Attachment of different soil bacteria to arbuscular mycorrhizal fungal extraradical hyphae is determined by hyphal vitality and fungal species

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Introduction

Arbuscular mycorrhizal (AM) symbiotic associations are formed between roots of most terrestrial plants and fungi of the phylum Glomeromycota (Schüssler et al., 2001). The extraradical hyphae of these fungi explore the soil, thereby increasing the nutrient absorptive surface area of their host plant root systems (Rhodes & Gerdemann, 1975). In addition, mycorrhizal fungal hyphae constitute an important pathway for the translocation of energy-rich assimilates from the plant to the soil (Johnson et al., 2002). This, in combination with the ubiquitous distribution of mycorrhizal fungi, and the high surface area of the extraradical mycelium in relation to that of the roots, renders hyphae important sites for interactions with other microorganisms in soil ecosystems.

It is fairly well established that AM may influence the composition of associated bacterial communities in the mycorrhizosphere. Many mechanisms for this interaction have been proposed, including the supply of energy-rich compounds via the extraradical mycelium (Andrade et al., 1997), changes in soil structure (Tisdall & Oades, 1979), competition for nutrients (Ravnkov et al., 1999), and changes in root exudation patterns (Söderberg et al., 2002). It has also been suggested that bacteria in the mycorrhizosphere influence the growth and establishment of AM fungi, and, more recently, that certain bacteria may specifically stimulate processes such as spore germination (Xavier & Germida, 2003) or colonization of roots by AM fungi (Budi et al., 1999). Although it seems logical that these interactions are facilitated by the close proximity of bacteria to the fungus, little is known about the extent to which bacteria associate with hyphae of AM fungi, as well as the specificity of these interactions, and what possible ecological significance such associations could have. Bianciotto et al. (1996a) found that spores and hyphae of several AM fungal species of the genus Gigaspora harboured endosymbiotic bacteria closely related to the genus Burkholderia. These bacteria were later found to be transmitted

Abstract

Attachment of certain bacteria to living arbuscular mycorrhizal fungal extraradical hyphae may be an important prerequisite for interactions between these microorganisms, with implications for nutrient supply and plant health. The attachment of five different strains of gfp-tagged soil bacteria (Paenibacillus brasilensis PB177 (pnf8), Bacillus cereus VA1 (pnf8), Pseudomonas fluorescens SBW25-: gfp/lux, Arthrobacter chlorophenolicus A6G, and Paenibacillus peoriae BD62 (pnf8)) to vital and nonvital extraradical hyphae of the arbuscular mycorrhizal fungi Glomus sp. MUCL 43205 and Glomus intraradices MUCL 43194 was examined. Arthrobacter chlorophenolicus did not attach to hyphae, whereas the other bacterial strains did to a varying degree. Only P. brasilensis showed greater attachment to vital hyphae than nonvital hyphae of both Glomus species tested. Pseudomonas fluorescens showed a higher attachment to vital compared with nonvital Glomus sp. MUCL 43205 hyphae, whereas this relationship was opposite for attachment to G. intraradices. Both B. cereus and P. peoriae showed higher attachment to nonvital hyphae. This study provides novel evidence that under laboratory conditions soil bacteria differ in their ability to colonize vital and nonvital hyphae and that this can also be influenced by the arbuscular mycorrhizal fungal species involved. The significance of bacterial attachment to mycorrhizal fungal extraradical hyphae is discussed.
Attachment of different soil bacteria to AM fungal extraradical hyphae

Fungal hyphae may aid in the distribution and dispersal of could involve signalling or metabolic exchange. In addition, fungal hyphae and bacteria facilitates interactions, which mechanisms. Presumably, direct contact between mycorrhizal unknown, and it is therefore important to understand the significance of bacterial–AM–fungal interactions is largely between bacteria and mycorrhizal fungi are common in soils. The uniqueness of bacterial–AM–fungal interactions is largely unknown, and it is important to understand the mechanisms. Presumably, direct contact between mycorrhizal fungal hyphae and bacteria facilitates interactions, which could involve signalling or metabolic exchange. In addition, fungal hyphae may aid in the distribution and dispersal of bacterial populations in the soil environment.

In a previous study (Artursson & Jansson, 2003), we identified active bacteria in fallow field soil. One of the active bacteria identified, Bacillus cereus strain VA1, was isolated from the soil and tagged with the gfp gene expressing green fluorescent protein (GFP). Using confocal microscopy, this bacterium was shown to attach to hyphal fragments of the AM fungus Glomus dussii; however, the viability of the hyphae was not determined. As attachment of bacteria to living AM fungal hyphae may be an important prerequisite for interactions between these microorganisms, with implications for nutrient supply and biocontrol in sustainable agriculture (Johansson et al., 2004), we were interested in further investigation of these interactions.

The aim of this study was to compare in vitro attachment of different gfp-tagged soil bacterial strains to vital and nonvital extraradical hyphae of two AM fungal species. We chose five bacterial strains with a range of functional characteristics and modes of growth. Our hypothesis was that specific bacterial strains would show preferential attachment to either vital or nonvital AM fungal hyphae. In addition, we aimed to determine whether the degree of attachment was influenced by fungal species.

Materials and methods

Mycorrhizal ribosomal T-DNA-transformed carrot roots

Ribosomal T-DNA-transformed carrot roots (Daucus carota L.) (Bécard & Fortin, 1988) were colonized with either fungal mycobiont Glomus sp. MUCL 43205 (previously described as G. claroideum) or G. intraradices Schenck & Smith MUCL 43194 (fungal isolates supplied by GINCO, Louvain-la-Neuve, Belgium) and were grown in Petri dishes (60 × 15 mm), each containing c. 10 mL liquid minimal medium (Bécard & Fortin, 1988), without gellan gum. Mycorrhizal root cultures were grown in a growth chamber at 25 °C. Actively growing mycorrhizal root cultures with ample amounts of extraradical hyphae were used in this study.

Bacterial strains and culture conditions

Five different strains of soil bacteria, previously tagged with the gfp gene encoding GFP, were used in this study. These were chosen to represent bacteria with a range of functional properties and types of growth. Paenibacillus brasiliensis PB177 and Paenibacillus peoriae NRRL BD62, both nitrogen fixers with known biocontrol properties (Montefusco et al., 1993; von der Weid et al., 2002), and B. cereus VA1, a potential saprotroph known to attach to degraded parts of AM hyphae (Artursson & Jansson, 2003), were all previously transformed (Artursson & Jansson, 2003; von der Weid et al., in press) with the puf8 plasmid containing gfp under the control of the strong constitutive promoter from Listeria (Fortin et al., 2000). Arthrobacter chlorophenolicus A6G, a biodegrader of 4-chlorophenol, dominant in bulk soil but not typically colonizing roots (Elvang et al., 2001) and Pseudomonas fluorescens SBW25::gfp/lux, an aggressive root colonizer (Unge et al., 1999), were previously chromosomally tagged with gfp or both gfp and luxAB genes (encoding bacterial luciferase), respectively, using mini-transposon vectors. All of the bacterial strains were routinely grown in Luria–Bertani medium. Erythromycin (10 μg mL⁻¹) was added as selection pressure to the medium for growth of those bacteria that were tagged with the puf8 plasmid to ensure stability of the plasmid and the GFP phenotype. Cultures were grown until late log phase and the cell concentration was determined by microscopic counting on an Axioshot Epifluorescence Microscope (Zeiss, Jena, Germany) using a Bürker counting chamber (Boeco, Hamburg, Germany).

Vital and nonvital hyphae

Nonvital hyphae were produced by aseptically excising hyphae from colonized carrot root cultures prior to the experiments. These hyphae were transferred to new Petri dishes containing sterile 0.1 M pH 5.8 phosphate buffer and incubated in a growth chamber at 25 °C, together with the remaining intact mycorrhizal cultures. After 7 days, the vitality of the excised nonvital hyphae and vital hyphae from intact cultures was verified in an Axioshot (Oberchem, Germany) fluorescence microscope (510–570 nm excitation) using the vital stain fluorescein diacetate according to Söderström (1979).

Experimental treatments

Vital and nonvital Glomus hyphae, sterile glass fibres, and vital hyphae from a nonmycorrhizal fungus (Clitocybe sp.)
were transferred separately to the centre well of a FALCON® 
_In Vitro_ Fertilization Dish (60 × 15 mm) (Becton Dickinson 
Labware, Franklin Lakes, NJ) containing 2 mL of sterile 
0.1 M pH 5.8 potassium phosphate buffer. Bacterial strains 
that had been washed twice in phosphate buffer (same as 
above) were resuspended in buffer and added to the above 
dishes containing vital hyphae, nonvital hyphae, sterile glass 
fibres, or the nonmycorrhizal fungus so that the final 
bacterial concentration was 10⁷ cells mL⁻¹. For each of the 
five bacterial strains, six replicates of each of the four 
treatments (vital hyphae, nonvital hyphae, glass fibres and 
hyphae of nonmycorrhizal fungus) were performed. The 
glass fibres, which were used as a nonbiological control, had 
a diameter of c. 8 μm, similar to that of the _Glomus_ hyphae 
used in this study. Dishes with added bacteria were incu-
bated at room temperature for 3 h on a rotary shaker 
(40 rpm). To remove electrostatically attached bacteria, the 
phosphate buffer was removed from the dishes, and the 
samples were washed with 2 mL of a strong salt solution 
following the methods of Zuberer (1994), with the modifi-
cation that the salt concentration was increased 2 × to 
17 g NaCl L⁻¹. The dishes were gently shaken for 1 min. 
Washing with the salt solution was repeated twice. The salt 
solution was then replaced with 2 mL phosphate buffer and 
the dishes were gently shaken for 1 min. Washing with 
phosphate buffer was repeated twice, and 2 mL fresh buffer 
was finally added to each dish. By using this method, 
bacterial biofilms not attached directly to the hyphae could 
be removed in order to quantify cells directly attached to 
hyphae.

Quantification of bacterial attachment

Hyphae or fibres from each replicate were randomly col-
lected and mounted on microscope slides. Slides were 
examined in an Axioplan epifluorescence microscope 
(Zeiss) equipped with filters for detection of GFP 
(450–490 nm excitation). Using an Axiocam Color camera 
(Zeiss), high-resolution digital photographs were taken at 
200 times magnification of the first five hyphae/fibres 
encountered on each slide. Sometimes several photographs 
of the same view, but at different focal planes, were taken 
to ensure good rendering of the field of view. Photographs were 
imported to Adobe® Photoshop® 7.0 and the number of 
attached bacterial cells per unit length of hyphae was 
counted.

Statistics

Differences between bacterial attachment to vital and non-
vital hyphae as well as between different bacterial strains 
were tested for statistical significance by one-way ANOVA of 
log-transformed data.

Results

General observations

The _gfp_-tagged bacterial strains _Paenibacillus brasiliensis_, 
_Bacillus cereus_, _Pseudomonas fluorescens_, and _Paenibacillus 
peoriae_ all exhibited different levels of attachment to vital 
and nonvital _Glomus_ hyphae (Figs 1 and 2 and Table 1). 
_Arthrobacter chlorophenolicus_ did not attach to hyphae at all 
(Table 1). None of the tested bacterial strains attached to 
_Clitocybe_ hyphae or to the sterile glass fibres. When con-
sidering attachment data for all bacterial strains together, 
there was significantly (_P_ = 0.0001) higher attachment to 
vital hyphae of _Glomus_ sp. MUCL 43205 compared with 
_Glomus intraradices_; however, there was no significant 
difference in attachment to nonvital hyphae between _Glo-
mus_ species.

Effects of hyphal vitality on attachment of 
individual bacterial strains

_Paenibacillus brasiliensis_ showed higher attachment to vital 
hyphae than nonvital hyphae of both _Glomus_ sp. MUCL 
43205 and _G. intraradices_ hyphace; however, this difference 
was only significant for _Glomus_ sp. MUCL 43205 (_P_ = 0.004) 
(Figs 2a and b and Table 1). The number of _P. brasiliensis_ cells 
attaching to _Glomus_ sp. MUCL 43205 vital hyphae was up to 
40 times higher than the number attaching to nonvital 
hyphae (Table 1). The attachment of _Ps. fluorescens_ to vital 
hyphae was significantly (_P_ = 0.002) higher than attachment 
to nonvital hyphae of _Glomus_ sp. MUCL 43205, whereas this 
relationship was reversed but nonsignificant for _G. intrarad-
ices_ (Figs 2c and d and Table 1). Numbers of attached _Ps. 
fluorescens_ were up to 52 times higher for vital compared 
with nonvital _Glomus_ sp. MUCL 43205 hyphae. _Bacillus 
cereus_ showed a higher attachment to nonvital hyphae of 
both _Glomus_ species. However, this trend was significant 
only for _G. intraradices_ (_P_ = 0.0001) (Figs 2e and f and Table 
1). Similarly, _P. peoriae_ showed a significantly (_P_ = 0.003) 
higher attachment to nonvital _G. intraradices_ hyphae, but 
did not attach to either vital or nonvital hyphae of 
_Glomus_ sp. MUCL 43205 (Fig. 2g and Table 1).

Discussion

The bacterial strains tested in this study differed in their 
overall ability to attach to hyphae of _Glomus_ sp. MUCL 
43205 and _Glomus intraradices_. Differences in their attach-
ment to vital vs. nonvital hyphae as well as differences in 
their attachment to either fungal species were observed. 
Only _Paenibacillus brasiliensis_ PB177 (_pfn8_) consistently 
showed a higher attachment to vital hyphae, irrespective of 
AM fungal species involved. _Budi et al_. (1999) previously 
demonstrated that a different, unidentified _Paenibacillus_
isolate ‘B2’ caused both stimulation of mycorrhizal root colonization by *Glomus mosseae* as well as antagonistic effects against a range of fungal plant pathogens. Attachment of these bacteria to living AM fungal hyphae could be important for the subsequent beneficial effects that *Paenibacillus* bacteria may have on mycorrhizal colonization and plant growth. In addition, it is possible that the bacteria could exert a biocontrol effect on pathogenic fungi, which could explain the biocontrol activity that some paenibacilli exhibit (see Budi *et al.*, 1999; von der Weid *et al.*, in press). *Paenibacillus brasiliensis*, along with many other paenibacilli, is a nitrogen fixer (Achouak *et al.*, 1999; von der Weid *et al.*, 2002). Nitrogen fixation in roots and AM colonization are processes known to act synergistically on each other (Puppi *et al.*, 1994), and direct contact between nitrogen fixing bacteria and mycorrhizal fungal hyphae may have a positive influence on the mutualistic interactions between these organisms.

Some bacteria associating with mycorrhizal fungal hyphae may also act as saprotrophs and derive carbon and other nutrients through degradation of fungal hyphae. In our study, *Bacillus cereus* VA1 (*pfn8*) showed higher attachment to nonvital hyphae of both *Glomus* species. In another recent study (Artursson & Jansson, 2003), we noted that *B. cereus* was often associated with AM fungal hyphae. Hyphal vitality was not determined in that investigation; however, it was observed that bacteria were frequently associated with damaged parts of hyphae. Staddon *et al.* (2003) demonstrated a rapid turnover of AM fungal hyphae in soil. Their results suggested that AM fungal hyphae in soils live on average only 5–6 days, contributing to a rapid pathway for carbon in soil ecosystems. The results of the present study suggest that *B. cereus* may behave as a saprotroph. Saprotrophic activity contributing to the degradation of hyphae and utilization of compounds released by damaged hyphae contributed to the rapid hyphal turnover observed by Staddon *et al.*

Interactions between soil bacteria and AM fungi can be specific and attachment of certain bacterial strains may occur to vital hyphae of some fungal species but not others. This was observed for attachment to *Glomus* sp. MUCL 43205 by the *Pseudomonas fluorescens* strain used in this study. In plant cultivation systems, the biocontrol effect of many strains of *Ps. fluorescens* (including strain SBW25) has been ascribed to their ability to form biofilms and colonize roots (Lugtenberg *et al.*, 2001). The biofilm itself consists of bacteria covered in mucilaginous material and enables anchorage to surfaces for whole bacterial aggregates, but does not necessarily involve direct cell contact between individual bacteria and the surface material. A number of studies have shown that bacteria forming extracellular mucilaginous material are better colonizers of roots and hyphae (Bianciotto *et al.*, 1996b, 2001; Matthysse & McManus, 1998). For example, Bianciotto *et al.* (1996b) studied the colonization of spores and hyphae of the AM fungus *Gigaspora margarita* by different strains of *Pseudomonas* and *Rhizobium*. Bacteria adhering to fungal tissues typically also adhered to nonliving substrata. They concluded that extracellular material of bacterial origin may mediate fungal/bacterial interactions and that specific receptors are not

**Fig. 1.** Examples of *gfp*-tagged bacteria attaching to extraradical *Glomus* hyphae. *Paenibacillus brasiliensis* PB177 on vital (a) and nonvital (b) *Glomus* sp. MUCL 43205 hyphae; *Pseudomonas fluorescens* SBW25 on vital (c) and nonvital (d) *Glomus* sp. MUCL 43205 hyphae; *Bacillus cereus* VA1 on vital (e) and nonvital (f) *Glomus* sp. MUCL 43205 hyphae; *Paenibacillus peoriae* BD62 on vital (g) and nonvital (h) *Glomus intraradices* MUCL 43194 hyphae. Bars = 10 μm.
involved in the observed attachment. In our experiments both *P. brasilensis* and *P. fluorescens* produced mucilage, causing bacterial cells to clump together and form loose aggregates surrounding hyphae and glass fibres. Most of the bacteria in these aggregates were, however, not attached to the hyphae or fibres themselves, and were therefore washed off in the subsequent washing procedure. This enabled us to quantify bacteria attached directly to the hyphal surface. Following washing of hyphae, *P. fluorescens* exhibited differences in attachment to vital and nonvital hyphae that
also appeared to vary according to the fungal strain involved. This suggests that mechanisms other than extracellular material of bacterial origin could also have a role in the attachment of bacteria to hyphae.

Specific attachment mechanisms of bacteria to hyphae may depend on which organisms are involved and could involve bacterial cell surface interactions. Sen et al. (1996) suggested that polar flagella of *Ps. fluorescens* strains could play a role in the attachment of these bacteria to the hyphal cell walls of some ectomycorrhizal fungi. In our study, *Ps. fluorescens* and *B. cereus* were both efficient colonizers of hyphae. Although these bacteria have polar flagella, which could play a role in enhancing their colonization of hyphae, motility itself does not infer specificity in attachment to certain substrates, such as roots or living or dead hyphae. Other mechanisms such as chemotaxis to components of exudates may also play a role (see review by Lugtenberg et al., 2001).

It is also possible that hyphal characteristics influence attachment. In this study, there were overall higher numbers of bacteria attaching to vital hyphae of *Glomus* sp. MUCL 43205 than to *G. intraradices*, whereas attachment to nonvital hyphae was more similar between the two *Glomus* species. Although some bacterial strains showed similar patterns of attachment between fungal species, other strains showed different attachment patterns depending on fungal species. These observations indicate that in addition to hyphal vitality, there was an influence of fungal species on attachment. The mycorrhizal fungal species used in this study are morphologically different, e.g. in terms of branching of hyphae and formation of spore-bearing structures. Though it remains a possibility that cell wall structures in the AM fungus influence bacterial attachment, there were no obvious differences on a microscopic level that could explain differential bacterial colonization of fungi in the current study. It is further possible that differences in quantity or quality of mycelial exudates could specifically promote or inhibit growth and attachment of certain bacteria. In an ongoing study we identified low-molecular weight carbohydrates in *Glomus* sp. MUCL 43205 mycelial exudates that induced changes in the composition of a bacterial community (J.F. Toljander et al., unpublished data). However, it remains to be investigated whether mycelial exudates differ between AM fungal species, and whether AM fungi are able to produce antibiotic compounds that specifically antagonize certain microorganisms.

Our study suggests that the mycorrhizal fungal extraradical mycelium constitutes an important interface for interactions with other microorganisms. It has previously been established that carbon flow via this interface provides a significant source of energy that can be utilized by opportunistic or mutualistic soil organisms. This study provides novel evidence of different colonization patterns of bacteria on vital and nonvital hyphae of two different AM fungal species *in vitro*. These observations add to our understanding of microbial interactions and the fate of bacterial inoculants; however, the underlying mechanisms at the cellular level still require further study.

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### References


