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Distinct Ectomycorrhizospheres Share Similar Bacterial Communities as Revealed by Pyrosequencing-Based Analysis of 16S rRNA Genes

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Analysis of the 16S rRNA gene sequences generated from *Xerocomus pruinatus* and *Scleroderma citrinum* ectomycorrhizospheres revealed that similar bacterial communities inhabited the two ectomycorrhizospheres in terms of phyla and genera, with an enrichment of the *Burkholderia* genus. Compared to the bulk soil habitat, ectomycorrhizospheres hosted significantly more *Alpha-*, *Beta-*, and *Gammaproteobacteria*.

Ectomycorrhizal fungi are important actors of nutrient cycling in the forest ecosystems. They enhance the nutrient uptake capacity of plants due to their ability to mobilize carbon from organic matter and access chemical elements with low mobility in the soil, such as nutritive cations, phosphorus, and nitrogen (5, 15, 32). They also connect tree roots to the surrounding soil and form a very specific ecological environment, the ectomycorrhizosphere (19). Apart from its physical and chemical characteristics, which differ from those of the rhizosphere (26, 28), the ectomycorrhizosphere is characterized by diverse fungal and bacterial communities that inhabit the same environment. Consequently, the functioning of the ectomycorrhizal symbiosis is influenced by each partner of the ectomycorrhizal (ECM) complex (6, 8, 9).

Cultivation-dependent and -independent studies have demonstrated the structuring effect of the mycorrhizal fungi on the soil bacterial communities. They revealed that the bacterial communities colonizing the ectomycorrhizal roots differed from those of uncolonized roots and that the ectomycorrhizal species differentially impacted the structure of ectomycorrhizosphere bacterial communities (2, 11, 12, 22, 35, 37). One mycorrhizal species could be colonized by either very similar or contrastingly different bacterial communities (2, 11, 13). Certain ectomycorrhizal species associated with *Betula pubescens*, such as *Piloderma fallax* or *Pseudotomentella tristis*, were colonized by distinct bacterial communities, whereas the bacterial communities colonizing the ectomycorrhizosphere of *Tomentellopsis submollis* or *Lactarius torminosus* were more similar (13), thus suggesting that the taxonomic classification of the host mycorrhizal fungi was not always the main structuring parameter of the bacterial communities. Within this context, a comprehensive description of the ectomycorrhizosphere bacterial communities using a pyrosequencing-based approach would detail the structure and diversity of the bacterial communities coexisting in this specific ecological habitat as well as impart access to the rare taxonomic groups.

In a recent study, Uroz et al. (36) used pyrosequencing of 16S rRNA fragments to compare the composition of bacterial communities inhabiting the oak (*Quercus petraea*) rhizosphere and surrounding bulk soil. In the study presented here, we investigated in the same soil core the composition and structure of bacterial communities inhabiting the ectomycorrhizosphere, which is a specific subhabitat of the rhizosphere. Pyrosequencing tags spanning the V5 to V6 hypervariable regions of the 16S rRNA gene were used to compare the bacterial communities colonizing the *Scleroderma citrinum* and *Xerocomus pruinatus* ectomycorrhizospheres. The species richness and the numbers of operational taxonomic units (OTUs) in the ectomycorrhizosphere bacterial communities were compared between the two ectomycorrhizospheres. They were also compared to the data obtained for the rhizosphere and the surrounding bulk soil.

Soil samples were recovered from three independent soil cores in an oak (*Quercus petraea*) forest located in Breuil-Chenue, France. Dominant ectomycorrhizal morphotypes in each soil core were collected in separated tubes to avoid contaminations between morphotypes. These morphotypes were identified as *Scleroderma citrinum* (samples C3B, C4A, and C4B) and *Xerocomus pruinatus* (samples C1B and C3A) according to Agerer's (1987 to 1998) descriptions and the sequencing of the fungal internal transcribed spacer. The mycorrhizal tips, which are a combination of the soil-ectomycorrhiza interface and the symbiotic fungal mantle, were considered ectomycorrhizosphere samples in this study. Samples analyzed in this study were compared to the surrounding bulk soil (BS) and rhizosphere (R) samples described previously (36). DNA was extracted from three mycorrhizal tips of *S. citrinum* and two mycorrhizal tips of *X. pruinatus* using the PowerSoil DNA isolation kit (MO BIO Laboratories, Inc.). Amplicon libraries were generated as recommended for 454 pyrosequencing using a combination of two tagged primers targeting the V5 and V6 variable regions of the 16S rRNA gene, using the primers 787r (5′-*Axxx*ATTAGATACCYTGTAGTCC-3′) (23) and 1073f (5′-*B*-ACGAGCTGACGACARCCATG-3′) (25) to generate PCR 16S rRNA fragments of ca. 250 bp, where A and B represent the linkers CCATCTCATCCCTGCGTGTCTCCGACTCAG and CCTATCC CCTGTGTGCCTTGGCAGTCTCAG and *xxx* represents the sample identification bar coding key (tag). Pyrosequencing resulted in 158,690 reads (average size, 286 bp) which passed the length and quality criteria (7). MOTHUR was used to trim, denoise, and align the reads and to generate the operational taxo-

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TABLE 1 Relative abundances of the taxonomic groups present in the oak ectomycorrhizosphere, rhizosphere, and surrounding bulk soil[a]

Taxonomic group	Relative abundance (%) in each soil source											Statistics (*P* value)
	BS			R			Myc Xp		Myc Sc			
	BS1	BS2	BS3	R1	R2	R3	C1B	C3A	C3B	C4A	C4B	
Proteobacteria	35.57	35.85	37.76	41.03	37.81	40.04	**64.32**	**58**	**56.8**	**48.40**	**46.95**	Myc > BS = R (0.004)
Acidobacteria	26.45	25.98	21.95	23.10	26.59	19.52	**16.85**	**13.36**	**14.06**	**25.66**	**26.82**	NS
Unclassified bacteria	21.00	20.44	22.16	18.27	18.59	19.99	**10.09**	**8.64**	**8.31**	**14.19**	**12.42**	Myc < R < BS (0.0007)
Actinobacteria	11.29	12.72	11.43	11.38	11.52	10.79	**3.85**	**3.86**	**3.99**	**5.01**	**5.24**	Myc < BS = R (0.0001)
Bacteroidetes	1.13	1.17	1.88	2.30	1.91	2.09	**2.47**	**6**	**6.23**	**3.18**	**5.87**	Myc Sc > BS (0.02)
Gemmatimonadetes	1.14	0.74	1.58	1.09	0.88	0.92	**0.18**	**0.28**	**0.26**	**0.34**	**0.17**	Myc < BS = R (0.0013)
Verrucomicrobia	1.18	1.07	1.09	1.01	1.28	2.43	**0.60**	**0.93**	**0.86**	**1.02**	**0.85**	NS
Planctomycetes	0.66	0.63	0.54	0.47	0.52	2.29	**0.25**	**0.51**	**0.52**	**0.38**	**0.31**	NS
Firmicutes	0.54	0.50	0.40	0.44	0.29	0.78	**0.29**	**0.24**	**0.27**	**0.29**	**0.20**	NS
Chlamydiae	0.46	0.57	0.75	0.40	0.31	0.72	**0.16**	**0.13**	**0.12**	**0.09**	**0.14**	Myc < BS = R (0.002)
Nitrospira	0.31	0.15	0.23	0.22	0.07	0.13	**0.01**	**0.02**	**0**	**0.06**	**0.02**	Myc < BS = R (0.008)
Genera incertae sedis OP10	0.10	0.06	0.12	0.14	0.10	0.12	**0.04**	**0.05**	**0.04**	**0.05**	**0.10**	NS
Genera incertae sedis TM7	0.06	0.06	0.07	0.08	0.06	0.13	**0.01**	**0.01**	**0.01**	**0.03**	**0.03**	NS

[a] The data generated in this study are presented in bold in the table. These data are compared with the data published by Uroz et al. (36) on the distribution of the bacterial communities in the rhizosphere and in the surrounding bulk soil. Because all the samples have been collected at the same time and treated using the same methods, a one-factor (niche) ANOVA at a threshold level of $P = 0.05$ and a Bonferroni-Dunn test were applied on the relative distribution values after an arcsine transformation. The results are presented in the column entitled "Statistics." The taxonomic groups for which a significant or nearly significant effect of the niche was found are presented. NS, nonsignificant differences; Myc, both *Xerocomus pruinatus* and *Scleroderma citrinum* ectomycorrhizospheres; Myc Xp, *Xerocomus pruinatus* ectomycorrhizosphere; Myc Sc, *Scleroderma citrinum* ectomycorrhizosphere; BS, bulk soil; R, rhizosphere; >, significantly more present; <, significantly less present.

nomic units (OTUs; 97% sequence similarity) as well as to perform the nonparametric analyses (31). Taxonomic assignments were obtained with the metagenomics RAST server (MG-RAST) (21) using an 80% confidence threshold according to the methods of Santelli et al. (30). The impact of the fungal species on the relative distributions of the phyla and genera was determined by analysis of variance (one-factor ANOVA) using the SuperANOVA software (Abacus Concepts, Inc., Berkeley, CA).

Nonparametric analyses revealed that the total numbers of OTUs observed were not significantly different between the ectomycorrhizospheres (see Table S1 in the supplemental material). However, for a similar number of 16S rRNA tag sequences corresponding to sample C1B ($n = 28{,}516$ reads), a higher number of OTUs was obtained for the *Scleroderma citrinum* ectomycorrhizosphere (12,523 OTUs) (see Table S1 in the supplemental material). As for other soil studies (27, 36), the rarefaction curves generated for each ectomycorrhizosphere did not reach a plateau (see Fig. S1 in the supplemental material). The Chao1 index estimated that the ectomycorrhizosphere samples contained between 31,000 and 39,000 OTUs (see Table S1 in the supplemental material). Altogether, these analyses highlighted the richness of the ectomycorrhizosphere bacterial communities and the relative overlap existing between the different ectomycorrhizospheres analyzed.

Taxonomic assignments demonstrated that the same 13 phyla were present in each sample, regardless of their ecological origin (*Xerocomus pruinatus* or *Scleroderma citrinum* ectomycorrhizosphere) and sampling location (Table 1). In each ectomycorrhizosphere, four major phyla were dominant, the *Proteobacteria* (mean value, 54.89% ± 3.22%; $n = 5$), the *Acidobacteria* (mean value, 19.35% ± 2.87%; $n = 5$), the *Bacteroidetes* (mean value, 4.75% ± 0.79%; $n = 5$), and the *Actinobacteria* (mean value, 4.39% ± 0.30%; $n = 5$) (Table 1). Similarly, the same genera were detected in both ectomycorrhizospheres (see Fig. S2 in the supplemental material). Analysis of the 10 most abundant genera showed that the genera *Acidobacterium* (mean value, 19.35% ± 2.88%; $n = 5$), *Burkholderia* (mean value, 6.46% ± 1.11%; $n = 5$), *Rhodoplanes* (mean value, 4.70% ± 0.38%; $n = 5$), *Chitinophaga* (mean value, 4.28% ± 0.77%; $n = 5$), and *Bradyrhizobium* (mean value, 4.37% ± 0.22%; $n = 5$) were dominant (Table 2), regardless of the type of ectomycorrhizosphere (*S. citrinum* or *X. pruinatus*). For both levels (phyla or genera), no significant differences were observed between the *X. pruinatus* and *S. citrinum* ectomycorrhizospheres ($P > 0.05$) (see Fig. S3A in the supplemental material).

Overall, the 158,690 read sequences generated in this study defined 47,292 OTUs. The 10 most abundant OTUs detected in the ectomycorrhizosphere, regardless of the type of ectomycorrhizal fungus, belonged to *Rhizobiales*, *Gammaproteobacteria* (*Steroidobacter* spp.), *Burkholderia*, *Acidobacteria*, *Bacteroidetes*, and *Actinobacteria* (data not shown). The detailed list of the 10 most abundant OTUs for each ectomycorrhizosphere is presented in Table 3. An in-depth analysis revealed that 42% of the total sequences (66,951 sequences distributed in 1,266 OTUs) were common to all the ectomycorrhizosphere samples. This result suggests a common bacterial core between the two mycorrhizal species but also a relative heterogeneity between the different samples regardless of the fungal species. Around 1% of the total sequences appeared specific to the *X. pruinatus* ectomycorrhizospheres, corresponding to 584 OTUs (1,694 sequences). Similarly, 0.85% of the total sequences were specific to the *S. citrinum* ectomycorrhizospheres, corresponding to 265 OTUs (1,367 sequences). Both ectomycorrhizospheres appeared dominated by OTUs related to *Proteobacteria* and *Acidobacteria*. These relatively low proportions of phylotypes specific to the *X. pruinatus* or *S. citrinum* ectomycorrhizospheres suggest a low impact of the fungal species on the associated bacterial communities, a finding which is in line with those of previous studies which have suggested that other environmental factors, such as soil characteristics, strongly influence bacterial distribution (2, 13).

The most abundant bacterial genera detected in the ectomycorrhizospheres of *X. pruinatus* and *S. citrinum*, including *Acido-*

TABLE 2 Relative abundances of the 10 most abundant genera in each of the ectomycorrhizospheres[a]

	Most abundant genera (relative abundance [%]) in each soil source				
	Xerocomus pruinatus mycorrhizosphere		*Scleroderma citrinum* mycorrhizosphere		
Rank	C1B	C3A	C3B	C4A	C4B
1	*Acidobacterium* (16.85)	*Acidobacterium* (13.36)	*Acidobacterium* (14.06)	*Acidobacterium* (25.66)	*Acidobacterium* (26.82)
2	*Burkholderia* (7.99)	*Burkholderia* (8.33)	*Burkholderia* (8.44)	*Burkholderia* (3.30)	*Burkholderia* (4.24)
3	*Chitinophaga* (2.03)	*Chitinophaga* (5.61)	*Chitinophaga* (5.82)	*Chitinophaga* (2.86)	*Chitinophaga* (5.09)
4	*Rhodoplanes* (3.42)	*Rhodoplanes* (5.14)	*Rhodoplanes* (4.82)	*Rhodoplanes* (5.72)	*Rhodoplanes* (4.42)
5	*Bradyrhizobium* (3.90)	*Bradyrhizobium* (4.97)	*Bradyrhizobium* (4.75)	*Bradyrhizobium* (3.87)	*Bradyrhizobium* (4.37)
6	*Caulobacter* (6.40)	*Caulobacter* (1.95)	*Caulobacter* (1.87)	*Caulobacter* (0.46)	*Caulobacter* (0.40)
7	*Curtobacterium* (1.58)	*Curtobacterium* (0.92)	*Curtobacterium* (1.02)	*Curtobacterium* (1.61)	*Curtobacterium* (2.02)
8	*Alterococcus* (2.70)	*Alterococcus* (0.95)	*Alterococcus* (0.86)	*Alterococcus* (1.34)	*Alterococcus* (0.74)
9	*Phenylobacterium* (0.35)	*Planctomyces* (0.38)	*Planctomyces* (0.39)	*Gemmatimonas* (0.34)	*Pedobacter* (0.58)
10	*Brevundimonas* (0.34)	*Conexibacter* (0.32)	*Rhodanobacter* (0.31)	*Acidimicrobium* (0.26)	*Nocardia* (0.29)

[a] Shading indicates the position of the genus *Burkholderia*.

bacterium, *Burkholderia*, *Chitinophaga*, *Rhodoplanes*, and *Bradyrhizobium*, have also been reported among the most abundant genera in different agricultural or forest soils (10, 27, 36), as well as in association with ectomycorrhizal roots (13, 14, 17, 33) (Table 2). Our knowledge on some of them, such as the *Acidobacterium* or *Chitinophaga* genus, is limited due to the fact that bacteria from these genera remain hitherto unculturable (10, 24, 29). In contrast, culturable approaches have revealed that the *Burkholderia* and *Bradyrhizobium* genera were frequently detected in mycorrhizospheres (1, 16, 20, 22, 34, 35). Notably, the *Burkholderia* OTUs defined in the present study showed high similarity (99 to 100%) with sequences of *Burkholderia glathei* strains coming from the same experimental site and characterized for their ability to weather minerals, a process of high importance in nutrient-poor forest soils (3, 4, 35). The pyrosequencing approach has also permitted the detection of rare phylotypes related to genera, such as *Nitrospira*, *Collimonas*, or *Streptomyces*, that are known to be involved in nitrogen and nutrient cycling or antibiotic production (18) (see Table S2 in the supplemental material). Our results thus reinforce the interest in performing a pyrosequencing approach to detect rare taxonomic groups.

Comparative analysis with the 16S rRNA sequences generated from the same soil cores (36) but from the rhizosphere and the surrounding bulk soil revealed specificities of the mycorrhizosphere bacterial communities. For the same number of sequences, the ectomycorrhizosphere bacterial communities were characterized by a higher number of OTUs than those of the rhizosphere and the surrounding bulk soil. Although similar taxonomic groups were detected in all the soil habitats considered (ectomycorrhizosphere, rhizosphere, or bulk soil), the ectomycorrhizosphere was significantly enriched in *Proteobacteria* ($P = 0.004$) (Table 1) and depleted in *Actinobacteria* ($P = 0.0001$), *Gemmatimonadetes* ($P = 0.001$), *Chlamydia* ($P = 0.002$), *Nitrospira* ($P = 0.008$), and unclassified bacteria ($P = 0.0007$). Closer investigation of proteobacterial sequences showed that the ectomycorrhizosphere was significantly dominated by *Betaproteobacteria* ($P = 0.002$) and *Gammaproteobacteria* ($P = 0.003$), in contrast to the surrounding bulk soil environment (Fig. 1). Significantly fewer *Alphaproteobacteria* ($P = 0.0006$) and *Deltaproteobacteria* ($P = 0.002$) were detected in the ectomycorrhizosphere than in the surrounding bulk soil environment. At the genus level, our analysis also revealed a significant predominance of sequences related to *Burkholderia* in ectomycorrhizosphere compared to that of the rhizosphere and the surrounding bulk soil ($P = 0.0018$), thus suggesting selection of this bacterial genus in the ectomycorrhizosphere. On the contrary, significantly fewer sequences related to *Rhodoplanes* were detected in the ectomycorrhizosphere than in the surrounding bulk soil ($P = 0.02$). Detailed comparisons at the

TABLE 3 Relative abundances of the 10 most abundant phylotypes in each of the ectomycorrhizospheres[a]

	Most abundant phylotypes (relative abundance [%]) in each soil source				
	Xerocomus pruinatus mycorrhizosphere		*Scleroderma citrinum* mycorrhizosphere		
Rank	C1B	C3A	C3B	C4A	C4B
1	*Steroidobacter* sp. (1.14)	*Burkholderia* sp. (0.93)	*Rhizobiales* (0.72)	*Steroidobacter* sp. (0.65)	*Bradyrhizobium* sp. (0.74)
2	*Bradyrhizobium elkanii* (0.92)	*Steroidobacter* sp. (0.64)	*Burkholderia* sp. (0.57)	*Bradyrhizobium* sp. (0.58)	*Flavosolibacter* sp. (0.52)
3	*Steroidobacter* sp. (0.88)	*Rhizobium miluonense* (0.64)	*Burkholderia* sp. (0.54)	*Mesorhizobium* sp. (0.57)	*Bradyrhizobium* sp. (0.50)
4	*Burkholderia glathei* (0.63)	*Rhizobiales* (0.60)	*Bradyrhizobium* sp. (0.50)	*Bradyrhizobium* sp. (0.55)	*Steroidobacter* sp. (0.49)
5	*Burkholderia glathei* (0.55)	*Burkholderia* sp. (0.59)	*Bradyrhizobium* sp. (0.49)	*Mesorhizobium* sp. (0.52)	*Acidobacteria* (0.46)
6	*Steroidobacter* sp. (0.55)	*Steroidobacter* sp. (0.54)	*Acidobacteriaceae* (0.47)	*Steroidobacter* sp. (0.52)	*Acidobacteria* (0.46)
7	*Steroidobacter* sp. (0.49)	*Bradyrhizobium elkanii* (0.51)	*Rhizobiales* (0.46)	*Acidobacteria bacterium* (0.49)	*Bradyrhizobium* sp. (0.45)
8	*Caulobacter* sp. (0.46)	*Pseudolabrys taiwanensis* (0.50)	*Steroidobacter* sp. (0.46)	*Steroidobacter* sp. (0.46)	*Rhizobiales* (0.42)
9	*Curtobacterium flaccumfaciens* (0.45)	*Steroidobacter* sp. (0.49)	*Bradyrhizobium* sp. (0.43)	*Bradyrhizobium elkanii* (0.44)	*Steroidobacter* sp. (0.41)
10	*Steroidobacter* sp. (0.45)	*Bradyrhizobium* sp. (0.42)	*Bradyrhizobium* sp. (0.42)	*Bradyrhizobium* sp. (0.36)	*Flavosolibacter* sp. (0.40)

[a] Based on the OTUs generated with a threshold of 97%.

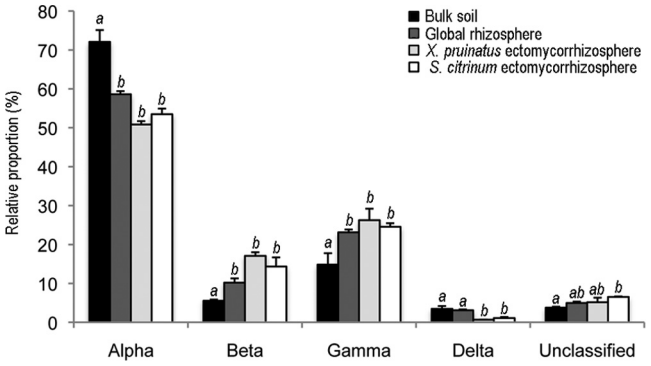

FIG 1 Relative abundances of *Proteobacteria* in the ectomycorrhizosphere, the rhizosphere, and the surrounding bulk soil. A one-factor (habitat) ANOVA was applied on the relative distribution values after an arcsine square root transformation for each proteobacterial group. Different letters for each proteobacterial group indicate that the values are significantly different.

phylotype (OTU) level were not as clear, highlighting a high heterogeneity between samples. The ectomycorrhizosphere did not cluster with the rhizosphere or the bulk soil (see Fig. S3B in the supplemental material), demonstrating once again that it is a specific ecological habitat of the soil. In this sense, the ectomycorrhizosphere OTUs represented 45% of the total number of OTUs (64,492 reads). A detailed analysis revealed that slightly more OTUs were common with the surrounding bulk soil (4.5%; 16,136 reads) than with the rhizosphere (3%; 17,054 reads) (see Fig. S4 in the supplemental material).

In conclusion, this study highlights for the first time, through 454 pyrosequencing, the richness and diversity of the ectomycorrhizosphere bacterial communities. We show that the bacterial communities inhabiting the ECM complex of two ectomycorrhizal fungi associated with oak, *S. citrinum* and *X. pruinatus*, are very similar at the phylum or genus level but clearly different at the OTU (phylotype) level. Our analysis demonstrates that the ectomycorrhizosphere bacterial communities qualitatively resemble those of the rhizosphere or bulk soil environments at the phylum and genus levels but present significant quantitative differences, illustrating the specificity of the ectomycorrhizosphere.

Nucleotide sequence accession numbers. The sequences determined in this study have been deposited in the Sequence Read Archive (SRA) service of the GenBank database under the accession numbers SRA029325.1 (mycorrhizosphere samples) and SRA029106.2 (for the rhizosphere and bulk soil samples of the study of Uroz et al. [36]).

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