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Bacterial community structure and colonization patterns of *Fagus sylvatica* L. ectomycorrhizospheres as determined by fluorescence *in situ* hybridization and confocal laser scanning microscopy

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Abstract The bacterial community structure of ectomycorrhizospheres on beech (*Fagus sylvatica*) grown in natural forest soil in southern Germany was examined by fluorescence in situ hybridization (FISH) using fluorescent oligonucleotide probes, targeting phylogenetic relevant sequences of the 16S and 23S rRNA. *Lactarius subdulcis*, *L. vellereus*, *L. rubrocinctus* and *Laccaria amethystina* were found to be the prevalent fungi forming ectomycorrhizae with *F. sylvatica*. For FISH studies using confocal laser scanning microscopy, oligonucleotide probes labeled with carboxymethylindocyanine-succinimidyl ester allowed detection of associated bacteria, because the autofluorescence of ectomycorrhiza samples could be overcome in the infrared. Bacteria of the α-, β and γ-subclasses of the proteobacteria were detected in high numbers on mantle surfaces, while members of other phylogenetically defined groups were found in smaller numbers. This contrasts with previous published results on the cultivation of mycorrhiza-associated bacteria. Hybridizing bacteria were also found within damaged cells of the hyphal mantle of *L. rubrocinctus*, as well as on emanating hyphae of *L. amethystina*. Using a newly developed extraction protocol for bacteria associated with ectomycorhizas, the two most common fungi on *F. sylvatica*, *L. vellereus* and *L. subdulcis,* were mostly associated with members of the α- and β-subclasses of the proteobacteria. The proportion of hybridizing bacteria varied between the two ectomycorrhizae, which were thus host to distinct populations of bacteria.

Key words Beech · Ectomycorrhizae · FISH · CLSM · Bacterial community structure

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Introduction

Studies of bacteria associated with the mycorrhizosphere are frequent and mainly focused on mycorrhization helper effects by bacteria which are enriched and isolated on artificial media (Garbaye and Bowen 1989; Duponnois and Garbaye 1991; Frey-Klett et al. 1997). However, bearing in mind that at least 95% of all soil bacteria can not be taken into culture (Torsvik et al. 1990; Ovreås and Torsvik 1998), the reported mycorrhization helper bacteria very likely represent only a small proportion of total mycorrhizosphere bacteria.

Molecular techniques offer the possibility to describe the structural diversity of bacterial communities in different habitats without cultivation (Hartmann et al. 1997). Fluorescence in situ hybridization (FISH) of bacteria, applying 16S and 23S rRNA directed oligonucleotide probes, has allowed the detection and identification of bacterial cells directly in their habitat without prior cultivation (Amann et al. 1995). FISH has been used to determine the bacterial community structure of various habitats (Snaidr et al. 1997; Zarda et al. 1997; Chatzinotas et al. 1998; Llobet-Brossa et al. 1998). Assmus et al. (1995) described the occurrence of plant growth-promoting rhizobacteria in the rhizosphere of wheat. They also showed that FISH is an excellent method for detecting bacterial cells in plant-soil ecology studies, especially in combination with confocal laser scanning microscopy (CLSM) (Hartmann et al. 1998).

We carried out the study described here to obtain information about the diversity and distribution patterns of bacteria in situ, in functionally different and intact parts of various ectomycorrhizae of beech (*Fagus sylvatica* L.).

Materials and methods

Site and ectomycorrhizae

The investigated beech forest soil was a sandy brown earth near Munich (pH_{CaCl_2} 4.3). Roots from *F. sylvatica* were sampled from the O-horizon of the soil on six sampling dates during winter and spring 1996/97. Ectomycorrhizal mantles were prepared according to Agerer (1991) and were identified on a morphological basis (Agerer 1987–1997). After cleaning, roots were examined with a stereomicroscope and photographed with an MC100 microscope camera (Zeiss Oberkochen, Germany) using EPY64T (Kodak) film to describe and document morphological features.

Preparation of ectomycorrhizae for qualitative investigations by FISH and CLSM

Mantle pieces of ectomycorrhizae were fixed immediately after sampling for 2–4 h on ice using 3% paraformaldehyde-phosphate-buffered saline (PBS: composed of 0.13 M NaCl, 7 mM Na_2HPO_4 and 3 mM NaH_2PO_4 at pH 7.2 in water) or ethanol-PBS (1 : 1). The paraformaldehyde-fixed samples were washed in PBS and stored in ethanol-PBS (1 : 1) at −20 °C. Samples were mixed with 0.3% agarose, dropped onto glass slides and dried at room temperature. Glass slides were immersed in 50, 80 and 96% ethanol for 3 min each and stored at room temperature until investigation. In some cases, the samples were incubated prior to fixation overnight with yeast extract and nalidixic acid to enhance probe-conferred signal intensity (Roszak and Colwell 1987).

Quantitative determination of the bacterial community structure by FISH

Bacteria associated with the most common ectomycorrhizae of *F. sylvatica* were extracted from fresh field samples. Samples were kept at 4 °C during transport and in the laboratory within 1 h of sampling. All extraction steps were conducted with sterile solutions on ice. An aliquot (1 g) of ectomycorrhiza sample was washed with PBS and suspended in 10 ml 0.1% sodium cholate solution. The suspension was treated at high speed for 4 min in a Stomacher 80 (Seward Medical, London). After transfer into Erlenmeyer flasks, 0.25 g polyethyleneglycol 6000 and 0.2 g chelex 100 were added and bacteria were separated from ectomycorrhizae by stirring for 1 h at 4 °C (repeated three times). Remnants of plant tissue, fungal structures and soil particles were removed by filtration through gauze (40 µm mesh) and subsequent filtration with 5-µm syringe filters (Sartorius No. 17549, Göttingen, Germany). Bacteria in the suspension obtained were fixed overnight at 4 °C with 2% formaldehyde or for 1 h with ethanol-PBS (1 : 1). Bacteria in 100 to 1000-µl aliquots were concentrated in three replicates on 0.2-µm polycarbonate filters. Dehydration of cells was with 50, 80 and 96% ethanol for 3 min each. The filters were stored at room temperature until investigation.

Probes and stains

Oligonucleotide probes (Table 1) were synthesized with a C6-TFA aminolinker [6-(trifluoroacetyl-amino)-hexyl-(2-cyanoethyl)-(*N*,*N*-diisopropyl)phosphoramidite] at the 5′-end (MWG-Biotech, Ebersberg, Germany). They were labeled with tetramethylrhodamine-5-isothiocyanate (TRITC; Molecular Probes, Eugene, Ore) or 5(6)-carboxyfluorescein-*N*-hydroxysuccinimide ester (FLUOS; Boehringer Mannheim, Penzberg, Germany). The dye-oligonucleotide conjugate (1 : 1) was purified from unreacted components and stored at −20 °C in distilled water at a concentration of 50 ng μl^{-1} (Amann et al. 1990). Oligonucleotide probes labeled with carboxymethylindocyanine-succinimidyl ester (Cy5) were obtained from MWG-Biotech, Ebersberg, Germany. All probe sequences, hybridization conditions, and references are given in Table 1. The DNA-specific dye 4′,6-diamidino-2-phenylindole (DAPI; Sigma, Deisenhofen, Germany) was stored as an aqueous stock solution (0.35 mg ml^{-1}) at −20 °C.

In situ hybridization

Ethanol-fixed cells were treated with lysozyme prior to hybridization to enhance permeation of probes into Gram-positive cells (Amann et al. 1992, modified). Hybridizations were performed as described by Wagner et al. (1993) at 46 °C for 90 min in hybridization buffer containing 0.9 M NaCl, formamide at the percentage shown in Table 1, 20 mM Tris-hydrochloride (pH 7.2), 0.01% SDS and 5 mM EDTA. Hybridization was followed by a stringent washing step at 48 °C for 15 min. Washing buffer was removed by rinsing the slides with distilled water. If counterstaining with DAPI was desired, the stock solution was diluted 500-fold in PBS and 20 µl of this solution was applied to each filter or ectomycorrhiza piece. The slides were incubated for 10 min on ice, rinsed with distilled water, air dried and mounted in Citifluor antifading solution to avoid bleaching (AF1; Citifluor Ltd., UK). For each hybridization, a positive control experiment with appropriate bacterial strains was conducted to test the specificity of the probes.

Image production

For qualitative investigations of bacteria an LSM 410 inverted confocal laser scanning microscope (Zeiss) was used, equipped with two lasers (Ar-ion visible and HeNe) having excitation wavelengths of 488, 543 and 633 nm to record optical sections. An oil immersion lense (NA 1.3 × 100) was used. Monochrome sequences of images were taken along the optical axis (z-axis) with an increment of 1 µm. Sequential monochrome images or projections of monochrome z-sequences were assigned to their respective fluorescence colours and then merged to a true colour display (red/green/blue). All image combining and processing was performed with the standard software provided by Zeiss.

Counting of bacteria on filters was performed with a Zeiss Axiophot 2 epifluorescence microscope (Zeiss, Oberkochen, Germany) equipped with filter sets F31-000, F41-001 and F41-007 (Chroma Tech. Corp., Battleboro, Vt.).

Statistical analysis

Total cell counts were determined by evaluating at least 20 microscopic fields with 20–100 cells per field (3 replicates). Probe-positive cells were compared to DAPI-stained cell counts. Means were compared using the non-parametrical U-test ($P<0.05$). For each parameter in Table 2, values not marked with the same letter are significantly different.

Table 1 Oligonucleotide probes

	Probe sequence (5′-3′)	Target site[a], and rRNA position	Specificity	FA[b] (%)	Reference
Eub338	GCTGCCTCCCGTAGGAGT	16S rRNA, 338–355	Bacteria	0	Amann et al. (1990)
Eub785[c]	CTACCAGGGTATCTAATCC	16S rRNA, 785–803	Bacteria	0	Lee et al. (1993)
Eub927[c]	ACCGCTTGTGCGGGCCC	16S rRNA, 927–942	Bacteria		Giovannoni et al. (1988)
Eub1055[c]	CACGAGCTGACGACAGCCAT	16S rRNA, 1055–1074	Bacteria		Lee et al. (1993)
Eub1088[c]	GCTCGTTGCGGGACTTAACC	16S rRNA, 1088–1107	Bacteria		Lee et al. (1993)
Alf1b	CGTTCG(C/T)TCTGAGCCAG	16S rRNA, 19–35	α-subclass of Proteobacteria	20	Manz et al. (1992)
Bet42a[d]	GCCTTCCCACTTCGTTT	23S rRNA, 1027–1043	β-subclass of Proteobacteria	35	Manz et al. (1992)
CF319a	TGGTCCGTGTCTCAGTAC	16S rRNA, 319–336	Cytophaga-flavobacterium cluster of CFB-phylum	35	Manz et al. (1996)
Gam42a[e]	GCCTTCCCACATCGTTT	23S rRNA, 1027–1043	γ-subclass of Proteobacteria	35	Manz et al. (1992)
HGC69a[f]	TATAGTTACCTCCGCCGT	23S rRNA, 1901–1918	Gram-positive bacteria with high DNA G+C content	35	Roller et al. (1994)
LGC354a/b	(C/T)GGAAGATTCCCTACTGC	16S rRNA, 354–371	Gram-positive bacteria with low DNA G+C content	25	Meier et al. (1999)
Pla5a	GACTTGCATGCCTAATCC	16S rRNA, 45–62	Members of the planctomycetes group	25	Zarda et al. (1997)

[a] *E. coli* numbering, Brosius et al. (1981)
[b] Percentage formamide in the hybridization buffer
[c] Used in combination with probe Eub338 and three other domain-specific probes for quantification of bacterial cells on filters (Eub mix)
[d] Used with an equimolar amount of unlabeled competitor oligonucleotide Gam42a
[e] Used with an equimolar amount of unlabeled competitor oligonucleotide Bet42a
[f] After pretreatment with lysozyme (Amann et al. 1992, modified)

Table 2 Specific bacterial populations as a percentage of DAPI-stained cells [g^{-1} fresh wt.]. Bacteria were hybridized with group specific probes after extraction of ectomycorrhiza samples. The values are means ± standard deviation; different letters indicate significant differences, U-test, $n = 60$, $P<0.05$

Probes*	*Lactarius subdulcis*	*Lactarius vellereus*	
	–5 °C, under snow (April 1997)	–5 °C, under snow (April 1997)	+5 °C (May 1997)
DAPI ($\times 10^8$)	2.5 (±1.1)	7.0 (±3.4)	5.8 (±0.9)
DAPI	100	100	100
Eub-mix	22 (±15)	31 (±17)	27 (±17)
Alf1b	2 (±2)[a]	7 (±7)[b]	7 (±4)[a]
Bet42a	3 (±4)[a]	12 (±9)[a]	7 (±2)[a]
Gam42a	2 (±2)[a]	3 (±3)[c]	1 (±1)[b]
Pla5a	2 (±4)[b]	n.d.	<1
LGC353a/b	2 (±4)[b]	<1	<1

* Values for probes HGC69a and CF319a were below the detection limit set at 1%

Results

Detection of bacteria on hyphal mantles and emanating hyphae with FISH/CLSM

The most common ectomycorrhizal fungi on *F. sylvatica* in winter and spring 96/97 were "*Fagirhiza pallida", L. amethystina, L. rubrocinctus, L. subdulcis*, *L. vellereus*, and *Sphaerozone ostiolatum.* Hybridizing bacteria detected in situ with FISH/CLSM were present in all mycorrhizal samples studied. Representative photomicrographs, showing the specific detection of defined bacterial populations on hyphal mantle mounts, are given in Fig. 1. Ectomycorrhizal mantles harboured large numbers of phylogenetically different bacteria on their surfaces. Bacteria belonging to the α-, β- and γ-subclasses of proteobacteria were detected using the probes Alf1b, Bet42a, and Gam42a, respectively, and were localized on the surface of hyphal mantles (Fig. 1a–d). Only a few bacteria of the flavobacteria-cytophaga cluster and Gram-positive bacteria

were detected after hybridization with probes CF319a, HGC69a, and LGC353a/b.

For *L. vellereus,* intercellular spaces and the surface of the hyphal mantle were often found to be colonized by bacteria hybridizing with probe Eub338 (Fig. 1e). An intracellular colonization by bacteria was only observed within damaged cells of the hyphal mantle of *L. rubrocinctus* (Fig. 1f); bacteria were never detected in intact hyphae. Regardless of the ectomycorrhiza investigated, bacterial colonization of emanating hyphae was evident, as shown for *L. amethystina* in Figure 1g.

Bacteria were detected on mantle surfaces of ectomycorrhizae by CLSM with all fluorochromes used. After excitation with lasers at 488 and 543 nm the ectomycorrhizal mantle itself showed an extremely high autofluorescence (e.g. 543 nm: Fig. 2a). Excitation at 633 nm strongly reduced such autofluorescence (Fig. 2b), facilitating the detection of bacterial cells with Cy5-labeled oligonucleotides.

Analysis of bacterial community structure after extraction of ectomycorrhiza samples

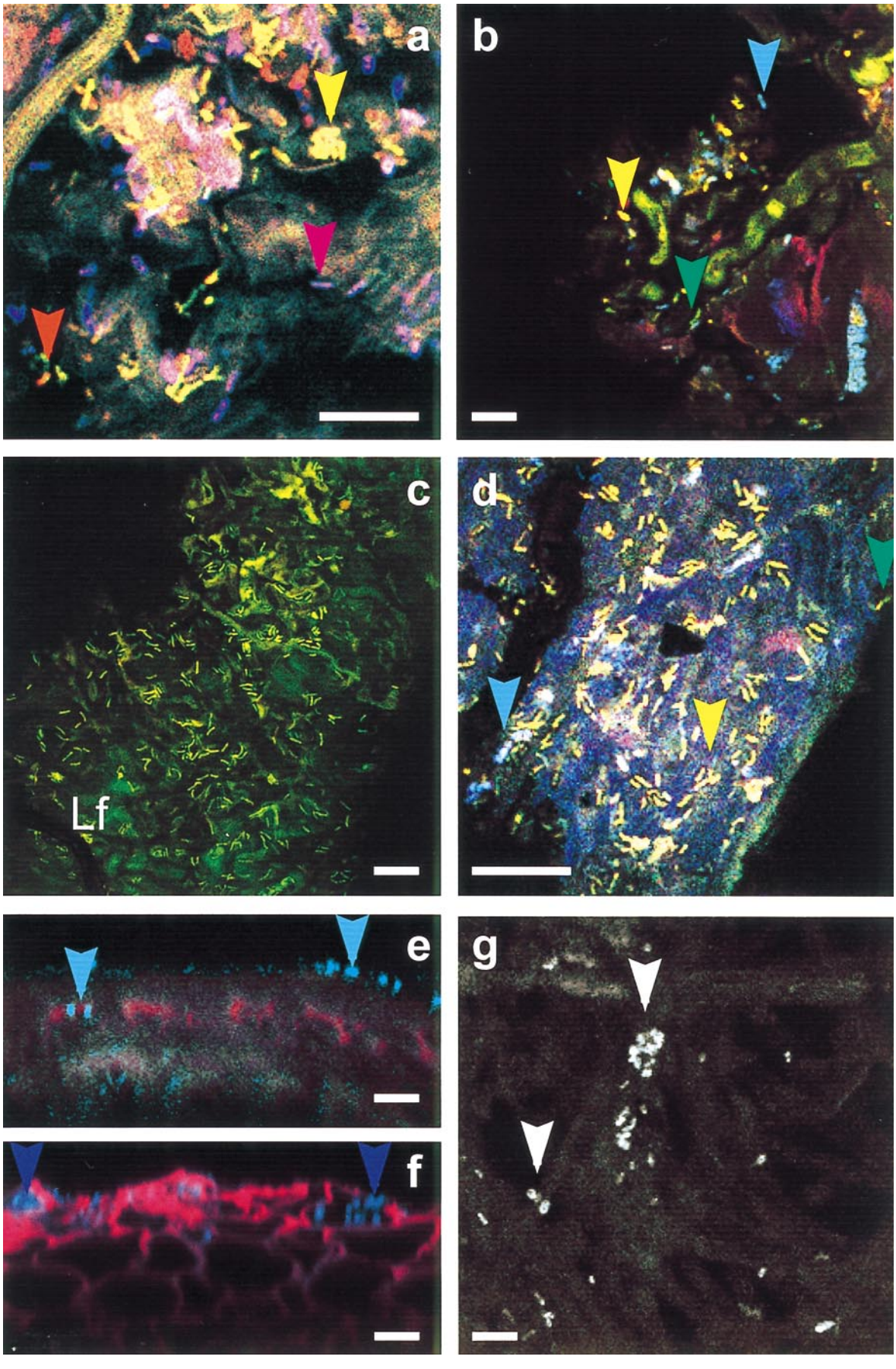
FISH was also used to analyse the bacterial community structure after extraction from ectomycorrhizospheres of *L. vellereus* (*L. vellereus*-EM) and *L. subdulcis* (*L. subdulcis*-EM). At all sampling dates, both ectomycorrhizae were found in sufficiently high amounts for extraction (at least 1 g each). The extraction protocol proved to be very efficient since no bacteria were detectable on extracted ectomycorrhizae using CLSM (data not shown). Significant differences were obtained for associated bacteria between the two ectomycorrhizae in April 1997 when the forest soil was still snow-covered (Table 2). The proportion of hybridizing bacteria on DAPI-stained cells was 31 and 22% for the *L. vellereus*-EM and *L. subdulcis*-EM, respectively. Of the bacteria detected by probe EubMix, 70 and 50%, respectively, could be assigned to the groups analysed (α, β, and γ-subclasses of proteobacteria, Gram-positive bacteria with a low DNA G+C content, and the planctomycetes). Members of the Gram-positive bacteria with a high DNA G+C content as well as bacteria of the cytophaga-flavobacterium cluster of the CFB phylum did not occur at levels above the detection limit, set at 1% of the DAPI-stained cells. For the *L. vellereus*-EM, the number of bacteria hybridizing with phylogenetic probes decreased in the order from β- to the α- and γ-subclasses of proteobacteria ($P<0.05$, $n=60$, U-test). The bacterial community of the *L. subdulcis*-EM were dominated in similar proportions by members of all the subdivisions of proteobacteria investigated. By comparison, the fractions of the α- and β-classes of proteobacteria were significantly higher in *L. vellereus*-EM than in *L. subdulcis*-EM ($P<0.05$, $n=60$, U-test).

To obtain information about temporal changes in colonization patterns of hybridizing bacteria, a further sample of the *L. vellereus*-EM was investigated in April 1997 after the snow had melted (air temperature 5 °C). No obvious differences between the sampling dates were observed (Table 2).

Discussion

CLSM enabled a direct insight into the bacterial colonization of the ectomycorrhizae of *F. sylvatica*. The previously described background fluorescence (Timonen 1995) could be overcome via single-spot excitation and the use of Cy5-labeled oligonucleotide probes. Thus CLSM is a very powerful tool for in situ studies in microbial ecology, especially in combination with specific molecular markers (Hartmann et al. 1998).

Fig. 1a-g Confocal laser scanning micrographs of bacteria colonizing ectomycorrhizae on *Fagus sylvatica* after fluorescence *in situ* hybridization; *bar* 10 μm **a** xy-scan of the hyphal mantle of *Lactarius vellereus*-EM. Hybridization with oligonucleotide probes Bet42a-FLUOS, Eub338-TRITC, and Alf1b-Cy5. Excitation wavelengths: 488 nm (FLUOS), 543 nm (TRITC), and 633 nm (Cy5) for green, red and infrared fluorescence, respectively. Signals are displayed as an rgb image. β-Proteobacteria appear in yellow, α-proteobacteria in violet, others in red (*arrows*) **b** xy-scan of the hyphal mantle of *L. vellereus*-EM incubated with nalidixic acid and yeast extract. Hybridization with oligonucleotide probes Eub338-FLUOS, Gam42a-TRITC, and Bet42a-Cy5. Excitation wavelengths: 488 nm (FLUOS), 543 nm (TRITC), and 633 nm (Cy5) for green, red and infrared fluorescence, respectively. Signals are displayed as an rgb image. γ-Proteobacteria appear in yellow, β-proteobacteria in turquoise, others in green (*arrows*) **c** xy-scan of the hyphal mantle of *L. vellereus*-EM incubated with nalidixic acid and yeast extract. Hybridization with oligonucleotide probe Bet42a-FLUOS. Excitation wavelengths: 488 nm (FLUOS), 543 nm for green and red fluorescence, respectively. Signals are displayed as an rg image. β-Proteobacteria have aggregated on the mantle surface (*Lf* laticifer) **d** xy-scan of the hyphal mantle of *L. vellereus*-EM incubated with nalidixic acid and yeast extract. Hybridization with oligonucleotide probes Eub338-FLUOS, Gam42a-TRITC and Bet42a-Cy5. Excitation wavelengths: 488 nm (FLUOS), 543 nm (TRITC), and 633 nm (Cy5) for green, red and infrared fluorescence, respectively. Signals are displayed as an rgb image. β-Proteobacteria appear in turquoise, γ-proteobacteria in yellow, others in green (*arrows*) **e** z-scan of the hyphal mantle of *L. vellereus*-EM. Hybridization with oligonucleotide probe Eub338-Cy5. Excitation wavelengths: 543 nm and 633 nm (Cy5) for red and infrared fluorescence, respectively. Signals are displayed as an rgb image by overlay of infrared fluorescence in both channels, blue and green. Turquoise-coloured bacterial cells colonizing the outer cells and the surface of the hyphal mantle (*arrows*) **f** z-scan of the hyphal mantle of *L. rubrocinctus*-EM. Hybridization with oligonucleotide probe Eub338-Cy5. Excitation wavelengths: 543 nm and 633 nm (Cy5) for red and infrared fluorescence, respectively. Signals are displayed as an rb image. Note the intracellular colonization of damaged hyphae by bacteria (*arrows*) **g** Bacteria (*arrows*) colonizing emanating hyphae of *L. amethystina*-EM incubated with nalidixic acid and yeast extract. Hybridization with oligonucleotide probe Eub338-TRITC. Excitation with 543 nm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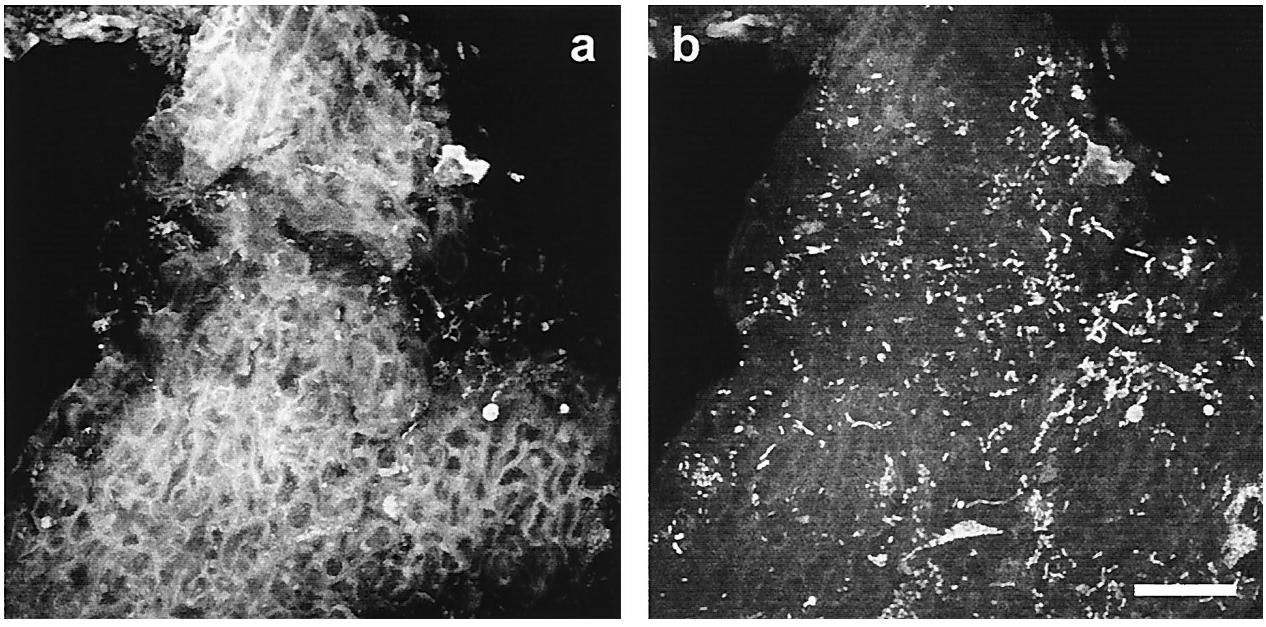

Fig. 2a,b Effectiveness of Cy5-labeled oligonucleotide probes in the detection of bacterial cells. Projection of a z sequence (10 μm) of the hyphal mantle of *L. vellereus*-EM. Hybridization with oligonucleotide probe Eub338-Cy5 (excitation wavelengths were **a** 543 nm **b** 633 nm). Note the autofluorescence of the ectomycorrhiza at the two wavelenghts; *bar* 20 μm

The general observation that mantle surfaces of *F. sylvatica* ectomycorrhizae are densely colonized by bacteria is in agreement with previously published results (Foster and Marks 1966; Malajczuk 1979; Garbaye 1994). Using scanning and transmission electron microscopy, Nurmiaho-Lassila et al. (1997) observed that the *Pinus sylvestris* mycorrhizospheres of two different fungi could be different habitats for associated bacteria. Schelkle et al. (1996) found bacterial cells primarily on the surface of the mantle and in the interhyphal spaces of a *Tuber* sp. mycorrhiza but not intracellularly within hyphae. Large amounts of intracellular localized bacteria (*Burkholderia* sp.), which have been observed for the arbuscular mycorrhizal fungus *Gigaspora margarita* (Bianciotto et al. 1996) as well as endobacteria observed in hyphae of *Morchella elata* (Buscot 1994), were found within hyphae of ectomycorrhizae of *F. sylvatica*. This might be an artefakt due to the limited magnification of the CLSM or to the low penetration of oligonucletide probes into paraformaldehyde-fixed mycorrhiza samples (Assmus et al. 1995).

The intracellular colonization of damaged cells of *L. rubrocinctus* by hybridizing bacteria is similar to the results of Filippi et al. (1995), who found an *Azospirillum*-like bacterium within epidermal cells containing fungal hyphae in senescing mycorrhizas. The intracellular colonization of these cells can be interpreted as a saprotrophic status of the bacteria observed. Hyphae emanating from ectomycorrhizae form a large surface area and probably provide a good habitat for bacterial colonization, as shown for *L. amethystina*. External hyphae play an important role in the mobilization of organic nutrients (N and P) and in the translocation of plant-derived carbon into the soil (Smith and Read 1997).

From our results, FISH/CLSM can be recommended if the determination and localization of distinct bacterial populations on ectomycorrhizae is desired. Pre-incubation with nalidixic acid and yeast extract did not enhance the number or intensity of signals and, therefore, seems unnecessary. With FISH, we were also able to quantify the bacterial community structure from *F. sylvatica* ectomycorrhizae in natural forest soil. Zarda et al. (1997) and Chatzinotas et al. (1998) used FISH to determine the community structure in organic soils from Switzerland and Norway. They found that about 40% of the bacterial cells stained with DAPI hybridized with the domain-specific probe Eub338. Our results for *L. subdulcis* and *L. vellereus* are in the same range (22–31%). The authors showed that most of the hybridizing bacteria belonged to the α- and δ-subclasses of the proteobacteria as well as the planctomycetes. The occurrence of planctomycetes in soils was first reported using PCR-based methods (Liesack and Stackebrandt 1992) but little is known about their ecological relevance in soils. Our results confirm their presence in the *L. subdulcis*-EM. Gram-positive bacteria with high DNA G+C content (probe HGC69a) and bacteria of the *cytophaga-flavobacterium* cluster (probe CF319a) were not detected (with the detection limit set at 1%), as shown by others (Chatzinotas et al. 1998; Llobet-Brossa et al. 1998). A detection yield of approximately 30% using a mixture of probes targeted to the domain bacteria implies that 70% of the bacterial community were not recognized in our studies. Zarda et al. (1997) used var-

ious fixation and pre-treatment protocols prior to hybridization with no substantial increase in hybridization with probe Eub338. Thus, the relatively low detection yield was probably due to the low soil temperature in winter and early spring and the resulting reduced metabolic activity.

So far, studies of bacterial populations on mycorrhizae focused mainly on the mycorrhization helper effects by a few bacteria isolated with culture-dependent methods (Garbaye 1994; Frey et al. 1997). Members of the γ-subclass of the proteobacteria (e.g. the fluorescent pseudomonads *Pseudomonas fluorescens* and *P. putida*) and Gram-positive bacteria with a low DNA G+C content (e.g. *Bacilli*) were often found enriched and stimulatory of root infection with arbuscular and ectomycorrhizal fungi (Meyer and Linderman 1986; Garbaye and Bowen 1989; Duponnois and Garbaye 1991; Garbaye 1994; Gryndler and Vosátka 1996; Frey-Klett et al. 1997; Timonen et al. 1998). However, culture experiments in our laboratory with King's B agar were found to overestimate the relative proportion of these bacteria compared with using oligonucleotide probe Gam42a (data not shown). Partial and strongly biased results due to culture-dependent community structure analysis have also been shown for other environments and phylogenetic levels, especially when the proportion of hybridizing cells was high (e.g. activated sludge: Wagner et al. 1993; Kämpfer et al. 1996; Snaidr et al. 1997).

Timonen et al. (1998) used the Biolog bacterial identification system for a detailed description of the culturable bacteria of *Suillus bovinus*-EM and *Paxillus involutus*-EM on *P. sylvestris.* Dendrograms generated from the carbon source utilization patterns divided their isolates on King's B agar into different groups. Members of the γ- but also of the α- and β-subclasses of the proteobacteria were isolated from ectomycorrhizospheres of *P. sylvestris*. The α- and β-subclasses of the proteobacteria contain, among others, numerous nitrogen-fixing and plant growth-promoting rhizobacteria, e.g. members of the genera *Burkholderia, Azospirillum, Acetobacter and Herbaspirillum* (Tabacchioni et al. 1993; Assmus et al. 1995; Hartmann et al. 1995). Thus, abundance of these proteobacteria on the ectomycorrhizae of *F. sylvatica* in winter and early spring and of *P. sylvestris* (Timonen et al. 1998) should be taken into account in future experiments on the mycorrhization helper bacteria.

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