RESEARCH ARTICLE

Evidence for specificity of cultivable bacteria associated with arbuscular mycorrhizal fungal spores

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Abstract

Bacteria associated with arbuscular mycorrhizal (AM) fungal spores may pl functional roles in interactions between AM fungi, plant hosts and defence again plant pathogens. To study AM fungal spore-associated bacteria (AMB) with reg to diversity, source effects (AM fungal species, plant host) and antagoni properties, we isolated AMB from surface-decontaminated spores of *Glom intraradices* and *Glomus mosseae* extracted from field rhizospheres of *Festuca ov* and *Leucanthemum vulgare*. Analysis of 385 AMB was carried out by fatty a methyl ester (FAME) profile analysis, and some also identified using 16S rRN gene sequence analysis. The AMB were tested for capacity to inhibit growth *in vit* of *Rhizoctonia solani* and production of fluorescent siderophores. Half of the AM isolates could be identified to species (similarity index 0.6) within 16 genera and 3 species. AMB were most abundant in the genera *Arthrobacter* and *Pseudomona* and in a cluster of unidentified isolates related to *Stenotrophomonas*. The AM composition was affected by AM fungal species and to some extent by plan species. The occurrence of antagonistic isolates depended on AM fungal species but not plant host, and originated from *G. intraradices* spores. AM fungal spores appear to host certain sets of AMB, of which some can contribute to resistance by AM fungi against plant pathogens.

Introduction

About 80% of plant families from all phyla of land plants are colonized by arbuscular mycorrhizal (AM) fungi, which belong to the fungal phylum *Glomeromycota* (Schüßler *et al.*, 2001). AM fungi provide numerous benefits to their host plants by improving the supply of plant nutrients and increasing protection from drought and soil pathogens (Smith & Read, 1997; Ruiz-Lozano *et al.*, 2001; Harrier & Watson, 2004). In addition, AM fungi provide specific niches such as spores, extraradical hyphae and intraradical mycelia for populations of bacteria (Scannerini & Bonfante, 1991; Schüßler, 2002). A number of studies have reported interactions between AM fungi and bacteria associated with their extraradical phase and the existence of endocellular bacteria, which live inside certain fungal isolates (Bianciotto *et al.*, 1996, 2003; Bianciotto & Bonfante, 2002; Roesti *et al.*, 2005; Toljander *et al.*, 2006). Bacteria such as *Paenibacillus brasilensis* and *Pseudomonas fluorescens* are associated with extraradical hyphae of *Glomus intraradices* (Toljander *et al.*, 2006) while *Alcaligenes*, *Bacillus*, *Corynebacterium* and *Pseudomonas* are associated with the spores of *Glomus versiforme* (Mayo *et al.*, 1986) and spore walls of *Glomus clarum* NT4 (Xavier & Germida, 2003). Recently, *Cellvibrio*, *Chondromyces*, *Flexibacter*, *Lysobacter* and *Pseudomonas* spp. were found to be associated with spores of *Glomus geosporum* and *Glomus constrictum* (Roesti *et al.*, 2005). A *Burkholderia* sp. bacterium was found in the cytoplasm of spores of *Gigaspora margarita*, which has recently been assigned the new taxonomical identity *Candidatus* Glomeribacter gigasporarum (Bianciotto *et al.*, 1996, 2003). Several kinds of associations occur between AM fungi and bacteria and depend on species and strains (Bianciotto *et al.*, 1996; Perotto & Bonfante, 1997; Johansson *et al.*, 2004). Very little is known about the mechanisms controlling these associations and their interactions with plant roots in the mycorrhizosphere (Johansson *et al.*, 2004; Artursson *et al.*, 2006).

The composition of bacterial populations in the mycorrhizosphere has been suggested to be affected by nutrient competition, soil structure and exudates from plant roots and from extraradical mycelium of AM fungi (Tisdall & Oades, 1979; Mayo *et al.*, 1986; Meyer & Linderman, 1986; Andrade *et al.*, 1997; Ravnskov *et al.*, 1999; Söderberg *et al.*, 2002). The bacteria associated with AM fungal spores (hereafter referred to as arbuscular mycorrhiza-spore-associated bacteria, AMB) has been found to stimulate the germination of spores and the colonization of roots (Mayo *et al.*, 1986; Budi *et al.*, 1999; Xavier & Germida, 2003) and stimulate the growth of AM fungi up to the formation of fertile spores in the absence of a host (Hildebrandt *et al.*, 2002, 2006). This influence has been suggested to be due to the production of stimulatory volatile compounds such as CO_2, erosion of the spore walls and phosphorous acquisition by bacteria (Carpenter-Boggs *et al.*, 1995; Filippi *et al.*, 1998; Ruiz-Lozano & Bonfante, 2000). Certain AMB can also hydrolyse biopolymers such as protein, chitin and cellulose (Filippi *et al.*, 1998; Roesti *et al.*, 2005) and could therefore help in AM fungal colonization by degrading plant cell walls and inhibit fungal pathogens by degrading fungal cells. Budi *et al.* (1999) reported that a *Paenibacillus* strain isolated from surface-sterilized *Glomus mosseae* spores inhibited a number of different plant fungal pathogens including *Rhizoctonia solani*, thus showing a broad spectrum of activity. The AMB can thus apparently play an important role in the development and adaptation of AM fungi. We, therefore, decided to investigate the impact of plant host and AM fungal species on AMB populations and their functional activities. The AM fungal spore was chosen as the fungal structure because it is an important long-term reproductive structure and could act as a habitat for bacteria that may play important roles in the mycelium of germinating spores during the formation of the AM–plant symbiosis.

In a previous study comparing the impact of 12 different plant species grown in monoculture on AM fungal sporulation and root colonization, we found that potato roots became highly colonized by AM fungi present in the rhizosphere soils of *Festuca ovina* and *Leucanthemum vulgare* (Bharadwaj *et al.*, 2007). Furthermore, the dominant AM fungal species occurring in these soils were identified as *G. intraradices* and *G. mosseae*. In the present study, we investigated the identity and diversity of cultivable bacteria associated with two morphologically different types of AM fungal spores obtained from *F. ovina* and *L. vulgare*. This study thus tested the hypothesis that the composition and biological activity of bacteria associated with AM fungal spores are both plant and fungal species-dependent. The biological activity of AMB was investigated with respect to production of fluorescent siderophores and growth inhibition of *R. solani*, a soil-borne pathogen of several economically important agricultural crops, including potatoes.

Materials and methods

Plant species and AM fungal spores

AM fungal spores were extracted from the rhizosphere soil of two plant species, *F. ovina* L. and *L. vulgare* Lam., grown in monoculture at a BIODEPTH site, Umeå, Sweden (Hector *et al.*, 1999; Mulder *et al.*, 2002). In brief, 250 g of rhizosphere soil from each plant was collected, suspended in aerated tap water and sieved through a set of sieves (Bharadwaj *et al.*, 2007). The dominant spores were collected and sorted into the following four groups, i.e. two AM fungal spore types from the two plant host species: Group 1, large yellow spores from *F. ovina* (FY); Group 2, small white spores from *F. ovina* (FW); Group 3, large yellow spores from *L. vulgare* (LY); Group 4, small white spores from *L. vulgare* (LW). Twenty spores from each group were separated and identified on the basis of their morphology, colour, size and surface ornamentation (Bharadwaj *et al.*, 2007, INVAM: http://invam.caf.wvu.edu/).

Microorganisms – isolation and culturing conditions

The four groups were considered to be the different source environments for the bacteria to be isolated. A total of 500 healthy spores from each source environment were used for isolation of bacteria. Spores were repeatedly and aseptically washed at least 30 times with sterile potassium phosphate buffer solution [phosphate-buffered saline (PBS), 0.14 M NaCl, 0.003 M KCl and 0.01 M phosphate buffer, pH 7.4]. In a preparatory study, the first and last washings were spread on sterile diluted tryptic soy broth agar medium (TSA10, 10 g tryptic soy broth and 15 g bacto agar; Difco, in 1000 mL distilled water) and checked for surface decontamination. Multiple aseptic washings proved to effectively decontaminate the spores. The spores were crushed aseptically in 10 mL PBS after the last washing and homogenized, and appropriate dilutions were spread on TSA10 in three replicates. Plates were incubated in the dark at 25 °C for 48 h. In order to obtain a representative range of bacteria from each source environment, colonies were selected using two methods. In Method 1 bacterial colonies were picked to represent different morphology, while in Method 2 the colonies present on a straight line across the centre of the plate were picked. In total, 394 AMB thus selected were purified and maintained in a sterile mixture of glycerol and tryptic soy broth (1 : 4) at − 20 °C until use.

Rhizoctonia solani Kühn anastomosis group 3 (AG-3) was selected as a test pathogen for investigating the antagonistic effects of AMB. This organism causes black scurf and stem canker of potato. The pathogen was obtained from our own culture collection and maintained on potato dextrose agar (PDA, 39 g; Oxoid, in 1000 mL distilled water) at 21 °C. Only fresh culture was used for the experiment.

Identification of AMB based on fatty acid methyl ester (FAME) profiles

All 394 AMB isolates were characterized for their FAME profiles. In brief, 50 mg fresh cell mass of each isolate cultured on TSA (TSA, 30 g tryptic soy broth and 15 g bacto agar; Difco, in 1000 mL distilled water) for 24 h at 24 °C was used for extraction of fatty acids. Saponification, methylation and extraction were carried out according to Sasser (1990). The FAME profiles of all isolates were generated by a Hewlett Packard 5890 series II gas chromatograph equipped with MICROBIAL IDENTIFICATION software (MIS, version 4) and then matched with a library containing the profiles of 2500 species (MIDI Inc., Delaware). A similarity index (SI) ≥ 0.6 was chosen for reliable identification to species level. We considered a SI < 0.6 to give a less reliable identification and these isolates were considered as unidentified AMB.

Identification of AMB based on 16S rRNA gene

A total of 25 isolates were selected for further analysis using PCR and 16S rRNA gene sequencing. These represented each of the four source environments FW, FY, LW and LY, and some of them were unidentified by FAME. The results from the analysis by 16S rRNA gene were compared with those from the FAME analysis. A total of 10 mg fresh biomass of each isolate was suspended in 1 mL sterile MilliQ water. The suspension was heated for 15 min at 95 °C in a water bath, 2 μL of lysis solution (0.2 M NaOH, 1% sodium dodecyl sulphate) was added, and the suspension was vortexed and centrifuged at 20 000 g for 2 min. One microliter of the supernatant containing the crude DNA was used for amplification of 16S rRNA gene. The PCR reaction mixture in a total volume of 20 μL consisted of 1 μL of crude DNA, 1 μL each of eubacterial primers (10 pmol μL^{-1}) SSU-bact- 27f (5′-AGAGTTTGATC(A/C)TGGCTCAG-3′; Lane, 1991) and 16S-rD1(5′-AAGGAGGTGATCCAGCC-3′; Weisburg *et al.*, 1991) and 17 μL of Platinum® blue PCR Supermix (Invitrogen). Control tubes contained the PCR reaction mixture but without crude DNA. The PCR (PCT-200, M.J. Research Inc., MA) cycling conditions were initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 52 °C for 30 s and elongation at 72 °C for 90 s, and finally cycling was completed by a final extension period of 72 °C for 5 min. The PCR products were fractionated by electrophoresis in 1.2% agarose gel and visualized by staining with ethidium bromide. The PCR products were purified using the QIAquick PCR purification kit (Qiagen GmbH, Hilden, Germany). Purified PC ducts were sequenced in both directions, using the primers as above in the PCR reaction (Macrogen Inc., Korea). Sequences from both directions were align obtain a consensus sequence. Sequences were aligned to 16S rRNA gene of *Escherichia coli*, trimmed to cover nuc tide positions 80–870 in the *E. coli* sequence. The numb nucleotides obtained in this interval ranged between and 805. Sequences were searched against the Ribosom Database Project II (http://rdp.cme.msu.edu/) database against the NCBI GenBank using BLASTN (htt www.ncbi.nlm.nih.gov/blast).

Taxonomic analysis of isolates and their relat to source environment

To find taxonomic relationships among identified unidentified isolates, we used the FAME data for 385 394 isolates to carry out Hierarchical Cluster ana Euclidian distances and Farthest neighbour in the prog PC-ORD, version 5.0 (McCune & Mefford, 1999). The rem ing nine isolates were not included in the analysis bec the amount of fatty acids extracted from them was very Each isolate was indicated by its descriptive data with reg to its identity obtained from the FAME profile analysis a 16S rRNA gene sequencing, spore type, host plant speci pathogen inhibitory activity and fluorescence productio To test the relationship between bacterial taxonomy a source environment (spore, plant host) and the trait inhi tion of *R. solani*, χ^2-tests were performed with equ distribution among clusters or among the traits as a nu hypothesis. Probability values below 0.05 were considered indicate statistically significant differences.

Antagonistic activity of AMB against *R. solani*

Antagonistic activity of AMB isolates was assessed agains *R. solani* in a dual culture assay *in vitro* using the meth described by Montealegre *et al.* (2003). In this assay, a disc o uniform size of fresh culture of *R. solani* was inoculated i the centre of a Petri dish (9 cm) containing PDA. After 24 of incubation at 21 °C, each isolate was inoculated in circ around the fungal disc in a uniform manner in all plat Control plates were inoculated with sterile distilled wat only. The growth diameter of the pathogen was measu after 1 week by measuring the radial colony growth of t fungus. Radial growth of fungus < 5.0 cm was conside as strong inhibition, between 5.0 and 7.0 cm as moder inhibition and above 7.0 cm as no inhibition.

Fluorescence production

The ability of all AMB to produce fluorescent pigment wa tested by culturing them on King's medium B agar (KBA ing *et al.*, 1954). After incubation at 25 °C for 48 h, plates were inspected for fluorescence under UV light (366 nm).

Results

All the white and small spores extracted from the soils of both plant species were identified as *G. intraradices*, while all the yellow and large spores were identified as *G. mosseae*.

Among a total of 394 AMB isolates, 53% originated from the *G. intraradices* spores and 47% from the *G. mosseae* spores from both plant species. With regard to plant species, approximately half of the isolates originated from the spores extracted from *L. vulgare* and the remaining half from *F. ovina*. Using the two methods to select a representative range of bacteria, 267 isolates were selected using method 1 and 127 isolates using method 2.

Identification of AMB based on FAME profiles

Of 394 AMB isolates, 52% could be identified by FAME to species level at SI > 0.6. The identified isolates were taxonomically distributed in three phyla, five classes, 12 families, 16 genera and 36 species (Table 1). *Micrococcaceae* containing 77 isolates and *Pseudomonadaceae* containing 41 isolates were two dominant families, representing 54% of all identified taxa. *Arthrobacter* (66 isolates) and *Pseudomonas* (41 isolates) were the most common genera isolated. In addition, many bacteria from a wide range of taxa with single or a few isolates were also identified (Table 1).

Identification based on 16S rRNA gene

Among 26 sequenced isolates, 16 showed similar identity to that revealed by FAME analysis. The remaining 10 isolates, which were considered unidentified by FAME analysis at SI ≥ 0.6, were identified as *Paenibacillus* sp. (1), *Pseudomonas putida* (1), *S. maltophilia* (7) and an alphaproteobacterium (1) (Table 2).

Distribution of AMB based on cluster analysis of FAME profiles

The hierarchical cluster analysis and identification based on FAME analysis using 91 different fatty acids and the MIDI database demonstrated that the bacteria were distributed over several clusters coherent with bacterial taxonomy. The 385 isolates were distributed into two major clusters, denoted A and B (Fig. 1).

Cluster A contained three subclusters, denoted 1, 2 and 3, with isolates belonging to the phylum *Proteobacteria*, while Cluster B contained two subclusters, denoted 4 and 5, with isolates belonging to the phyla *Actinobacteria* and *Firmicutes* (Table 1, Fig. 1), respectively. The genera *Pseudomonas* and *Acidovorax* represented subcluster 1 and *Agrobacterium* represented subcluster 3 in Cluster A, while *Arthrobacter*, *Cellulomonas*, *Micrococcus* and *Bacillus* represented subclusters 4 and 5 in Cluster B. Surprisingly, subcluster 2 constituted mostly unidentified isolates and those that were identified belonged to one genus, *Stenotrophomonas*. All the 23 isolates producing fluorescence belonged solely to subcluster 1 (Fig. 1).

Relation to spore type and plant host source environment

To test the relationship between bacterial taxonomy at different levels of the clusters and their original environment (spore, plant host), we performed a series of χ^2-tests on all isolates (Table 3). The relationship between Clusters A and B and the source environment was tested with the null hypothesis that within each source environment the isolates should be equally distributed among the major Clusters A and B, i.e. what could be expected if the bacterial taxonomy was not related to the source environment. This analysis revealed that the distribution of bacteria among the two Clusters A and B was influenced by both AM fungal species (*G. mosseae*, $P = 0.012$, *G. intraradices*, $P = 4.2 \times 10^{-10}$) and the plant species *F. ovina* ($P = 3.06 \times 10^{-6}$), but not by *L. vulgare* ($P = 0.42$).

Within Cluster A, the majority (66%) of all AMB isolates originated from *G. intraradices* spores (Tables 1 and 3). In addition, the AM fungal spores, irrespective of type extracted from the plant species *F. ovina* contributed the majority (60%) of the AMB isolates. In contrast, the majority (65%) of all AMB isolates in Cluster B originated from *G. mosseae* spores. Furthermore, the AM fungal spores extracted from the plant species *L. vulgare* contributed the majority (59%) of the Cluster B isolates (Table 3).

With regard to distribution between the four different source environments, there seemed to be AM fungal and host plant interaction effects on the occurrence of the various AMB taxa with the spores, because 50% of a total of 220 isolates in Cluster A originated from the FW environment only (Fig. 1). The remaining half of the isolates was from the other three environments FY (10%), LW (16%) and LY (24%). Furthermore, in Cluster B the LY source environment dominated as the source of AMB with 48% of 165 isolates, while the remainder was from the FW (23%), FY (18%) and LW (11%) environments.

Subclusters that contained identified genera were further analysed to test whether the distributions among the spore types and among the plant hosts were different from an equal distribution, i.e. what could be expected if the bacterial taxonomy was not related to the source environment. The analysis of spore type effects showed that subclusters within Cluster A were dependent on a certain spore type (Table 3). In contrast, in Cluster B one of the two subclusters did not depend on a certain spore type. Genera

Table 1. Taxonomic position and number of bacterial isolates (AMB) associated with two types of AM fungal spores

			Source environment*			
Phylum/class	Family/species	Total isolates	LY	FY	LW	FW
Actinobacteria/Actinobacteria	***Micrococcaceae***	**77**				
	Arthrobacter atrocyneus	1	1	–	–	–
	A. globiformis	3	1	–	–	2
	A. ilicis	21	10	1	4	6
	A. oxydans	32	14	8	4	6
	A. protophormiae/ramosus	5	2	–	–	3
	A. ramosus	3	1	–	–	2
	Micrococcus halobius	1	1	–	–	–
	M. kristinae	2	1	–	1	–
	M. luteus GC subgroup C	4	–	–	–	4
	M. roseus	5	2	2	–	1
	Microbacteriaceae	**5**				
	Aureobacterium saperdae	3	3	–	–	–
	Clavibacter michiganense insidiosum	1	–	–	–	1
	Curtobacterium citreum	1	1	–	–	–
	Cellulomonadaceae	**21**				
	Cellulomonas flavigena	21	14	2	1	4
	Corynebacteriaceae	**1**				
	Corynebacterium bovis	1	–	–	1	–
Firmicutes/Bacilli	***Bacillaceae***	**7**				
	Bacillus brevis	3	1	–	1	1
	B. chitinosporus	1	–	–	–	1
	B. megaterium GC subgroup A	1	1	–	–	–
	B. megaterium GC subgroup B	1	1	–	–	–
	B. subtilis	1	–	–	–	1
Proteobacteria/Alphaproteobacteria	***Rhizobiaceae***	**18**				
	Agrobacterium radiobacter	18	–	1	3	14
Betaproteobacteria	***Alcaligenaceae***	**4**				
	Achromobacter piechaudii	2	–	–	–	2
	A. xylosoxydans denitrificans	2	–	–	2	–
	Burkholderiaceae	**1**				
	Burkholderia picketti	1	1	–	–	–
	Comamonadaceae	**19**				
	Acidovorax delafieldii	16	3	13	–	–
	Hydrogenophaga pseudoflava	3	–	3	–	–
	Oxalobacteraceae	**1**				
	Janthinobacterium lividum	1	–	–	–	1
Gammaproteobacteria	***Pseudomonadaceae***	**41**				
	Pseudomonas alcaligenes	2	1	–	–	1
	P. chlororaphis	1	–	1	–	–
	P. fluorescens biotype F	1	–	–	–	1
	P. pseudoalcaligenes	20	19	–	1	–
	P. putida biotype A	8	–	–	3	5
	P. putida biotype B	4	–	1	1	2
	P. syringae tagetes	2	–	–	–	2
	P. stutzeri	3	3	–	–	–
	Xanthomonadaceae	**7**				
	Stenotrophomonas maltophilia	7	1	–	1	5
	Total identified	202	82	32	23	65
	Total unidentified	192	53	19	35	85
	Grand total	394	135	51	58	150

The AM fungal spores were extracted from *Festuca ovina* and *Leucanthemum vulgare* plant species grown as monoculture in field conditions.
*FW, white spores of *Festuca ovina*; FY, yellow spores of *F. ovina*; LW, white spores of *Leucanthemum vulgare*; LY, yellow spores of *L. vulgare*. See text for details.

Table 2. Comparison of identification of 26 AMB isolates by FAME profiles and by 16S rRNA gene sequencing

Strain	FAME	SI	16S rRNA gene*	% Identity
LWC9	*Agrobacterium radiobacter*	0.459	Alphaproteobacterium	93
FWC45	*Achromobacter piechaudii*	0.810	*Ac. piechaudii*	99
LWD36	*Ac. xylosoxidans denitrificans*	0.630	*Ac. xylosoxidans*	99
FWC110	*Arthrobacter ilicis*	0.710	*Arthrobacter* sp.	100
FWD4	*Ar. oxydans*	0.629	*Ar. oxydans*	100
LYC55	*Bacillus brevis*	0.674	*B. benzoevorans*	98
FWC83	*B. chitinosporus*	0.546	*Bacillus* sp.	100
FYD11	*B. circulans*	0.244	*Paenibacillus* sp.	100
LYC54	*B. megaterium* C subgroup B	0.625	*B. benzoevorans*	99
FWC42	*B. subtilis*	0.583	*B. subtilis*	99
FYC15	*Paenibacillus pabuli*	0.133	*Paenibacillus* sp.	96
FWC30	*Pseudomonas fluorescens* biotype F	0.377	*P. putida*	99
LWC17	*P. putida* biotype A	0.651	*P. putida*	97
FWC66	*P. putida* biotype A	0.630	*P. putida*	98
FWC70	*P. putida* biotype A	0.625	*P. putida*	99
LWD2	*P. putida* biotype B	0.545	*Pseudomonas* sp.	99
FWC16	*P. putida* biotype B	0.439	*P. putida*	99
LYC7	*P. pseudoalcaligenes*	0.834	*Pseudomonas* sp.	100
FWC94	*Stenotrophomonas maltophilia*	0.549	*S. maltophilia*	95
FWC39	*S. maltophilia*	0.304	*S. maltophilia*	99
LWD12	*S. maltophilia*	0.193	*S. maltophilia*	100
LYC39	*S. maltophilia*	0.185	*S. maltophilia*	98
FWC101	*S. maltophilia*	0.184	*S. maltophilia*	99
LWC2	*S. maltophilia*	0.163	*S. maltophilia*	100
FWC51	*S. maltophilia*	0.154	*S. maltophilia*	100
LWD23	*S. maltophilia*	0.094	*S. maltophilia*	99

*GenBank accession numbers are EU833932–EU833957.
SI, similarity index.

dependent on the spore type *G. mosseae* were found to be *Acidovorax* and nonfluorescent *Pseudomonas*, while fluorescent *Pseudomonas*, *Agrobacterium* and *Stenotrophomonas* were dependent on *G. intraradices* spores within Cluster A (Fig. 1, Table 3). The *Cellulomonas* subcluster as well as subcluster 4 *Arthrobacter* and *Micrococcus* depended on the spore type *G. mosseae*. With respect to plant host effects, all subclusters in Cluster A, but only one in Cluster B, were dependent on a certain plant species (Table 3). In Cluster A, most *Acidovorax*, fluorescent *Pseudomonas*, *Stenotrophomonas* and *Agrobacterium* depended on *F. ovina*, while nonfluorescent *Pseudomonas* depended on *L. vulgare*. Similarly, most isolates in the *Cellulomonas* subcluster in Cluster B depended on *L. vulgare*.

In the case of the subcluster *Arthrobacter*, the effect of spore type was significant but less than that for other clusters, and for plant host it was not significant when analysed separately (Table 3). However, the data in Fig. 1 suggest that many AMB isolates originate from a certain spore type–plant host combination, and this was significant ($P = 3.1 \times 10^{-6}$) when each of the four types of origin (FW, FY, LW and LY) were taken into account. Isolates from the genus *Arthrobacter* thus co-occurred with spores from the *G. intraradices–F. ovina* combination and also from the *G. mosseae–L. vulgare* combination, but significantly less so for the other two AM–plant combinations.

Interestingly, at the species level some AMB appeared only with one spore type and one plant species or with one spore type–plant species combination (Table 4). Ten species were exclusively associated with *G. mosseae* spores and ten other species were exclusively associated with *G. intraradices* spores. Similarly, 10 species found with both spore types extracted from *F. ovina* were not found with spores extracted from *L. vulgare*. In contrast, 11 other species found with both spore types extracted from *L. vulgare* were not found with spores from *F. ovina*. With regard to distribution among the four source environments, two species were exclusively found with FY, eight with FW, seven with LY and two with LW (Table 4). Spore type–plant species interaction effects observed as the presence or absence of certain species were found for *Aureobacterium*, *Curtobacterium* and *Burkholderia*, *Hydrogenophaga*, *Clavibacter* and *Janthinobacterium* (Table 4). Furthermore, eight species appeared exclusively when selected by Method 1, while 10 different species appeared exclusively with Method 2 (Table 4). The phytopathogenic bacteria *P. syringae* pv. *tagetes* and *Clavibacter michiganense* ssp. *insidiosum* were found associated with *G. intraradices* spores extracted from *F. ovina* (Table 4).

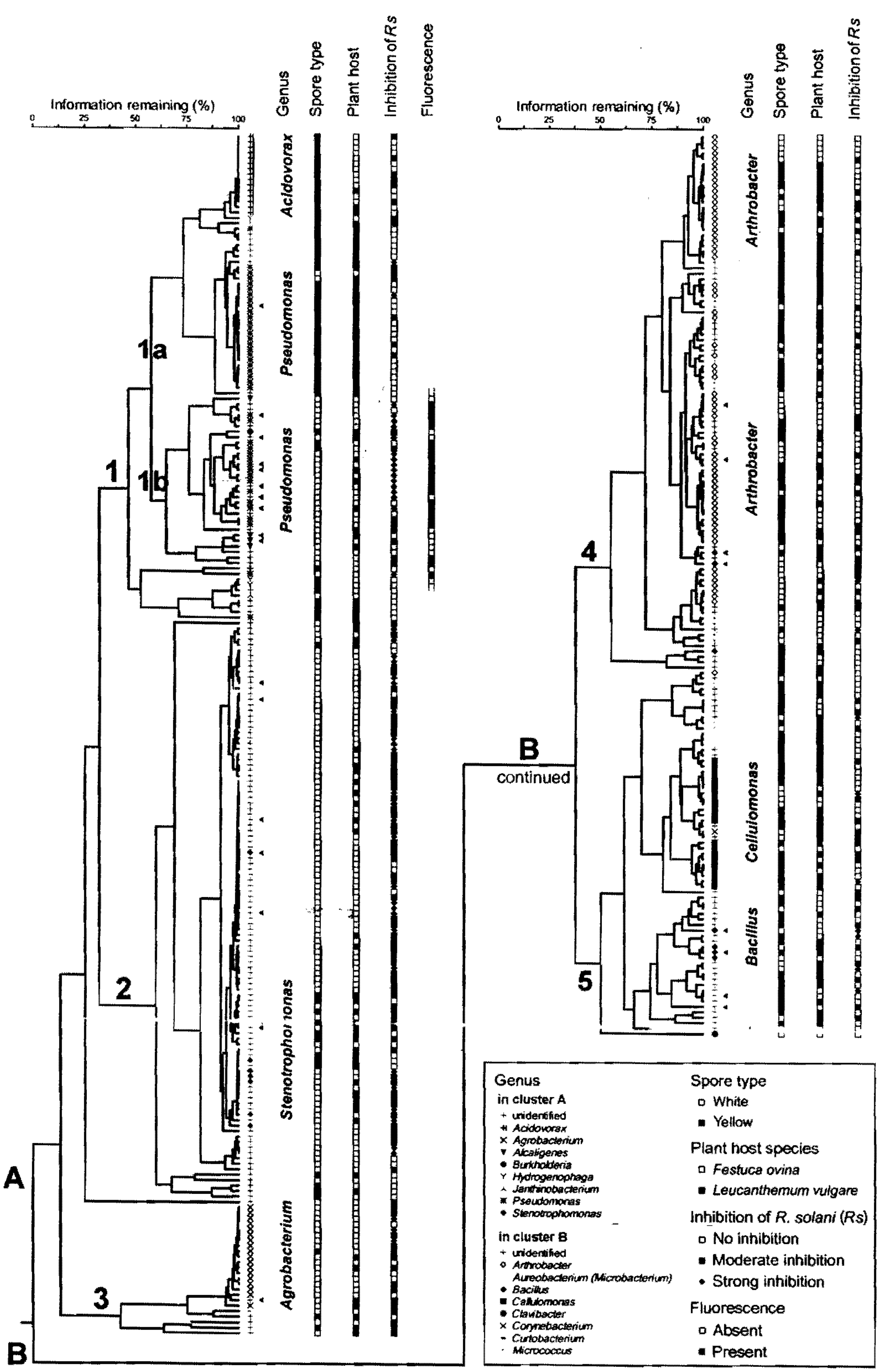

Fig. 1. Dendrogram based on cluster analysis of whole-cell fatty acid profiles of bacterial isolates showing the taxonomic distribution of identified (SI ≥ 0.6) isolates in relation to unidentified isolates. Major genera are indicated. Source environments (spore type and plant host species) of the isolates and their phenotypes inhibition *in vitro* of *Rhizoctonia solani* growth and production of fluorescent pigments are presented. White spores were identified as *Glomus intraradices* and yellow spores as *Glomus mosseae*. Isolates also identified using partial sequencing of the 16S rRNA gene are indicated by arrows (◀).

Table 3. Relationships among bacterial taxa and their source environment (spore type, plant host) and trait inhibition of *Rhizoctonia solani*

	Probability values from χ^2 analyses		
Bacterial taxonomic level	Spore type	Plant host	Inhibition of *R. solani*[*]
Major Cluster A (220)	$P < 0.0001$	$P = 0.019$	$P < 0.0001$
Major Cluster B (165)	$P < 0.0001$	$P = 0.016$	$P < 0.0001$
Within Cluster A (179)[†]			
Subcluster 1a, *Acidovorax* (15)	$P < 0.0001$	$P = 0.020$	$P = 0.439$
Subcluster 1a, *Pseudomonas*, nonfluorescent (27)	$P < 0.0001$	$P < 0.0001$	$P = 0.034$
Subcluster 1b, *Pseudomonas*, fluorescent (25)	$P < 0.0001$	$P = 0.028$	$P < 0.01$
Subcluster 2, *Stenotrophomonas* (93)	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$
Subcluster 3, *Agrobacterium* (19)	$P < 0.0001$	$P = 0.012$	$P = 0.039$
Within Cluster B (148)[†]			
Subcluster 4, *Arthrobacter* and *Micrococcus* (94)	$P = 0.039$	$P = 0.216$	$P < 0.0001$
Subcluster 5, *Cellulomonas* (29)	$P < 0.01$	$P < 0.01$	$P = 0.095$
Subcluster 5, *Bacillus* (25)	$P = 0.071$	$P = 0.841$	$P = 0.841$

Results are presented as probabilities from χ^2 analyses where the null hypothesis is equal distribution among each spore type, plant host or inhibition trait, respectively. See Fig. 1 for cluster analysis. Numbers in parentheses show the numbers of isolates used in each analysis.

[*] Equal distribution of inhibitory (strong + moderate) and non-inhibitory isolates.

[†] Isolates unidentified by FAME but closely related in the cluster analysis are included in the χ^2 analyses.

Functional characteristics of AMB

Approximately half of the AMB isolates exhibited different degrees of inhibition of *R. solani* growth *in vitro*. Of the 394 isolates tested, only 14% (57) strongly inhibited the pathogen, while 39% (152) were only moderately antagonistic (Table 5). A large proportion of the isolates (47%) showed weak or practically no inhibition. The occurrence of most antagonistic isolates was spore type-dependent and not plant host-dependent and they originated from *G. intraradices* spores (Table 5).

Antagonistic isolates appeared in both major Clusters A and B, but significantly higher than expected in Cluster A and was significantly lower in Cluster B when compared with an equal distribution among antagonists and nonantagonists (Table 3). Within Cluster A, the antagonistic isolates occurred among the fluorescent *Pseudomonas*, *Stenotrophomonas* and *Agrobacterium* subclusters (Table 3) in contrast to the nonfluorescent *Pseudomonas*, which were significantly nonantagonistic. Within the *Acidovorax* subcluster, the relative proportions of antagonistic and nonantagonistic isolates were about equal (Table 3, Fig. 1). In Cluster B, the antagonistic isolates occurred mainly in subcluster 4 *Arthrobacter* and *Micrococcus*. Within the *Cellulomonas* and *Bacillus* subclusters the pattern was the same as for the *Acidovorax* subcluster (Table 3, Fig. 1). Species showing strong inhibition were identified as *P. putida* (11), *Agrobacterium radiobacter* (3), *Cellulomonas flavigena* (2), *Arthrobacter oxydans* (1), *Bacillus subtilis* (1), *Micrococcus kristinae* (1), *Pseudomonas pseudoalcaligenes* (1) and *S. maltophilia* (1) and 36 isolates were considered as unidentified.

Of a total of 57 strongly antagonistic isolates, only 16 were fluorescent (Table 5). In total, 23 isolates were fluorescent. Most of these were obtained from *G. intraradices* (21) and *F. ovina* (19). The fluorescent AMB belonged to *Pseudomonas putida* (12), *Pseudomonas fluorescens* (1), *Pseudomonas chlororaphis* (1) and *Pseudomonas syringae* pv. *tagetes* (2), and the remainder were unidentified. None of the 135 isolates obtained from *G. mosseae* spores of the LW source environment were fluorescent (Table 5).

Discussion

AM fungal spores are important long-term reproductive structures that could act as a habitat and provide a niche for certain bacteria. In this study, AM fungal spores were extracted from field-grown plants rather than trap cultures. The spores had thus been exposed to and developed in the natural soil-plant environment and were able to host a relevant AMB flora.

The AMB were isolated from surface-decontaminated AM fungal spores. This method makes it possible to isolate cultivable bacteria tightly associated with spores or present inside the spores, and to avoid interference in bacterial composition by chemicals normally used for surface sterilization (Xavier & Germida, 2003). A low-strength medium, e.g. diluted TSA as in this study, can help in isolating greater numbers of bacterial species (Walley & Germida, 1996) by retarding fast-growing bacteria and allowing slow-growing bacteria to proliferate. However, the possibility cannot be excluded that using a different type of growth medium for isolation might have revealed other bacterial taxa.

To ensure a good representation of AMB of different origins, we used two different selection methods and found differences in occurrence of certain low-abundance species, depending on the selection method (Table 4). When using

Table 4. Identified AMB isolates specific for particular source environment (plant host, spore type and combinations) and isolation method

Plant species	
Festuca ovina (10)	*Leucanthemum vulgare* (11)
Achromobacter piechaudii, Bacillus chitinosporus, B. subtilis, Clavibacter michiganense insidiosum, Hydrogenophaga pseudoflava, Janthinobacterium lividum, Micrococcus luteus, Pseudomonas chlororaphis, P. fluorescens, P. syringae pv. *tagetes*	*Achromobacter xylosoxydans denitrificans, Arthrobacter atrocyneus, Aureobacterium saperdae, Bacillus megaterium, Burkholderia pickett Corynebacterium bovis, Curtobacterium citreum, Micrococcus kristin M. halobius, Pseudomonas pseudoalcaligenes, P. stutzeri*
Spore type	
Glomus intraradices, W (10)	*Glomus mosseae*, Y (10)
Achromobacter xylosoxydans, A. piechaudii, Bacillus chitinosporus, B. subtilis, Clavibacter michiganense insidiosum, Corynebacterium bovis, Janthinobacterium lividum, Micrococcus luteus, Pseudomonas fluorescens, P. syringae pv. *tagetes*	*Acidovorax delafieldii, Arthrobacter atrocyneus, Aureobacterium saperdae, Bacillus megaterium, Burkholderia pickettii, Curtobacterium citreum, Hydrogenophaga pseudoflava, Micrococcus halobius, Pseudomonas chlororaphis, P. stutzeri*
Source environment	
FW (8)	FY (2)
Achromobacter piechaudii, Bacillus chitinosporus, B. subtilis, Clavibacter michiganense ssp. *insidiosum, Janthinobacterium lividum, Micrococcus luteus, Pseudomonas syringae* pv. *tagetes, P. fluorescens*	*Hydrogenophaga pseudoflava, Pseudomonas chlororaphis*
LW (2)	LY (7)
Corynebacterium bovis, Achromobacter denitrificans ssp. *xylosoxydans*	*Arthrobacter atrocyneus, Aureobacterium saperdae, Bacillus megaterium, Burkholderia pickettii, Curtobacterium citreum, Micrococcus halobius, Pseudomonas stutzeri*
Isolation method	
Method I (8)	Method II (10)
Arthrobacter protophormiae/ramosus, A. ramosus, Bacillus subtilis, Corynebacterium bovis, Curtobacterium citreum, Pseudomonas chlororaphis, P. stutzeri, P. syringae tagetes	*Achromobacter xylosoxydans denitrificans, Arthrobacter atrocyneus, Aureobacterium esteroaromaticum, Burkholderia pickettii, Clavibacte michiganense insidiosum, Janthinobacterium lividum, Micrococcus halobius, M. varians, Pseudomonas fluorescens, P. putida*

Numbers in parentheses show number of isolates.

Table 5. Distribution of AMB according to *in vitro* inhibition of *Rhizoctonia solani* and production of fluorescent pigment

	Isolates inhibiting *R. solani* growth[+]						
	Strong		Moderate		None		
Source environment of AMB*	No.	%	No.	%	No.	%	Fluorescent
FY	3	7	9	22	29	71	2
FW	33	22	73	49	44	29	17
LY	4	3	23	17	108	80	0
LW	17	29	37	64	4	7	4
Total isolates	57	14	152	39	185	47	23
Fluorescent	16	70	4	17	3	13	

Data presented are number of isolates and percentages within each source environment and range of pathogen inhibition.

*See Materials and methods for details.

[+]Colony diameter size in presence of AMB: ≤ 5.0 cm was considered as strong, 5.0–7.0 cm as moderate and ≥7.0 cm as no inhibition.

morphology as the criterion (Method 1), the choice of colonies can be biased by overlooking colonies with similar appearance but different identity. However, the advantage is that low-abundance colonies with a characteristic appearance can be detected. In contrast, the straight-line method (Method 2) ensures that colonies that may be from different species but with similar appearance are represented in the selection. Hence, the use of more than one method provides a better representation of the range of culturable AMB from the original habitat than a single method.

We used FAME analysis for bacterial identification, as it a fast, reliable and less expensive method that is widely us for the identification of bacteria from agricultural soi marine water and clinical isolates. More than 50% of th AMB isolates were identified to species level, using FAM analysis, at SI ≥0.6. However, the identification of bacte to genus and species level is normally acceptable at SI 0. and 0.5, respectively, (MIDI Inc.), but we used SI ≥0.6 obtain confirmed identity. When a lower SI value (≥0. was used, it was possible to identify up to 69% of AMB genus level, thus still leaving a large fraction unidentified. To find out more about the identity of the isolates that were not firmly identified at SI ≥ 0.60, the FAME data of most isolates (385) were subjected to hierarchical cluster analysis. Most unidentified isolates were found to be related to the genus *Stenotrophomonas* or *Bacillus* and in some cases to *Pseudomonas*, *Paenibacillus* and uncultured *Alpha*- and *Betaproteobacteria* (Fig. 1). Hence, the closest position of unidentified AMB to the neighbouring identified AMB in cluster analysis based on FAME can be worth considering for obtaining suggested identity. Comparing the results from FAME analysis with those obtained from 16S rRNA gene sequencing showed that 16 out of 26 isolates had the same identity at species level. The identity of *Paenibacillus* revealed by FAME at SI = 0.133 was also confirmed using 16S rRNA gene. Hence, the use of two approaches can give firm identification of bacteria (Thurlow & Gillock, 2005).

The genera *Acidovorax*, *Agrobacterium*, *Arthrobacter*, *Achromobacter*, *Bacillus*, *Burkholderia*, *Cellulomonas*, *Clavibacter*, *Corynebacterium*, *Micrococcus*, *Paenibacillus* and *Pseudomonas* have all previously been reported to be associated with either AM fungal spores or mycelia (Mayo *et al.*, 1986; Bianciotto *et al.*, 1996; Mansfeld-Giese *et al.*, 2002; Xavier & Germida, 2003; Roesti *et al.*, 2005; Toljander *et al.*, 2006). In the present study we found all of them to be associated with spores. In addition, we found the genera *Aureobacterium*, *Curtobacterium*, *Hydrogenophaga*, *Janthinobacterium* and *Stenotrophomonas* to be associated with spores (Table 1).

This study demonstrates that the species composition of cultivable AMB in AM fungal spores is affected by their source environment, i.e., AM fungal species, plant species and the AM–plant species combination. Support for this was found in the differences in distribution among the two major AMB clusters. Also, the differences in distribution among source environments within clusters at the genus level and the exclusive occurrence of certain AMB species with specific AM fungi or plant species and their combinations (Fig. 1, Tables 3–5) support this conclusion.

Pseudomonas, *Stenotrophomonas* and *Arthrobacter* were the dominant genera in this study. The genus *Pseudomonas* has been reported to be naturally associated with spores of *G. versiforme* (Mayo *et al.*, 1986) and the genus *Arthrobacter* is frequently found in AM fungi-inoculated soil (Mansfeld-Giese *et al.*, 2002). In addition, *Pseudomonas* and *Arthrobacter* are reported to be more attracted by exudates of mycorrhizal tomato roots than by exudates of nonmycorrhizal tomato roots (Sood, 2003). A hypothesis explaining the high abundance of these genera in our study of AMB in AM fungal spores could therefore be that these genera are favoured by the environment created by the mycorrhizae present in the plant host environment, possibly through differences in exudates between plant species and their mycorrhization status. Support for this can be found in the plant host effect on the nonfluorescent *Pseudomonas* subcluster and the *Stenotrophomonas* subcluster (Fig. 1, Table 3). The frequent occurrence of *Arthrobacter*, specifically in the AM–plant combinations *G. intraradices*–*F. ovina* and *G. mosseae*–*L. vulgare*, also provides support for the idea that the bacterial composition in the mycorrhizosphere, here the AMB composition in particular, could depend on the nature of the exudates from roots and from AM fungal hyphae and spores. It has been shown previously that the quantity and composition of plant root exudates differ depending upon the plant species (Lynch & Whipps, 1990) and can determine the composition of rhizosphere bacterial communities (Bais *et al.*, 2006). The mycorrhizosphere exudates are also modified both qualitatively and quantitatively by the presence of an active AM symbiosis (Bansal & Mukerji, 1994), can affect the chemical composition and pH of the soil (Filion *et al.*, 1999) and can include antimicrobial compounds produced by AM fungi (Ravnskov *et al.*, 1999). One possible explanation for the abundance of certain bacterial species being increased by specific AM fungi may be that those bacteria are activated by the modified exudate pattern (Artursson *et al.*, 2006). Thus, differences in plant root exudates and AM fungal exudates could play an important role in the selection of bacteria in AM–plant associations.

The taxonomic composition of AMB isolates was more influenced by spore type than plant host species (Fig. 1, Table 3). This was more evident for the isolates in Cluster B than in Cluster A and, depending on the subcluster, the AM fungal species was either *G. intraradices* or *G. mosseae* (Fig. 1, Table 3). Roesti *et al.* (2005) also reported that the composition of communities of spore-associated bacteria was more dependent on AM fungal identity (*G. geosporum* or *G. constrictum*) than on plant host (*Plantago lanceolata* or *Hieracium pilosella*) identity. Interestingly, these two studies reached the same conclusions, although our study investigated only cultivable bacteria from surface-decontaminated spores while the Roesti *et al.* (2005) study used PCR-denaturing gradient gel electrophoresis and surface-sterilized spores, which should be able to detect both cultivable and putative uncultivable AMB. The benefit of the molecular method is that it investigates the occurrence of AMB *in situ*, but it may be biased towards the more abundant species and limited by PCR primer recognition and the short sequence data obtained. On the other hand, isolation of cultivable AMB may be limited by the ability of AMB to grow without the presence of AM fungi depending on isolation conditions, but may also allow AMB of small populations to grow and allow identification using multigene inherited traits such as FAME analysis. Clearly, a set of different methods for these types of studies is necessary for investigating the full diversity of AMB.

One major factor controlling the abundance and occurrence of AMB could be traits of the spores themselves. Filippi *et al.* (1998) found holes in the outer layer of the *G. mosseae* spores that contained the bacteria. Our results clearly show that the composition of AMB differed among the two fungal species investigated. Reasons for this could be differences in the potential of the spores to host different species of AMB, depending on their requirements, on their habitat. The surface structure as well as attachment mechanisms of AMB to surface depending on surface molecules, would affect the population sizes and composition of AMB on spores. In fact, the spore morphology of the two AM fungi species studied is known to differ significantly in terms of size and the surface roughness that occurs due to differences in spore wall development (INVAM: http://invam.caf.wvu.edu/). Alternatively, it might be that some bacteria can use the spore as a growth substrate to obtain nutrients from AM fungal spores and hyphae. Roesti *et al.* (2005) reported that cell wall-degrading bacteria represented the major bacterial community associated with spores. In our study, we also found several *Bacillus* isolates and other isolates that showed chitinase activity (D.P. Bharadwaj *et al.*, unpublished data), and *Bacillus* spp. are known to have fungal cell wall-degrading properties (Aktuganov *et al.*, 2003). The association of bacterial populations with AM fungal spores can also be affected by the age of the spore. These possibilities deserve further studies to identify the precise mechanisms behind the potential of spores to favour certain AMB.

We found that a large proportion of the AMB isolates had the ability to inhibit the growth of *R. solani* in an *in vitro* assay. Budi *et al.* (1999) also found an inhibitory effect of the AMB *Paenibacillus* sp. against several fungal pathogens, including *R. solani*. The inhibition of *R. solani* by bacteria may be due to the production of antibiotics or siderophores or both (O'Sullivan & O'Gara, 1992). In the present study, we found that 16 of the 57 strongly antagonistic isolates produced fluorescent siderophores and that they belonged to the group fluorescent *Pseudomonas*. Bacterial siderophores bind to ferric iron faster than the siderophores of fungal pathogens and thus make it unavailable to the pathogens. Hence competition for iron uptake by fluorescent AMB might be one mechanism behind the growth inhibition of *R. solani*. This study did not investigate whether the antagonistic ability was due to production of antibiotics or resistance mechanisms involving plant defence mechanisms. However, antagonism through cell wall-degrading properties is another possible mechanism. In fact, many of the other strongly antagonistic isolates belonged to the genera *Agrobacterium* spp. and *Bacillus* and *Stenotrophomonas*-related isolates, which are known to inhibit the growth of bacteria and fungi by producing cell wall-degrading enzymes, e.g., chitinases, proteinases as well as nonfluorescent siderophores, volatile compounds an antibiotics (Kerr, 1980; Jakobi *et al.*, 1996; Aktuganov *et al.* 2003; Kai *et al.*, 2007). There are some reports on multi functional characteristics of AMB, indicating that some of these can be proteolytic, chitinolytic and/or cellulolytic in the natural environment (Filippi *et al.*, 1998; Roesti *et al.* 2005).

The ecophysiological role of AMB in the fitness of AM fungi and in mycorrhizal symbiosis requires more investigation. However, taken together, our results suggest that a mechanism for the often found positive effect of AM fungi against plant pathogens (Yao *et al.*, 2002; Herre *et al.*, 2007) could be provided by the antagonistic AMB. The inhibitory effect appears to differ among the AM fungal species, which may be due to different spore types offering protected niches for the survival and establishment of different kinds of bacteria. Through direct applications of AMB, as well as by providing the AM fungal species most effective for hosting efficient AMB communities, these AMB have a potential to be exploited as biological control agents.

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