Mycorrhiza helper bacterium *Streptomyces* AcH 505 induces differential gene expression in the ectomycorrhizal fungus *Amanita muscaria*

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Summary

• The interaction between the mycorrhiza helper bacteria *Streptomyces* nov. sp. 505 (AcH 505) and *Streptomyces annulatus* 1003 (AcH 1003) with fly agaric (*Amanita muscaria*) and spruce (*Picea abies*) was investigated.

• The effects of both bacteria on the mycelial growth of different ectomycorrhizal fungi, on ectomycorrhiza formation, and on fungal gene expression in dual culture with AcH 505 were determined.

• The fungus specificities of the streptomycetes were similar. Both bacterial species showed the strongest effect on the growth of mycelia at 9 wk of dual culture. The effect of AcH 505 on gene expression of *A. muscaria* was examined using the suppressive subtractive hybridization approach. The responsive fungal genes included those involved in signalling pathways, metabolism, cell structure, and the cell growth response.

• These results suggest that AcH 505 and AcH 1003 enhance mycorrhiza formation mainly as a result of promotion of fungal growth, leading to changes in fungal gene expression. Differential *A. muscaria* transcript accumulation in dual culture may result from a direct response to bacterial substances.

Key words: acetoacyl coenzyme A synthetase, *Amanita muscaria*, cyclophilin, ectomycorrhiza, mycorrhiza helper bacteria, streptomycetes, suppression subtractive hybridization (SSH).

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Introduction

The majority of plants form symbiotic associations, termed mycorrhizas, with filamentous fungi. Mycorrhizal symbiosis improves plant nutrient and water uptake, while the fungal partner gains carbohydrates from its host plant (Smith & Read, 1997). Bacteria have been visualized inside mycorrhizas and as colonies on soil-colonizing fungal hyphae (Foster & Marks, 1966; Nurmiaho-Lassila *et al.*, 1997), and the plant partner selects for bacterial strains beneficial for the symbiosis and for the plant (Frey-Klett *et al.*, 2005). Specifically, the growth of ectomycorrhizal fungi and mycorrhiza formation are promoted by some of the mycorrhizosphere bacteria, termed 'mycorrhiza helper bacteria' (MHB; Garbaye, 1994). MHB have predominantly been reported from fluorescent pseudomonads (Garbaye & Bowen, 1989; Founoune *et al.*, 2002) and from *Burkholderia* and *Rhodococcus* (Poole *et al.*, 2001).

Actinomycetes are known for their capacity to control plant diseases. A number of investigations have reported antagonism of soil actinomycetes to root pathogenic fungi (Rose *et al.*, 1980; Crawford *et al.*, 1993), and that streptomycetes in particular provide a rich source of antifungal compounds (Mousavi & Robson, 2004). When 12 actinomycete isolates were tested for their effects on mycelial growth of ectomycorrhizal fungi (Richter *et al.*, 1989), the bacterial isolates inhibited, promoted or showed no significant effects on hyphal extension in dual culture (DC). Three fungal species

were tested in DC, *Laccaria bicolor*, *Laccaria laccata* and *Thelephora terrestris*, of which the slowest growing fungus, *T. terrestris*, was most sensitive to both growth-promoting and antagonistic actinomycetes (Richter *et al.*, 1989). Some antagonistic actinomycetes are also producers of plant growth-promoting substances, helping plants to withstand adverse conditions and attacks by pathogens (Igarashi *et al.*, 2002). Thus, the opportunity exists to select for actinomycetes stimulatory to certain symbiotic fungi and plant growth but antagonistic to pathogenic organisms.

We have isolated a collection of actinomycetes from the hyphosphere of a spruce (*Picea abies*) stand (Maier, 2003). One of these isolates, *Streptomyces* sp. nov. 505 (AcH 505), significantly increases the mycelial growth and mycorrhiza formation rate of *Amanita muscaria* but suppresses the mycelial extension of the plant pathogens *Armillaria obscura* and *Heterobasidion annosum* (Hampp & Maier, 2004; Maier *et al.*, 2004). In contrast to AcH 505, the second MHB isolated, *Streptomyces annulatus* 1003 (AcH 1003), does not affect the growth of the tested plant pathogenic fungi.

The aim of the experiments presented in this study was to compare the fungus specificity of AcH 505 to that of AcH 1003, and to test the MHB function of these bacteria in a seminatural perlite-moss culture system. Given the present lack of information regarding fungal genes expressed in response to the interaction with MHB, we have used suppression subtractive hybridization (SSH; Diatchenko *et al.*, 1996) to identify genes of *A. muscaria* that are preferentially expressed in DC with AcH 505. For subtraction we used the first 1 cm from the actively growing hyphal front of 9-wk-old DC, as AcH 505 most strongly promotes mycelial growth at this stage. Here we report several *A. muscaria* genes that are differentially transcribed in DC with the bacterium AcH 505.

Materials and Methods

Influence of streptomycetes on fungal growth in axenic conditions

The conditions for bacterial and fungal single and dual cultures, and the screening of bacterial effects on fungal growth, were essentially as described in Maier *et al.* (2004). *Amanita muscaria* growth-promoting *Streptomyces* sp. nov. 505 (AcH 505) and *Streptomyces anulatus* (Beijerinck) Waksman 1003 (AcH 1003), and the control strain *Streptomyces argenteolus* (Tresner *et al.*, 1961) 504 (AcH 504) exerting no effect on hyphal growth in DC (Maier, 2003), isolated from the hyphosphere of *Picea abies* (L.) Karst (Norway spruce) in Haigerloch, Germany (Maier *et al.*, 2004), were cultivated on ISP-2 agar (Shirling & Gottlieb, 1966) in the dark at 20°C.

Amanita muscaria (Fr.) Hooker strains 6 and MEII, isolated from fruiting bodies collected from the Schönbuch forest near Tübingen, Germany, *Amanita muscaria* strain CS83 (Schaeffer, 1995), *Hebeloma cylindrosporum* Romagnesi strain H1-H7 (Debaud & Gay, 1987), *Paxillus involutus* (Batsch: Fr.) Fr. strain ATCC 200175, originally isolated from a fruiting body associated with 15–30-yr-old *Betula pendula* trees growing on coal waste in Midlothian, UK, and *Suillus bovinus* (L.: Fr.) O. Kuntze strain K3 (Sen, 1990) were cultivated in the dark at 20°C on MMN (modified Melin–Norkrans medium) agar (Molina & Palmer, 1982) with 10 g l^{-1} glucose. Cultures for growth experiments were started with 4-mm-diameter inoculates, obtained from the actively growing rims of single fungal colonies placed in the centre of a sterile cellophane sheet (exclusion limit 10 kDa; Folia, Wendelstein, Germany) on MMN agar (Maier *et al.*, 2004).

The bacterial effect on fungal growth was tested in DC without direct contact between fungus and bacterium on MMN medium [see Maier *et al.* (2004) for details]. A single thin streak (4 cm) of bacteria, obtained from 1-wk-old culture, was applied to the edge of a Petri dish (outside the cellophane sheet) with a sterile inoculation loop. Ten replicates were used. Culture periods were between 6 and 11 wk, depending on the mycelial growth rate of the fungal species in question. At harvest, the first 1 cm of mycelium was rapidly scraped off the cellophane layer, and the material was immediately frozen in liquid nitrogen and stored at −80°C.

Suspension culture experiments

Fungal suspension cultures were started from actively growing hyphae which were finely cut, transferred to MMN medium, homogenized and incubated at 20°C on a rotary shaker at 80 rpm. The MMN medium was exchanged once a week. Carbon-based induction of *A. muscaria* acetoacyl coenzyme A synthetase (*AmAacs*) expression was investigated according to Nehls *et al.* (1999). For *A. muscaria* cyclophilin 40 kDa (*AmCyp40*) induction experiments, suspension cultures of *A. muscaria*, grown for 5 d after MMN medium exchange, were used. Streptomycete suspension cultures were started by transferring an appropriate amount of 1-wk-old bacterial culture to MMN medium and grown for 7 d.

For DC, 10 ml of a filtered (0.22-μm mesh) cell-free 7-d-old streptomycete culture supernatant, taken at an optical density of 600 nm of 0.2, was added to 15 ml of a fungal suspension culture and grown for 3 h. An untreated fungal suspension culture served as a control. All suspension cultures were kept at 20°C on a rotary shaker at 80 rpm. At harvest, suspension cultures were filtered through 100-μm mesh nylon membranes. The material was immediately frozen in liquid nitrogen and stored at −80°C.

Synthesis of ectomycorrhizas and analysis of seedlings

Seeds of Norway spruce (*P. abies*) were obtained from Staatsklenge Nagold (Nagold, Germany) and those of Scots pine (*Pinus sylvestris* L.) from Forelia (Häkkilä, Finland). Seedlings were cultivated until 4 wk old according

to Schaeffer *et al.* (1996). Ectomycorrhizas were synthesized using a Petri dish method modified from Poole *et al.* (2001). Moss peat (Botanical Garden, Tübingen, Germany) and perlite (Knauf Perlite GmbH, Dortmund, Germany) were mixed in a 1 : 1 ratio, crushed, moistened with double-distilled water (10 g water : 15 g mixture) and autoclaved. A slit was cut into the side of the Petri dishes and their cover using a hot scalpel. One part MMN medium (without glucose) was mixed with 4 parts peat–perlite mixture and evenly spread onto the Petri dishes. Plants and fungal inocula (*A. muscaria* 6 or *S. bovinus* K3) were positioned according to Poole *et al.* (2001). Briefly, plants were inserted into the slit, with the root systems placed on the peat–perlite mixture and the shoots exposed to the atmosphere. Three fungal inocula, consisting of 5-mm-diameter discs cut from the growing margin of an MMN agar culture, were placed upside-down along each side of the root at 15-mm intervals, at a distance of 5 mm from the root. For bacterial inoculations, 1-wk-old AcH 505 or AcH 1003 culture in MMN medium was centrifuged (1500 ***g*** for 15 min) and the pellets were resuspended at OD 0.6 in MMN medium without glucose. Two 500-µl aliquots of bacteria were pipetted between the roots and the fungal plugs. Four-month-old seedlings were harvested for analysis. For all inoculations, 10 replicates were used. Significance was analysed with Student's *t*-test.

Nucleic acid extractions and construction of a subtracted cDNA library

The first 1 cm of the actively growing rims of 9-wk-old *A. muscaria* 83 mycelia inoculated with AcH 505 was used as starting material for cDNA subtraction. Total RNA from *A. muscaria* was extracted according to Nehls *et al.* (1998). cDNA was synthesized from 0.2 µg of total RNA (Superscript II; Invitrogen, Groningen, the Netherlands) using a Smart PCR cDNA synthesis kit (Clontech, Palo Alto, CA, USA) and then PCR-amplified for 17–18 cycles using conditions recommended by the manufacturer (Clontech). Suppression subtractive hybridization (SSH) was performed between *A. muscaria*–streptomycete 505 DC and *A. muscaria* pure culture using the PCR-Select cDNA Subtraction Kit (Clontech). Subtracted gene fragments were cloned into the TOPO TA cloning vector and transformed into TOPO cells (Invitrogen).

Dot blot analysis of the subtracted library

Samples of bacterial cultures were used for PCR amplification of cDNA inserts from the subtracted library with the primers sense, 5′-TCGAGCGGCCGCCCGGGCAGGT-3′ and antisense, 3′-AGCGTGGTCGCGGCCGAGGT-5′. The conditions for the PCR reaction were as follows: 98°C for 3 min, and 30 cycles of 94°C for 30 s, 68°C for 30 s and 72°C for 2 min. cDNA inserts (200) from randomly selected clones were arrayed on duplicate positively charged nylon membranes (Roche, Mannheim, Germany). For probe synthesis, digoxigenin-UTP (DIG) labelled total cDNA was amplified from 100 ng total cDNA from *A. muscaria*–AcH 505 DC or *A. muscaria* pure culture using the Smart PCR cDNA synthesis kit (Clontech). The strength of the DIG label in the cDNA populations was equalized by dilution series to allow semiquantitative comparisons of hybridization intensities between filters. The dots were quantified using Quantity One software (Bio-Rad, Munich, Germany). Hybridizations were performed at 68°C for 16 h, and membranes were treated according to the manufacturer's instructions (Roche). Array analyses were replicated two times using first-strand cDNA from two independent cultures. The Thermo Sequence fluorescent-labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech, Freiburg, Germany) was used to obtain sequence information from differentially hybridized inserts. Software for the sequencing was Base Imagir 4.0 (Li-Cor, MWG-Biotech, Ebersberg, Germany). DNA sequences we compared with already known entries using the BLASTX algorithm (Altschul *et al.*, 1990) of NCBI (http://www.ncbi.nlm.nih.gov/BLAST).

Rapid amplification of cDNA ends (RACE) PCR

The SMART RACE cDNA Amplification Kit (Clontech) was used according to the manufacturer's instructions. The *Aacs* cDNA was completed using the gene-specific primer 5′-TGCCGCTGGCCCGATAAGATAGC-3′ for 3′-RACE. Primers for *Cyp40* RACE reactions were 5′-CCTCTATGGTATAA-GGGAAG-3′ for 3′-RACE and 5′-CCTCGAATGATGAA-CAGGGAAACC-3′ for 5′-RACE. All RACE reactions were conducted using the respective primers for 3′- and 5′-RACE provided by the SMART RACE cDNA Amplification Kit and under standard PCR conditions.

Fragments obtained were cloned into pCR2.1 according to instructions given for the TOPO TA Cloning Kit (Invitrogen).

Nucleic acid hybridizations

For northern blot analyses, 2–3 µg of total RNA was separated on a 1% agarose gel in the presence of formamide and formaldehyde in 20 mM MOPS [3-(4-Morpholino) propane sulphonic acid] buffer, and transferred to a nylon membrane. To confirm dot blot results, membranes were hybridized with DIG-labelled cDNA inserts from the subtracted library. For gene-specific hybridizations, DIG-labelled PCR fragments were obtained from the noncoding 3′ regions, using the forward primer 5′-AACTGCCGGAAAA-TTCTC-3′ and the reverse primer 5′-TTTCCCATGTCTT-GTTCC-3′ for *AmCyp40*, and the forward primer 5′-CATCCTGTTCGTCAAGC-3′ and the reverse primer 5′-GCTGGGGCAGCTTAC-3′ for *AmAacs*, with the following PCR programme: 94°C for 3 min; 32 cycles of 94°C for 30 s, 46°C for 30 s and 72°C for 2 min; 72°C for 5 min. PCR-based

Table 1 Cyclophilin (Cyp) and acetoacyl coenzyme A synthetase (Aacs) homologues from the genomic DNA sequences of *Phanerochaete chrysosporium* and *Coprinus cinereus*

Designation	Organism	Scaffold	Position (bp)	Theoretical isoelectric point	Theoretical MW (kDa)	% identity to *Amanita muscaria* protein
AmCyp40	*Amanita muscaria*			5.77	40 735	
CcCyp40	*Coprinus cinereus*	11	48300–50100	5.39	40 769	70
PcCyp40	*Phanerochaete chrysosporium*	102	74800–77000	5.51	40 222	63
CcCyp18	*Coprinus cinereus*	17	77100–78100	7.97	18 303	82
PcCyp18	*Phanerochaete chrysosporium*	44	67000–69000	8.96	17 563	64
AmAacs	*Amanita muscaria*			6.7	77 738	
CcAacs	*Coprinus cinereus*	10	748200–751100	6.04	77 153	66
PcAacs	*Phanerochaete chrysosporium*	89	70350–74200	6.01	78 580	66

The properties of the predicted amino acid sequences are shown in comparison to the deduced *Amanita muscaria* proteins. Cyp40 and Cyp18 indicate cyclophilin proteins with the predicted theoretical molecular weights (MWs) of 40 and 18 kDa, respectively.

labelling with DIG and hybridization procedures under high-stringency conditions were performed according to instructions provided in the PCR DIG Probe Synthesis Kit (Roche).

Amino acid sequence analysis

Web-based algorithms provided by ExPASY Molecular Biology Server (PFSCAN; http://www.isrec.isb-sib.ch/software/PFSCAN_form.html) and NCBI (conserved domain search; Marchler-Bauer *et al.*, 2003) were used to analyse the protein sequences. The predicted full-length protein sequences of AmCyp40 and AmAacs were used for phylogenetic analyses. For the alignments, homologous sequences from other fungi detected by BLASTX were selected. In addition, the genomic DNA sequences of *Coprinus cinereus* (http://www.broad.mit.edu/annotation/fungi/coprinus_cinereus/) and *Phanerochaete chrysosporium* (Martinez *et al.*, 2004) were used to search for homologues using the tBLASTN (AmCyp40 protein vs genomic nucleotide sequence) search. The detected amino acid sequences and their properties in comparison with the predicted *A. muscaria* homologues are shown in Table 1.

The peptidyl-prolyl isomerase domains of cyclophilins and full-length Aacs sequences were used for phylogenetic analyses. Sequences were aligned in ClustalX (Jeanmougin *et al.*, 1998). To estimate the phylogenetic relationships, alignments were analysed using a Bayesian approach based on Markov chain Monte Carlo (MCMC; Larget & Simon, 1999) as implemented in the program MrBayes 3.0b4 (Huelsenbeck & Ronquist, 2001). Four incrementally heated simultaneous Monte Carlo Markov chains were run over 2×10^6 generations using random starting trees and assuming a percentage of invariable alignment sites with gamma-distributed substitution rates of remaining sites. The amino acid substitution model was sampled from the substitution models implemented in MrBayes. Trees were sampled every 100 generations, resulting in an overall sampling of 20 000 trees. The stationarity of the process was controlled using Tracer software, version 1.0 (Rambaut & Drummond, 2003). The first 8000 trees were discarded ('burn in') and the remaining 12 000 were used to compute a 50% majority rule consensus tree to obtain estimates of posterior probabilities. Branch lengths were averaged over the sampled trees and scaled in terms of expected numbers of amino acid substitutions per site. To test the independence of the results, the Bayesian MCMC phylogenetic analyses were performed twice for each data set. Resulting MCMC trees were rooted with *Streptomyces coelicolor* Aacs as outgroup for the Aacs tree and with *Streptomyces antibioticus* Cyp18 as outgroup for the cyclophilin tree.

GenBank/EMBL/Swissprot accession numbers for the cyclophilin sequences are as follows. PcCyp40: *P. chrysosporium* (not annotated); AmCyp40: *A. muscaria* (AY773481); CcCyp40: *C. cinereus* (not annotated); ScCyp40: *Saccharomyces cerevisiae* (NP_013317); AtCyp40: *Arabidopsis thaliana* (NP_565381); NcCyp40: *Neurospora crassa* (Q9P3 × 9); SpCyp40: *Schizosaccharomyces pombe* (Q11004); MmCyp40: *Mus musculus* (Q9CR16); HsCyp40: *Homo sapiens* (Q08752); HsCyp18: *H. sapiens* (P62937); MmCyp18: *M. musculus* (P17742); DlCyp18: *Digitalis lantana* (T50769); PcCyp18: *P. chrysosporium* (not annotated); CcCyp18: *C. cinereus* (not annotated); ScCyp18: *S. cerevisiae* (NP_010439); PoCyp18: *Pleurotus ostreatus* (CAD10797); PaCyp18: *P. abies* (CAC81066); StraCyp18: *Streptomyces antibioticus* (P83221). Aacs sequences derived from public databases were as follows. AmAacs: *A. muscaria* (DQ089688); CnAacs: *Cryptococcus neoformans* (CNBE4890); UmAacs: *Ustilago maydis* (UM05131.1); RnAacs: *Rattus norvegicus* (AB026291); AnAacs: *Aspergillus nidulans* (CAE47912); StrcAacs: *S. coelicolor* (AL939108.1); CcAacs: *C. cinereus* (not annotated); PcAacs: *P. chrysosporium* (not annotated).

Results

Effects of bacteria on fungal growth and mycorrhiza formation

The two streptomycete isolates, AcH 505 and AcH 1003, showed similar fungus specificities in axenic culture (Table 2).

Table 2 The effect of streptomycete isolates AcH 505 and AcH 1003 on the mycelial growth of a selection of ectomycorrhizal fungi

	Colony area (cm^2)		
	Bacterial isolate		
Fungal isolate	None	AcH 505	AcH 1003
Amanita muscaria 6	24.6	34.7 (+)	37.04 (+)
Amanita muscaria 83	29.8	36.8 (+)	38 (+)
Amanita muscaria MEII	44.5	48.2	50.3
Suillus bovinus K3	16.6	23.5 (+)*	21.01 (+)*
Paxillus involutus 031	30.3	30	33.7
Hebeloma cylindrosporum H1–H7	35.1	24.2 (–)*	21.7 (–)*

Values followed by a sign are significantly higher (+) or lower (–) than the value corresponding to the control treatment in the same line of the table using Student's *t*-test ($P < 0.001$). Dual culture results (AcH 505 vs AcH 1003) with differing ($P < 0.1$) values are marked with asterisks.

The growth of all three tested *A. muscaria* isolates was promoted, but significant changes in mycelial growth rates were only detected for the slowly growing (6 and 83) strains of this fungus. The extent of response to either AcH 505 or AcH 1003 varied significantly in *S. bovinus* and *H. cylindrosporum*. Growth of the *S. bovinus* K strain was promoted, yet both bacteria suppressed *H. cylindrosporum*. The margins of the fungal colonies remained isodiametric in all culture experiments. A mycorrhiza formation test in a peat–perlite culture system was conducted to determine whether AcH 505 and AcH 1003 increase the mycorrhiza formation rate (Table 3). Consistent with the results on mycelial growth promotion in DC, AcH 1003 promoted mycorrhiza formation more strongly than AcH 505. The number of mycorrhizal second-order lateral roots in Norway spruce was increased with AcH 505 (20%) and with AcH 1003 (39%) in comparison with the control treatment. In pine seedlings the number of mycorrhizal first-order lateral roots was increased by 27% with AcH 505 and by 39% with AcH 1003, and the number of second-order lateral roots was increased by 50% with AcH 505 and by 72% with AcH 1003. Furthermore, AcH 505 induced a significant increase in secondary lateral root formation in pine, and a slight increase in formation of these roots in spruce seedlings. No other significant effects on seedling growth of either plant were recorded.

The use of the cDNA subtraction technique to select preferentially expressed genes

As hyphal growth promotion was found to be an important factor for the MHB effect, we performed an SSH analysis with the aim of isolating fungal genes preferentially expressed in DC, i.e. the genes that are related to the induction of

Table 3 Effects of AcH 505 and AcH 1003 on lateral root and mycorrhiza formation in individual seedlings of Norway spruce (*Picea abies*) and Scots pine (*Pinus sylvestris*)

Bacterial isolate	No. of first-order uninfected lateral roots	No. of second-order uninfected lateral roots	No. of first-order mycorrhizal lateral roots	No. of second-order mycorrhizal lateral roots	Total no. of roots	First-order root mycorrhizal rate (%)	Second-order root mycorrhizal rate (%)	Total Mycorrhizal rate (%)
Picea abies-Amanita muscaria 6								
None	28	31.3	ND	14.3	59.3	ND	45.2	24.3
	(8,23)	(9,37)		(4,69)	(16,79)		(3,5)	(2)
AcH 505	28.6	38.5	ND	21	67.1	ND	54.6 (+)	29.3
	(8,83)	(19,3)		(10,8)	(24,7)		(3,34)	(5,83)
AcH 1003	25.4	40	ND	25.1	65.4	ND	63.5 (+)	35.7 (+)
	(10,9)	(20,9)		(12,5)	(25,4)		(5,64)	(11,2)
Pinus sylvestris–Suillus bovinus K3								
None	15.2	42.9	4.5	4.3	58.1	32.3	10.6	15.8
	(6,12)	(16,2)	(2,12)	(1,16)	(19,2)	(14,3)	(3,13)	(3,05)
AcH 505	18.4	95	8.1 (+)	14.8 (+)	113.4 (+)	41.1	15.9 (+)	21 (+)
	(4,88)	(52,5)	(3,25)	(7,41)	(55,4)	(9,06)	(2,77)	(3,8)
AcH 1003	16.4	52.2	7.5 (+)	9.3 (+)	67.5	45.9	18.3 (+)	26.1 (+)
	(3,63)	(15,82)	(1,96)	(1,77)	(16,6)	(8,52)	(2,67)	(3,14)

Picea abies was grown with *Amanita muscaria* for 12 wk and *Pinus sylvestris* with *Suillus bovinus* K3 for 16 wk. Values significantly higher than the control treatment ($P < 0.01$) are indicated with (+). Mean values of 10 replicates are given, with standard deviations in parentheses underneath.

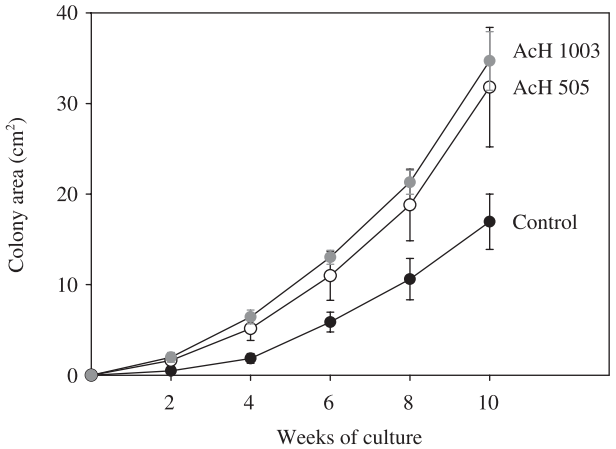

Fig. 1 Time course of *Amanita muscaria* 83 growth induction in dual culture. The colony area of control mycelium (black circles) is compared with that of mycelium in dual culture with AcH 505 (open circles) or with AcH 1003 (grey circles). The interaction with bacteria leads to significantly promoted growth at and after 2 wk of culture, according to Student's *t*-test ($P < 0.01$).

growth of *A. muscaria* hyphae through AcH 505. First, the time course of *A. muscaria* growth promotion was analysed to determine the appropriate stage for mRNA isolations (Fig. 1). The strongest induction with AcH 505 and AcH 1003 was detected between 8 and 10 wk of growth. Nine-wk-old AcH 505-induced *A. muscaria* 83 mycelia were used for gene expression analysis. Differentially expressed sequences were cloned to obtain a subtracted *Am-505* cDNA library. The average size of DNA inserts in the subtracted library was 450 bp.

Altered fungal gene expression in DC

Two hundred randomly picked clones from the subtracted library were analysed by dot blot analysis (data not shown). Fifty-eight clones (28%) showed at least 2-fold increase or decrease in hybridization intensities, 45 of them being up-regulated and 13 down-regulated. Twenty-one of these differentially regulated genes showed statistically significant matches against database sequences (expected value < e-10) (Table 4). Differential expression of nine genes was validated by northern analysis. These genes were related to important aspects of the fungus–bacterium interaction, or were unknown genes with significantly altered hybridization intensities. The observed changes in expression rates were comparable to those detected in dot blot analysis (data not shown). For genes of known function, the highest differential expression (6-fold up-regulated) was observed for cyclophilin 40, and the lowest (3-fold down-regulated) for the gamma-aminobutyric acid/polyamine permease. Among the genes of unknown function, clones with strongly increased (6-fold up-regulated; CV632830) and decreased (5-fold down-regulated; DN779411) levels of transcription were found (Fig. 2).

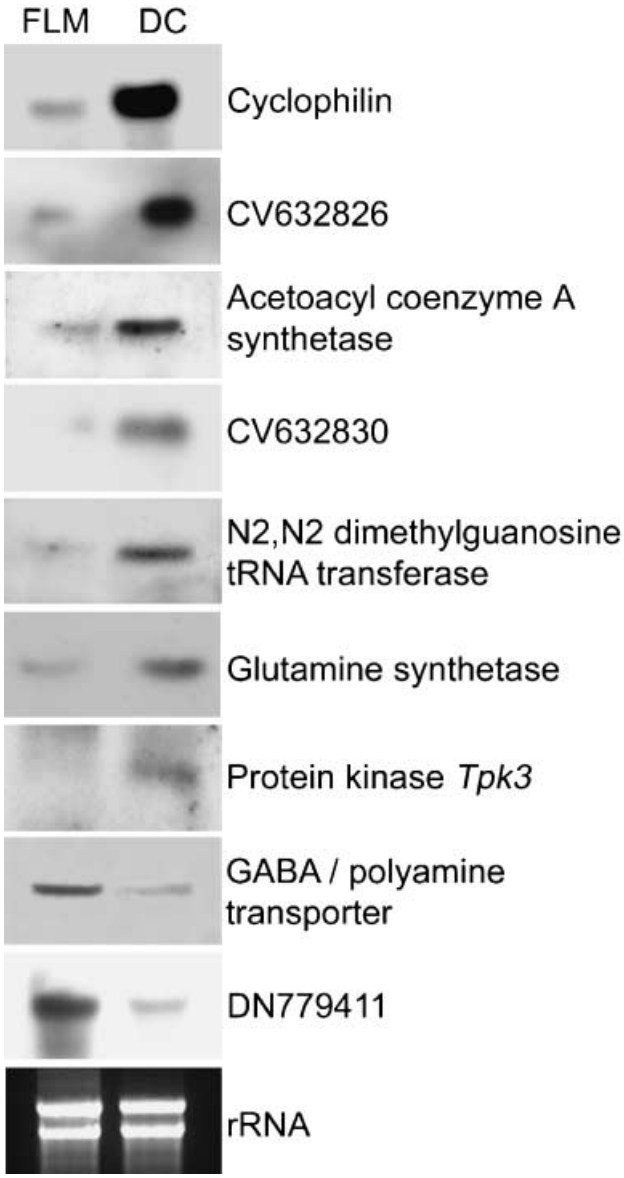

Fig. 2 Transcript levels of subtracted genes in free-living *Amanita muscaria* 83 mycelium (FLM) and in dual culture (DC) with AcH 505 according to northern analysis. Cultures were harvested after 9 wk of growth on MMN medium.

The majority of the genes with known function were related to signal transduction pathways (seven genes; protein kinases and transcription factors) and metabolism (five genes; carbon-metabolism: acetoacyl coenzyme A synthetase; sulphur-metabolism: adenylyl sulphate kinase and sulphite reductase; nitrogen metabolism: glutamine synthetase and protease). In addition, genes coding for proteins involved in cell growth or cell stress (five genes; cyclophilin 40, gamma-aminobutyric acid/polyamine transporter, ribosomal protein genes and histone H4) and cell structure [two genes; beta-glucosidase and p21-activated kinase (PAK)] were identified.

Table 4 *Amanita muscaria*–AcH 505 interaction-specific library clones

Accession number	cDNA length (bp)	Best database match determined by BLASTX	Species	Expected value	Relative transcript ratio Am–AcH 505/Am
CV632831	537	Hypothetical protein	*Coprinus cinereus*	8e-18	6
CV632829	564	Peptidyl-prolyl *cis-trans* isomerase	*Neurospora crassa*	1e-48	6
CV632830	399	Histone H4	*Phanerochaete chrysosporium*	9e-37	4
CV632827	697	ND	ND	ND	4
CV632828	490	N2,N2-dimethylguanosine tRNA methyltransferase	*Saccharomyces cerevisiae*	7e-11	3
CV632826	343	ND	ND	ND	3
CV632832	401	ND	ND	ND	3
CV632847	470	Ribosomal L27e protein	*Cryptococcus neoformans*	2e-28	2
CV632846	391	Acetoacyl coenzyme A synthetase	*Danio rerio*	5e-15	2
CV632845	512	Hypothetical protein	*Coprinus cinereus*	7e-20	2
CV632844	382	Cellulose-binding beta-glucosidase	*Phanerochaete chrysosporium*	7e-25	2
CV632842	587	Flavin-binding monooxygenase-like	*Aspergillus nidulans*	7e-21	2
AJ428992	286	Glutamine synthetase	*Amanita muscaria*	3e-49	2
CV632834	653	Adenylylsulphate kinase	*Saccharomyces cerevisiae*	1e-49	2
CV632835	471	Ribosomal protein gene	*Gibberella zeae*	2e-58	2
CV632839	494	Sulphite reductase	*Cryptococcus neoformans*	1e-58	2
DN779412	645	PAK kinase (Don3)	*Ustilago maydis*	9e-65	2
CV632841	693	Protein kinase Tpk3	*Saccharomyces cerevisiae*	1e-18	2
CV632825*	373	Glutamic protease eqolisin; carboxyl peptidase A	*Talaromyces emersonii*	1e-20	0.5
CV632837*	500	Transcription initiationfactor	*Ustilago maydis*	3e-16	0.5
CV632840*	266	Reverse transcriptase	*Tricholoma matsutake*	3e-23	0.5
CV632824*	481	DEAD box RNA helicase	*Ustilago maydis*	9e-73	0.5
CV632833*	520	Transcription factor	*Cryptococcus neoformans*	4e-12	0.5
CV632836*	300	Protein kinase	*Cryptococcus neoformans*	4e-12	0.5
CV632843*	470	γ-aminobutyric acid/ polyamine permease	*Saccharomyces cerevisiae*	7e-28	0.3
DN779411*	211	ND	ND	ND	0.2

The GenBank accession numbers of the nucleotide sequences are given. BLASTX searches gave differing degrees of identity to genes in other organisms (expected values). The first clone has the highest and the last clone the lowest apparent *Am*–AcH 505/*Am* transcript ratio, detected by dot blot analysis. Clones marked with an asterisk are less strongly expressed in the interacting fungal hyphae.
Am, *Amanita muscaria*; PAK, p21-activated kinase; Tpk3, Protein kinase A catalytic subunit 3.

Two up-regulated genes were selected for further analysis: *Aacs*, to analyse whether the increased *Aacs* expression was related to growth activation of *A. muscaria* mycelia, and *Cyp40*, to investigate whether *Cyp40* expression increased as a result of the addition of bacterial culture supernatants.

Isolation and expression analysis of Aacs

Aacs catalyses the activation of acetoacetate, which is needed for efficient sterol biosynthesis (Bergstrom *et al.*, 1984). Increased expression of a functionally related gene, acetyl coA acetyl transferase (*Acat*), was observed in *L. bicolor* hyphae grown in contact with pine root exudates (Podila *et al.*, 2002). We investigated whether the *AmAacs* transcription level was increased as a result of activation of fungal mycelium, as was suggested for *L. bicolor Acat* by Podila *et al.* (2002).

As no previous information about fungal Aacs proteins existed, we isolated the full-length *AmAacs* cDNA by RACE. The predicted 702 amino acid AmAacs protein sequence includes two conserved and overlapping regions according to conserved domain search (CD-Search; Marchler-Bauer *et al.*, 2003), namely the AMP-binding enzyme region (amino acids 124–577) and the acyl coenzyme A synthetase/AMP (fatty) acid ligase region (amino acids 81–667). Using the predicted AmAacs amino acid sequence, we detected homologue sequences in the published genomes of the homobasidiomycetes *C. cinereus* and *P. chrysosporium* (66% and 66% identity at the amino acid level, respectively), which are both close relatives of *A. muscaria*. Several AmAacs homologues were found by BLASTX, such as the hypothetical protein CNBE4890 from the human opportunistic pathogen *C. neoformans* (48% identity), the hypothetical protein UM05131.1 from the maize pathogen *Ustilago maydis* (45%), and the rat Aacs (41%; Iwahori *et al.*, 2000). By contrast, no AmAacs homologue was found in the budding yeast genome database. The predicted amino acid sequences of the AmAacs homologues had comparable molecular weight, pI, AMP-binding and acyl coenzyme A synthetase domain sequences (data not shown). As expected, the homobasidiomycete

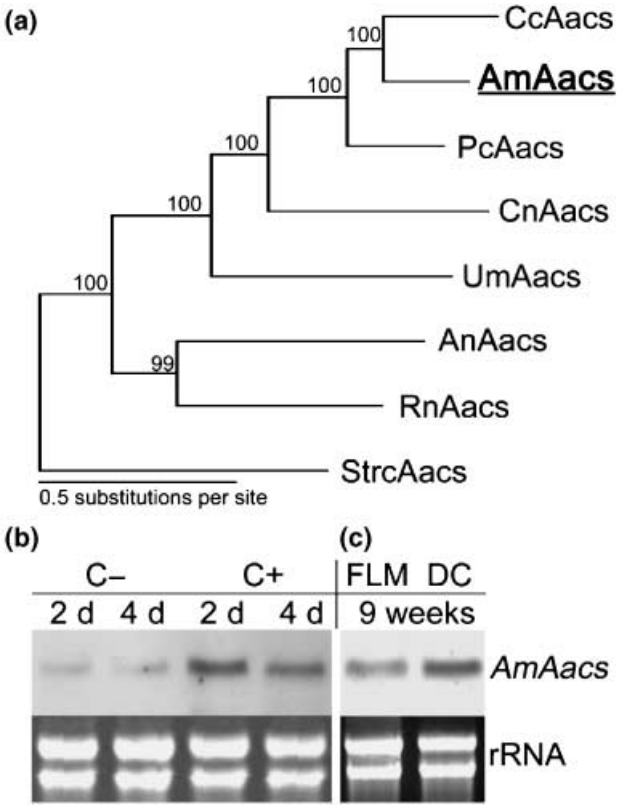

Fig. 3 Phylogenetic and gene expression analysis of *Amanita muscaria* acetoacyl coenzyme A synthetase (AmAacs). (a) Rooted tree illustrating the phylogenetic relationship between AmAacs and other acetoacyl coenzyme A synthetases. In the genomes of homobasidiomycetes *Coprinus cinereus* (CcAacs) and *Phanerochaete chrysosporium* (PcAacs), one Aacs homologue was detected according to sequence homology to AmAacs. *Streptomyces coelicolor* Aacs (StrcAacs) was included as an outgroup. Other protein sequences used for the analysis were: CnAacs, *Cryptococcus neoformans* Aacs; UmAacs, *Ustilago maydis* Aacs; AnAacs, *Aspergillus nidulans* Aacs; RnAacs, *Rattus norvegicus* Aacs. Bayesian Markov chain Monte Carlo analysis was performed using full-length Aacs sequences. Numbers at branches are *a posteriori* probabilities regarding the monophyly of the respective protein. Branch lengths are proportional to evolutionary distances. The scale bar indicates an evolutionary distance of 0.5 amino acid substitutions per position in the sequence. (b) Carbon-based induction of *AmAacs* expression. Changes in the transcript level of *AmAacs* during fungal growth in submerged culture were investigated. No (C–) or 100 mM (C+) glucose was added to mycelia depleted of glucose. Levels of *AmAacs* gene expression were analysed by northern analysis after 2 or 4 d of culture. (c) For a comparison, the *Aacs* expression rate at 9 wk of Petri dish culture in free-living mycelium (FLM) and dual culture (DC) is shown.

(*A. muscaria*, *C. cinereus* and *P. chrysosporium*) Aacs proteins grouped together in phylogenetic analysis (Fig. 3a).

We used submerged *A. muscaria* culture to analyse whether the changed *AmAacs* expression in DC is a result of the activation of dormant hyphae (Podila *et al.*, 2002). The effect of addition of glucose on *Aacs* expression was determined in *A. muscaria* suspension culture. The glucose-supported (100 mM) fungal culture showed a 3-fold increase in *Aacs* expression in comparison with glucose-starved hyphae (Fig. 3b). A 2-fold up-regulation of *Aacs* expression was observed in DC at 9 wk of culture (Fig. 3c).

Isolation and characterization of Cyp40 cDNA

The Cyp40 proteins are prolyl isomerases, which are necessary for efficient cell growth and endurance of cell stress in *S. cerevisiae* (Duina *et al.*, 1996). Of all subtracted genes of known function, the level of *A. muscaria Cyp40* transcription is most significantly increased in DC hyphae, and we decided to investigate whether comparable differential expression occurs already in a short-term culture.

Using RACE PCR, the complete cDNA sequence of the first cyclophilin of *A. muscaria* was determined and denoted *AmCyp40* for the *A. muscaria* cyclophilin 40-kDa protein. Homology searches using genome sequences or the BLASTX algorithm revealed related proteins belonging to the cyclophilin family, most notably from *C. cinereus* and *P. chrysosporium* genomes (70% and 63% identity at the amino acid level, respectively).

A motif scan of the predicted AmCyp40 protein sequence by PFSCAN detected a cyclophilin-type peptidyl-prolyl *cis-trans* isomerase domain of 166 amino acids. In addition, the profile scan also detected the presence of two putative tetratricopeptide repeat (TPR) motifs for protein–protein interactions and a putative calmodulin-binding site. An analysis using the 'PESTfind' algorithm revealed the presence of two putative PEST sequences. A study of 12 other fungal and mammalian 40-kDa cyclophilins obtained from the GenBank/EMBL database showed that only the fungal cyclophilins contain PEST sequences. TPR motifs are only absent from the 40-kDa cyclophilin wis2 of *S. pombe*, and all investigated proteins contain the putative calmodulin-binding site. A phylogenetic analysis of AmCyp40 based on the conserved peptidyl-prolyl *cis-trans* isomerase region (166 amino acids) of the deduced protein sequence was used for the determination of the phylogenetic position of AmCyp40 among the eukaryotic cyclophilins. Cyclophilins clustered (i) according to the size of the entire protein and (ii) according to the relatedness of organisms. AmCyp40 grouped with the other homobasidiomycete 40-kDa cyclophilins (Fig. 4a).

AmCyp40 is highly expressed in the DC hyphae with both AcH 505 and AcH 1003 (data not shown). In order to determine whether the increase in *AmCyp40* transcript is a growth effect, the expression was analysed in short-term experiments in suspension culture. *A. muscaria* suspension cultures were incubated with a cell-free supernatant of the streptomycete strains AcH 505 and AcH 1003 and cultivated for 6 h. Incubation with supernatant of the strain AcH 504, which exerts no effects on *A. muscaria* growth (Maier *et al.*, 2004), served as a control. Levels of *AmCyp40* transcription were strongly increased in the presence of AcH 505 and AcH 1003 supernatants after 6 h, while there was no effect with control strain AcH 504 (Fig. 4b).

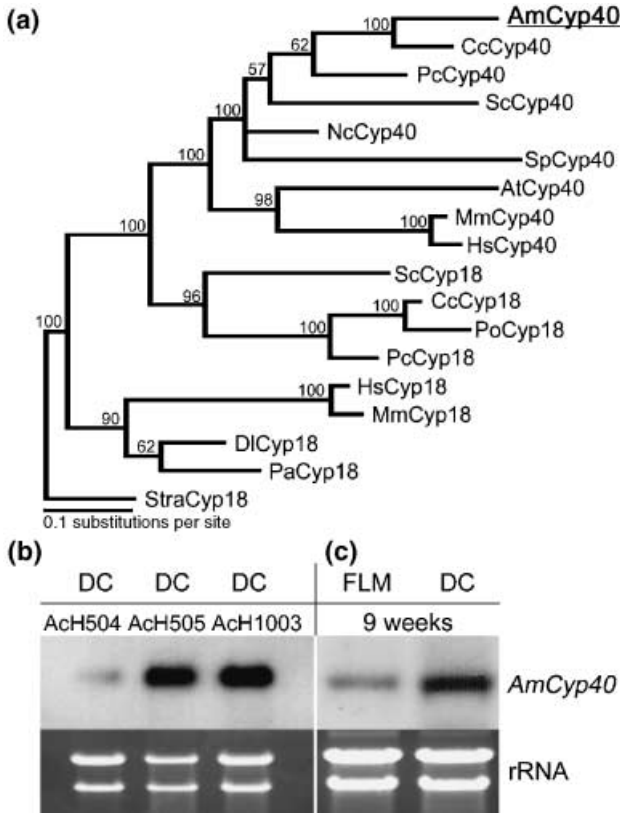

Fig. 4 Phylogenetic analysis and levels of *Amanita muscaria* cyclophilin 40-kDa (*AmCyp40*) gene expression. (a) Phylogenetic analysis of AmCyp40. The conserved region of the peptidyl-prolyl *cis-trans* isomerase (PPIase) domain of a collection of 40-kDa and 18-kDa cyclophilins was used to perform Bayesian Markov chain Monte Carlo analysis. Numbers at branches are *a posteriori* probabilities regarding the monophyly of the respective groups of proteins. *Streptomyces antibioticus* cyclophilin is shown as an outgroup (StraCyp18). The cyclophilin homologues from the homobasidiomycetes *Coprinus cinereus* (CcCyp40 and CcCyp18) and *Phanerochaete chrysosporium* (PcCyp40 and PcCyp18) were identified according to sequence homology to AmCyp40. The other 40- and 18-kDa cyclophilins used in the analysis were from the following organisms: ScCyp40, *Saccharomyces cerevisiae*; AtCyp40, *Arabidopsis thaliana*; NcCyp40, *Neurospora crassa*; SpCyp40, *Schizosaccharomyces pombe*; MmCyp40, *Mus musculus*; HsCyp40, *Homo sapiens*; HsCyp18, *Homo sapiens*; MmCyp18, *Mus musculus*; DlCyp18, *Digitalis lantana*; ScCyp18, *Saccharomyces cerevisiae*; PoCyp18, *Pleurotus ostreatus*; PaCyp18, *Picea abies*. (b) Short-term induction of *AmCyp40* transcription by AcH 505 and AcH 1003 culture supernatants. *A. muscaria* suspension cultures were incubated for 6 h with culture supernatants of streptomycete strains AcH 504 (supernatant control), AcH 505 and AcH 1003. (c) For a comparison, the level of *AmCyp40* expression at 9 wk of Petri dish culture is represented.

Discussion

The two streptomycete strains investigated in this contribution have similar specificities for ectomycorrhizal fungi. This is surprising, as Maier *et al.* (2004) showed previously that AcH 505 suppresses the growth of *H. annosum* and *Armillaria abietina*, whereas AcH 1003 has no effect on these plant pathogens. This indicates an important application for ectomycorrhiza helper bacteria such as AcH 505: the simultaneous growth promotion of mycorrhizal and growth suppression of pathogenic fungi.

Resistance to antifungal substances can be an important characteristic for fungal species colonizing the rhizosphere (Whipps, 2001). *H. cylindrosporum* is inhibited in its growth and mycorrhiza formation by MHB (*Bacillus*, *Pseudomonas*) associated with *L. laccata* (Garbaye & Duponnois, 1992). Accordingly, the streptomycetes of this study suppressed *H. cylindrosporum* growth in DC experiments, indicating that *H. cylindrosporum* is sensitive to antifungal substances produced by very different bacterial species.

Fungi and bacteria were spatially separated in DC, although a continuum of nutrient solution existed in the Petri dish cultures. The shape of fungal colonies remained isodiametric in all cultures, indicating that the substances that promote (*A. muscaria* and *S. bovinus*) mycelial growth must readily diffuse in the culture medium, more rapidly than the mycelial growth rate of responsive fungi. Such an effect has been previously shown for the ECM fungus *Pisolithus tinctorius*, as even a local application of the small fungal growth-promoting plant secondary substance rutin did not result in asymmetric colony extension of this fungus (Lagrange *et al.*, 2001). Although streptomycetes are known to produce a variety of volatile compounds (Schöller *et al.*, 2002), volatiles from AcH 505 and AcH 1003 did not show any effects on the mycelial extension rates of the six tested fungal isolates. To investigate the differential responses of the fungal species in detail, we are currently analysing mycelial growth-stimulating and -suppressing substances from the AcH 505 culture supernatant.

Differential gene expression in DC

Subtractive hybridization is a well-accepted method to detect differentially regulated genes in ectomycorrhizal fungi. It has been used to select fungal genes expressed preferentially in the presymbiotic phase (Podila *et al.*, 2002), in ectomycorrhizal tissues (Voiblet *et al.*, 2001; Peter *et al.*, 2003) and in extramatrical hyphae (Morel *et al.*, 2005). We present the first detailed report on differential fungal gene expression during DC with mycorrhiza helper bacteria. Becker *et al.* (1999) showed modulation of *in vitro* translation products after inoculation of *L. bicolor* with culture filtrates from a growth-promoting and a growth-suppressing streptomycete strain, but no information concerning the identity of the regulated mRNAs was given in their contribution. The presented data thus identify the first fungal genes with altered expression rates in DC with ectomycorrhiza helper bacteria, as well as providing the first clues to the mechanisms that lead to growth promotion of fungal mycelia. Three aspects of mycorrhiza helper bacteria were chosen for a more detailed analysis:

enhancement of the fungal growth rate, the stress response and signalling between bacteria and fungi.

Accelerated fungal growth and cell stress The presented data show that *A. muscaria* is able to change its gene expression in response to bacterial stimuli. The largest number of induced genes (five) are related to fungal metabolism. These include *AmAacs*, the first acetoacetyl-coA synthetase cDNA isolated from fungi. Aacs activity (Bergstrom *et al.*, 1984) and expression profiles (Ohgami *et al.*, 2003) suggest that this enzyme is involved in cholesterol biosynthesis. We suggest that part of the response of *A. muscaria* to AcH 505 is the induction of increased expression of *AmAacs*, leading to higher production of ergosterol, which is needed for promoted mycelial growth. As carbon, especially glucose, is a key regulator of fungal gene expression in ECM symbiosis (Nehls *et al.*, 1999; Nehls, 2004), the levels of *AmAacs* expression were analysed in carbon-depleted (dormant) and in well-supported (activated) *A. muscaria* suspension cultures. We found an up-regulation of *Aacs* expression in the glucose-supplemented culture, comparable to that seen in DC experiments at 9 wk of growth. After 9 wk of DC, the fungal mycelium nearly covered the Petri dish, and it had probably consumed most of the available glucose. As no agar-degrading activity that might lead to an additional carbon supply has been observed in bacterial cultures, the observation suggests that (i) the fungus is able to change its gene expression in response to bacterial stimuli, and (ii) an effective bacterial compound acting in a similar manner to glucose could be produced by the bacterium.

The transport of polyamines and gamma-aminobutyric acid are mediated by the vacuolar Uga4 permease in budding yeast *S. cerevisiae* (Uemura *et al.*, 2004). The inhibitor of polyamine biosynthesis difluoromethylornithine decreases the mycelial growth rate of *P. involutus* (Zarb & Walters, 1994). The down-regulation of *Uga4* in DC may thus reflect changes in individual polyamine concentrations in the DC hyphae, necessary for the faster growth of fungal mycelia.

Peptidyl-prolyl *cis-trans* isomerases (PPIases) are enzymes that bind to mature proteins to control their activity, subunit assembly and localization (Dolinski & Heitman, 1997). Cyp40 proteins are involved in the regulation of cell proliferation and differentiation and in the control of cell stress. The closest characterized homologues of *AmCyp40* are the yeast Cyp40 family members encoded by *cpr6* and *cpr7* genes. Yeast strains with disrupted *cpr7* show a significant impairment of the rate of cell division, leading to the slow growth phenotype (Duina *et al.*, 1996). The elevation of *AmCyp40* transcription in coculture could thus play a role in the enhanced hyphal growth rate observed in DC. As the streptomycete culture supernatants were added to submerged fungal cultures, an increased level of *AmCyp40* expression was observed with both AcH 505 and AcH 1003 supernatants. By contrast, the use of the culture supernatant of *S. argenteolus* 504, a streptomycete strain exerting no effects on *A. muscaria* growth, resulted in basal levels of *AmCyp40* expression. This indicates that additional factors, perhaps the presence of stimulatory compounds in the supernatants of AcH 505 and AcH 1003, lead to increased *AmCyp40* expression.

ECM fungi are known to release extracellular enzymes, such as mono-oxidases, capable of oxidizing aromatic (Gramss *et al.*, 1998) and nonaromatic (Takakura & Kuwata, 2003) substances. *A. muscaria* mono-oxidase expression was observed to be increased in DC hyphae, suggesting an activation of specific steps in either secondary metabolism or catabolism in *A. muscaria.*

Signalling between bacteria and fungi Changes in gene expression levels occur in parallel with modulated transcription of signalling-related genes such as PAK and cAMP-dependent protein kinase (PKA). PAK is a central regulator of polarized growth in eukaryotes (Leveleki *et al.*, 2004), and the induced level of PAK expression suggests modulated organization of the actin cytoskeleton in DC hyphae. PKA regulates the cell cycle and metabolism in yeast and mammalian cells. The increased expression level of the gene related to the catalytic subunit of PKA, *Tpk3*, in *A. muscaria* indicates altered growth-related signal transduction in DC.

The differentially expressed genes detected in this *in vitro* approach could serve as a basis for the analysis of *A. muscaria* gene expression in more natural culture systems such as non-sterile microcosms or pot cultures. Analysis of comparable fungal genes in the growth-promoted cultures of *S. bovinus*, and in the growth-inhibited cultures of *H. cylindrosporum* and *H. annosum*, would provide information about the specificity of the fungal response to AcH 505.

Mycorrhiza formation tests

Our findings support the view of Poole *et al.* (2001) that MHB have a ubiquitous distribution, and that the main helper mechanism is fungal growth promotion. This widens the scope of MHB investigations, as previous studies mainly focused on *Bacillus* and *Pseudomonas* species (Garbaye, 1994; Founoune *et al.*, 2002), and resulted in the isolation of MHB from *Burkholderia* and *Rhodococcus* (Poole *et al.*, 2001).

As the ability of the bacteria to stimulate mycelial growth correlated with their effect on mycorrhiza formation, we suggest that the effective mechanism for MHB is predominantly hyphal growth promotion. When *Bacillus* and *Pseudomonas* MHB were investigated, the behaviour of fungal mycelium in DC correlated with mycorrhiza formation tests (Garbaye & Duponnois, 1992; Frey-Klett *et al.*, 1997; Founoune *et al.*, 2002), and Poole *et al.* (2001) suggested that promoted mycelial growth of *Lactarius rufus* is the probable mechanism for the MHB outcome in the Scots pine model system. According to the observations of Richter *et al.* (1989) with *T. terrestris*, the *A. muscaria* strain with the slowest observed growth rate (strain 6) was the most sensitive to the streptomycete isolates

in *in vitro* tests. This suggests that MHB may compensate for slow mycelial growth rates in receptive fungal isolates.

For *A. muscaria*, however, we have observed that the fungal mycelial density decreases in DC with both streptomycete isolates (Maier, 2003; M.T., unpublished data). This suggests either that the observed MHB effect is caused by a faster spreading of fungal mycelia on the peat–perlite culture system (but not by the higher mycelial biomass) or that additional, plant-related factors are involved. As originally suggested by Garbaye (1994), the latter could include increased receptivity of the roots to the fungal hyphae, or improved fungal colonization of the fine roots produced by plant cell wall modifying bacterial substances. According to our results, and a previous study on mycorrhiza formation in Norway spruce on a MMN agar medium conducted by Maier (2003), AcH 505, but not AcH 1003, promotes root branching. Plant growth promoters with auxin-like activity have previously been isolated from *Streptomyces hygroscopicus* (Igarashi *et al.*, 2002).

To conclude, the present data confirm that the streptomycetes AcH 505 and AcH 1003 are MHB, exerting their positive action mainly through the activation of fungal metabolism. As a result of the changed gene expression levels, fly agaric mycelia activated in DC should have a modulated physiology. The question now arises as to which bacteria-generated substances give rise to the improved growth of selected mycorrhizal fungi and inhibit preferentially the growth of phytopathogenic fungi.

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