

# Occurrence and distribution of endobacteria in the plant-associated mycelium of the ectomycorrhizal fungus *Laccaria bicolor* S238N

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## Summary

**Fluorescence *in situ* hybridization, associated with confocal laser scanning microscopy or epifluorescence microscopy with deconvolution system, has allowed the detection of a community of intracellular bacteria in non-axenic samples of the ectomycorrhizal fungus *Laccaria bicolor* S238N. The endobacteria, mainly  $\alpha$ -proteobacteria, were present in more than half of the samples, which consisted of ectomycorrhizae, fungal mats and fruit bodies, collected in the glasshouse or in the forest. Acridine orange staining suggests that the endobacteria inhabit both live and dead fungal cells. The role of these endobacteria remains to be clarified.**

## Introduction

In the soil, mycorrhizal fungi and bacteria undergo complex interactions that influence dramatically the biology of the fungus and the nutrition of the plant (Frey-Klett *et al.*, 2005). Many mycorrhizosphere bacteria are in close contact with mycorrhizal fungi, either in the fruit bodies (Sbrana *et al.*, 2000), or with the mycorrhizal mats or hyphae in soil (Filippi *et al.*, 1995; Mogge *et al.*, 2000). Some bacteria attach to the spores (Walley and Germida, 1996) or to the hyphae (Nurmiaho-Lassila *et al.*, 1997;

Sbrana *et al.*, 2000), and some are even present inside the fungal cells, as discussed here below.

The occurrence of such intracellular bacteria is a well-established fact among the Glomeromycota fungi, a phylum consisting mostly of endomycorrhizal fungi (Schüßler *et al.*, 2001). The cyanobacteria *Nostoc* colonizing the fungal bladders of *Geosiphon pyriforme* have been known since the 19th century (Schüßler *et al.*, 1994), and different morphotypes of bacteria-like organisms have been detected with electron microscopy in many genera of the Glomeromycota since the seventies (MacDonald *et al.*, 1982). In some *Gigaspora* and *Scutellospora* species, Bianciotto and colleagues (2003) recently showed that the rod-shaped endosymbionts belonged to a new taxon within the  $\beta$ -proteobacteria. These endosymbionts are transmitted vertically in their fungal host, from generation to generation (Bianciotto *et al.*, 2004). They colonize all the life-stages of the fungus: the spores, the external and the intraradical mycelium (Minerdi *et al.*, 2002). In contrast, *Nostoc* colonizes only the bladders of *G. pyriformis*, i.e. specialized structures developing on the soil surface. *Geosiphon pyriformis* acquires its cyanobacterial endosymbionts cyclically from the environment through endocytosis (Schüssler *et al.*, 1994).

Several studies propose that the ectomycorrhizal fungi could also harbour intracellular bacteria (Bonfante-Fasolo and Scannerini, 1977; Buscot, 1994; Nurmiaho-Lassila *et al.*, 1997; Barbieri *et al.*, 2000; Mogge *et al.*, 2000; Bertaux *et al.*, 2003). However, some electron microscopy studies have provided contrasting data concerning the viability of the colonized fungal cells, which sometimes questions the endosymbiotic status of the bacteria. For the ectomycorrhizae of *Pinus strobus-Endogone flamicolora* (Bonfante-Fasolo and Scannerini, 1977), *Picea abies*-'Type F' ectomycorrhizae (Buscot *et al.*, 1994) and *P. sylvestris-Suillus bovinus* (Nurmiaho-Lassila *et al.*, 1997), the endobacteria were observed in live cells. However, the 'Type F' ectomycorrhizae were senescing, and in the case of *Fagus sylvatica-Lactarius rubrocinctus* (Mogge *et al.*, 2000), endobacteria were detected only inside damaged cells. Intracellular bacteria were also observed in axenic cultures of *Tuber borchii* (Barbieri *et al.*, 2000) and *Laccaria bicolor* S238N (Bertaux *et al.*, 2003), and in axenically synthesized ectomycorrhizae of

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*Tilia platyphyllos* Scop.-*T. borchii* (Barbieri *et al.*, 2000). But in these two studies, the viability of the fungal cells was not assessed. The endobacteria were identified by fluorescence *in situ* hybridization (FISH) and/or polymerase chain reaction (PCR) techniques only in the case of the pure cultures.

To sum up, very little is known about the ecology and the diversity of the endobacteria of the ectomycorrhizal fungi, and their permanent or sporadic relation towards the fungal host. Recently, we reported the existence of rare intracellular bacteria affiliated to the Firmicutes inside the mycelium of pure cultures of *L. bicolor* S238N, an ectomycorrhizal basidiomycete cultured axenically since 1976 (Bertaux *et al.*, 2003). Here, we present FISH observations on plant-associated mycelium of *L. bicolor* S238N collected in the glasshouse and in the forest, which provide new information about the occurrence and distribution of the endobacteria of this fungus under non-axenic conditions.

## Results

### *Diversity of the intracellular bacteria*

Bacteria were detected with 16S rRNA-targeted oligonucleotide probes (FISH), and/or with the general DNA stain 4,6-diamidino-2-phenylindole (DAPI). Their intracellular location was checked by examining optical sections obtained with confocal laser scanning microscopes (CLSM) or with the Zeiss ApoTome deconvolution system. Both devices produced images of similar resolution. The samples of *L. bicolor* S238N collected in the glasshouse and in the forest contained intracellular bacteria of variable shape: mostly spherical and oval ones, and occasionally rod-shaped ones (Fig. 1), measuring about 1 µm in diameter on average. The endobacteria in one colonized cell always had the same morphology, and were all labelled with the same FISH probes. A total of 309 FISH and/or DAPI observations was recorded in a database to enable a semiquantitative analysis of the microscopic observations. Most of the endobacteria were affiliated with the  $\alpha$ -proteobacteria: from 120 images, 92.5% showed a positive signal with EUBmix and ALF1b, whereas 7.5% showed a positive signal with EUBmix only. The use of other phylum- and group-specific probes tested (Table 1) did not allow the identification of the remaining 7.5%, with the exception of a single hybridization of the probe BET42a, specific for  $\beta$ -proteobacteria. Another  $\alpha$ -proteobacterial probe was occasionally used, ALF968, which has a slightly different spectrum from the probe ALF1b. It hybridized with the intracellular bacteria only in some cases, but always together with ALF1b (six observations out of 24). This cohybridization of ALF1b and ALF968 indicates that the corresponding endobacteria

were not  $\delta$ -proteobacteria or Spirochaetes, which can also hybridize with ALF1b. To further identify the intracellular  $\alpha$ -proteobacteria, we used Rhi1247, G Rb and SPH120 probes, specific for the *Rhizobia*, *Rhodobacter* group and *Sphingomonas* (Table 1). However, when these probes were used at the recommended stringency, no hybridization was observed.

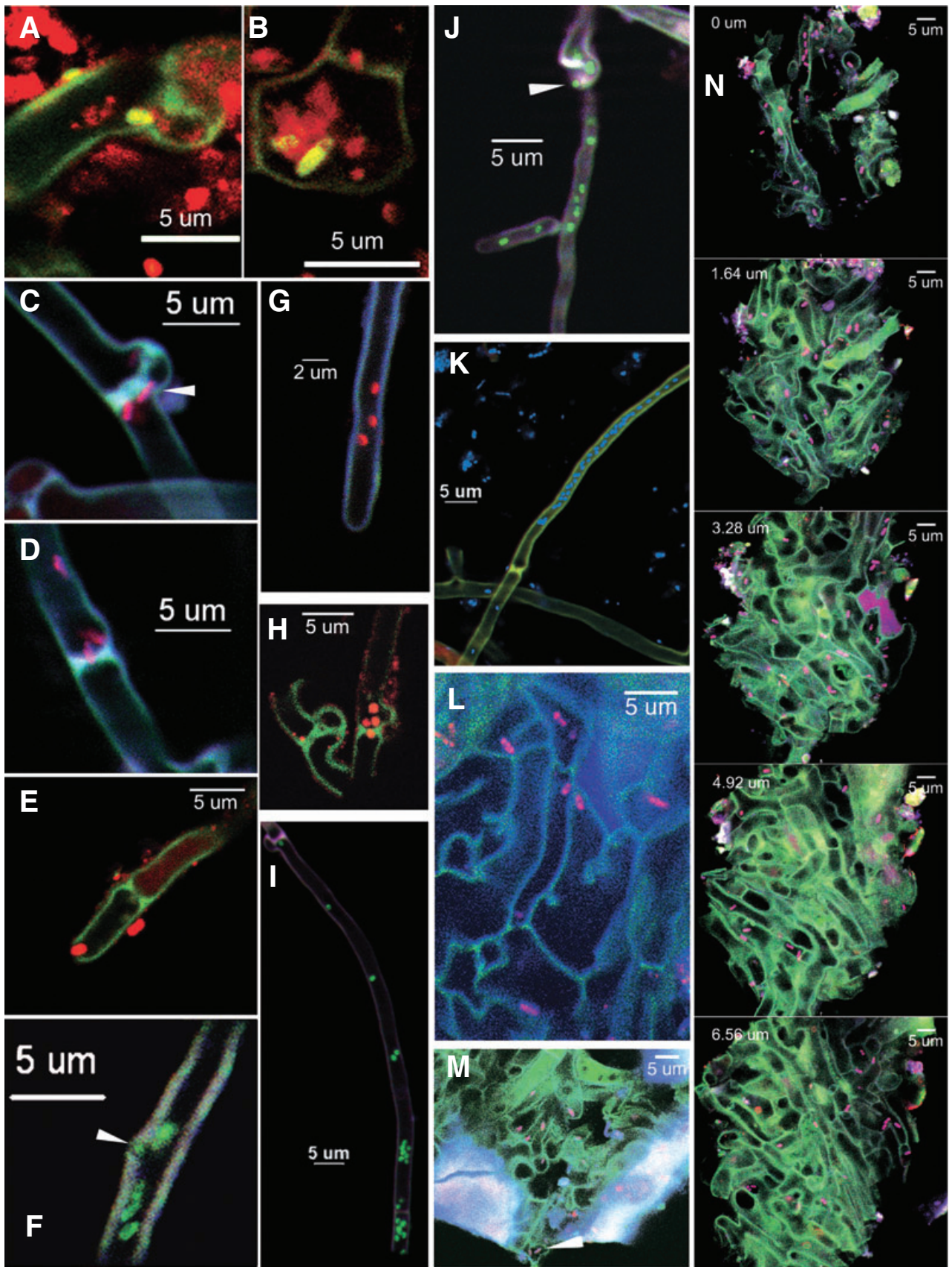
### *Colonization pattern at the fungal cell level*

Acridine orange, which stains the RNA in red and the DNA in green, showed that some fungal cells colonized by bacteria contained RNA (12 observations with Acridine orange, Fig. 1A and B). With DAPI staining, it was sometimes possible to see bacteria within fungal cells with nuclei. However, colonized cells without nuclei were seen more frequently. Intracellular bacteria were also often observed in the clamp connections of the fungus. Clamp connections are characteristic for Basidiomycetes. These are mycelial loops that develop when the apical cell divides. Depending on the growth stage, the apical cell is separated from the previous cell and the loop by cross-walls: three Y-shaped cross-walls during the formation of the loop (Fig. 1H), and only two when the loop is completed, an arm of the Y disappearing (Fig. 1A). On 118 observations of colonized clamp connections, it was possible to identify 58 developing ones and 31 fully formed ones. The intracellular bacteria could be seen in the loop of the clamp connections even when it was closed by two cross-walls. Apical cells with intracellular bacteria were also detected (15 observations out of a total of 309, Fig. 1E and G).

Endobacteria were often observed against the dolipores from the septum of the loop or from the septum separating the cells. Sometimes, bacteria were seen across the dolipore holes (10 observations out of a total of 309, Fig. 1C and D). Bacteria were also occasionally seen in holes in the fungal cell walls of the apex (Fig. 1E), of the clamp connection (Fig. 1J), or of the hypha (Fig. 1F) (three observations out of a total of 309). Cut hyphae that contained bacteria, as well as the next colonized intact fungal cell, were observed (Fig. 1H and I).

### *Colonization pattern at the fungal tissue level*

Endobacteria were found in more than half of the analysed samples (Table 2). Concerning the negative samples, endobacteria could be either not detectable or absent. When considering which tissue of the fungal host was colonized, 19 out of 29 ectomycorrhizae and 10 out of 12 fungal mats investigated contained endobacteria. In the ectomycorrhizae, intracellular bacteria were frequently observed in the extramatricial mycelium (193 out of a total



**Table 1.** Oligonucleotide probes used in this study.

Probe name	Position <sup>a</sup>	Sequence (5'-3')	Stringency (% formamide)	Specificity	Target	Reference
EUBI	338–355	GCTGCCTCCCGTAGGAGT	35	Eubacteria	16S rRNA	Amann <i>et al.</i> (1990)
EUBII	338–355	GCAGCCACCCGTAGGTGT	35	<i>Planctomycetales</i>	16S rRNA	Daims <i>et al.</i> (1999)
EUBIII	338–355	GCAGCCACCCGTAGGTGT	35	<i>Verrucomicrobiales</i>	16S rRNA	Daims <i>et al.</i> (1999)
LGC354A	354–371	TGGAAGATTCCCTACTGC	35	Firmicutes (low GC content Gram-positive bacteria)	16S rRNA	Meier <i>et al.</i> (1999)
LGC354B	354–371	CGGAAGATTCCCTACTGC	35	Firmicutes (low GC content Gram-positive bacteria)	16S rRNA	Meier <i>et al.</i> (1999)
LGC354C	354–371	CCGAAGATTCCCTACTGC	35	Firmicutes (low GC content Gram-positive bacteria)	16S rRNA	Meier <i>et al.</i> (1999)
HGC69a	1901–1918	TATAGTTACCACCGCCGT	25	Actinobacteria (high GC content Gram-positive bacteria)	23S rRNA	Roller <i>et al.</i> (1994)
BET42a	1027–1043	GCCTTCCCACCTTCGTTT	35	$\beta$ -proteobacteria	23S rRNA	Manz <i>et al.</i> (1992)
GAM42a	1027–1043	GCCTTCCCACATCGTTT	35	$\gamma$ -proteobacteria	23S rRNA	Manz <i>et al.</i> (1992)
CF319	319–336	TGGTCCGTGTCTCAGTAC	35	<i>Cytophaga-Flavobacteria</i>	16S rRNA	Manz <i>et al.</i> (1996)
ALF1b	19–35	CGTTCGYTCTGAGCCAG	20	$\alpha$ -proteobacteria, several members of $\delta$ -proteobacteria, most spirochetes	16S rRNA	Manz <i>et al.</i> (1992)
ALF968	968–985	GGTAAGGTTCTGCGCGTT	35	$\alpha$ -proteobacteria, except for Rickettsiales	16S rRNA	Neef (1997)
RHI1247	1247–1251	TCGCTGCCCACTGTC	45	Rhizobia	16S rRNA	Ludwig <i>et al.</i> (1998)
G Rb	626–645	GTCAGTATCGAGCCAGTGAG	35	Group Rhodobacter	16S rRNA	Eilers <i>et al.</i> (2000)
SPH120	120–137	GGGCAGATCCCACGCGT	30	Sphingomonas	16S rRNA	Eilers <i>et al.</i> (2000)
SUBU1237	1237–1254	CCCTCTGTCCGACCATT	35	<i>Burkholderia</i> spp. and <i>Sutterella</i> spp.	16S rRNA	Stoffels <i>et al.</i> (1998)
PLA46	46–63	GACTTGCATGCCTAATCC	30	Planctomycetales	ARNr 16S	Neef <i>et al.</i> (1998)
Ppu	1432–1446	GCTGGCCTAACCTTC	20	<i>Pseudomonas putida</i>	23S rRNA	Schleifer <i>et al.</i> (1992)
BIF216	216–233	GCCCATCCCCGAGTAACA	35	<i>Paenibacillus</i> isolates	ARNr 16S	Bertaux <i>et al.</i> (2003)

a. According to the study by Brosius and colleagues (1981).

**Fig. 1.** A and B. Acridine orange staining of a fresh Douglas fir mycorrhiza. A yellow intracellular bacterium (superposition of green DNA coloration and red RNA coloration) colonizes (A) a clamp connection in formation containing fungal RNA (red), (B) a fungal cell from the mantle containing fungal RNA (red). Bio-Rad CLSM.

C–N. The images are the result of the superimposition of photos taken in different wavelengths. The colour of the bacteria reflects the colocalization or on the non-colocalization of the probes used in the experiments, according to the following pattern: FITC = green, Cy3 = red, Cy5 = blue, FITC + Cy3 = yellow, FITC + Cy5 = turquoise, Cy3 + Cy5 = purple and FITC + Cy3 + Cy5 = white. C and D.  $\alpha$ -proteobacteria (purple rods) hybridized with EUB338mix-Cy3 and ALF1b-Cy5, (C) across the dolipore of a clamp connection in formation, as was checked with a z-scan (data not shown), or (D) across the dolipore of the septum between two fungal cells. These images originate from Douglas fir ectomycorrhizae sample B. E. Red coccoid eubacterium hybridized with EUB338mix-Cy3 but not with ALF1b-Cy5, in a hole of the fungal cell wall at the apex of a hypha. An extracellular bacterium labelled similarly can be seen outside of the fungal cell, the sample being non-axenic. This image originates from Douglas fir ectomycorrhizae sample 2-16. F. Green coccoid eubacterium (arrow) hybridized with EUB338mix-FITC but not with Ppu-Cy3, in a hole of the fungal cell wall of a hypha. This image originates from Douglas fir ectomycorrhizae sample B. G. Red coccoid eubacteria hybridized with EUB338mix-Cy3 but not with BET42a-Cy5 and GAM42a-FITC, colonizing the apex of a fungal cell. This image originates from Douglas fir ectomycorrhizae sample B. H. Red coccoid eubacteria hybridized with EUB338mix-Cy3 but not with SPH120-FITC, in between a cut fungal cell and the next cell. This image originates from the mycelium mat at the base of the stem of a fruit body under a Douglas fir, sample D. I. Green coccoid eubacteria hybridized with EUB338mix-FITC but not with SUBU1237-Cy3, in a cut fungal cell and in the next intact cell. This image originates from Douglas fir ectomycorrhizae sample B. J. Green coccoid eubacteria hybridized with EUB338mix-FITC but not with Pla46-Cy3, intracellular and extracellular. The arrowhead points to a bacterium that could be embedded in the fungal cell wall, according to the z-scan (data not shown). This image originates from Douglas fir ectomycorrhizae sample B. K. Blue DAPI-stained bacteria, both intracellular and extracellular. The hypha in the centre contains more than 30 intracellular bacteria. This image originates from Douglas fir ectomycorrhizae sample 13-4. L and M.  $\alpha$ -proteobacteria (purple rods) hybridized with EUB338mix-Cy3 and ALF1b-Cy5, (L) in the mantle of an ectomycorrhiza, or (M) in an ectomycorrhiza. The presence of plant cells (three bluish areas) indicates proximity with the Hartig net. Arrows show noteworthy bacteria, which colonize fungal cells between two plant cells, at the beginning of the Hartig net. These images originate from Douglas fir ectomycorrhizae sample 3-16. N. Gallery of optical sections showing the distribution of  $\alpha$ -proteobacteria (purple rods) hybridized with EUB338mix-Cy3 and ALF1b-Cy5 in the mantle of an ectomycorrhiza, from the outer part to the inner part. Both intracellular and extracellular bacteria can be seen. These images originate from Douglas fir ectomycorrhizae sample 3-16. (C, D, F, I, J, K) Zeiss Apotome; (E, H, L, M, N) Bio-Rad CLSM; (G) Zeiss CLSM.

**Table 2.** Frequency of the intracellular bacteria in the fungal samples.

Tree host	Substrate	Sample	Detection of intracellular bacteria	
			Positive samples <sup>a</sup>	No. of images <sup>b</sup>
Douglas fir	Artificial	Mycorrhizae	3/4	138
		Mycelium mat	1/1	31
		Mycelium mat at the base of the stem of the fruit body	7/7	43
		Stem of the fruit body	0/1	0
		Cap of the fruit body	0/3	0
		Whole fruit body	1/3	5
	Natural	Mycorrhizae	9/11	76
		Mycelium mat at the base of the stem of the fruit body	0/1	0
		Stem of the fruit body	0/1	0
		Cap of the fruit body	0/1	0
		Whole fruit body	0/4	0
		Total Douglas fir		21/37
Oak	Artificial	Mycorrhizae	2/7	3
		Mycelium mat	2/3	4
	Natural	Mycorrhizae	5/7	9
Total oak		9/17	16	

a. Number of samples containing endobacteria versus the number of analysed samples for each category of samples.

b. Number of images in the database for the whole category.

of 309 observations), but also in the mantle (33 out of a total of 309 observations, Fig. 1L and N), sometimes very close to the plant cells, in the outer part of the Hartig net (7/33 observations in the mantle, Fig. 1M). Only one fruit body presented occasional endobacteria (five out of a total of 309 observations). This counting could be underestimated, because of methodological problems originating from a particular autofluorescence of the fungal cells in the fruit bodies, which made it more difficult to confirm the intracellular position of the bacteria. Besides, some difficulties for fixing the fruit bodies were suspected. They could have limited the FISH detection of bacterial cells. However, further investigations using the general DNA fluorescence dye DAPI did not provide more observations of endobacteria.

Images showing single colonized cells represented more than half of the observations (Table 3). In the other observations, the bacteria were spread in several neighbouring cells, which were rarely more than five (9.7%, Table 3), as observed in some of the richer samples, and in the mantle of the ectomycorrhizae (Fig. 1L–N), where the fungal cells were smaller. Within one fungal cell, the

bacteria were not always homogeneously spread, but rather clustered in small numbers, either distributed along the cell or grouped at one end (Fig. 1I). On average, the number of endobacteria per cell was between two and 20 (Table 3), occasionally more than 20 (Fig. 1K).

## Discussion

Very few studies have shown the occurrence of endobacteria in ectomycorrhizae, with contrasting data regarding the vitality of the fungal cells colonized. In two studies the colonized fungal cells were alive (Bonfante-Fasolo and Scannerini, 1977; Nurmiaho-Lassila *et al.*, 1997). In a third study, the cells were alive but belonged to a senescing mycorrhiza (Buscot *et al.*, 1994). In contrast, Mogge and colleagues (2000) detected endobacteria in fungal cells that were definitely dead. The contrast persists in our study, where endobacteria were detected in both live and dead cells of *L. bicolor* S238N, in the same samples. Indeed, many colonized fungal cells had no RNA visible, and others lacked nuclei, suggesting that these cells could be inactive or dead. However, endobacteria were found

**Table 3.** Distribution of the intracellular bacteria in the fungal cells.

No. of bacteria per image	No. of colonized cells per image			Total no. of images
	1 cell	2–5 cells	>5 cells	
1	48/309 (15.5%)	0/309 (0%)	0/309 (0%)	48
2–20	135/309 (43.7%)	95/309 (30.7%)	14/309 (4.5%)	244
>20	1/309 (0.3%)	0/309 (0%)	16/309 (5.2%)	17
Total no. of images	184	95	30	309

inside cells that should be alive, that is cells containing fungal RNA, as proved by acridine orange staining. Some endobacteria were also found in supposedly growing parts of the fungus (clamp connections in formation and apical cells). To date, it is not clear whether the endobacteria inhabiting the live and the dead cells are related. Indeed we were unable to connect the occurrence of the endobacteria within live or dead fungal cells with the phylum to which they belong nor with a particular morphological type. They could be the same bacteria, being pathogens inducing the death of the fungal cells when colonizing them, or there could be two distinct bacterial populations, symbionts versus saprobes.

In this study, because of the presence of many extracellular bacteria in natural *L. bicolor* S238N ectomycorrhizal complexes (Frey-Klett *et al.*, 2005), the classical 'top-to-bottom' approach (Amann *et al.*, 1995) could not be used to analyse the genotypic diversity of the endobacteria. Indeed, designing new FISH probes from bacterial DNA sequences retrieved by a PCR approach would have been irrelevant, as such sequences would have belonged not only to the endobacteria, but also to the numerous extracellular bacteria. Therefore, a wide range of phylum-specific probes were first used, then group-specific probes within the  $\alpha$ -proteobacteria, in an attempt to close in towards the identity of the endobacteria.

Following this, FISH has shown that 92.5% of the endobacteria were affiliated to the  $\alpha$ -proteobacteria. The use of other phylum-specific probes did not allow the identification of the remaining 7.5% of the detected endobacteria, with the exception of the single hybridization of BET42a, specific for  $\beta$ -proteobacteria. However, these probes were not used systematically enough to infer the absence of the corresponding bacterial phyla within the remaining 7.5%. Among the  $\alpha$ -proteobacteria, two kinds could be distinguished, thanks to the probes ALF1b and ALF968, which both target the  $\alpha$ -proteobacteria, but with a slightly different spectrum. When these probes were used together, only one quarter of the endobacteria hybridized with both. More specific probes within the  $\alpha$ -proteobacteria were also used, but unsuccessfully so far. No hybridization of the following probes was observed: RHI1247, specific for many members of the *Rhizobium-Agrobacterium* group, G Rb, for the *Rhodobacter* group and SPH120, for the *Sphingomonas* group. But these probes do not cover all the groups targeted by ALF1b and ALF968, particularly the *Rhodospila*, *Caulobacter*, *Hyphomonas* and *Rickettsia* groups. Probes for these groups are not available yet.

Interestingly, so far, the LGC354mix and the BIF216 probes have not allowed to detect any intracellular Firmicutes or *Paenibacilli* in the plant-associated samples, in contrast with the pure cultures of the same fungus (Bertaux *et al.*, 2003), although such pure cultures served to

inoculate the experiments. This could be because the intracellular *Paenibacilli* found in the pure cultures were very rare and heterogeneously distributed (Bertaux *et al.*, 2003). Such a distribution, which obviously hampers the sampling of the intracellular *Paenibacilli* when subculturing the fungal cultures, could explain why these endobacteria were not detected in the plant-associated samples. The intracellular *Paenibacilli* could even be absent in these samples.

Concerning the intracellular  $\alpha$ -proteobacteria detected in this study, they could originate from the pure cultures used to inoculate the systems. However, so far,  $\alpha$ -proteobacteria have not been detected in pure cultures of *L. bicolor* S238N. In contrast, many  $\alpha$ -proteobacteria were detected outside the hyphae, in addition to bacteria belonging to other phyla, such as Actinomycetes and *Cytophaga-Flexibacter* in particular (data not shown). Thus, the possibility of an environmental origin of the  $\alpha$ -proteobacteria should be considered, more especially as bacteria were occasionally observed in holes in the fungal cell wall of the hyphae. However, the corresponding images are static and lack movement information, and it is not possible to infer from them that the bacteria were either leaving or entering the fungal cell. Consequently, the hypothesis of an environmental origin of the endobacteria needs to be confirmed experimentally.

It is noteworthy that both the CLSM and the Zeiss Apo-Tome deconvolution system produced images of comparable, high resolution. With both devices, within the hyphae, bacteria were often seen against the septa of the hyphae, and even across the dolipore holes of the septa. These observations suggest that the endobacteria could move from cell to cell through the dolipore, similarly to mitochondria (Müller *et al.*, 2000), to colonize the fungus. This would contrast with the case of other  $\alpha$ -proteobacteria, the *Rhizobia*, which colonize the different cells of the host plant by moving through holes that they create by degrading the cell wall with enzymes (Mateos *et al.*, 2001).

The endobacteria detected in *L. bicolor* S238N do not seem to share many common points with the endosymbionts of the Glomeromycota. Apparently, the endobacteria of *L. bicolor* S238N do not constitute a conserved component of the mycelium: they were not homogeneously distributed and they remained undetected in 45% of the samples. Moreover, as the endobacteria were seldom observed in the fruit bodies, and so far not in the spores, the transmission of the bacteria from one generation of the fungus to the next via the spores seems unlikely, at least in the controlled conditions of our glasshouse experiments where we collected the sporocarps. However, if not permanent, the endobacteria of *L. bicolor* S238N were a recurrent phenomenon. Indeed, apart from the fruit bodies, the mycelium from fungal mats and from

ectomycorrhizae frequently contained endobacteria. Furthermore, the endobacteria were found in different systems, produced independently in different experiments: in artificial substrates, in a natural nursery soil, also in ectomycorrhizae produced in the forest by *in situ* inoculation of the fungus. It has to be noted that in all these samples,  $\alpha$ -proteobacteria were the predominant endobacterial group, like in many bacterial endosymbioses (Andersson and Dehio, 2000). Therefore, even if the number of endobacteria observed here is far from meeting the number of endobacteria detected in the Glomeromycota fungi (up to 250 000 per spore, Bianciotto *et al.*, 1996), the recurrence of predominant  $\alpha$ -proteobacteria inside *L. bicolor* S238N cells is noteworthy.

Even sporadic endobacteria can have an impact on the biology of their host. Indeed, Fritsche and colleagues (1993) have reported the occurrence of endosymbionts in 24% of the examined *Acanthamoeba* isolates. Among the phylogenetically diverse bacteria that can infect *Acanthamoeba*, Jeon (1995) has found obligate endosymbionts, which can become necessary to the survival of the host. In our case, it is interesting to note that the endobacteria were far more frequent in the plant-associated fungal samples than in the fungal pure cultures studied previously, where many samples remained negative (Bertaux *et al.*, 2003). Therefore, the culture conditions of the fungus could have an impact on the intracellular bacterial populations, as this seems to be the case for some endosymbionts in Aphids (Ferrari *et al.*, 2004; Tsuchida *et al.*, 2004).

Considering all our results, we now propose the hypothesis that when isolating the fungus in pure culture 28 years ago, the purification steps dramatically reduced the endobacteria frequency and diversity. In return, each time the fungus is confronted with natural bacterial communities after inoculation in the environment, the endobacterial community would increase in frequency and diversity, possibly through an environmental acquisition of new endobacteria. Future works will aim to demonstrate this hypothetical cycle and to investigate the role of these endobacteria, which recurrently colonize a fungus used commercially in France to promote the growth of the Douglas fir.

## Experimental procedures

### Biological material

The fungal strain *L. bicolor* S238N (Maire) P.D. Orton is an ectomycorrhizal basidiomycete belonging to the *Tricholomataceae*. It was originally isolated in Oregon, USA, by Molina and Trappe in 1976, from a fruit body collected under *Tsuga mertensiana* (Bong.) Carr. (Di Battista *et al.*, 1996). In 1980, after its transfer to the 4°C fungal collection of INRA Nancy (Institut National de la Recherche Agronomique,

France) the original strain, S238O (Oregon) took the name of S238N (Nancy). *Laccaria bicolor* S238N is routinely grown on modified Pachlewski medium (Pachlewski and Pachlewski, 1974). The inoculum used for mycorrhizal inoculation was obtained from these cultures, and prepared as described in the study by Brulé and colleagues (2001).

Two tree species were inoculated with *L. bicolor* S238N mycelium: Douglas fir (*Pseudotsuga menziensis*) and oak (*Quercus robur*). The Douglas seeds were thoroughly washed and stratified to break dormancy (Frey-Klett *et al.*, 1997). The mycorrhizal plants (Frey-Klett *et al.*, 1997) were grown on different substrates: artificial ones, such as peat-vermiculite (1/4 v/v), or peat-terragreen (1/4 v/v), and a natural one, a sandy loam soil from a forest nursery (Peyratle-Château, Limousin, France). The substrates were not maintained in sterile conditions. In addition, ectomycorrhizae were collected from an oak stand, on *Quercus petraea* and *Q. robur* roots that had been inoculated *in situ* with *L. bicolor* S238N in March 2003 (P.-E. Courty, pers. comm.). The *L. bicolor* S238N identity of the samples was confirmed by PCR as described in the study by Selosse and colleagues (1996), by observing the presence on acrylamide gel of a heteroduplex specific for the S238N strain. Ectomycorrhizae, fungal mycelium mats growing on the side of pots and fruit bodies were collected from 5 to 11 months (50 samples) and occasionally more than 2 years (five samples) after inoculation. All these systems were provided by the following persons, as material from their own experiments: D. Bouchard, P.-E. Courty and M. Peter.

### Fixation

Samples were usually fixed in 2 ml of fixation solution, by immersing them immediately in 3% paraformaldehyde-1 X Phosphate Buffered Saline (130 mM NaCl, 7 mM Na<sub>2</sub>HPO<sub>4</sub>, 3 mM NaH<sub>2</sub>PO<sub>4</sub>; pH 7.3) 3:1, for 3 h at 4°C, as described in the study by Bertaux and colleagues (2003). In the case of fruit bodies, except for the very small ones measuring only several millimetres, all the others were divided into three distinct parts that were fixed individually: the mycelium mat growing at the base of the stem, the stem and the cap. Fixing bigger, whole fruit bodies in a greater volume of fixation solution (50 ml) overnight was also tried.

### Fluorescence *in situ* hybridization

Fluorescence *in situ* hybridization was performed as described in the study by Bertaux and colleagues (2003). Small pieces of mycelium or of ectomycorrhizae were detached from the samples with scalpel and pins before being deposited on gelatine-coated slides (0.075% gelatin-0.01 CrK(SO<sub>4</sub>)<sub>2</sub>). Hybridization was performed for 1 h 30 min at 46°C, then the slides were washed at 48°C for 10 min. The stringency of the hybridization buffer was adjusted according to the probes used (Table 1). ALF1b was also used at the stringency of 35% formamide. The probes EUBI, EUBII, EUBIII and the probes LGC354A, LGC354B, LGC354C were used in equimolar mixtures, called EUBmix and LGCmix, as each single probe of the mix is not sufficient to detect all eubacteria or all Firmicutes (Daims *et al.*, 1999). Oligonucle-

otide probes labelled at the 5' end with Cy3, Cy5, or fluorescein isothiocyanate (FITC) were purchased from Thermo Hybaid, Division Interactiva now Thermo Electron Corporation (GmbH, Ulm, Germany). Up to three probes were used together, with different labelling allowing to discriminate between them. As a control, it was checked that probes having different specificities never hybridized together on the same bacterium.

#### DNA and RNA staining

4,6-diamidino-2-phenylindole (DAPI) staining was performed on fixed samples, occasionally after FISH. A 20- $\mu$ l drop of DAPI (0.7  $\mu$ g ml<sup>-1</sup>) was deposited on each well and incubated in the dark at room temperature for 10 min. The slides were rinsed quickly with distilled water and air-dried before mounting with Citifluor AF1 antifading reagent (Citifluor, England). Alternatively, DAPI was mixed with Citifluor to a final concentration of 2.5  $\mu$ g ml<sup>-1</sup> and used for mounting (two drops per slide).

Acridine orange staining was performed on fresh Douglas ectomycorrhizae from the sandy loam soil cultures. The protocol was adapted from the study by Darzynkiewicz and Juan (2003). Acridine orange (stock solution 10 mg ml<sup>-1</sup> in water) was added for a final concentration of 1 mg ml<sup>-1</sup> to one of the following solutions: 1:3 permeabilizing solution PS (0.1% Triton X-100, 80 mM HCl, 150 mM NaCl) and staining solution STS (37 mM citric acid, 126 mM Na<sub>2</sub>HPO<sub>4</sub>, 150 mM NaCl, 1 mM Na<sub>2</sub>EDTA), or 1:3:1 permeabilizing solution PS, staining solution STS and NaCl 5 M. Three to four mycorrhizae were plunged in the mixture, and were incubated for 10–30 min, then rinsed quickly with water. They were dilacerated on a slide before observation. Acridine orange stains DNA in green when excited at 488 nm, and RNA in red when excited at 457 nm.

#### Observation and imaging

Observations were made with two CLSM, from Zeiss (LSM 510 Axiovert 100 M) and from Bio-Rad (Radiance 2100 Rainbow), and with the Zeiss ApoTome microscope equipped with a deconvolution system. The Zeiss CLSM was equipped with the following laser lines: Argon (488 nm) for FITC excitation and two Helium Neon lasers providing the wavelength for Cy3 (543 nm) and Cy5 (633 nm) excitation. The Bio-Rad CLSM, built on a Nikon Eclipse TE2000-U, was equipped with Argon (457 nm, 488 nm) and Helium Neon (543 nm) laser lines, and two diodes: blue (405 nm) for DAPI excitation and red (637 nm) for Cy5 excitation. ApoTome consists of a stative Axiovert 200 M Microscope with fluorescence device and the add-on module 'ApoTome' for structured illumination. Images were taken using the software package Axiovision Version 4.1. The microscope associated with the Zeiss ApoTome system was equipped with a mercury lamp and specific filters for FITC (Zeiss Filter Set 10), Cy3 (Zeiss Filter Set 20), Cy5 (Zeiss Filter Set 26) and DAPI (Zeiss Filter Set 49). Plan-Neofluar 100 $\times$ /1.3 (Zeiss) and Plan-Apo 60 $\times$ /1.4 (Nikon) oil immersion objectives were used. Images were taken as described in the study by Bertaux and colleagues (2003). The images were analysed with the Zeiss LSM 5 Image Browser

and with ImageJ 1.3. An image was taken each time intracellular bacteria were detected, whatever the probe set used. When necessary, serial optical sections along a z-axis were realized to check that the bacteria were intracellular, i.e. surrounded by fungal cell wall. If there was any doubt, the image was excluded from the database created to enable a semiquantitative analysis of the microscopic observations. A total of 309 images were recorded in this database. The 10 images corresponding to acridine orange staining of unfixed samples were not included.

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