; Frey-Klett *et al*., 2007; Uroz *et al*., 2007). The roles of fungi in soil can be separated into three broad groups of functions, namely (1) saprotrophy, (2) animal/plant pathogenicity and (3) plant symbiosis (Finlay, 2007). Saprotrophy is certainly an important fungal role in soil, as fungi are largely responsible for the breakdown and recycling of plant material (litter), for example cellulose, lignocellulose and hemicellulose. Plant pathogenicity is another ecological role of certain soil fungi, with obvious consequences for the plant. Ecologically speaking, it temporarily enhances the release of C compounds into the soil microbiota. Finally, symbiotic soil fungi such as mycorrhizae are ubiquitous components of most soil systems throughout the world, playing key roles in plant and soil processes (Smith & Read, 1997; Founoune *et al*., 2002a). The roles of bacteria in soil are largely akin to those of fungi, and thus saprotrophy (saprophytic bacteria), pathogenicity and symbiosis can all be distinguished as defined roles for different bacteria. Concerning saprotrophy, the bacteria in soil are key organisms in the further decomposition steps of smaller molecules that are often produced by soil fungi, as well as in important steps of the nitrogen cycle, such as nitrification and nitrogen fixation.

Members of both the fungi and the bacteria also play roles in the maintenance of soil structure as a result of their cementing/aggregating action on soil particles. There are constraints posed to the extent of functioning of, in particular, nonhyphal soil bacteria by the soil matrix, which acts as a natural barrier to bacterial migration. However, specific groups of soil bacteria, classified as (filamentous) actinomycetes (*Actinobacteria*), can – much like the soil fungi – cross air-filled soil voids as a result of their hyphal/mycelial growth mode (Schafer *et al*., 1998). Another property that makes these filamentous organisms (in particular the fungi) successful in soil, is their ability to transport carbonaceous compounds over longer distances, allowing these to provide resources to distant cells in the hyphal matrix. Thus, nutrient-poor sites in the soil can be crossed by the hyphal network (Jennings, 1987). As mycorrhizal fungi form symbiotic structures with plant roots, this allows the latter to extend their sphere of influence in the soil. The mycorrhizal fungi, along with plants, are responsible for the release of various carbonaceous compounds into the soil environment, the mycorrhizosphere as well as mycosphere (Fig. 1). The compounds may vary from simple substrates to more complex molecules, which can be used by the soil bacteria, as well as other microorganisms, as carbon and energy sources (Bais *et al*., 2006).

Interactions

In soil, many bacteria and fungi will often occupy a shared microhabitat, which is hereafter called the bacterial–fungal interface (Johansson *et al*., 2004). Traditional studies have indeed revealed the presence of bacterial cells in the interface, for example on top of fungal hyphae and spores, on mycorrhized roots and in association with fungal fruiting bodies (de Boer *et al*., 2005). Thus, ample bacterial occupation of the bacterial–fungal interface has been shown. In the interface, the organisms are either ecologically neutral (inactive), they compete with or antagonize each other or, alternatively, they cooperate, in order to cope with the presence of the partner. Hence, interactions between the two partners in this interface may vary in accordance with their ecophysiology and the local conditions in the soil, as outlined in Fig. 2. Such putative interactions, for instance in the degradation of recalcitrant soil organic matter, have not yet been extensively investigated (de Boer *et al*., 2005), one of the reasons being that such studies in soil are inherently difficult. Moreover, most fungal-associated bacteria are as yet uncultured and therefore phenotypically still undescribed (Barbieri *et al*., 2005). However, for bacteria to cope with fungal-affected soil microhabitats, they need to at least survive under the local conditions established by the fungal partner. Moreover, and predictably, in cases in which beneficial conditions are established by the fungal partner (for instance, with respect to nutrient availability), it is likely that local bacteria are selected that optimized their mode of interaction with the fungus (Fig. 2), allowing them to dominate the fungal-associated communities.

Among the bacteria that occur at the interface, different roles, interactions with and effects on their host may thus be supposed. Although our understanding of these roles and interactions is increasing (Garbaye, 1994; Finlay, 2007; Frey-Klett *et al*., 2007), we still need to boost the knowledge of the types of ecological niches that are offered by the fungus to the bacteria. Clearly, the niches are primarily defined by the types and rates of release of the carbonaceous compounds present in fungal exudates (Toljander *et al*., 2007). In addition, the bacterial strategies that allow the efficient exploration of the niches have long remained enigmatic. The occurrence of often abundant bacteria on the surface of fungal hyphae lends credit to the assumption of a role of these bacteria in the system. Moreover, they may have the possibility to cross nutrient-poor spots in soil along with the extending fungal hyphae, and thus to gain access to distant nutrient resources (de Boer *et al*., 2005). In the following, our current understanding of the mechanisms and strategies involved in the bacterial–fungal interactions in soil is examined.

Fungi as selectors of bacteria in soil

Given the fact that saprotrophic, pathogenic as well as mycorrhizal fungi are all able to form hyphal networks, new interfaces are continuously being created by fungal activity

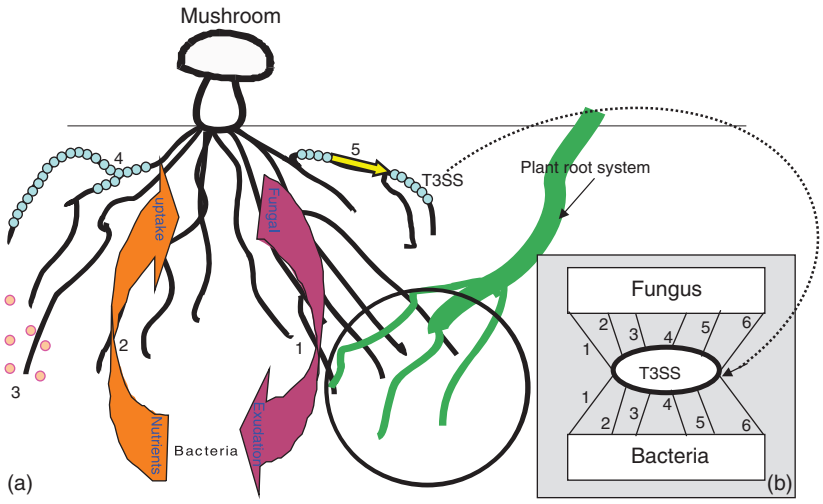

Fig. 2. Conceptual depiction of salient bacterial interactions with soil fungi and the strategies involved. Round circle represents the mycorrhizosphere to the left of which is the mycosphere. Interactions and mechanisms highlighted in the mycosphere are also expected in the mycorrhizosphere. (a) General ecological effects: 1, fungal exudation; 2, supply of phosphorus and nitrogen compounds to fungus; 3, change in microhabitat (e.g. pH); 4, bacterial biofilm formation; 5, migration along fungal hyphae. (b) Putative involvement of T3SS; 1, bacterial attachment to hyphal surface; 2, injection of effector proteins; 3, suppression of fungal defense system; 4, fungal exudation; 5, facilitation of migration along fungal hyphae; 6, bacterial biofilm.

in the soil. Particular bacteria in soil may thus become associated with all three functional groups of fungi. Given their capacity to continuously shunt part of the photosynthate of the plant partner to its hyphal network, the mycorrhizal fungi offer particularly suitable interfaces for heterotrophic soil bacteria. The conditions in the local microhabitats that are thus created in the mycorrhizosphere and mycosphere also imply that different interactive strategies will be required in the bacteria in order to gain a benefit from the newly emerged interfaces (Johansson *et al.*, 2004; Duponnois *et al.*, 2005; Frey-Klett *et al.*, 2007).

The mycorrhizosphere and the mycosphere

As outlined in Fig. 1, the mycorrhizosphere constitutes a specific microhabitat in soil that offers specific niches to adapted microbial soil inhabitants. It encompasses the mutual effects of plant roots and their associated fungal hyphae, resulting in a locally defined microhabitat shaped by the two partners. This microhabitat is often quite stable, because, as a result of their beneficial effects, mycorrhizal associants have become indispensable for many plants (Frey-Klett *et al.*, 2007). In contrast, the mycosphere may be more ephemeral, as it is strongly affected by the growth, aging and death of local fungal hyphae. For instance, underneath freshly formed mushrooms, dense hyphal networks can be discerned that provide new colonization sites for soil bacteria. Following aging, such sites may show a shifted chemistry in terms of the compounds that are present. Thus, particular carbonaceous compounds may become available in a dynamic fashion at these sites, spurring bacterial growth and survival. The conditions in this microhabitat are likely shaped, in different ways, by both the fungal and the bacterial partners. The ecological effects of the mycorrhizosphere and mycosphere on local soil bacteria may be beneficial, neutral or deleterious, depending on how the local conditions are affected by the fungus. Whereas in the former case the provision of carbon sources is a key factor, in the latter case the local conditions may be turned hostile to bacteria by the release of antibacterial compounds, thus limiting bacterial growth and/or survival. Hereafter, we examine the state of the art of our knowledge on how mycorrhizal fungi affect bacterial assemblages in soil.

Bacterial communities in the mycorrhizosphere and mycosphere

In-depth analysis of bacterial communities present in the mycorrhizosphere and of bacterial interactions with mycorrhizal fungi basically started in the 1990s (Tylka *et al.*, 1991; Garbaye, 1994; Toro *et al.*, 1996; Budi *et al.*, 1999). These early studies already showed that mycorrhizal fungi can profoundly influence the mycorrhizosphere-inhabiting bacteria and, vice versa, soil bacteria may locally exert an influence on their fungal host (Johansson *et al.*, 2004). Recently, the arbuscular mycorrhizal fungus (AMF) *Glomus mosseae* was found to significantly stimulate bacterial communities in soil, in particular *Paenibacillus* sp. and uncultured *Gammaproteobacteria* (Artursson *et al.*, 2005). That mycorrhizal fungi are major factors shaping bacterial communities in the grass mycorrhizosphere was convincingly shown by Singh *et al.* (2008). These authors examined the bacterial and AM fungal assemblages on grass roots and concluded that AM fungi were indeed major determinants of the local bacterial assemblages. In this context, mycorrhizal fungi often release substances such as α-ketoglutaric acid that, in addition to solubilizing phosphate from surrounding minerals, also affect the local microbial communities (Duponnois *et al.*, 2005) by stimulating their growth. Thus, bacteria associated with mycorrhizal fungi are likely driven by the fact that suitable carbon and energy sources are provided and colonization sites are available. In retribution, soil bacteria may be involved in activities that provide benefits for the fungus such as the aforementioned phosphate

solubilization, nitrogen fixation and the acquisition of minerals. In particular cases, there may even be specificity between the fungus and the associated bacteria. Artursson *et al.* (2006) discussed that specific bacteria were activated in the mycosphere by fungal exudates. Mansfeld-Giese *et al.* (2002) investigated the culturable bacterial communities in the mycorrhizosphere of cucumber colonized or not by *Glomus intraradices*. The results showed that *Paenibacillus* spp. were more frequently found in the mycorrhizal treatment, indicating a close association of these bacteria with the fungal host. *Glomus intraradices* was also found to alter the population density of different bacteria, as for example *Pseudomonas chlororaphis* increased as a result of the presence of the fungus whereas *Pseudomonas putida* did not (Mansfeld-Giese *et al.*, 2002). On the other hand, no significant differences between mycorrhizal and nonmycorrhizal treatments were found in total bacteria (mainly encountered were *Pseudomonas*, *Arthrobacter* and *Burkholderia*). During an investigation of the bacterial diversity in the mycorrhizosphere of *Medicago trunculata*, strains belonging to the *Oxalobacteriaceae* were found to be more abundant in mycorrhizal than in nonmycorhizal roots (Offre *et al.*, 2007, 2008).

Studies of the bacterial communities in the mycosphere at the base of fungal fruiting bodies are quite recent. Warmink & van Elsas (2008) showed that the bacterial numbers in the mycosphere of the ectomycorrhizal fungus *Laccaria proxima* can be significantly higher than those in corresponding bulk soil. Using cultivation-independent and cultivation-based analyses, they also reported the selection of specific bacterial groups in the mycosphere. On the basis of both approaches, particular pseudomonads were shown to be selected by the fungus, whereas on the basis of cultivation, the following four other bacterial genera were also found to be selected (Warmink & van Elsas, 2008): *Variovorax*, *Chryseobacterium*, *Arthrobacter* and *Mycobacterium*. Later, Warmink and colleagues (2009) extended the number of fungi examined and again found an enhancement of the numbers of culturable bacteria in the vicinity of fungi, thus reinforcing the concept of selective force exerted by the fungi on the soil bacteria that are locally present. The cultivation-independent part of this study (Warmink *et al.*, 2009) supported the viewpoint that the mycosphere indeed exerts – in most cases – a selective effect on particular soil bacteria, which show some diversity. Hence, the fungal selective effect is not only widespread, but it also appears to affect members of a limited number of bacterial types. For instance, clear selective effects were found to be exerted by the fruiting bodies of the ectomycorrhizal fungi *L. proxima* and *Russula exalbicans* growing in forest soil on members of the *Sphingomonadaceae* (Boersma *et al.*, 2009). Using 16S rRNA gene-based analysis, these authors reported that the major *Sphingomonas* groups from the examined mycospheres did not cluster with *Sphingomonadaceae* in the public databases, which indicates that novel groups of this family are present in these poorly investigated environments. Interestingly, similar bacterial community structures were observed for the same fungal species from different sampling sites, whereas the corresponding bulk soil communities differed from each other. This indicates a strong fungal selective effect on similar groups of soil bacteria. Furthermore, the two fungi selected different bacteria of the same family, indicating that different fungi exert different selective forces on soil bacteria. In line with this contention, different bacteria may behave differently in their association with (by attachment) fungal hyphae. Toljander *et al.* (2006) studied five different bacterial strains and two *Glomus* species and found that the ability of bacteria to adhere to the fungal tissue depended on the hyphal activity and on the type of fungal species. Very recently, Levy *et al.* (2009) reported the specific association of members of the genus *Burkholderia* – especially *Burkholderia pseudomallei* – with AM fungal spores in soil. However, no data on the specific adherence/interactive behavior of these bacteria with the fungal partner were reported.

Effects of bacteria on (mycorrhizal) fungi in soil

A range of bacterial effects on fungi in soil is possible. In particular, the mycorrhization of plant roots is often affected by the bacteria that are locally present (Garbaye, 1994; Frey-Klett *et al.*, 2007; Pivato *et al.*, 2009) in either positive, neutral or negative ways. A key issue is the positive effect of some soil bacteria on mycorrhizae. During the free-living stage, mycorrhizal fungi may interact with specific bacterial populations in the rhizosphere, and such bacteria (especially pseudomonads) may enhance mycorrhizal establishment (Garbaye, 1994; Pivato *et al.*, 2009). Hence, these bacteria are called mycorrhization helper bacteria (MHB; Garbaye, 1994). The MHB can increase the mycorrhization of the plant from 1.2 up to 17.5 times (Frey-Klett *et al.*, 2007). MHB are not plant-specific, but are rather selective for the fungal species (Garbaye, 1994; Pivato *et al.*, 2009). Different mechanisms by which MHB enhance the mycorrhization of the plant have been hypothesized. Bianciotto *et al.* (1996) proposed a two-step mechanism for the physical interaction of bacteria with fungal hosts, as follows: weak bacterial–fungal binding may be operational during the first stage of the interaction, which is governed by general physicochemical parameters, such as electrostatic attraction. In a second stage, more stable binding may ensue, involving attachment and the production of bacterial extracellular polymers. To support this hypothesis, they studied bacterial mutants inhibited in the production of extracellular polysaccharides (Bianciotto *et al.*, 2001). These mutants were less able to attach to the fungal surface compared with the wild-type strain, indicating the importance of an active

bacterial adhesion process. According to Deveau *et al.* (2007), the MHB *Pseudomonas fluorescens* BBc6R8 promotes the presymbiotic survival and growth of the ectomycorrhizal fungus *Laccaria bicolor* S238N in soil. Specifically, the bacterium increases the radial fungal growth, hyphal apex density and branching angle. These changes are coupled with pleiotropic alterations of the fungal transcriptome. *Pseudomonas fluorescens* BBc6R8 thus induces a shift in mycelial physiology, from saprotrophy to the so-called presymbiotic status (Deveau *et al.*, 2007). Moreover, *P. fluorescens* BBc6R8 was shown to be able to affect mycorrhization through the improvement of fungal viability, especially when the fungus is growing under unfavorable conditions (Brule *et al.*, 2001).

During mycorrhization, the proliferation of bacteria in the rhizosphere before the symbiosis can improve the receptivity of the roots to mycorrhizal formation (Aspray *et al.*, 2006). Such proliferating bacteria may also promote growth of the fungus in its saprotrophic state in the soil or at the root surface, triggering or accelerating the germination of fungal propagules in soil (Garbaye, 1994). In this respect, Tylka *et al.* (1991) suggested that certain volatile compounds produced by soil bacteria (in this case *Streptomyces* spp.) positively influenced the germination of AM fungal spores. In later work, particular compounds, such as auxofurans, were found to be produced during the cocultivation of *Streptomyces* sp. and the fungus *Amanita muscaria*. These compounds are probably released by bacteria and positively affect fungal development. Auxofuran has recently been shown to indeed affect fungal metabolism, as it stimulated lipid metabolism-related gene expression (Riedlinger *et al.*, 2006).

The MHB effect is usually measured by assessing the ergosterol contents of the mycorrhizospheric soil. Founoune *et al.* (2002a) observed a significant increase in the ergosterol contents of fungal plugs taken from the mycorrhizosphere, resulting from bacterial coinoculation. The bacteria – introduced together with the fungal symbiont – stimulated the growth of the fungus as well as the production of phenolic compounds and ectomycorrhiza formation, along with significant effects on plant shoot and/or root mass (Founoune *et al.*, 2002b). Bharadwaj *et al.* (2008) also reported that AM fungal root colonization increased up to ninefold in the presence of associated bacteria. The introduced bacteria were also found to significantly increase the gallic acid content of fungal plugs and to stimulate radial growth of the fungus compared with the control (Founoune *et al.*, 2002b).

The presence of *P. putida* was also shown to be necessary for the initiation of fruiting body formation in the fungus *Agaricus bisporus* (Rainey *et al.*, 1990). The exact mechanism behind this phenomenon was not determined, but it has been suggested that the fungal mycelium produces self-inhibiting compounds, which are removed by the associated bacteria. There are also several other reports about the stimulation of fungal spore germination by spore-associated bacteria (de Boer *et al.*, 2005).

Another effect of soil bacteria on fungi is based on the release by bacterial cells of compounds such as C, N and/or P sources during the interaction. Some organic acids excreted by MHB represent carbon sources that are as good as glucose for fungal growth (Duponnois & Garbaye, 1992). Moreover, the provision by MHB of reduced nitrogen acquired via nitrogen fixation is also likely to play a stimulatory role in growth or mycorrhization by mycorrhizal fungi (Garbaye, 1994). Early research had already shown the presence of nitrogen-fixing bacteria in the fruiting bodies of different ectomycorrhizal fungi (Spano *et al.*, 1982). This suggested a role for these bacteria in nitrogen provision, supporting fungal growth during ascocarp development. Also, phosphate-solubilizing bacteria that associate with AM fungi may access the soil phosphate sources by the excretion of phosphatases and/or organic acids (Artursson *et al.*, 2006). There are several indications of other mutualistic relationships between soil fungi and their associated bacteria. This issue has been reviewed by de Boer *et al.* (2005) and will not be further treated here.

Finally, fungal-associated bacteria can also play roles in detoxification of the fungal microhabitat. For instance, such bacteria may remove fungal-released waste products, or change the pH and the level of siderophores, facilitating mycorrhizal growth and colonization (Garbaye, 1994). A particular case is formed by the bacteria associated with decaying wood (Clausen, 1996).

Effects of (mycorrhizal) fungi on their associated bacteria

As argued in the foregoing, the main mechanism underlying the effect of mycorrhizal fungi on soil bacteria is nutritional, i.e. bacteria may benefit from the fungal partner by obtaining resources from it. In a recent review, Leveau & Preston (2008) described three ways by which soil bacteria achieve this: (1) extracellular necrotrophy – nutrient release by local killing of fungal cells, (2) extracellular biotrophy – nutrients becoming available due to the release by actively growing fungal hyphae and (3) endocellular biotrophy – existence of bacteria inside fungal hyphae. Examples of all three mechanisms are known in mycorrhizospheres and mycospheres. In addition, fungi may modify root exudates or serve as vectors for migration through soil, as examined below.

The mycorrhizosphere

The establishment of mycorrhizal fungi in the plant rhizosphere, yielding a mycorrhizosphere, exerts a range of positive or negative effects on the local soil bacteria. First,

the fungus can change the chemical composition of the root exudates, which contain resources for root-associated bacteria (Artursson *et al.*, 2006). Thus, the selective force exerted on local bacterial communities is changed. In addition, additional nutrients may be released by the fungus, on which local bacteria are able to grow. Filion *et al.* (1999) investigated the interaction between the AM fungus *Glomus intraradices* and soil microorganisms, including bacteria and other fungi. As a result of the fungal presence, they found an increased growth of *P. chlororaphis* as well as germination of *Trichoderma* conidia. On the other hand, the germination of *Fusarium* conidia was reduced in the presence of AM fungal extract (Filion *et al.*, 1999). The results obtained by Frey *et al.* (1997) suggested that the ectomycorrhizal fungus *L. bicolor* releases trehalose into the mycorrhizosphere, thus exerting nutrient-mediated selection on the local bacteria, including fluorescent pseudomonads. In another study, the number of fluorescent pseudomonads and their metabolic activities were significantly affected in the mycorrhizosphere of *G. intraradices* with or without mineral phosphate amendments (Duponnois *et al.*, 2005). The release of soluble fungal storage sugars such as trehalose as well as polyols such as mannitol has thus been suggested as the mechanism behind the selection of fungus-associated bacteria by the ectomycorrhizal fungus. Organic acids may also contribute to the selection (de Boer *et al.*, 2005).

With respect to potential negative effects exerted by soil fungi on the fungus-associated bacteria, the exudation of inhibitory chemicals by mycorrhizal fungi has been invoked as a key mechanism. In this respect, the exudation of antibiotics may have, next to negatively affecting antibiotic-sensitive bacteria, spurred the selection of fungus-specific antibiotic-resistant bacteria (de Boer *et al.*, 2005).

The mycosphere

Currently, data on the mechanisms behind the effects of the mycosphere on associated bacteria are sparse, and, until very recently, our understanding of mycosphere bacterial communities has mostly been based on studies of culturable bacteria. For instance, typical fluorescent pseudomonads were found in the mycosphere of *Cantharellus cibarius* (Rangel-Castro *et al.*, 2002a). The authors hypothesized that the selection was based on the utilization of the trehalose and mannitol, which they found to be secreted by the fungus (Rangel-Castro *et al.*, 2002b). In their study, compounds such as erythritol, arabitol and amino acids such as glutamate and asparagine were found to be secreted by the fungus (Rangel-Castro *et al.*, 2002b). Furthermore, Sahin (2003) observed that *Methylobacterium* spp. from the mycosphere were particularly able to degrade oxalic acid (a compound often exuded by mycorrhizal fungi), while Timonen *et al.* (1998) reported fructose and mannitol as the selective agents for *Pseudomonas* spp. in the myco(rrhizo)sphere of *Paxillus involutus* and *Suillus bovinus*, respectively. Without knowing the exact mechanism of selection, Warmink & van Elsas (2008) found *P. fluorescens*, *Chryseobacterium piscium* and *Mycobacterium* sp. to be specifically selected among the culturable bacteria associated with *L. proxima*. Later, Warmink *et al.* (2009) showed the selection of bacteria in the mycosphere of different fungi and introduced the concept of universal 'fungiphiles' (bacteria adapted to the use of common fungal exudates as carbon sources and found in two or more mycospheres) vs. species-specific fungiphiles (bacteria presumably adapted to the use of unique fungal exudates and found in one specific mycosphere).

On the other hand, the information on the quality and quantity of the carbonaceous compounds that are released by fungi in the surrounding mycosphere is still limited. Hence, the idea of a substrate-mediated selection of bacteria by fungi still needs experimental support. For instance, Olsson *et al.* (1996) found no support for the hypothesis that the mycelia of ectomycorrhizal fungi can stimulate the growth of bacteria via carbon exudation. Furthermore, for AM fungi, it has been suggested that the effect of fungal exudates on the bacterial populations is qualitative (i.e. related to species and strain composition) rather than quantitative (Andrade *et al.*, 1997).

Very recent work shows that fungal hyphae growing through the soil can create novel hospitable microhabitats for local soil bacteria (Warmink & van Elsas, 2009), a phenomenon also seen with growing plant roots (Marschner *et al.*, 2001). The mycosphere formed by the fungus upon movement through the soil was shown to exert significant selective effects on particular bacteria that had been added to the soil (Warmink & van Elsas, 2009). Such bacteria were apparently attracted toward these sites, being able to colonize them up to the presumed carrying capacity. Kohlmeier *et al.* (2005) analyzed the capacity of soil fungi to serve as vectors for the dispersion of specific pollutant-degrading bacteria and found that this vector action is possible for a selection of these. Bacterial motility was absolutely necessary for this phenomenon, showing the key role of bacterial movement along fungal hyphae (denoted the fungal 'highway').

Besides the release of nutrients and the provision of a fungal highway, soil fungi can also affect the associated bacteria by local pH changes, the secretion of inhibitory or stimulatory compounds and/or adaptations of the soil structure (Johansson *et al.*, 2004).

Endomycotism

A very interesting finding has been the (obligate) endomycotic occurrence of specific bacteria (Bonfante & Anca, 2009). Such endomycotic occurrence has been observed in a range of fungal species belonging to the AM (Salvioli *et al.*, 2008), ectomycorrhizal (Bertaux *et al.*, 2005) and plant

pathogenic fungi (Partida-Martinez & Hertweck, 2005). It was also found recently that in the rice seedling blight fungus *Rhizopus microsporus*, particular *Burkholderia* spp. were responsible for the production of a potent toxin. Partida-Martinez *et al.* (2007b) cured the fungus from the bacterium and found that, in the absence of the endosymbiont, the fungal host was incapable of vegetative reproduction. Even the addition of crude extracts from symbiont cultures did not induce sporulation of the cured fungus. The formation of sporangia and spores was only restored upon reintroduction of the endobacterium. Hence, reproduction of the fungal host was dependent on endobacteria, which also provided a toxin for defending the habitat and accessing nutrients from decaying plants (Partida-Martinez *et al.*, 2007b). Such a persistent association is also found for the AM fungus *Gigaspora margarita* and the endobacterium '*Candidatus Glomeribacter gigasporarum*'; here, it was found that the endobacteria have positive impacts on fungal fitness during the presymbiotic phase (Anca *et al.*, 2009). In this specific system, bacterial cell division is dependent on fungal metabolism. The authors analyzed an *ftsZ* (a marker gene for bacterial division) clone and found that this gene is highly expressed during extraradical extension of mycelia, when the fungus was associated with the plant (Anca *et al.*, 2009). Endosymbiotic *Burkholderia* cells were also found to be responsible for the production of the phytotoxin 'rhizoxin' (Partida-Martinez & Hertweck, 2005) as well as the mycotoxin 'rhizonin'(Partida-Martinez *et al.*, 2007a). The mechanisms by which the *Burkholderia* symbionts invade fungal cells are still unknown. However, Valdivia & Heitman hypothesized (2007) that effector proteins of *Burkholderia* translocated by a type III secretion system (T3SS) control a range of interactions of the bacterium with its fungal host. In the *Rhizopus–Burkholderia* system (Partida-Martinez & Hertweck, 2005; Partida-Martinez *et al.*, 2007a, b), it was obvious that the fungal host benefits from the biosynthetic capabilities of its endosymbiont in order to access particular nutrient sources. The endosymbiosis may have become possible through a parasitism-to-mutualism shift, in which a hypothetical zygomycotic ancestor of *R. microsporus* developed resistance against the bacterial antimitotic agent 'rhizoxin,' enabling a bacterial–fungal alliance against rhizoxin-sensitive rice seedlings for mutualistic nutrient acquisition (Schmitt *et al.*, 2008).

Bacterial mechanisms that enhance mycosphere competence

Bacteria that interact with hosts such as mycorrhizal fungi may depend on a range of particular mechanisms for ecologically successful interactions (Table 1). Evident bacterial capacities such as the ability to contact and interact with the fungal host and to deal with the specific resources that become available in the mycorrhizosphere and mycosphere are likely to be consistent myco(rhizo)sphere competence features. Here we examine our current understanding of these competence-enhancing capacities.

Bacterial movement via chemotaxis toward or along with fungal hyphae

Next to resource capturing and utilization, there are clearly other mechanisms that determine the ecological success of bacteria interacting with soil fungi (Table 1). Bacterial motility and chemotaxis, for instance, are thought to be involved. Chemotaxis toward fungal hyphae has been observed in several studies (de Weert *et al.*, 2004; Kamilova *et al.*, 2008; Warmink & van Elsas, 2009). Kohlmeier *et al.* (2005) revealed that the movement of bacteria through soil, allowing them to occupy the microhabitats at the fungal hyphae, occurs by virtue of a thin water layer that surrounds the fungal hyphae. This viewpoint has recently been experimentally supported by Warmink & van Elsas (2009), who observed the migration of bacteria from an inoculation spot at a hyphal growth front in soil microcosms to a distant spot, in the form of a biofilm around growing hyphae of the saprotroph *Lyophyllum karsten*. A simplifying model of this biofilm-mediated movement is shown in Fig. 3. It is likely to involve motility, attachment, growth and possibly swarming motility phases. In support of the role of motility, Kohlmeier *et al.* (2005) observed that intrinsic (swimming and/or swarming) motility of the bacteria was required for bacterial translocation along fungal highways, as only their flagellated bacterial strains could move along the hyphal surface. On the other hand, Warmink & van Elsas (2009) reported that not all flagellated bacteria could move through soil with growing hyphae of *L. karsten*. Hence, motility was clearly not the only factor required for successful migration. It has been suggested by Sen *et al.* (1996) that *P. fluorescens* strains interacting with soil fungi could use their polar flagella to anchor to fungal hyphal surfaces. Toljander *et al.* (2006) conducted an experiment on soil bacteria tagged with green fluorescent protein to analyze the variability of bacterial attachment to AM fungal extraradical hyphae. They concluded that bacteria differ in their ability to colonize vital and nonvital hyphae and attachment is also influenced by the fungal species involved (Toljander *et al.*, 2006). As bacterial motility is positively – albeit one-sided – correlated with the ability to comigrate with the growing fungal partner, a role for chemotaxis is indicated (Warmink, 2009), as theoretically, nutrients should be available on/around vital fungal hyphae that are extending right behind the tip.

Capacity to utilize particular fungal-released nutrients

In purified sand, fungal hyphae were found to significantly increase the numbers of associated bacteria (de Boer *et al.*,

Table 1. Possible mechanisms involved in bacterial–fungal interactions

Fungal partner	Associated bacteria	Mechanism involved	Remarks	References
AM fungus	*Rhizobium leguminosarum* and *Azospirillum brasilense*	Physical attachment; electrostatic attraction and extracellular polymers	Preliminary evidence Experimentally proven	Bianciotto *et al*. (1996) Bianciotto *et al*. (2001)
Glomus mosseae	Different AMB	Bacterial multifunctionality*	Circumstantial evidence	Bharadwaj *et al*. (2008)
Heterobasidion annosum	Different bacteria	Growth factors	increased efficiency of white rot fungi	Murray & Woodward (2003)
Glomus sp.	Different bacteria	Fungal exudates	more bacterial species in the mycosphere than in the rhizosphere	Andrade *et al*. (1997)
Basidiomycetous fungi	Pseudomonads	Fungal exudates	Fungal-specific compounds utilized in biolog assay	Warmink *et al*. (2009)
Agaricus bisporus	*Pseudomonas putida*	Removal of self-inhibiting compounds	Experimental evidence	Rainey *et al*. (1990)
Tuber brochii	*Pseudomonas fluorescens* and *Bacillaceae*	Chitinolytic and cellulolytic weakening of spore wall	Enzymatic and EM analysis	Citterio *et al*. (2001)
AM fungi	Different bacteria	Change in pH	Hypothetical mechanism	Johansson *et al*. (2004)
AM fungi	*Streptomyces* sp.	Production of volatile compounds	Growth stimulus	Tylka *et al*. (1991)
Lyophyllum karsten	*Burkholderia terrae*	Biofilm formation	Bacterial migration along fungal hyphae	Warmink & van Elsas (2009)
Laccaria proxima	Different bacteria	T3SS	Mycosphere selection of T3SS harboring bacteria	Warmink & van Elsas (2008)

*Multifunctionality means the production of various extracellular enzymes and bioactive compounds. Thus, bacteria may perform a multitude of functions, for example growth inhibition of pathogens, mycorrhization and plant growth promotion.
AM, arbuscular mycorrhizal (fungus); AMB, 'arbuscular mycorrhizal bacteria' (bacteria associated with AM fungi); EM, electron microscopy.

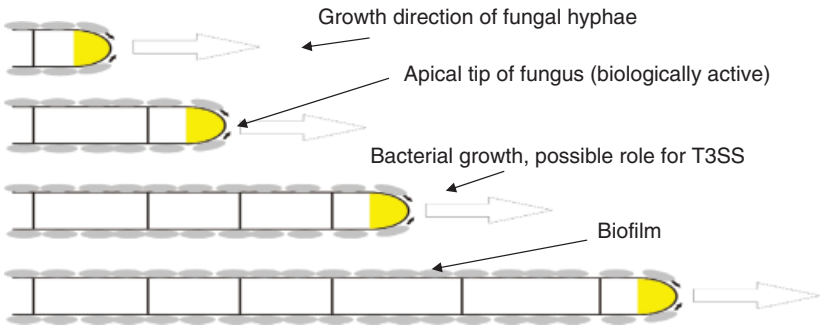

Fig. 3. Hypothetical model proposed for bacterial movement on fungal hyphae.

2001; Mansfeld-Giese *et al*., 2002), suggesting the acquisition by the bacterial community of nutrients from the fungus (Leveau & Preston, 2008). The fungal exudates may have a qualitative and/or a quantitative impact on the bacterial community. The growth of *P. chlororaphis* was stimulated in the presence of an extract of a culture of the AM fungus *G. intraradices* (Filion *et al*., 1999). Van Hees *et al*. (2006) reported oxalate and ferricrocin as the main compounds identified in the exudates of the ectomycorrhizal fungus *Hebeloma crustuliniforme* in symbiosis with *Pinus sylvestris*. The oxalate exudation rate was as high as 19 fmol per hyphal tip h^{-1} or 488 fmol per hyphal mm^2 h^{-1}. They also identified malonate and acetate in fungal exudate, albeit in lower amounts than oxalate (van Hees *et al*., 2006). Oxalate and acetate were also found, next to carbohydrates and peptides, in material released by the ectomycorrhizal fungus *S. bovinus* (Sun *et al*., 1999). Their analysis showed inositol, xylitol, mannitol and ribose among the main sugars and polyols. Oxalate or oxalic acid may feed bacteria as there are several bacteria reported as oxalotrophs (Sahin, 2003), whereas mannitol-specialized bacteria have also been found, in this case in association with *S. bovinus* growing in soil (Timonen *et al*., 1998). Glycine, glutamic acid and aspartic acid were the main amino acids present in fungal exudates examined by Sun *et al*. (1999). Toljander *et al*. (2007) reported formiate, acetate, α and β glucose, and glycogen, along with di- and oligosaccharides and some polymeric compounds, in the exudates of *Glomus* sp. MUCL 43205. Thus, one can posit that mycosphere-adapted bacteria utilize a range of specific compounds that are made available

by fungal hosts in the vicinity of their hyphae. The mycelial exudates were shown to not only increase bacterial growth and vitality but also influence the bacterial community compositions (Toljander *et al.*, 2007). This suggested that some bacteria preferentially utilized different compounds available in exudates. Warmink *et al.* (2009) analyzed the potential utilization of fungus-related compounds by mycosphere vs. soil pseudomonads using the BIOLOG assay. They then correlated the utilization of potentially fungal-released compounds as carbon sources with bacterial habitat, and posited that preferential resource utilization might be a key selective mechanism in the fungal niche. Recently, H-nuclear magnetic resonance analysis of fungal compounds produced by *L. karsten* revealed that this fungus releases, next to some other compounds, glycerol as a main carbonaceous compound. This glycerol is preferentially utilized as a carbon and energy source by the mycosphere-specific bacterium *Variovorax paradoxus* related strain HB44 (F. G. H. Boersma, unpublished data), as glycerol peaks were completely absent from the fungal exudate medium in which strain HB44 had grown. To capture and utilize resources from the fungal partner, particular enzyme complexes may be necessary for the fungal-associated bacteria (de Boer *et al.*, 2005). Thus, the efficient use of such enzyme systems to obtain essential energy and carbon sources from the fungal partner emerges as a key mechanism involved in the bacterial interaction with soil fungi.

Change of conditions of the local microhabitat

Specific effects exerted by soil fungi on the microhabitat at the fungal hyphae will also affect the local bacteria. For instance, the microhabitat pH may be changed by the fungus by a change in the balance of extruded protons or anions. Thus, given pH changes to extreme or moderate values, either inhibitory or even stimulatory effects may be exerted on the local bacterial communities (Johansson *et al.*, 2004; F. G. H. Boersma, unpublished data). Recently, Singh *et al.* (2008) also reported that bacterial assemblages in the mycorrhizosphere are affected by the local pH. Their results also suggest that the relationship between bacterial and fungal assemblages might be influenced, to some degree, by soil pH (Singh *et al.*, 2008). In addition, changes in the structure of the local (soil) habitat (for instance by the production of extracellular polysaccharides) by either of the partners (Andrade *et al.*, 1997) and/or the production of antibacterial substances by the fungal partner (de Boer *et al.*, 2005) may play pivotal roles. Moreover, the study by Singh *et al.* (2008) also showed that the fungal rhizosphere assemblages were influenced by plant species, whereas the bacterial ones were not. This suggested an effect of local environment as a result of either soil, plant and/or the interaction among the two microbial groups (Singh *et al.*, 2008). Common to all these effects is the paradigm that when the habitat changes, the mode by which local bacteria colonize the changed habitat will also undergo changes. This bacterial adaptation may also involve bacterial signaling such as in quorum sensing (QS), which is an effector of a broad range of bacterial activities with environmental relevance, including colonization of a substrate (Miller & Bassler, 2001). For instance, QS has been found to play a role in the interaction of the soil bacterium *Rhizobium* sp. in its symbiosis with plants (Daniels *et al.*, 2002; Pongsilp *et al.*, 2005). However, in spite of the likelihood of its involvement, there is no current evidence for the role of QS activity among bacteria inhabiting the myco(rrhizo)sphere.

Bacterial protein secretion systems

Bharadwaj *et al.* (2008) reported a set of 10 different bacteria isolated from the spores of AMF to be potentially multifunctional in the mycorrhizosphere. In detail, they showed that the production of diverse specific extracellular enzymes and bioactive compounds forms the basis for this multifunctionality (Table 1). This indicates the importance of bacterial protein secretion systems in habitats associated with mycorrhizal fungi. Such systems affect the translocation of proteinaceous macromolecules from the cytoplasm across the membrane(s) onto the surface of the bacterial cell or into the extracellular environment, which may include fungal cells. There are six distinct protein secretion systems in Gram-negative bacteria, designated types I through VI, while Gram-positive bacteria (such as the mycobacteria) have a T7SS as well (Tseng *et al.*, 2009). The T1SS is required for the expression of effector proteins in the rice pathogen *Xanthomonas oryzae* (da Silva *et al.*, 2004). The T2SS (Sec pathway) and T5SS (tat pathway) are generic secretion systems found in all kinds of bacteria. The key T3SS is required for the virulence of several human as well as plant pathogens (Tseng *et al.*, 2009). The T4SS plays an important role in a number of plant and animal pathogens, secreting proteins and nucleoproteins into host cells (Dale & Moran, 2006). In this respect, *Agrobacterium tumefaciens* C58 has been a model system to study T4SS (Christie & Cascales, 2005). The T6SS was first characterized for *Vibrio cholerae*, but it is now known to be widespread in Gram-negative bacteria (reviewed in Bingle *et al.*, 2008). For instance, some *A. tumefaciens* strains apparently use T6SS, next to T4SS, to secrete specific proteins and cause virulence in plants (Wu *et al.*, 2008).

T3SS and its involvement in bacterial–fungal interactions

The T3SS forms a complex organelle in the envelope of many Gram-negative bacteria. The system requires a cytoplasmatic membrane-associated ATPase (Hueck, 1998) and

is thus energy demanding. The genes encoding the T3SS are often found together on the genome and may be located on bacterial genomic islands, which indicates their proneness to horizontal gene transfer. During the interaction between a bacterium and its host cell, physical contact is required for efficient functioning of the T3SS. A mutant of *Sodalis glossinidius* without the *invC* gene (one gene of the T3SS) could not enter cells of its host, the tsetse fly (Dale *et al.*, 2001). This indicated that the functional T3SS is important in the mutualism between this bacterium and its host. A reacquired T3SS was found to be essential for the (re)establishment of the symbiosis between *S. glossinidius* and the host (Ochman & Moran, 2001).

The T3SS functions as a molecular syringe and can deliver bacterial effector proteins into host cells to modulate host cellular functions (He *et al.*, 2004; Rezzonico *et al.*, 2005), for example for suppression of the host defense system. T3SS is used for different purposes in different bacteria, for example supporting the invasion of host cells or the release of nutrients from epithelial cells. It is actually the T3SS that promotes the interaction of opportunistically infectious and mutualistic/symbiontic bacteria with their hosts (Preston, 2007). In fact, the T3SS represents an ancient system in bacteria that may – over evolutionary time – have served diverse ecological goals (Coombes, 2009), including symbiosis and pathogenicity. It now yields a wonderful organelle that enables bacteria to successfully occupy the nutrient-rich niches provided by eukaryotic hosts (He *et al.*, 2004). Coombes (2009) discussed the contribution of T3SS to the adaptation of (pathogenic and/or nonpathogenic) bacteria to their host. Effector proteins translocated from T3SS-positive bacteria to their host provide unique opportunities to modulate the host physiology, and specific ratios of these effector proteins may dictate the outcome of host colonization (Coombes, 2009).

Systems such as T3SS may also contribute to other ecological roles played by bacteria. For instance, the protein encoded by *espA* (a gene of the T3SS machinery), as well as pili, are involved in biofilm formation by enteropathogenic *Escherichia coli* (Moreira *et al.*, 2006). In addition, enterohemorrhagic *E. coli* has been reported to use the T3SS needle as an anchor for attachment to plant leaves (Shaw *et al.*, 2008). Furthermore, T3SS was found to be required for aggregative multicellular behavior of enterobacterial *Erwinia chrysanthemi* (Yap *et al.*, 2005). Bleasdale *et al.* (2009) recently reported that SPI-2 (one of the two T3SS) is essential for the survival of *Salmonella enterica* in free-living amoebae. Even in the mutualistic symbiosis of *Rhizobium* and legumes, one underlying molecular mechanism is T3SS (Freiberg *et al.*, 1997). Furthermore, Mazurier *et al.* (2006) reported that the *hrcRST* genes (of T3SS) are conserved in *Bradyrhizobium* isolated from nodules of soybean.

There is a lack of knowledge about the potential role of the T3SS in bacterial–fungal interactions, but there is some emerging evidence for a role. First, Rezzonico *et al.* (2005) found that functional T3SS genes may play a role in the biocontrol activity of *P. fluorescens* KD against the phytopathogenic oomycete *Pythium ultimum*. In fact, the expression of these genes was induced by the presence of the host. Moreover, Mazurier *et al.* (2004) assessed the distribution of the *hrcRST* gene cluster in fluorescent pseudomonads from rhizosphere vs. bulk soil. They found that among the total strains isolated from rhizosphere vs. bulk soil, 35–52% were positive for this gene region in the rhizosphere vs. 22–39% in the bulk soil. The rhizosphere examined may in fact have contained a mycorrhizal inhabitant, and might therefore be akin to a mycorrhizosphere. Interestingly, *P. fluorescens* BS053, a representative of a major group inhabiting the mycosphere of the ectomycorrhizal fungus *L. proxima*, was positive for *hrcR*, used as a marker of the T3SS (Warmink & van Elsas, 2008). Furthermore, in the same study, a selection of specific T3SS types by this mycosphere was revealed by direct molecular analyses of the *hrcR* gene. In addition, a significant enhancement of the incidence of culturable T3SS-positive bacteria was found in this mycosphere as compared with the respective bulk soil (Warmink & van Elsas, 2008). Specifically, the T3SS-containing bacterial species made up 13.4% of cultured isolates from the mycosphere of *L. proxima*, whereas this was only about 2% in bulk soil. However, the precise role of T3SS in these bacteria in the (mycor)rhizosphere (Mazurier *et al.*, 2004) or the mycosphere (Warmink & van Elsas, 2008) is not yet known.

In recent work (Warmink & van Elsas, 2009), all bacteria migrating through soil with the hyphal front of the saprotrophic fungus *Lyophyllum* strain *karsten* were found to be positive for the T3SS. Hence, it was hypothesized that the T3SS plays a key role in the bacterial migratory response to an emerging mycosphere. Migration via fungal hyphae using flagellar movement and attachment via the T3SS may be involved in the probably complex phenomenon, which may further include bacterial growth. We have so far ignored whether attachment to the fungal wall and injection of effector molecules are involved as well. Warmink & van Elsas (2009) proposed a model in which, minimally, flagella-mediated and T3SS-supported bacterial motility and attachment are required, next to growth, for successful biofilm formation along the growing fungal hyphae (Fig. 3). However, clear-cut proof for the validity of this model, for example by testing the behavior of the respective knock-out mutants, is still needed.

The role of plasmids and T4SS

Mobile genetic elements, in particular plasmids, impact bacteria by both affecting their behavior and the organization of their genomes (Sota & Top, 2008). Plasmids are capable of self-transfer between closely as well as distantly

related bacteria and even from bacteria to eukaryotic hosts such as yeast or plants (Mazodier & Davies, 1991). They consist of a backbone and may carry accessory genes that are responsible for key phenotypic traits that affect host behavior. The accessory genes are actually the main players in the plasticity of plasmids and consequently also of genomes (Thomas, 2000). They may be quite different between otherwise similar plasmids (Schluter *et al.*, 2007). Known phenotypic traits conferred on the host include antibiotic resistance (Sota & Top, 2008), heavy metal resistance (Silver, 1996; Silver & Phung, 2005) and the degradation of organic or xenobiotic compounds (Dennis, 2005), but other ecologically relevant traits, for example those that allow enhanced fitness under a range of stressful conditions, are bound to be discovered. There are also plasmids that possess genes involved in symbiosis – such as the nitrogen-fixing genes of rhizobia (Young *et al.*, 2006) – or virulence – i.e. the toxin-encoding genes of *Bacillus anthracis* (Okinaka *et al.*, 1999). There are studies reporting environmentally relevant plasmids in the rhizospheres of wheat (van Elsas *et al.*, 1998), alfalfa (Schneiker *et al.*, 2001) and also from the phytopathogen *Xylella fastidiosa* (Marques *et al.*, 2001). Recently, an IncP1beta plasmid (denoted pHB44) was found in a *V. paradoxus* related strain, HB44, which specifically inhabited the mycosphere of *L. proxima* (F. G. H. Boersma, unpublished data). The about 60-kb plasmid is self-transferable and contained the canonical backbone of IncP1beta plasmids. This included a full T4SS, which is likely involved in self-transfer. Plasmid pHB44 further contained about 14 kb of accessory sequence. However, the function of the estimated 14-odd genes of pHB44 in the *V. paradoxus* like host in the *L. proxima* mycospheres is as yet unknown.

The plasmid conjugation machinery – mediating the spread of genes (Thomas & Nielsen, 2005) – is often based on T4SS. A key example of an ecological function for a plasmid-borne T4SS in soil is the infectious process of *A. tumefaciens*. In this process, plasmid transfer to the plant mediated by T4SS is key for the ecological success of the bacterium. T4SS also plays a role as a protein secretion system in other (animal) pathogens, and so it is challenging to investigate its involvement (and plasmids as secreted nucleoproteins) in the interaction of bacterial plasmid hosts with soil fungi.

How selection in the fungus-determined microhabitat may function to favor plasmid-borne genes is currently unknown. For instance, it is unclear whether a situation arises in the mycosphere in which bacteria harboring a plasmid that encodes genes offering a unique catabolic capacity are favored (De Rore *et al.*, 1994; Top & Springael, 2003).

However, there may be a plasmid-borne asset in biofilm formation. In natural environments, most bacteria attach to surfaces on which they form biofilms. Interestingly, the presence of particular plasmids in bacterial hosts stimulates the formation of biofilms, in which so-called type-IV pili have been found to be important (Ghigo, 2001). Because we do not yet know whether the IncP1beta plasmid pHB44 is involved in any aspect of biofilm formation of *V. paradoxus* related strain HB44 on its fungal partner, it is a challenge for future research to examine this in the mycosphere.

Discussion and future perspectives

The study of bacterial–fungal interactions in soil is not only of interest from a fundamental perspective, but it is also important from an applied point of view. We have witnessed the enhanced understanding of the role of several key mechanisms in bacteria, such as T3SS, motility, biofilm formation and nutrient acquisition, which allow these bacteria to competently interact in the fungal–bacterial interface. Bacteria possessing these capabilities, which can be shown to thrive in the mycosphere or mycorrhizosphere, may be called *mycosphere-competent*. It is likely that the universal as well as specific fungiphiles, as proposed by Warmink *et al.* (2009), fall in this category. However, with respect to the ecology and physiology of the mechanisms involved, we are largely ignorant of the details of the complexity and dynamics of these interactions. Although there are several current (Toljander *et al.*, 2006; Offre *et al.*, 2008; Singh *et al.*, 2008; Levy *et al.*, 2009) as well as older (Tylka *et al.*, 1991; Toro *et al.*, 1996; Budi *et al.*, 1999) studies that address the interactions between soil fungi and bacteria, the actual mechanisms behind these associations are in general not very well understood (Artursson *et al.*, 2006). The indications for a possible role of the T3SS (outlined in Fig. 2) in soil bacterial–fungal interactions (Warmink & van Elsas, 2008, 2009) open up new opportunities for studying the fundamental ecological questions posed. During functional secretion, bacterial effector proteins (He *et al.*, 2004) can potentially change the biochemical pathways of the affected host cells. Is this the mechanism that also functions in at least some of the interactions of the T3SS-positive bacteria with soil fungi? In this way, it will make the fungus serve as a nutrient source for the bacteria, as the likely outcome of the T3S is the enhancement of the release of nutrients to the bacterium. The T3SS-positive bacteria may also affect the physiology or the biochemistry of the fungal hyphae by changing their surface or by stimulating fruiting body formation, the latter being corroborated by the finding of an enrichment of T3SS-positive bacteria underneath mushrooms (Warmink & van Elsas, 2008). Another possible effect (corrollary) of active T3S might be the shutting down of fungal defense mechanisms against bacteria. In this way, the bacteria would create their own microhabitat at the surface of fungal hyphae, including an intimate interaction with these. However, definite proof for these hypotheses has

yet to be found and landmark studies on the role of T3SS in the interactions of specific soil bacteria with fungi are urgently needed in the future.

The role and specificity of fungal-released carbonaceous compounds as driving forces for the selection of specific bacteria in the mycosphere is also worthy of new research. Clearly, soil fungi do select bacterial species in their surroundings by releasing specific carbonaceous compounds, but how dynamic is this system? What type of (bacterial) succession can be envisioned in this fungal-dominated habitat? How is the dynamics of interaction of specific bacteria with the growing hyphae in soil? We largely ignore the intricacies of these interactions. Moreover, the impact of soil fungi on the selection of bacteria with otherwise antibiotic properties (e.g. against other fungi) or that can withstand antibiotics excreted by the fungal partner can be studied by experimental manipulation of fungal density. In this way, antibiotic-mediated selection of bacteria by fungi becomes worthy of increased attention.

Bacterial comigration with fungal hyphae in the soil (Warmink & van Elsas, 2009; Warmink, 2009) is also an interesting subject for future research, as details of the mechanisms involved in this phenomenon are not yet known. There is still an open question about how T3SS may play a role and how this role combines with motility/chemotaxis, biofilm formation and growth, or, for that matter, whether fungal exudates are the only incentive for this bacterial migratory capacity. Involvement of QS signaling during the bacterial colonization of growing fungal hyphae is also worthy of investigation. The bacterial migration and even the migration helper effect (Warmink, 2009) can aid other bacterial partners in their establishment in new locations along the hyphae. Furthermore, biofilm formation during bacterial migration with the fungus can provide protection against the hazardous environment, which may include antagonistic organisms such as fungi and predators such as protozoa. Thus, bacterial comigration with an extending fungus in soil may also be helpful in the establishment of mycorrhizal interactions with plants (Johansson *et al.*, 2004; Frey-Klett *et al.*, 2007). The bacteria may even move directly to the right location on the basis of their propensity to migrate. These hypotheses require future research in which more complex soil microcosm systems are used, which also include, next to specific bacteria and fungi, plants.

Finally, the ever-increasing availability of fungal and bacterial genome sequences will also help us obtain an improved picture of the impact that (mycorrhizal) soil fungi, since their emergence, have exerted on the evolution of fungal-associated soil bacteria. In particular, key functions encoded on the genomes of both partners that stand out as being interaction- or mycosphere-specific may become explicit, allowing the testing of hypotheses that can be built in respect of their potential function. Horizontal gene transfer between fungal-associated bacteria, but also between bacteria and fungal hosts (and vice versa), may also become apparent from comparative genome evaluations, also representing interesting fields of future research. The comparison of the genomes of fungal-associated bacterial strains with those of phylogenetically related free-living strains will also raise our understanding about the evolution of bacterial–fungal interactions, including the role of horizontal gene transfer.

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