Actinomycetales bacteria from a spruce stand: characterization and effects on growth of root symbiotic and plant parasitic soil fungi in dual culture

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The rhizosphere, the narrow zone of soil around living roots, is characterized by root exudates which attract soil microorganisms. Most importantly, certain soil fungi establish symbiotic interactions with fine roots which enhance nutrient availability for the plant partner (mycorrhiza). The establishment of such a symbiosis can be affected by soil bacteria. In this study we isolated Gram-positive soil bacteria from the rhizosphere of a spruce stand rich with fly agaric (*Amanita muscaria*) fruiting bodies. Using a coculture technique in Petri dishes, bacterial isolates were characterized by their effect on the growth of fungal hyphae. A group of bacterial strains were identified which significantly promoted growth of fly agaric hyphae. One of these strains was shown to additionally inhibit growth of pathogenic fungi such as *Armillaria obscura* (wide host range) and *Heterobasidion annosum* (causes wood decay in conifers). Taxonomic characterization of the effective bacterial isolates by their morphological appearance, by the analysis of diaminopimelic acid, cell wall sugars, and DNA sequencing (16S rDNA) identified them as actinomycetes, some of which are not yet contained in data banks.

Roots constitute important plant organs for water and nutrient uptake. They, however, also release a wide range of carbon compounds of low molecular weight. These compounds form the basis for an environment rich in diversified microbiological populations, the rhizosphere (HILTNER 1904); the rhizosphere has been defined as a narrow zone of soil which is influenced by living roots). Bacteria are an important part of these populations. In addition, roots of most terrestrial plants develop symbiotic structures (mycorrhiza) with soil-borne fungi. In these interactions, the fungal partner provides the plant with improved access to water and nutrients in the soil due to more or less complex hyphal structures which emanate from the root surface and extend far into the soil. The plant, in return, supplies carbohydrates for fungal growth and maintenance (HAMPP & SCHAEFFER 1998, SMITH & READ 1997). Due to leakage and the turnover of mycorrhizal structures, these solutes are also released into the mycorrhizosphere where they can be accessed by other microorganisms. It has been shown that microbial communities within the rhizosphere are distinct from those of non-rhizosphere soil (CURL & TRUELOVE 1986, WHIPPS & LYNCH 1986).

Interactions between soil bacteria and symbiotic as well as plant-parasitic fungi can be both positive and negative. Mycorrhiza-forming fungi have been shown to reduce bacterial viability (GREEN et al. 1999, MEYER & LINDERMAN 1986). Due to the transfer and exudation of plant-derived organic compounds to soil microsites not accessible to roots, fungi can promote bacterial growth and survival (FREY-KLETT, PIERRAT & GARBAYE 1997, HOBBIE 1992, SÖDERSTRÖM 1992). Furthermore, there is evidence that soil bacteria can also enhance the formation of mycorrhizal structures, either by promoting growth (mycorrhization helper bacteria, MHB: (BENDING et al. 2002, GARBAYE 1994)) or by protecting them from pathogenic microorganisms (PEDERSEN, REDDY & CHAKRAVARTY 1999, SCHELKLE & PETERSON 1996). Plant-growth-promoting rhizobacteria (PGPR; KLOEPPER, LIFSHITZ & ZABLOTOWICZ 1989) include species and strains which belong to the genera *Azotobacter, Pseudomonas, Burkholderia, Acetobacter, Herbaspirillum* and *Bacillus* (GLICK 1995, PROBANZA et al. 1996).

Most investigations on the interaction between PGPR and mycorrhizal fungi deal with Gram-negative bacteria, and their effects on mycorrhiza development and plant performance vary considerably (HAMPP & MAIER 2003).

With this background, it was the aim of this study to focus on spore-forming bacteria which constitute an abundant group of bacteria in forest soils of temperate zones (TIMONEN et al. 1998) and on their interaction with ectomycorrhiza-forming as well as pathogenic soil fungi. Using an axenic culture system, we show that a range of actinomycetes (Gram-positive, not investigated so far with regard to their effect on soil fungi) distinctly and highly reproducible interact with the development of hyphae of *Amanita muscaria*, a wide-spread ectomy-

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corrhiza-forming soil fungus. We further show, that some bacteria which promote *A. muscaria* growth inhibit growth of parasitic soil fungi such as *Heterobasidion annosum*. Bacteria which exerted clear cut effects were characterized chemotaxonomically and by 16S rDNA sequencing.

Material and methods

Isolation of bacteria

Bacteria were extracted from soil under approx. 30-year-old spruces (Haigerloch, near Tübingen, Germany). The stand showed well mycorrhized roots and extensive fruiting body formation by *Amanita muscaria.* Soil samples were taken in spring from the respective "hyphosphere" which was defined as the fungal hyphae containing organic layer after removal of the uppermost undigested litter layer; between 3 to 8 cm from the surface and at a distance of approx. 2 m from the next spruce trunk. Five samples from an area covering 1.5 m^2 of surface area were mixed. Bacteria were separated from soil by membrane filtration (HIRSCH & CHRISTENSEN 1983).

For growth of bacteria, synthetic CMS medium was used. The CMS-medium had the following composition: citric acid, 2 mM; $MgCl_2 \cdot 6H_2O$, 1.25 mM; KCl, 10 mM; Na_2SO_4, 2 mM; $NaH_2PO_4 \cdot H_2O$, 10 mM; NH_4Cl, 100 mM; glucose, 200 mM; MES-Buffer, pH 7.2, 200 mM; trace elements solution (TS), 1 % v/v and agar, 2 % w/v. The composition of TS was as follows: $FeCl_3 \cdot 6H_2O$, 1 mM; $MnSO_4 \cdot H_2O$, 2.5 mM; $CuSO_4 \cdot 5H_2O$, 0.2 mM; $CaCl_2 \cdot 2H_2O$, 10 mM; H_3BO_3, 1 mM, $CoCl_2 \cdot 6H_2O$, 0.1 mM; $ZnCl_2$, 0.5 mM; and $Na_2MoO_4 \cdot H_2O$, 0.1 mM. TS was prepared in 0.1 N HCl. To suppress the growth of fungi, the medium contained 100 µg ml^{-1} each of cycloheximide and candicidin. The antibiotics, glucose, magnesium chloride, and TS were sterilized by filtration through a 0.2 µm membrane and added separately to autoclaved and cooled CMS before the plates were poured. The solidified medium was covered by a cellulose ester membrane (mesh size 0.3 µm; Millipore). Soil samples (0.5 g) were suspended in 100 ml sterilized, distilled water and vigorously shaken for 30 min. The membrane surface was inoculated with 200, 100 and 50 µl aliquots.

This approach selects for actinomycetes; only these are able to penetrate the membrane pores in order to grow into underlaying agar. In contrast, growth of other bacteria is restricted to the membrane surface. About 30 colonies could be optically distinguished. Their purity was verified by repeated culture of diluted samples on ISP 2 agar (SHIRLING & GOTTLIEB 1966).

Culture of fungi

Dual culture system

For testing the effect of bacteria on fungal growth, dual cultures were used. The fungal inoculum was excised from the actively growing edge of a fungal colony using the wide end of a Pasteur pipette and transferred to the center of a sterile Cellophan® (Folia, Wendelstein, Germany) sheet on top of ISP 2 agar contained in a 9-cm-Petri dish. This way, the fungal inoculum always had the same size, and fungal growth into the agar was prohibited, i.e. fungal growth could be determined as change in the area covered. In addition, the fungal hyphae could easily be collected at the end of the experiment for further analysis. Bacterial isolates were applied to the edge of the Petri dish (outside the Cellophan® layer) as a thin line of about 4 cm in length. The distance between both inocula was at least 3.5 cm, and both were physically separated by the medium.

Agar and membrane (exclusion limit about 10 kDa) allow for diffusion also of larger substances. In order to provide sufficient gas exchange, the Petri dishes were not sealed with Parafilm® (American National Can, Chicago, USA). Contamination was kept to a minimum by placing the Petri dishes in miniature plant culture chambers. Fungal growth was recorded by means of a digital image analysis system (DIAS, Bachofer, Reutlingen, Germany). For screening the effects of bacterial isolates on fungal growth, culture periods (20 °C, darkness; at least 2 independent trials with 4 parallels each) were between 15 and 42 days. With bacterial isolates which had been shown to promote fungal growth, culture experiments were extended for up to 76 days and 20 parallel trials. This way both the consistency of the bacterial effect could be checked and sufficient fungal hyphae could be obtained for biochemical analysis (e.g. changes in protein pattern).

Statistical treatment of data

Growth effects were validated by an analysis of variance (students t-test) using data of at least 4 independent culture experiments.

Taxonomic characterization of the bacterial isolates

The bacterial isolates were characterized by their morphological appearance (HÜTTER 1967) and by the analysis of diaminopimelic acid (STANECK & ROBERTS 1974) and cell wall sugars (CUMMINS & HARRIS 1956, LECHEVALIER & LECHEVALIER 1968).

Further characterization was carried out by a modified method of Tindall (TINDALL 1990) to determine the menaquinones. 0.1g freeze-dried cell material of each strain were extracted by a mixture of methanol-hexan [2+1] at 4 °C over night. After separation of the two phases at –80 °C the hexane phase was removed. The remaining methanol phase was extracted again with the same volume of hexane at 4 °C for 1 hour. After centrifugation the hexane extract was combined with the first extract and concentrated to dryness on a rotary evaporator. The residue was dissolved in 100 µl acetonitrile–*iso*-propanol [65+35]. A 10 µl-sample was injected onto a HPLC column filled with Nucleosil-100 C-18 (125 × 4.6 mm, particle size 5 µm; Maisch, Ammerbuch, Germany). The samples were analyzed by isocratic elution using acetonitrile–*iso*-

Tab. 1: Chemotaxonomical markers of 8 isolated *Streptomyces* strains

	AcH 504	AcH 505	AcH 506	AcH 1001	AcH 1003	AcH 2008	AcH 2009	AcH 20010
amino acid in peptidoglycan	LL-diaminopimelic acid in all strains tested							
cell wall sugars	glucose, galactose, ribose in all strains							
menaquinone	MK-9 (H_4,H_6)	MK-9 (H_4,H_6)	MK-9 (H_4,H_6,H_8)	MK-9 (H_4,H_6)	MK-9 (H_6,H_8)	MK-9 (H_4,H_6)	MK-9 (H_6)	MK-9 (H_8)

propanol [65+35] within 30 minutes and a flow rate of 1.3 ml min^{-1}. UV detection was carried out at 269 nm (HP 1050 liquid chromotograph, Agilent, Waldbronn, Germany).

For molecular characterization, DNA was prepared using a DNA-extraction kit (Qiagen, Hilden, Germany) according to the manufacturer´s instruction. Genomic DNA was used as template for PCR-amplification of a large fragment of the 16S rDNA region, flanked by a universal primer couple [pA: 5´-AGA GTT TGA TCC TGG CTC AG; position 8 to 27 and pH: 5´-AAG GAG GTG ATC CAG CCG CA; position 1541 to 1522 (based on *E. coli* numbering)] (Ramos et al. 1997). The PCR was carried out with *Taq* DNA Polymerase (Qiagen, Hilden, Germany). PCR products were purified using a E.Z.N.A. ® Cycle pure kit (peqLab, Erlangen, Germany). For sequencing of a fragment of the coding strand, primer pA was used. Sequencing was performed using the ABI PRISM® Dye Terminator Cycle Sequencing kit (Perkin-Elmer, Forster City, CA, USA) and the ABI 373A (Perkin-Elmer) sequencer.

Results

Selection for bacteria promoting fungal growth

Statistically significant growth promoting effects were obtained with the isolates AcH 505, AcH 506, AcH 2009, AcH 1006, AcH 1001, which increased the area covered by fungal mycelium up to 150 % within 36 days of culture. Four isolates caused a significant decrease of fungal growth (AcH 507, AcH 502, AcH 2008, AcH 20010), and three showed no effect (AcH 501, AcH 10010, AcH 2005). Others were slightly supportive or inhibitory (Fig. 1).

In order to investigate whether the observed effects are influenced by the composition of culture medium, 6 potentially growth promoting bacteria were co-cultured with *A. muscaria* on synthetic MMN medium (in relation to the ISP-2 medium: glucose as the only carbon source; ammonium as the only nitrogen source, vitamine B1 only). The data presented in Fig. 2 show that a decrease in the richness of the medium did not qualitatively alter the way of interaction.

Soil bacteria can produce volatiles such as ethylene (PRIMROSE & DILWORTH 1976) which can stimulate the formation of fruiting bodies (*e.g. Agaricus bisporus*; VISSCHER 1979). We thus investigated the effect of physically separating both organisms by an impermable barrier. For this purpose, compartmentalised Petri dishes were used. Under these conditions, neither of the most growth-promoting bacterial isolates (AcH 505, AcH 506, AcH 1003) caused any effect (not shown).

Interaction of bacteria with plant pathogenic soil fungi

For further characterization of bacterial effects, dual cultures were also perfomed using pathogenic fungi such as *Armillaria obscura* (attacks primarily declining plants, wide host range) and *Heterobasidion annosum* (causes wood decay in conifers) both of which can enter their host via the root system. None of the bacteria tested improved growth of *A. obscura*. Interestingly, one of the isolates (AcH 505) even caused some reduction of parasite growth (Fig. 3).

Taxonomic characterization of growth-promoting bacterial isolates

Eight of the investigated bacterial strains were assigned to the genus *Streptomyces* because of their morphological appearance and characteristic chemotaxonomic features, such as LL-diaminopimelic acid in the peptidoglycan together with the cell-wall sugars galactose, glucose and ribose, and the typical pattern of menaquinones. The data are summarized in Table 1.

For phylogenetic studies the 16S rDNA of the most effective isolates was partially sequenced. Two bacterial strains which did not cause significant growth effects were used as additional controls and were also sequenced (AcH 501 and AcH 504). A comparison of a highly variable 120 bp range within the first 300 base pairs at the 5`end (KATAOKA et al. 1997) with ribosomal data banks (MAIDAK et al. 2001) indicated sequence similarity values as follows (see also Table 2 and Fig. 4): AcH 501, 99 % with *Microbacterium oxydans*; AcH 504, 100 % with *Streptomyces argenteolus*; AcH 505, 93 % with *S. laceyi*; AcH 506, 95 % with *S. bobili*; AcH 1001, 100 % with *S. sampsonii;* and AcH 1006, 99 % with *Microbacterium liquefaciens*. A corresponding phylogenetic tree established with CLUSTALX and TreeView visualizes these relationships (Fig. 4). In the case of AcH 1003, the highly variable 120 bp region failed to identify this strain. Using a 532 bp fragment of the 16S rDNA sequence, AcH 1003 exhibited 98.5 % identity with *S. setonii.* α regions of AcH 504 and AcH

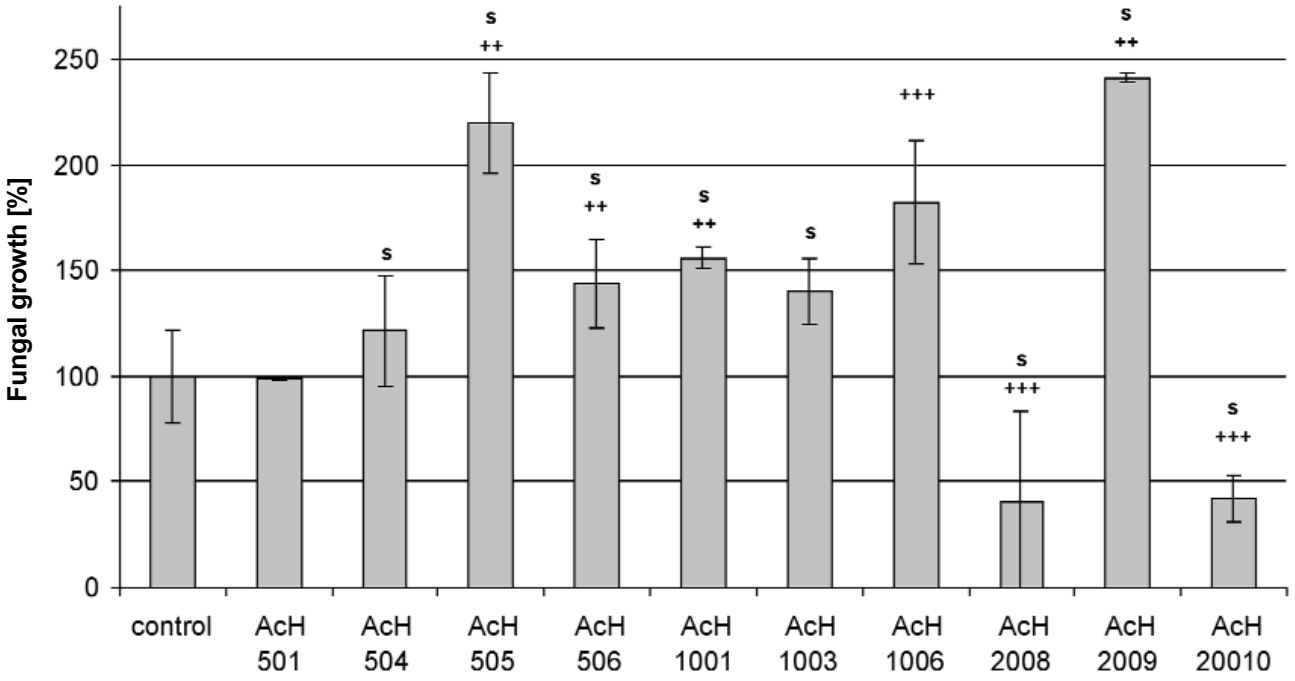

Fig.1: Growth of the *A. muscaria* mycelium (ectomycorrhizal fungus) as influenced by 10 bacterial strains.
Control: growth under axenic conditions. Streptomycetes are marked with s. (+/- SD; n = 4–8). Statistically significant growth effects are marked as ++ ($P < 0.01$) or +++ ($P < 0.001$).

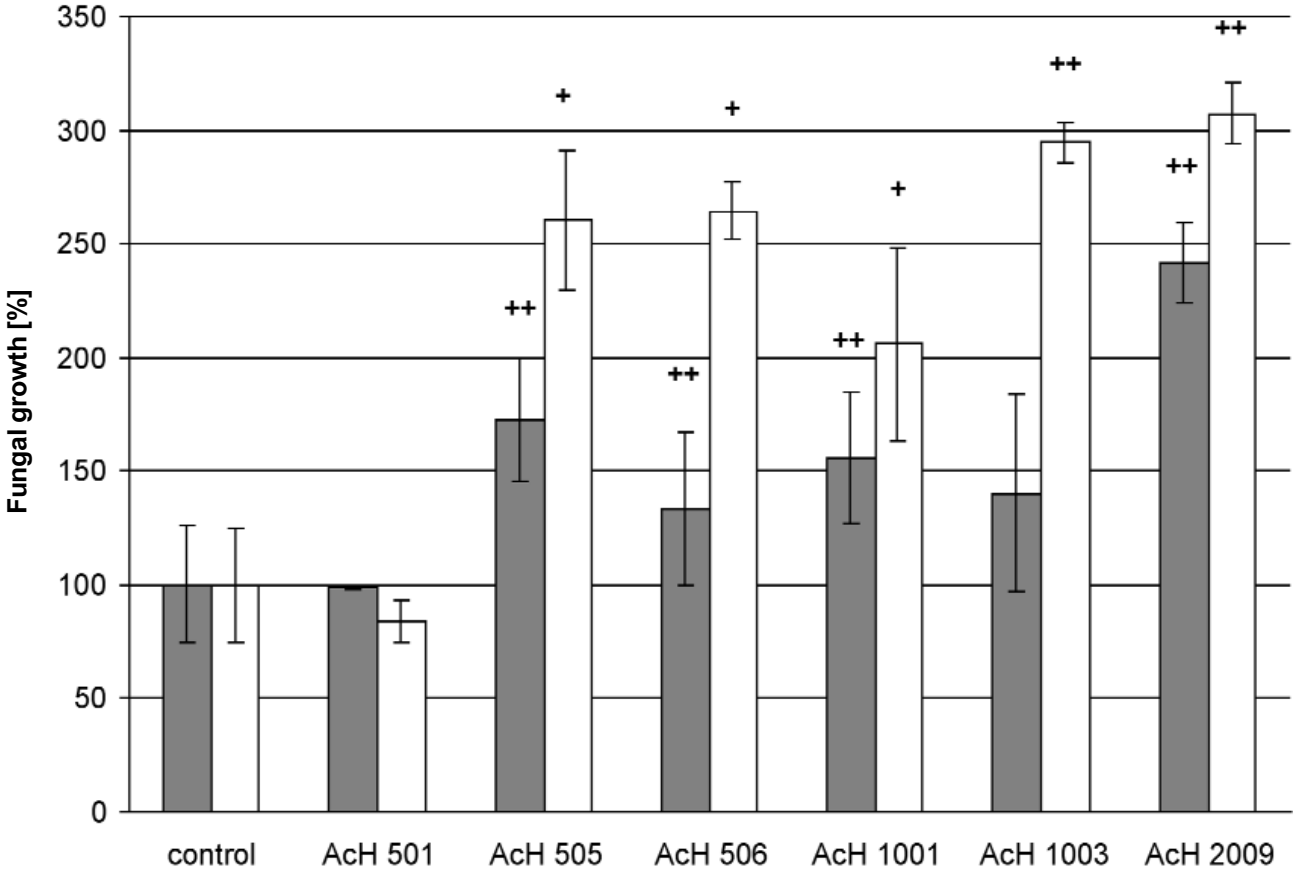

Fig. 2: Comparison of growth effects on complex ISP 2 (filled columns) and synthetic MMN (blank columns) medium.
(+/- SD; n = 4–8). + ($P < 0.05$) ++ ($P < 0.01$).

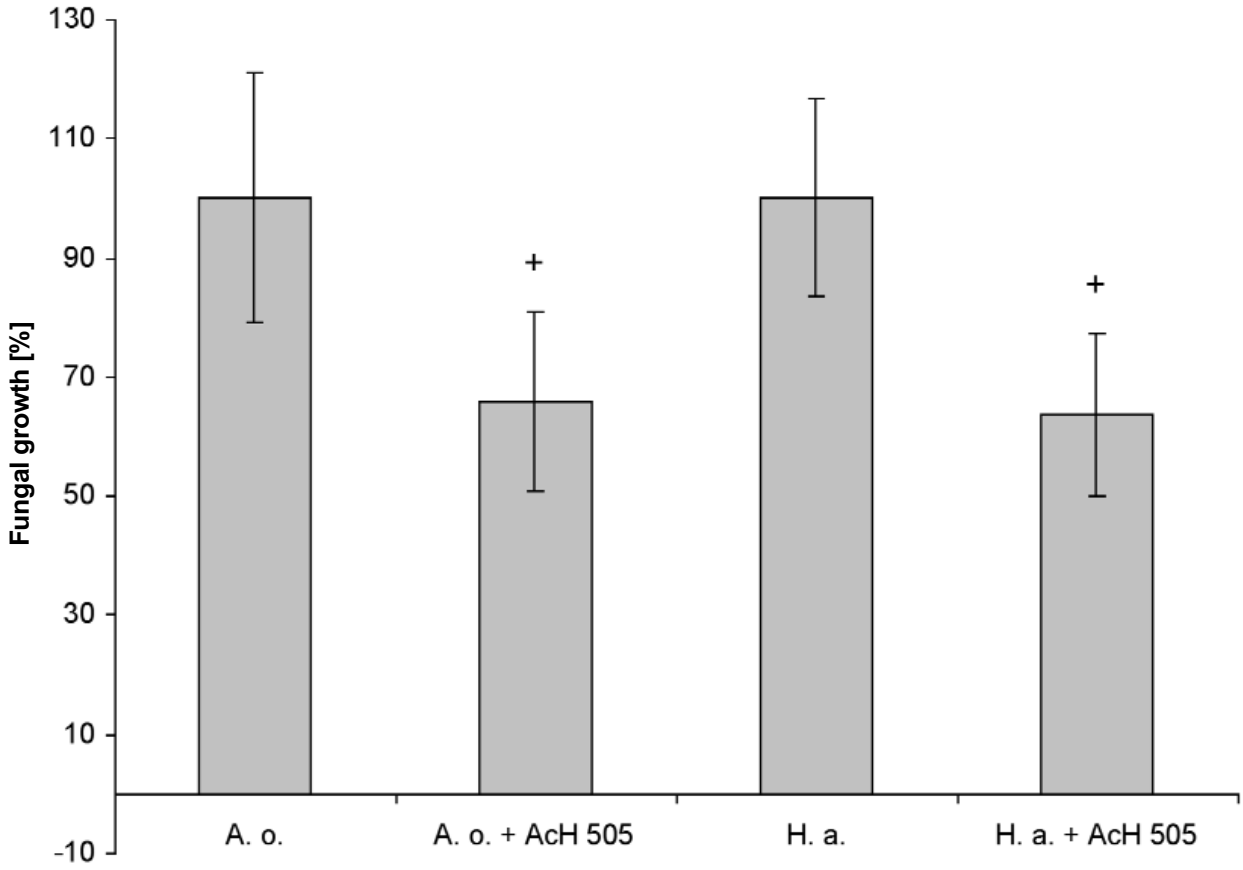

Fig. 3: Effect of the bacterial strain AcH 505 on the mycelial growth of two plant pathogens.
A.o. = *Armillaria obscura*; H.a. = *Heterobasidion annosum.* (+/- SD; n = 4–8).
+ Indicates a statistically significant difference in fungal growth compared to the control (P = 0.02).

2009 were identical, although these bacteria appeared different according to morphological features. By comparing the entire sequenced part of 16S rDNA, AcH 504 showed high homology with *S. argenteolus* (710 of 718 bp, 98.9 %) while AcH 2009 was more closely related to *S. cyaneus* (586 of 599 bp, 97.8 %).

Discussion

Our studies show that members of the actinomycetes, isolated from soil samples inhabited by mycelia of the ectomycorrhiza-forming soil fungus *A. muscaria* can excert distinguished effects in dual culture with *A. muscaria* and plant parasitic fungi such as *Heterobasidion annosum*. The most interesting microorganisms – under ecological as well as commercial aspects – are isolates such as AcH 505 which promote growth of the symbiotic fungus and inhibit the development of the parasite *H. annosum*. Chemical analysis of peptidoglycans showed patterns characteristic for actinomycetes which is in accordance with the morphology of the isolated bacteria.

Regarding the effective substances produced by the bacteria our data only indicate that no volatiles are involved, and work on the effect of bacterial culture filtrates on fungal growth, as well as their chemical analysis is under progress. Because some of the soil bacteria are producers of antibiotics, this class of compounds may be involved. Newer studies show that a variety of Gram-nagative and Gram-positive rhizobacteria (e.g. *Bacillus subtilis*, *Pseudomonas fluorescens*) can inhibit the growth of pathogenic fungi (*Fusarium oxysporum*; *Cylindrocarpon* sp.) in co-culture with ECM fungi such as *Laccaria bicolor*, *L. proxima* and *Suillus granulatus* (Schelkle & Peterson 1996). They can, however, also affect ECM fungi. *Burkholderia cepacia* significantly reduced the in vitro growth of mycelia of *Paxillus involutus*; *B. cepacia*, *Pseudomonas chlororaphis*, *Ps. fluorescens*, and *P. involutus* reduced the mycelial growth of the root pathogens *Fusarium moniliforme*, *F. oxysporum*, and *Rhizoctonia solani* (Pedersen, Reddy & Chakravarty 1999). *Burkholderia cepacia* also reduced the formation of ECM short roots by *P. involutus* on lodgepole pine and white spruce seedlings on the short term (2 months) but not upon longer incubation (4 months). *Pseudomonas chlororaphis* and *Ps. fluorescens* did not reduce mycelial growth and mycorrhiza formation. Treatment of the seedlings with either *B. cepacia* or *P. involutus* increased their survival in the presence of some of the root pathogens investigated. From the data given by Pedersen et al. (Pedersen, Reddy & Chakravarty 1999) it can thus be concluded, that the most simple protective system exists, when bacteria do not

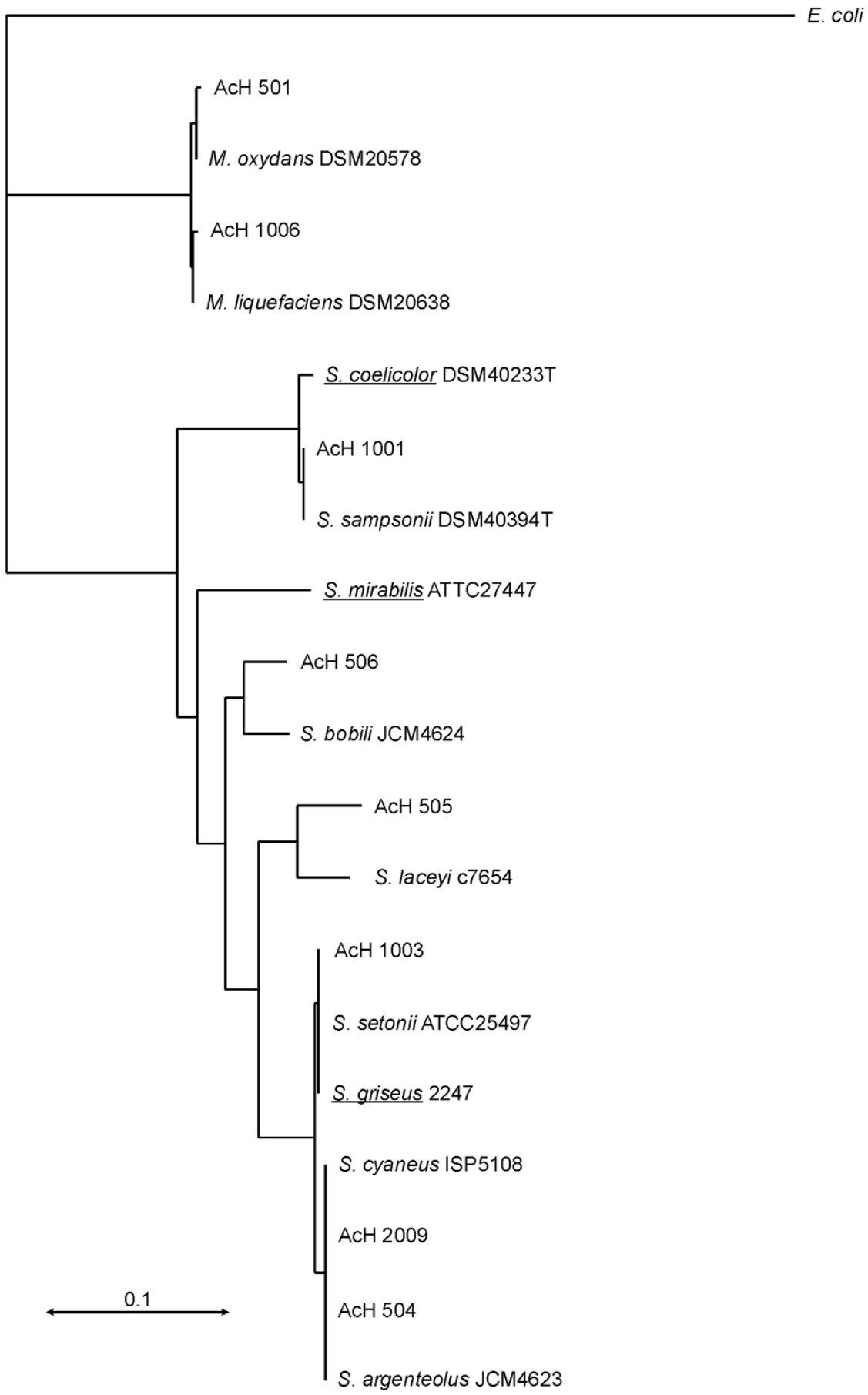

Fig. 4: Phylogenetic tree constructed from the 120-bp α region aligned by CLUSTALX, version 1.83. Alignment was extended by additional, published, streptomycete sequences (underlined). Outgroup: *Escherichia coli*.

Tab. 2: Sequence similarity of eight rhizobacteria with established species.

Bacterial isolate	next related species	Entrez Locus	Sequence similarity 120 bp α-Region [%]
AcH 501	*Microbacterium oxydans* DSM 20578	Y17227	99
AcH 504	*Streptomyces argenteolus* JCM 4623	AB045872	100
AcH 505	*Streptomyces laceyi* c7654	AY094367	93
AcH 506	*Streptomyces bobili* JCM 4624	AB045876	95
AcH 1001	*Streptomyces sampsonii* DSM 40394T	Z76680	100
AcH 1003	*Streptomyces setonii* ATCC25497	D63872	100
AcH 1006	*Microbacterium liquefaciens* DSM 20638	X77444	99
AcH 2009	*Streptomyces cyaneus* ISP 5108	AJ399460	100

inhibit fungal growth/mycorrhiza formation but affect potential root pathogens (FREY-KLETT et al. 2000). There are obviously also synergistic effects between these bacteria and ECM fungi such as *L. proxima* in inhibiting pathogens (SCHELKLE & PETERSON 1996).

Our finding that certain actinomycetes can stimulate mycelial growth of the root symbiotic fungus, *A. muscaria*, is also in agreement with other reports, although on Gram-negative bacteria (FREY-KLETT, PIERRAT & GARBAYE 1997, GARBAYE 1994). Generally, the effect ascribed to the presence of bacteria consists of a significantly increased number of infected root tips (DUNSTAN, MALAJCZUK & DELL 1998, POOLE et al. 2001).

Host plants can benefit from the stimulation of mycorrhiza formation by soil bacteria. Co-cultivation with *P. tinctorius* and PGPR belonging to the genus *Bacillus* enhanced growth of *Pinus pinea* (PROBANZA et al. 2001). A stimulation of shoot and root biomass production was also observed for *Acacia holoserica* seedlings, mycorrhizal with *Pisolithus alba* and after co-cultivation with two fluorescent pseudomonad strains (FOUNOUNE et al. 2002).

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