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ORIGINAL ARTICLE

Characterisation of microbial communities colonising the hyphal surfaces of arbuscular mycorrhizal fungi

Tanja R Scheublin[1,2], Ian R Sanders[1], Christoph Keel[2] and Jan Roelof van der Meer[2]
[1]*Department of Ecology and Evolution, University of Lausanne, Lausanne, Switzerland and* [2]*Department of Fundamental Microbiology, University of Lausanne, Lausanne, Switzerland*

Arbuscular mycorrhizal fungi (AMF) are symbiotic soil fungi that are intimately associated with the roots of the majority of land plants. They colonise the interior of the roots and the hyphae extend into the soil. It is well known that bacterial colonisation of the rhizosphere can be crucial for many pathogenic as well as symbiotic plant–microbe interactions. However, although bacteria colonising the extraradical AMF hyphae (the hyphosphere) might be equally important for AMF symbiosis, little is known regarding which bacterial species would colonise AMF hyphae. In this study, we investigated which bacterial communities might be associated with AMF hyphae. As bacterial-hyphal attachment is extremely difficult to study *in situ*, we designed a system to grow AMF hyphae of *Glomus intraradices* and *Glomus proliferum* and studied which bacteria separated from an agricultural soil specifically attach to the hyphae. Characterisation of attached and non-attached bacterial communities was performed using terminal restriction fragment length polymorphism and clone library sequencing of 16S ribosomal RNA (rRNA) gene fragments. For all experiments, the composition of hyphal attached bacterial communities was different from the non-attached communities, and was also different from bacterial communities that had attached to glass wool (a non-living substratum). Analysis of amplified 16S rRNA genes indicated that in particular bacteria from the family of Oxalobacteraceae were highly abundant on AMF hyphae, suggesting that they may have developed specific interactions with the fungi.
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Introduction

Arbuscular mycorrhizal fungi (AMF) are obligate plant symbionts that are able to colonise the roots of approximately two-thirds of all terrestrial plant species (Trappe, 1987; Smith and Read, 1997). AMF can have large effects on plant growth by nutrient acquisition and protection against pathogens or drought (Davies *et al.*, 1993; Newsham *et al.*, 1995; Borowicz, 2001). They are intimately associated with plant roots, colonising the root cortex as well as the surrounding soil. AMF hyphae are sometimes considered as an extension of the root system, taking up (immobile) nutrients such as phosphorus from that part of the soil space that is not accessed by the plant's roots. It is well known that bacterial colonisation of the rhizosphere can be crucial for many pathogenic as well as symbiotic plant–microbe interactions (Whipps, 2001; Weller, 2007). However, although extensive literature exists on rhizosphere colonisation, little is known regarding bacterial colonisation of AMF hyphae (the 'hyphosphere').

Those studies that have examined bacterial colonisation of AMF hyphae have revealed a number of important findings. First, pure cultures of a number of biocontrol, *Rhizobium* and soil bacteria (for example, *Pseudomonas fluorescens*, *Rhizobium leguminosarum*, *Paenibacillus brasilensis*, *Paenibacillus peoriae* and *Bacillus cereus*) are able to attach to AMF hyphae and differences in bacterial attachment have been found between vital and nonvital hyphae (Bianciotto *et al.*, 1996, 2001; Toljander *et al.*, 2006). This suggests that specific AMF–bacteria interactions may exist. Second, AMF hyphae seem to influence the composition of bacterial communities in their surroundings. For example, Andrade *et al.* (1998) observed an increase in fluorescent pseudomonads and an *Alcaligenes eutrophus* strain in the presence of AMF hyphae, while Ravnskov *et al.* (1999) found a decrease in *Pseudomonas fluorescens* strain DF57. In addition,

Correspondence: TR Scheublin, Department of Microbial Ecology, Netherlands Institute of Ecology (NIOO-KNAW), Boterhoeksestraat 48, 6666 GA Heteren, The Netherlands.
E-mail: t.scheublin@nioo.knaw.nl
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several studies found evidence that the bacterial community composition in soil is different in the presence or absence of AMF hyphae (Mansfeld-Giese *et al.*, 2002; Marschner and Baumann, 2003; Rillig *et al.*, 2006). Like plant roots, also AMF hyphae produce exudates and these exudates might explain the observed differences in soil bacterial communities. It has been found that extracted AMF hyphal exudates can stimulate bacterial growth (Filion *et al.*, 1999) and change the bacterial community composition (Toljander *et al.*, 2007).

One reason for the limited amount of information on hyphal colonisation by bacteria is that the hyphosphere is an environment, which is much more difficult to experimentally access than the rhizosphere. To analyse the rhizosphere, researchers generally take the soil adhering to roots. Unfortunately, the same approach is not feasible for the hyphosphere, because hyphae are too small and not sufficiently rigid to extract with adhering soil. Some researchers, therefore, have taken the total soil volume colonised by hyphae as the hyphosphere. In this approach there is no distinction between hyphosphere and bulk soil, and therefore it is likely to yield non-hyphosphere bacteria. Others have limited their studies to those bacterial cells directly attached to hyphae. However, although attachment to hyphae per definition means being in the hyphosphere, it is unlikely that hyphal influence would not go beyond attached cells, for example by leaked nutrients. Therefore, this approach seems too narrow. At the moment there is no solution for this problem and one has to choose between one of these non-optimal approaches.

The potential effect of bacterial hyphal colonisers on AMF and the AMF symbiosis is high. Several types of interactions between bacteria and AMF have been described (Bonfante and Anca, 2009). So-called mycorrhiza helper bacteria have been shown to promote mycelial growth and mycorrhiza formation (Garbaye, 1994; Frey-Klett *et al.*, 2007). Several studies have reported interactive effects between plant-growth-promoting bacteria, pathogens, rhizobia and AMF (Azcon-Aguilar and Barea, 1996; Requena *et al.*, 1997; Xavier and Germida, 2002; Wamberg *et al.*, 2003). Moreover, bacteria have been isolated from AMF spores and mycorrhizal cultures that promote or sometimes inhibit AMF spore germination, mycorrhisation and plant growth (Mayo *et al.*, 1986; Budi *et al.*, 1999; Xavier and Germida, 2003). Such data suggest that bacterial colonisers of AMF hyphae may have an important role for successful AMF plant colonisation and symbiosis. As a result of the tight association between AMF and plants, and the importance for plant ecology and (sustainable) agriculture (Johansson *et al.*, 2004), further insights into bacteria–AMF interactions are highly relevant.

The main goals of this project were to investigate colonisation of the AMF hyphal surface by bacteria, to determine whether attachment is specific, to identify possible main hyphal colonisers, and to study consequent changes in bacterial community composition after being in contact with AMF hyphae. The approach we take in this study is to use *in vitro* cultures of the AMF *Glomus intraradices* and *Glomus proliferum*, grown in compartmented plates in which the hyphae can be brought into contact with total bacterial communities extracted from agricultural soils. Microscopy and molecular community analysis methods based on amplified 16S ribosomal RNA (rRNA) gene diversity were then used to analyse specific attachment. To our knowledge this is the first study to take such an *in vitro* community approach to investigate bacterial attachment to AMF hyphae.

Materials and methods

Fungal isolates and preparation of soil bacterial suspension

We used four different AMF isolates, three isolates of the species *G. intraradices*, namely DAOM 181602 (Biosystematics Research Centre, Ottawa, Canada), C2 and C3 (Koch *et al.*, 2004), and one *G. proliferum* isolate, MUCL 41827 (Declerck *et al.*, 2000). Bacterial communities were isolated from top soil (0–10 cm) collected at an agricultural field on the campus of the University of Lausanne (clay-mineral dominated Cambisol, pH 5, see Supplementary methods for analysis of the soil composition). Ten grams of fresh soil was mixed with 40 ml of 0.1% $Na_4P_2O_7$ (pH 7) in a blender (A11 basic, IKA-Werke, Staufen, Germany), for four times during 7 s, with 2 min intervals on ice. The suspension was centrifuged for 5 min at $150 \times g$ and 4 °C to precipitate soil particles. The supernatant was filtered through a 30-μm sieve, and 5 ml of Nycodenz (Optiprep, Axis-Shield, Oslo, Norway) was pipetted below the aqueous phase filtrate. After centrifugation for 20 min at $3000 \times g$ and 4 °C, 5 ml of the interphase containing the bacteria was collected and diluted with 5 ml of 0.1% $Na_4P_2O_7$. A second Nycodenz centrifugation step was performed for 60 min at $3000 \times g$ and 4 °C. In all, 3 ml of the interphase was diluted with 9 ml of liquid M-medium without sucrose (Becard and Fortin, 1988; 3 mM $MgSO_4$, 0.79 mM KNO_3, 0.87 mM KCl, 35 μM KH_2PO_4, 1.2 mM $Ca(NO_3)_2$, 20 μM Fe(Na)EDTA, 4.5 μM KI, 30 μM $MnCl_2$, 9.2 μM $ZnSO_4$, 24 μM H_3BO_3, 0.52 μM $CuSO_4$, 9.9 μM Na_2MoO_4, Gamborg's vitamin solution (Sigma, St Louis, MO, USA)). After centrifugation for 15 min at $3000 \times g$, the pellet was resuspended in 3 ml of liquid M-medium without sucrose.

Attachment assays

AMF isolates were grown on Root tumor-inducing plasmid T-DNA-transformed *Daucus carota* roots in two-compartment plates, which consist of a root compartment with AMF-colonised carrot roots and

a hyphal compartment without roots (St-Arnaud *et al.*, 1996). When AMF hyphae started to grow in the hyphal compartment (after 46–75 days), a block of 2×5 cm was removed from the hyphal compartment and replaced with liquid M-medium without sucrose. After another 9–26 days, when hyphae visibly colonised the liquid compartment, 200 µl of soil bacterial suspension (see above) was added. Bacterial suspensions were always freshly prepared and inoculated directly after isolation. Hyphae and bacteria were incubated for 20 h at 25 °C. This period allows good attachment of bacteria to the hyphae, but is short enough to prevent other fungi (that is, contaminants introduced with the bacterial inoculum) to establish. Hyphae with attached bacteria were removed from the compartment using a flame-sterilised forceps and washed in liquid M-medium without sucrose. The rest of the liquid medium (approximately 4 ml) was collected and centrifuged for 15 min at $15\,000 \times g$ to collect the remaining bacteria. Bacterial pellets and hyphae with attached bacteria were stored separately at −20 °C until DNA isolation, or fixed in ethanol for microscopic observation. In one experiment, the total number of bacteria and their vitality was determined at the time of inoculation and after the 20-h incubation. Cells were stained with LIVE/DEAD *Bac*Light Bacterial Vitality Kit (Invitrogen, Carlsbad, CA, USA), and life and dead cells were counted in a Thoma chamber under an epifluorescent microscope (Axioskops 2, Zeiss, Jena, Germany).

A total of three attachment experiments were carried out. In the first experiment (experiment A), we used six replicate plates of *G. intraradices* isolate C3. The C3 isolate produces relatively high amounts of hyphae. A negative control without added bacteria was included in this experiment. In the second experiment (experiment B), we used four different AMF isolates, with nine replicate plates for isolate C3, five replications for isolate C2, seven for isolate DAOM 181602 and four for *G. proliferum*. The number of replications in experiment B is unequal because many plates failed to produce sufficient hyphal material in the liquid compartment. In the third experiment (experiment C), we compared bacterial attachment to AMF hyphae with attachment to glass wool as a non-living control substrate. Six replicate plates with isolate C3 were used and six replicate plates in which sterile glass wool was added to the liquid compartment (that is, these plates were not inoculated with AMF, and therefore the liquid compartment did not contain hyphae).

Microscopic observation of bacterial attachment to hyphae

Sub-samples of the collected hyphae and bacteria were fixed in ethanol and stained with 1 µg per ml 4′,6-diamidino-2-phenylindole (DAPI) for 15 min in the dark. Hyphae were mounted on a microscope slide and observed under an epifluorescent microscope (Axioskops 2, Zeiss) with blue filter (Excision 385 nm, beam splitter 395 nm, emission long pass 420 nm).

DNA isolation and terminal restriction fragment length polymorphism (T-RFLP)

DNA from hyphae plus bacteria, or soil bacteria alone was isolated by a bead-beating procedure according to the protocol described by Bürgmann *et al*. (2001) with small modifications. A PCR was performed with the general bacterial primers 27f (5′-AGAGTTTGATCCTGGCTCAG-3′; Lane, 1991) and 1492r (5′-GGTTACCTTGTTACGACTT-3′; Lane, 1991), of which primer 27f was labelled with hexachloro-6-carboxyfluorescein. After purification, a quantity of 500 ng of PCR product was digested with *Hae*III. T-RFLP community profiles were determined with 1 µl of digested product on an ABI 3100 Genetic Analyser (Applied Biosystems, Foster City, CA, USA). A detailed description of the DNA isolation procedure and T-RFLP analysis is found in the Supplementary methods.

Clone libraries

DNA isolated from experiment B was used to construct eight clone libraries, one for the bacterial fraction attached to hyphae and one for the non-attaching bacterial fraction, times four for the four different AMF isolates. DNA obtained from replicate plates was mixed in equal amounts and 16S rRNA gene fragments were amplified in a PCR using primers 27f and 534r (5′-ATTACCGCGGCTGCTGG-3′; Muyzer *et al.*, 1993). PCR fragments were ligated into pGEM-T Easy (Promega, Madison, WI, USA) and transformed into competent *Escherichia coli* DH5α cells (OneShot MaxEfficiency; Invitrogen). Using plasmid-specific primer T7, 48 clones were sequenced for each library, resulting in a total of 384 sequenced clones (see Supplementary methods for a detailed description of the cloning and sequencing procedure). Sequences were deposited in GenBank under accession numbers GQ403990 to GQ404373.

Analysis

T-RFLP profiles were analysed using the ABI GENEMAPPER software, version 3.7 (Applied Biosystems). Bins were automatically created using the automatic panel generation feature and then manually corrected. Total peak height of the T-RFLP profiles was at least 3000 relative fluorescent units and peaks smaller than 1% of the total peak height were removed from the analysis.

Statistical analyses were performed using the R software (http://www.r-project.org/). The community profiles were analysed by correspondence analysis (R function ‘cca (vegan)’). The relative

abundance of individual terminal restriction fragments (T-RFs) between samples was compared using a paired *t*-test (R function 't.test (stats)'). Separate tests were performed for each T-RF. As multiple testing increases the chance to find significant results, the significance levels are separately shown for $0.01 < P < 0.05$, $0.001 < P < 0.01$ and $P < 0.001$.

Sequences from the clone library were operationally assigned a taxonomic position in the Ribosomal Database Project (release 9, https://rdp.cme.msu.edu/index.jsp) using the CLASSIFIER option with a confidence threshold of 80% (Wang *et al.*, 2007). A theoretical digest with *Hae*III was performed for all sequences and the size of the terminal fragment was calculated. This, we called the predicted T-RF size. One to four representative plasmid clones of each predicted T-RF size and each operational taxonomic unit (OTU) were reanalysed for apparent T-RF size on the ABI sequencer. This allowed us to match the T-RFs in the community T-RFLP electropherograms to the corresponding OTUs from the clone library.

Results

To analyse bacterial colonisation of AMF hyphae, we developed an experimental system in which soil bacteria that were separated from soil particles could be incubated with axenically pre-grown AMF hyphae in liquid compartments. Microscopic observation of AMF hyphae from our experimental system confirmed bacterial attachment to the hyphae. This was most easily observed on samples stained with DAPI (Figure 1). In some cases, only few bacterial cells were found to be attached to the hyphae, whereas in other cases hyphae were covered almost completely with bacteria or with microcolonies of cells (Figure 1). At the time of inoculation the liquid compartment contained 3.3×10^6 bacteria per ml with 86% vital cells. After 20-h incubation, these numbers increased to $1.76 \times 10^7 \pm 0.13 \times 10^7$ bacteria per ml (average ± s.d.) with 91 ± 1% vitality.

Bacterial communities sticking to AMF hyphae were examined by T-RFLP analysis and compared

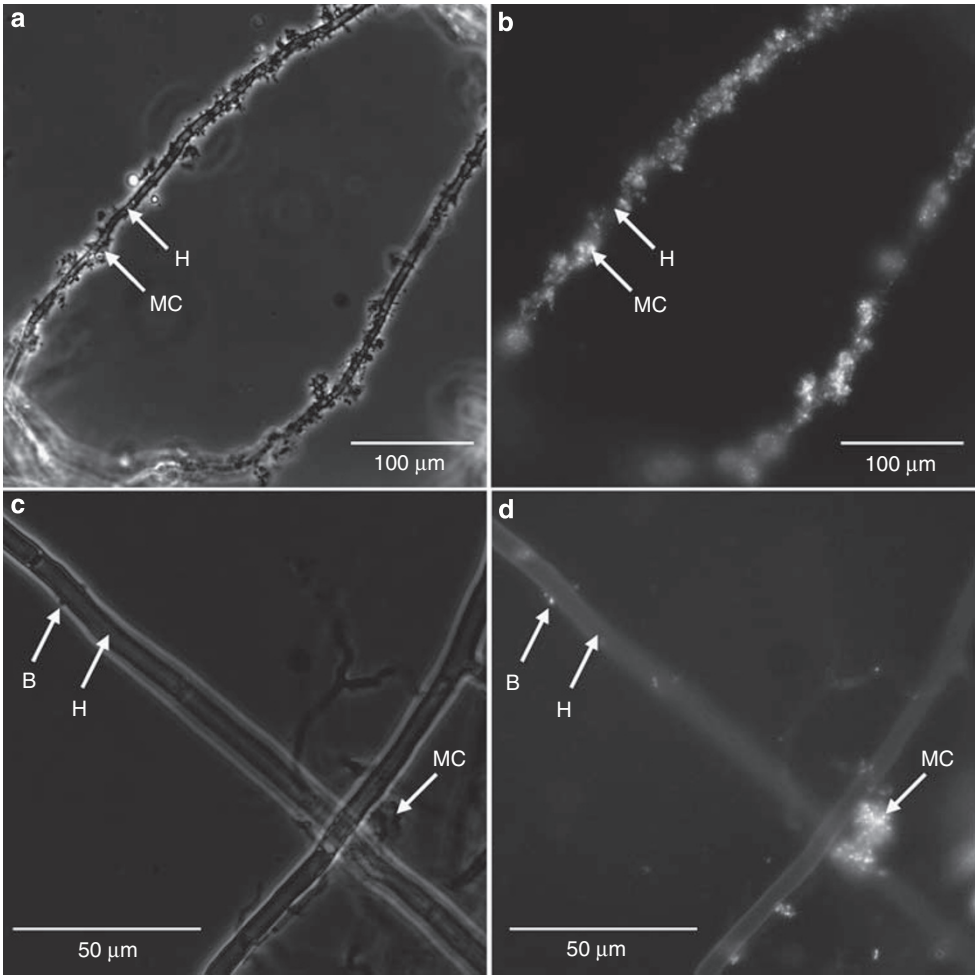

Figure 1 DAPI-stained AMF hyphae with attached soil bacteria. Pictures were taken with normal light (**a**, **c**) or fluorescence (**b**, **d**); H, hyphae; B, bacteria; MC, microcolony of bacteria. Hyphae in pictures a/b are densely colonised with bacteria, while pictures c/d show attachment of single bacteria and a microcolony.

with the community composition of bacteria remaining in solution. Three independent experiments were carried out with each time freshly isolated soil bacteria. All three showed a clear overall distinction between the T-RFLP profiles produced from bacteria that had attached to AMF hyphae and those that had remained in solution (Figures 2a–c). No significant differences were found between the T-RFLP profiles of bacterial communities attaching to hyphae of different AMF isolates (Figure 2b). T-RFLPs produced from attached bacterial communities also differed between AMF hyphae and glass wool as a non-living control substrate (Figure 2c). In this experiment, the communities that remained in solution were also different between compartments containing hyphae or glass wool. The negative control without bacteria did not result in a PCR product and the T-RFLP profile revealed no peaks, neither for DNA isolated from hyphae alone, nor from the AMF growth medium without bacteria added.

A comparison of the relative abundances of individual T-RFs showed that 6–8 T-RFs were more abundant in hyphae-attached communities than in non-attached communities, whereas 7–16 T-RFs were less abundant (Figure 3 and Supplementary Figure 1). To analyse which bacterial groups were represented by those T-RFs, we constructed clone libraries from the DNA of experiment B. A total of 384 clones were sequenced. Table 1 shows the operational taxonomic assignment of the clones and the number of assigned clones in each library.

As differences can occur between the size of a theoretical digest (predicted size) and the apparent T-RF size obtained from the capillary sequencer, we determined the T-RF sizes on cloned fragments in the library for one to four representatives of each fragment size and each OTU. The difference between the apparent T-RF size and the predicted size was usually between 1 and 2 bp, but could amount to 6 bp, in particular for the smaller-sized T-RFs (Table 2). We also observed that fragments of the same predicted size but from different OTUs could result in different apparent T-RF sizes in the electropherogram. For example, a predicted T-RF of 217 bp was found to produce T-RFs of 213, 215 and 216 bp for clone inserts from different OTUs (Table 2). Overall, the clone library contained 32 OTUs for 40 apparent T-RF sizes. Some OTUs were presented by several T-RFs whereas most T-RFs represented more than one OTU.

Many T-RFs were represented by one dominant OTU that could be identified up to the genus level. For example, T-RFs of 73, 197, 198, 216, 218 and 401 bp were deduced to represent members of the Oxalobacteraceae family (*Duganella*, *Janthinobacterium*, *Massilia* and unclassified Oxalobacteraceae). These T-RFs were generally more abundant in the T-RFLP profiles from hyphae-attached communities, except for T-RF 198. T-RFs 222 and 223, which represent *Streptomyces* spp., were also more

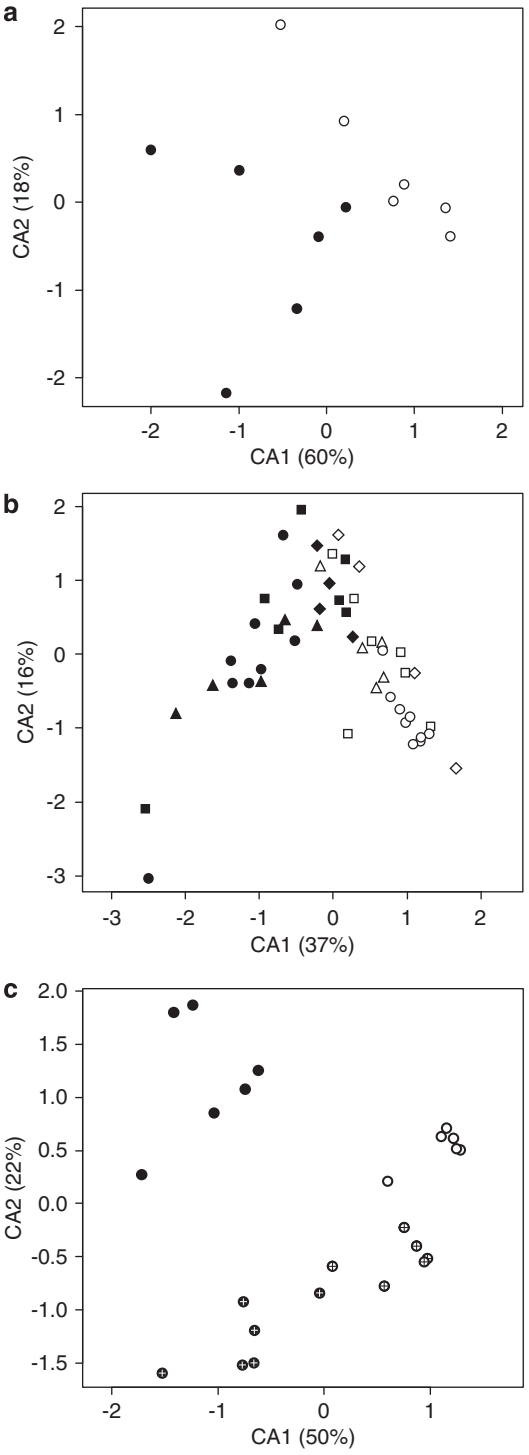

Figure 2 Correspondence analysis (CA) of the bacterial community composition identified by T-RFLP. Relative abundance data of T-RFs were used for CA analysis and the first two dimensions were plotted, (**a**) experiment A, comparing hyphae-attached and non-attached communities, (**b**) experiment B, comparing attachment between different AMF isolates and (**c**) experiment C, comparing attachment to AMF hyphae and glasswool. Black symbols, non-attached communities; white symbols, hyphae-attached communities; circle, *G. intraradices* strain C3; triangle, *G. intraradices* strain C2; square, *G. intraradices* strain DAOM 181602; diamond, *G. proliferum* strain MUCL 41827; crossed circles, glass wool. Shown in brackets is the percentage of variance explained by that axis.

Table 1 Number of clones per operational taxonomic unit from each library

Operational taxonomic unit	*Non-attaching communities*				*Attaching communitites*				*Total*
	C2	*C3*	*D*	*M*	*C2*	*C3*	*D*	*M*	
Bacillus	4	0	0	2	0	0	0	0	6
Paenibacillus	1	0	0	0	0	1	0	0	2
Caulobacter	0	0	0	0	1	0	0	0	1
Methylobacterium	0	0	0	0	1	0	0	0	1
Rhizobium	0	1	0	0	0	0	0	0	1
Unclassified Rhizobiaceae	0	0	0	0	0	0	1	0	1
Unclassified Rhizobiales	0	0	0	1	0	0	1	0	2
Novosphingobium	0	0	0	0	1	0	0	0	1
Sphingopyxis	0	0	1	0	0	0	0	0	1
Unclassified Sphingomonadaceae	0	0	0	0	0	0	1	0	1
Unclassified Alphaproteobacteria	0	0	0	1	1	0	0	0	2
Duganella	2	0	3	3	4	3	5	8	28
Janthinobacterium	2	0	0	0	0	0	0	0	2
Massilia	0	4	2	0	2	1	0	1	10
Unclassified Oxalobacteraceae	17	10	9	8	15	10	7	6	82
Pelomonas	0	0	0	0	0	0	0	1	1
Unclassified Incertae sedis 5	2	1	0	0	1	1	1	0	6
Ralstonia	0	0	0	0	0	0	1	0	1
Polaromonas	0	0	0	0	0	0	0	1	1
Rhodoferax	0	0	0	0	0	0	0	1	1
Unclassified Burkholderiales	0	0	0	0	0	1	0	0	1
Acinetobacter	3	0	4	0	0	0	1	0	8
Pseudomonas	7	10	18	12	15	5	19	22	108
Enterobacter	0	1	1	4	1	6	0	0	13
Erwinia	0	0	0	3	0	3	1	0	7
Serratia	1	1	0	0	0	0	0	0	2
Yersinia	0	0	0	0	0	1	0	0	1
Unclassified Enterobacteriaceae	1	0	4	1	1	1	1	0	9
Lysobacter	0	0	0	1	0	0	0	0	1
Unclassified Xanthomonadaceae	0	0	0	1	0	0	0	0	1
Streptomyces	4	18	3	7	3	10	8	6	59
Cellulomonas	0	0	0	0	0	0	1	0	1
Arthrobacter	0	0	2	0	0	2	0	0	4
Rhodococcus	0	1	0	0	0	0	0	0	1
Agromyces	0	1	0	1	0	0	0	0	2
Unclassified Microbacteriaceae	0	0	0	0	0	1	0	0	1
Marmoricola	0	0	0	0	1	0	0	0	1
Unclassified Nocardioidaceae	0	0	0	1	0	0	0	0	1
Unclassified Micromonosporaceae	0	0	0	0	0	0	0	1	1
Solirubrobacter	1	0	0	0	0	0	0	0	1
Unclassified Actinobacteria	0	0	0	0	0	1	0	0	1
Gp3	0	0	0	0	0	0	0	1	1
Gp6	1	0	0	0	0	0	0	0	1
Flavobacterium	2	0	0	0	0	0	0	0	2
Adhaeribacter	0	0	1	0	0	0	0	0	1
Unclassified Planctomycetaceae	0	0	0	0	1	0	0	0	1
Unclassified Bacteria	0	0	0	2	0	1	0	0	3
Total	48	48	48	48	48	48	48	48	384

C2, C3, D: *Glomus intraradices* isolate C2, C3 and DAOM181602, respectively; M: *Glomus proliferum* MUCL41827.

abundant in hyphae-attached communities. In experiment C, this T-RF was even completely absent from the non-attached bacterial communities and from the communities attached to glass wool (Supplementary Figure 1b). By contrast, T-RF 230 and T-RF 233, which are predicted to represent mostly *Bacillus* spp., were strongly decreased or absent from hyphae-attached communities. Also T-RF 255 was less abundant in hyphal-derived material across all four experiments. This T-RF is predicted to have originated from *Acinetobacter* spp. T-RF 33 was one of the most abundant T-RFs across all experiments and was also consistently less abundant in hyphae-attached communities. OTU assignments of this T-RF suggest that most of the DNAs originate from *Pseudomonas* spp., but this T-RF also contains several other OTUs. As the differences in abundance are relatively small, we cannot attribute the change in abundance of T-RF 33 to one OTU at species level.

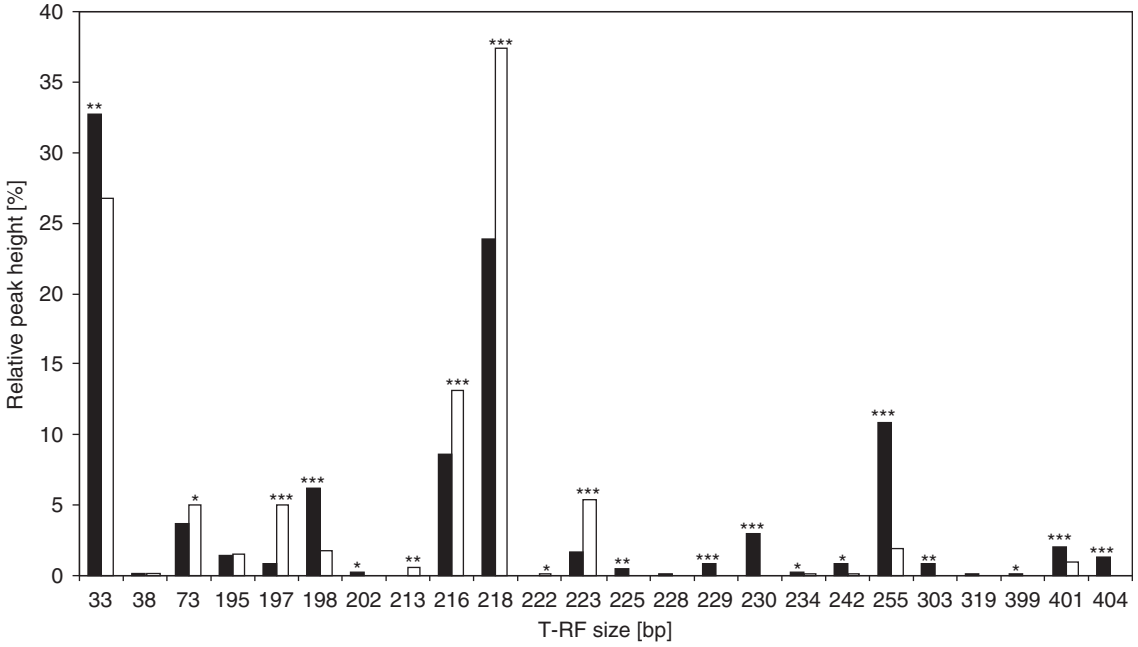

Figure 3 Relative peak heights of T-RFs from bacterial communities in experiment B. Each column represents the average peak height over all hyphae-attached or non-attached communities. Black bars, non-attached communities; white bars, hyphae-attached communities. For each T-RF a paired *t*-test was performed, $*0.01<P<0.05$, $**0.001<P<0.01$ and $***P<0.001$.

Table 2 Comparison of T-RF sizes calculated from the DNA sequence of 16S rRNA gene fragments in the clone libraries (predicted T-RF size), with the apparent T-RF size in capillary electrophoresis

Apparent T-RF size (bp)	*Predicted T-RF size (bp)*	*No. clones*	*%*	*Operational taxonomic unit*
33	**39**	**106**	**74**	**Pseudomonas**
		13	**9**	**Enterobacter**
		7	**5**	**Erwinia**
		2	**1**	**Serratia**
		1	**1**	**Yersinia**
		9	**6**	**Unclassified Enterobacteriaceae**
		1	**1**	**Lysobacter**
		1	**1**	**Unclassified Xanthomonadaceae**
		1	**1**	**Caulobacter**
		1	**1**	**Solirubrobacter**
		1	**1**	**Adhaeribacter**
64	67	1	25	*Rhodococcus*
		1	25	*Marmoricola*
		1	25	Unclassified Nocardioidaceae
		1	25	Unclassified Micromonosporaceae
66	70	1	100	Unclassified Bacteria
67	71	1	100	Unclassified Sphingomonadaceae
72	76	2	100	Unclassified Alphaproteobacteria
73	**77**	**3**	**38**	**Duganella**
		1	**13**	**Massilia**
		4	**50**	**Unclassified Oxalobacteraceae**
159	163	1	100	Unclassified Bacteria
163	165	1	100	*Methylobacterium*
167	170	1	100	Unclassified Actinobacteria
187	191	1	100	Unclassified Rhizobiales
192	193	2	67	*Duganella*
	195	1	33	Unclassified Rhizobiales
196/197	**198**	**2**	**25**	**Duganella**
		6	**75**	**Unclassified Oxalobacteraceae**
197	**199**	**1**	**100**	**Unclassified Oxalobacteraceae**
198	**200**	**9**	**43**	**Massilia**
		10	**48**	**Unclassified Oxalobacteraceae**
		1	**5**	**Rhodoferax**
		1	**5**	**Pseudomonas**

Table 2 (Continued)

Apparent T-RF size (bp)	*Predicted T-RF size (bp)*	*No. clones*	*%*	*Operational taxonomic unit*
205	206	1	100	*Ralstonia*
209	212	1	100	*Pelomonas*
212	214	1	100	Gp3
213	**217**	**2**	**100**	**Unclassified Incertae sedis 5**
215	217	4	100	Unclassified Incertae sedis 5
216	**217**	**16**	**64**	**Duganella**
		9	**36**	**Unclassified Oxalobacteraceae**
217	218	1	100	Unclassified Oxalobacteraceae
218	**219**	**5**	**9**	**Duganella**
		2	**3**	**Janthinobacterium**
		50	**86**	**Unclassified Oxalobacteraceae**
		1	**2**	**Unclassified Burkholderiales**
220	222	1	100	*Thermomicrobium*
222	**223**	**1**	**100**	**Streptomyces**
223	**224**	**58**	**100**	**Streptomyces**
224	224	1	33	Unclassified Planctomycetaceae
	225	1	33	*Rhizobium*
	225	1	33	Unclassified Rhizobiaceae
228	**227**	**1**	**25**	**Novosphingobium**
	228	**2**	**50**	**Arthrobacter**
	229	**1**	**25**	**Cellulomonas**
229	**230**	**1**	**100**	**Unclassified Microbacteriaceae**
230	**229**	**1**	**17**	**Agromyces**
	230	**2**	**33**	**Arthrobacter**
	231	**3**	**50**	**Bacillus**
231	230	1	100	*Agromyces*
232	233	1	100	Gp6
234	**233**	**3**	**100**	**Bacillus**
255	**253**	**8**	**100**	**Acinetobacter**
263	262	1	100	*Flavobacterium*
277	279	1	100	*Flavobacterium*
291	293	1	100	*Sphingopyxis*
303	**305**	**2**	**100**	**Paenibacillus**
312	313	1	100	*Polaromonas*
401	**404**	**1**	**100**	**Unclassified Oxalobacteraceae**
>500	>495	1	100	*Pseudomonas*

Abbreviations: rRNA, ribosomal RNA; T-RF, terminal restriction fragment.
For each apparent T-RF size, the corresponding predicted T-RF size(s) as well as the number of clones in each operational taxonomic unit (OTU) is given. The percentage of clones per OTUs is calculated for each (apparent) T-RF individually. In bold are indicated those T-RFs that were also found in the T-RFLP community profile, in regular font those that were present only in the clone library (compare with Figure 3). Note that not all T-RFs from the community profile were also found in the clone library.

Discussion

In this paper, we studied the types of bacteria from soil, which adhere specifically to AMF hyphae. As bacterial adherence to AMF hyphae is extremely difficult to examine *in situ* in the soil, we used an experimental setup in which AMF hyphae were first allowed to grow under sterile conditions. Bacteria were then freshly separated from agricultural soil samples and brought into contact with living sterile hyphae for a period of 20 h. Our hypothesis was therefore that bacteria, which would adhere to and perhaps start to multiply on the hyphae during the period of contact, might be indicative for bacteria that interact with AMF under real soil conditions. To identify those bacteria that would adhere 'specifically' to AMF hyphae, we compared compositional differences of amplified 16S rRNA gene fragments in DNA purified from bacterial fractions that had attached to hyphae during the incubation period, and the bacterial fraction remaining in the incubation solution. Importantly, our 16S rRNA gene diversity data show that bacterial communities that adhered to AMF hyphae were significantly different from non-attaching communities. One should realise that both community samples will overlap, because they are derived from one and the same community during the incubation. The result of this is that even for species that are enriched in the attached fraction, one will likely still find them in the non-attached fraction.

We also obtained some evidence regarding the specificity of the interactions. The composition of bacterial communities attached to AMF hyphae was different from that of communities attached to glass wool during the same incubation time, but no differences were observed between communities attached to four different AMF isolates. These AMF isolates were, however, closely related, and therefore a comparison between more distantly

related AMF species and/or other fungal species would be necessary to further elucidate the specificity of these interactions. Differences in communities attached to hyphae and glass wool suggest that not just physicochemical attraction was responsible for attachment to AMF hyphae, but that there may have been some population growth already on the hyphae during the incubation period or that there were specific signalling interactions that made certain types of bacteria attracted to the hyphae. As the communities that remained in solution were also different between compartments containing hyphae or glass wool, it is not unlikely that hyphal exudates have had a role in shaping these communities. A study by Toljander *et al.* (2007), using exudates isolated from AMF hyphae, have also shown a marked influence of AMF hyphal exudates on bacterial community composition. They detected several low-molecular-mass sugars and organic acids as well as some unidentified high-molecular-mass compounds in hyphal exudates.

Sequencing of clone library inserted 16S rRNA gene fragments amplified from the DNA of the bacterial communities allowed us to identify several of the T-RFs that increased or decreased in abundance on hyphal-attached compared with the non-attached communities. Operational taxonomic identification of such T-RFs suggested *Streptomyces* and members of the Oxalobacteraceae family, specifically *Duganella*, *Janthinobacterium* and *Massilia* to be particularly abundant on hyphae. By contrast, *Bacillus* and *Acinetobacter* were less abundant or absent from the hyphal-attached bacterial communities.

Members from the Oxalobacteraceae family are commonly found in soil and rhizosphere (Green *et al.*, 2007). Increasingly now, a number of reports have mentioned the possible interactions of this group of bacteria with (mycorrhizal) fungi. Oxalobacteraceae have been found to preferentially associate with mycorrhizal roots rather than with the roots of plant mutants that cannot form the AMF symbiosis (Offre *et al.*, 2007, 2008). The similarity between the findings of these experiments, which were performed in natural soil, and our results that were obtained from a more controlled, but artificial system is a strong indication that we are looking at realistic interactions. In another recent study, a bacterial strain belonging to the Oxalobacteraceae family was isolated from mycorrhizal roots. This isolate promoted spore germination, hyphal growth and root colonisation of *Glomus mosseae* (Pivato *et al.*, 2009). Furthermore, bacteria closely related to *Janthinobacterium lividum* have been isolated from AMF spores. These isolates showed strong antagonistic effects against several pathogenic fungi and were capable of phosphorus solubilisation (Cruz *et al.*, 2008). *Janthinobacterium* sp. have also been isolated from ectomycorrhizal fungi and it was shown that these bacteria utilise fungal-derived sugars more readily than plant sugars (Izumi *et al.*, 2006). Moreover, *Collimonas fungivorans*, a mycophagous bacterium that is able to feed on living fungal hyphae, also belongs to the Oxalobacteraceae family (de Boer *et al.*, 2004). Another point of evidence is that *J. lividum* can produce extracellular chitinases (Gleave *et al.*, 1995), which is suggestive for its ability to degrade fungal cell walls. *Janthinobacterium agaricidamnosum* is a mushroom pathogen (Lincoln *et al.*, 1999), while a *J. lividum* isolate with strong antifungal activity has also been isolated from salamander skin (Brucker *et al.*, 2008). Taken together these data suggest that the Oxalobacteraceae may have particular importance for both beneficial and parasitic bacteria–fungi interactions.

In addition to the enriched abundance of Oxalobacteraceae members on hyphae, certain T-RFs also pointed to significantly more *Streptomyces* spp. attached to hyphae than remaining in liquid suspension. Given their filamentous form, we cannot be completely sure that the 'attachment' of *Streptomyces* to AMF hyphae is specific or an entanglement effect. However, it is noteworthy that *Streptomyces* was completely absent from the attached and non-attached fractions of the glass wool treatment. Streptomycetes are often found in soil and have also been found in association with roots or fungal hyphae. Different types of interactions have been reported for streptomycete–fungal interactions. In some cases streptomycetes inhibit fungal growth. *Streptomyces griseoviridis* for example, has been shown to depress mycorrhiza formation (Wyss *et al.*, 1992). Other streptomycetes have a stimulatory effect on fungi, such as *Streptomyces orientalis*, which stimulates spore germination of some AMF (Tylka *et al.*, 1991).

In contrast to streptomycetes and members of Oxalobacteraceae, T-RFs representative for *Bacillus* spp. were significantly less abundant or even absent in material recovered from hyphae. This is in marked contrast to what has been found earlier. Artursson and Jansson (2003) reported good attachment of pure cultures of *B. cereus* to hyphae of *Glomus dussii*. Our data did not specifically suggest presence of *B. cereus* in the communities isolated from soil and therefore we cannot refute or confirm their findings. It could indicate that there are species-specific differences in attachment abilities for *Bacillus* spp. As Artursson and Jansson used single pure culture attachment experiments, it is also possible that *B. cereus* can colonise hyphae when it is alone, but is a relatively bad coloniser in competition with other bacteria. Vitality of the hyphae might also have had a role. Toljander *et al.* (2006) found the same *B. cereus* strain to preferentially attach to nonvital hyphae of *G. intraradices*. In addition to *B. cereus,* also *Paenibacillus* spp. have been mentioned in a number of reports to attach to AMF hyphae, to stimulate mycorrhiza formation and to proliferate in the presence of hyphae (Budi *et al.*, 1999; Mansfeld-Giese *et al.*, 2002; Artursson

and Jansson, 2003). In our experiments, we did find genetic signature evidence for *Paenibacillus* spp., notably from T-RF 303, but only in two cloned inserts. Interestingly, T-RF 303 was absent from bacterial communities attached to hyphae, which would suggest that *Paenibacillus* is not a good hyphal attacher. Again, however, it is important to realise that in our experimental conditions we separated attached bacteria from non-attached bacteria after 20 h of contact to hyphae and, therefore, it is possible that important 'slow-colonizers' under real-life soil conditions were not detected here. Finally, also T-RFs operationally defined as originating from *Acinetobacter* spp. were less abundant in hyphae-derived material. Despite a large amount of literature on *Acinetobacter* spp., no information is available on interaction or association with AMF, which would be in agreement with the observed lack of interaction in our data.

Given the difficulty to study AMF hyphae within their natural occurrence, it is not surprising that only a limited number of studies have attempted to investigate the role of bacteria in the hyphosphere. Some studies have investigated the ability of bacteria to attach to AMF hyphae, but only using pure and single cultures and sometimes with hyphae that were not connected to plant roots (Bianciotto *et al.*, 1996, 2001; Toljander *et al.*, 2006). Such studies may therefore have been biased towards less active AMF hyphae and non-competitive attachment. Others have investigated differences in bacterial community composition in soil in the presence or absence of AMF, in which case it is difficult to conclude which bacteria are attached to AMF (Andrade *et al.*, 1998; Ravnskov *et al.*, 1999; Mansfeld-Giese *et al.*, 2002; Marschner and Baumann, 2003; Rillig *et al.*, 2006). The system used here was successfully applied in the past to grow AMF but to guide their hyphae into compartments separately from the plant roots they are obligately attaching to. This permitted us to study bacterial attachment to AMF hyphae but without the interfering effects of plant root exudates. Bacteria were purified directly from agricultural soil without further cultivation, from which we believe that this approaches their natural activity and thus, their tendency to interact with the AMF hyphae. However, there are also some important limitations to our method and we do not claim to have achieved the complete picture of all bacteria that colonise AMF hyphae. The community approach allowed us to identify hyphal colonisers without *a priori* assumptions regarding which bacteria would be involved, but some steps in our experimental procedure, such as the extraction of bacteria from soil, the 20-h incubation and the PCR and cloning procedure, were probably selective for some bacterial groups, while it was also difficult to draw conclusions regarding lowly abundant bacteria. Exclusion of some bacterial groups is almost unavoidable in a controlled artificial system, and we can draw conclusions only regarding that part of the community that is amenable to our experimental procedure. The complete picture should arise by combining results of researchers using different experimental approaches.

In conclusion, we believe we have shown specific attachment to AMF hyphae by certain types of bacteria derived from agricultural soil. First, this indicates that the ability to attach to AMF hyphae is variable between different bacterial groups. Second, this specific attachment may be indicative for further colonisation and more complex types of interactions between bacteria and hyphae. Genetic signatures for several bacterial groups were obtained of which that for the family of Oxalobacteraceae is particularly interesting. Traces for this family were highly abundant on AMF hyphae after 20-h incubation, suggesting specific apt interactions with the fungi. This is in agreement with several recent reports, which pointed to the involvement of these bacteria in (mycorrhizal) fungal interactions. Further studies on their involvement in AMF-plant symbiosis seem therefore warranted.

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