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Black truffle-associated bacterial communities during the development and maturation of *Tuber melanosporum* ascocarps and putative functional roles

Sanjay Antony-Babu,[1,2†] Aurélie Deveau,[1,2*†] Joy D. Van Nostrand,[3] Jizhong Zhou,[3,4,5] François Le Tacon,[1,2] Christophe Robin,[6,7] Pascale Frey-Klett[1,2] and Stéphane Uroz[1,2]

[1]*INRA, Interactions Arbres – Microorganismes, UMR1136, F-54280 Champenoux, France.*

[2]*Interactions Arbres – Microorganismes, Université de Lorraine, UMR1136, F-54500 Vandoeuvre-lès-Nancy, France.*

[3]*Institute for Environmental Genomics, Department of Microbiology and Plant Biology, University of Oklahoma, Norman, OK 73072, USA.*

[4]*Earth Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA.*

[5]*State Key Joint Laboratory of Environment Simulation and Pollution Control, School of Environment, Tsinghua University, Beijing 100084, China.*

[6]*Agronomie & Environnement, Université de Lorraine, Nancy-Colmar, UMR 1121, F-54500 Vandoeuvre-lès-Nancy, France.*

[7]*INRA, Agronomie & Environnement, Centre INRA de Nancy-Lorraine, Nancy-Colmar, UMR 1121, F-54500 Vandoeuvre-lès-Nancy, France.*

Summary

Although truffles are cultivated since decades, their life cycle and the conditions stimulating ascocarp formation still remain mysterious. A role for bacteria in the development of several truffle species has been suggested but few is known regarding the natural bacterial communities of Périgord Black truffle. Thus, the aim of this study was to decipher the structure and the functional potential of the bacterial communities associated to the Black truffle in the course of its life cycle and along truffle maturation. A polyphasic approach combining 454-pyrosequencing of 16S rRNA gene, TTGE, *in situ* hybridization and functional GeoChip 3.0 revealed that Black truffle ascocarps provide a habitat to complex bacterial communities that are clearly differentiated from those of the surrounding soil and the ectomycorrhizosphere. The composition of these communities is dynamic and evolves during the maturation of the ascocarps with an enrichment of specific taxa and a differentiation of the gleba and peridium-associated bacterial communities. Genes related to nitrogen and sulphur cycling were enriched in the ascocarps. Together, these data paint a new picture of the interactions existing between truffle and bacteria and of the potential role of these bacteria in truffle maturation.

Received 6 August, 2013; accepted 20 September, 2013. *For correspondence. E-mail deveau@nancy.inra.fr; Tel. (+33) 038339 4088; Fax (+33) 038339 4069. †Sanjay Antony-Babu and Aurélie Deveau contributed equally to this work.

Introduction

Truffles are ascomycete hypogeous fungi, which establish ectomycorrhizal symbiosis with roots of gymnosperms and angiosperms. Although these fungi are distributed worldwide (Jeandroz *et al.*, 2008; Bonito *et al.*, 2010), only a few species are edible and recognized for the flavours they impart to food. Indeed, the fruiting bodies they produce are of high commercial value by virtue of their organoleptic properties (Mello *et al.*, 2006). Among those is the Périgord 'Black truffle' *Tuber melanosporum*. The high economic value of Black truffle has encouraged its cultivation through artificial inoculations of their hosts for about two centuries, and most of the current production is now obtained from cultivated truffle orchards (Mello *et al.*, 2006). Efforts taken to cultivate truffles have shown that truffle production strongly depends on soil characteristics and climate (Callot, 1999; Olivier *et al.*, 2012). Nevertheless, the production is far from being completely controlled because of the role of other factors badly known, notably biotic factors such as plant physiology and interactions with other soil fungi and bacteria.

In the last decade, our understanding of the reproductive biology and development of *T. melanosporum* has considerably increased (Martin *et al.*, 2010; Kues and Martin, 2011; Murat *et al.*, 2013). The life cycle of truffles starts at springtime with the germination of haploid spores and the growth of the haploid mycelium into the soil. This first saprophytic phase is followed by the establishment of a symbiotic association of the fungus with tree roots and

by the formation of ectomycorrhizae. Ectomycorrhizae are thought to give birth to antheridia and ascogones producing male and female gametes respectively. These structures of opposite mating type form after plasmogamy, an ascogenous heterokaryotic tissue surrounded by homokaryotic maternal tissues. The growth of these tissues gives birth *sensu stricto* to the ascocarp (the truffle) that is linked to the ectomycorrhizae through extrametrical hyphae until complete maturation (Le Tacon *et al*., 2013). In contrast to many epigeous fungal fruiting bodies, which develop and reach maturity within a few days, the maturation of truffles requires up to 6 months.

At each of these life-cycle steps (spores, hyphae, ectomycorrhizae or ascocarps), *T. melanosporum* is in close interaction with the surrounding bulk soil bacterial communities (Mello *et al*., 2013). Therefore, truffle is an excellent model to decipher the potential selective effect of the fungal ascocarp on the bacterial communities along the ascocarp maturation process. The development of the ascocarp starts by a primordium, a clear yellowish mycelial pellet of 80–350 μm covered by radiating hyphae in contact with the soil. After disappearance of these hyphae, the surface becomes defined and the cell walls of the external layers thicken, forming a peridium that starts to protect the inner part called gleba. Then the peridium becomes covered with small warts (Ascocarps of 400–500 μm) (G. Pacioni, pers. comm.). In the following months, the fruiting body grows up and sporogenesis occurs after differentiation of the ascogenous tissues. The expansion of the truffle body is thought to occur by repetitive breaks in the peridium. The successive stages of the ascocarps, from the primordium to the final ascocarp, as well as the repetitive breaks in the peridium during ascocarp maturation raise interlocked questions: does the ascocarp select bacterial communities from the surrounding bulk soil? Do the ascocarp-associated bacterial communities evolve independently from those of the surrounding bulk soil along the maturation process? Does the microbial community participate or impact the formation and the development of the fruiting body?

Presence of bacteria residing on the surface and within the truffle ascocarps has been reported in the last decades: bacterial populations ranging from 10^7 to 10^8 cfu g^{-1} have been recorded in *T. aestivum* (Gryndler *et al*., 2013), *T. borchii* (Sbrana *et al*., 2000; Barbieri *et al*., 2005), *T. magnatum* (Barbieri *et al*., 2007) and *T. melanosporum* (Rivera *et al*., 2010). Moreover, there have been indicative suggestions on the involvement of these associated bacterial communities in the growth or the nutrition of the fungus during the development and maturation of the ascocarps of *T. borchii* and *T. magnatum* (Sbrana *et al*., 2000; 2002; Barbieri *et al*., 2007; 2010), but no clear demonstration of their existence *in situ* has been provided yet. Only Barbieri and colleagues (2010) demonstrated a nitrogenase activity within *T. magnatum* ascocarps possibly attributed to *Bradyrhizobia*, suggesting a potential role of these bacteria in N_2 fixation by the ascocarp. It has also been suggested that some bacteria may play a role in the development of the characteristic truffle aroma of *T. borchii* (Barbieri *et al*., 2000; Splivallo *et al*., 2007). In contrast, very few is known regarding the natural communities colonizing the Black truffle and their potential impact on the life cycle of the fungus. The identification and characterization of bacterial strains stimulating the production of ascocarp primordia and their developments into mature truffles would surely be of great agronomic and economic interest.

The main objectives of this study were to determine whether the Black truffle *T. melanosporum* selects specific bacterial communities from the surrounding bulk soil, how these communities evolve along the ascocarp maturation and what could be the potential functional role of truffle-associated bacterial communities in the maturation process of the ascocarp. We hypothesized that particular bacterial communities which could participate in the truffle development are selected during the maturation of the ascocarp. To test this hypothesis, we collected *T. melanosporum* ascocarps at three stages of maturation (6a–6c) in the well-managed French truffle orchard of Rollainville from October 2010 to January 2011. In order to obtain the most comprehensive view of the bacterial communities inhabiting the ascocarp compared with the ectomycorrhizosphere and the surrounding bulk soil along the maturation process, we applied a combined approach based on TTGE fingerprinting and 16S rRNA gene amplicon pyrosequencing. Validation of the predominant taxa in the ascocarp was performed through *in situ* hybridization (FISH) microscopy. Lastly, we questioned the ecological meaning of the microbial assembly and the potential functional role of truffle-associated bacterial communities using functional GeoChip 3.0 microarray analyses.

Results

Ascocarps harbour a distinct bacterial community from truffle ectomycorrhizae and soil

In order to determine how specific the structure of the bacterial communities colonizing the ascocarp of *T. melanosporum* is compared with other organs such as ectomycorrhizae, 16S rRNA amplicon pyrosequencing was performed on samples collected in November 2010. These samples consisted of ectomycorrhizae of *T. melanosporum*, ectomycorrhizospheric and bulk soils on one hand, and of ascocarps of *T. melanosporum* (gleba and peridium), ascocarpic and bulk soils on the other hand.

Table 1. Taxonomic diversity of bacterial communities in the ascocarps, mycorrhizae and soil sample along maturation. Each value is the mean value of three biological replicates (± SE).

Sampling time	Origin	Number of observed phylotypes	Shannon index	Chao1 index
October	Gleba	880 ± 13	5.91 ± 0.13	4292 ± 1033
	Peridium	1511 ± 19	7.26 ± 0.04	15917 ± 6580
	Ascocarpic soil	1339 ± 61	6.96 ± 0.14	10442 ± 2801
	Bulk soil	1475 ± 42	7.22 ± 0.06	12109 ± 3129
November	Gleba	444 ± 107	3.78 ± 0.36	2006 ± 912
	Peridium	1334 ± 64	7.01 ± 0.06	7585 ± 5414
	Ascocarpic soil	1474 ± 27	7.22 ± 0.05	12072 ± 3944
	Bulk soil of ascocarpic compartment	1493 ± 31	7.25 ± 0.05	11787 ± 3163
	Bulk soil of mycorrhizal compartment	1494 ± 14	7.25 ± 0.04	10948 ± 2449
	Ectomycorhizosphere soil	1296 ± 373	7.07 ± 0.32	8245 ± 3224
	Ectomycorrhizae	1494 ± 122	7.25 ± 0.04	10948 ± 2449
December	Gleba	524 ± 24	4.23 ± 0.23	2111 ± 577
	Peridium	1122 ± 140	6.54 ± 0.32	7422 ± 2562
	Ascocarpic soil	1472 ± 21	7.21 ± 0.05	10641 ± 2602
	Bulk soil	1502 ± 20	7.27 ± 0.05	10358 ± 2194
January	Gleba	512 ± 83	4.57 ± 0.41	1938 ± 709
	Peridium	1047 ± 151	6.25 ± 0.45	6731 ± 2490
	Ascocarpic soil	1412 ± 111	7.02 ± 0.22	12107 ± 4555
	Bulk soil	1558 ± 19	7.32 ± 0.04	16569 ± 4949

The three main compartments considered – ascocarp, ectomycorrhizae and soil – were clearly differentiated as indicated by non-parametric analyses. The gleba was characterized by a significantly lower number of phylotypes (444 ± 107) and lower values of Shannon (3.78 ± 0.36) and Chao1 indices (2006 ± 912) than the other compartments ($P < 0.005$). The different soil compartments as well as the peridium of the ascocarp were characterized by significantly higher values; number of phylotypes: 1296–1494, Shannon index: 7.01–7.25 (Table 1).

The most abundant phyla in the different compartments considered were *Proteobacteria* (11–21%), *Bacteroidetes* (2–20%), *Actinobacteria* (0.6–20%), *Verrucomicrobia* (0.1–6.5%), *Firmicutes* (0.1–4.6%), *Acidobacteria* (0.1–2.9%) and *Planctomycetes* (0.1–1.7%) as shown by the taxonomic assignment (Fig. 1). Unassigned bacteria represented between 26% and 85% of the operational taxonomic units (OTUs) depending on the compartment considered. This distribution was not uniform among the compartments, as highlighted by the multivariate analysis performed on the relative distribution of these phyla. The ascocarp-associated bacterial communities were separated from those of the different soil samples (ascocarpic, ectomycorrhizospheric and bulk soil) according to the first axis which explained 51.6% of the variability and from those of the ectomycorrhizae according to the second axis which explained 25.1% of the variability (Fig. 2A). In addition, the bacterial communities of the gleba differentiated from those of the peridium at the phylum, family, class, order and genus levels (ANOVA, $P < 0.05$). The different soil samples considered (ascocarpic, ectomycorrhizospheric and bulk soil) clustered together. In accordance with these data, a very similar discrimination of the bacterial communities according to their ecological origin was obtained using the TTGE fingerprint method (Table S7). However, this method was not resolutive enough to differentiate the bacterial communities of the gleba from those of the peridium (Fig. S1).

In addition to the differences observed at the phylum level, a more detailed analysis showed that the compartments were significantly differentiated according to the relative distribution of the 16S rRNA sequences at the levels of classes, orders, families and genera (Table S1). The ectomycorrhizal compartment was significantly enriched in *Actinobacteria* sequences related to the *Streptomyces* and *Thermoleophilum* genera in comparison to soil and ascocarpic samples. The peridium compartment was enriched in *Bacteroidetes* sequences related to the *Pedobacter*, and *Sphingobacterium* genera in comparison to the gleba and the ectomycorrhizae (ANOVA, $P < 0.001$, Table 2). *Alphaproteobacteria* corresponding to sequences assigned to the genus *Bradyrhizobium* were significantly enriched in the peridium, as well as in the ectomycorrhizae compared with other compartments. Representatives of the *Betaproteobacteria*, especially sequences related to the *Variovorax* genus, appeared significantly enriched in the ascocarp (gleba and peridium) than in the other compartments. In contrast, representatives of the *Terrimonas*, *Microscilla* and *Flavobacterium* were significantly lower or even absent from the ascocarp (both gleba and peridium), while they were dominant in soil samples ($P < 0.0001$). Sequences assigned to *Pseudomonas* genus were

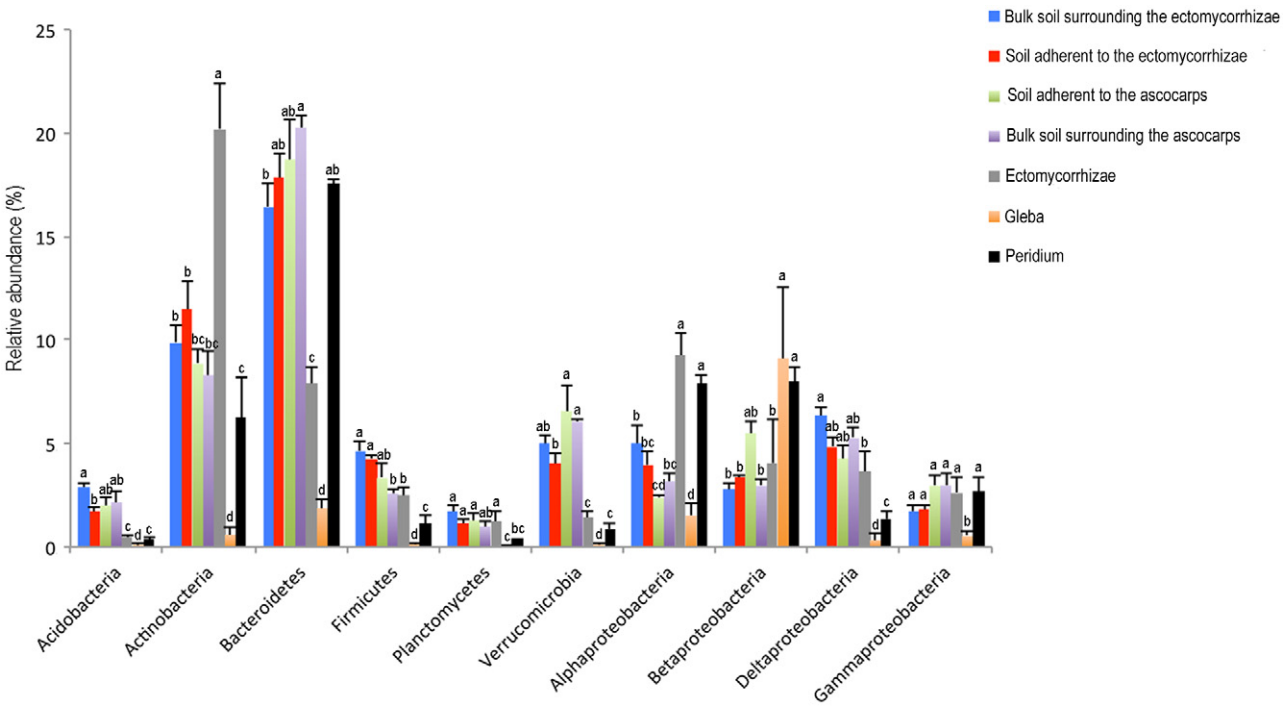

Fig. 1. Relative distribution of the seven most abundant phyla present in black truffle ectomycorrhizae, ascocarpic tissues, adhering soils from ectomycorrhizae and ascocarps, and bulk soil surrounding ectomycorrhizae and ascocarps in November 2010. Each bar represents the average value of three biological replicates. Bars associated with the same letters are not significantly different from each other according to a one-way ANOVA (with sample origin as factor, $P < 0.05$, Fisher test).

retrieved in very low percentage in the ascocarp (0.3%) as well as in the soil and ectomycorrhizospheric compartments. Top genera detected in samples are listed in Table 2.

Structure of ascocarp-associated bacterial communities evolves during fruiting body maturation

In a second step, we investigated the composition of the bacterial communities of the ascocarps collected at different stages of the maturation process (6a–6c, Table S2) from October 2010 to January 2011 to decipher the structure and the dynamic of the ascocarp-associated communities.

As observed for the November time point, the gleba was characterized by a significantly lower number of phylotypes and lower values of Shannon and Chao1 indices compared with the other compartments regardless of the truffle maturation stage (ANOVA, $P < 0.0001$). The highest values (phylotype, Shannon and Chao1) were measured in young ascocarps collected in October (Table 1). Soil samples (ascocarpic and bulk) showed significantly higher values than the ascocarpic samples (gleba and peridium), whatever the sampling time, except in October and November (stages 6a and 6b) for which the peridium compartment harboured similar number of phylotypes and diversity values (Shannon and Chao1) than the soil samples.

The ascocarp-associated bacterial communities were separated from those of the other compartments in samples collected from October to January in all the maturation stages considered, similarly to the November sampling time, as demonstrated by the multivariate analysis performed on the relative distribution of the phyla (Fig. 2B). Notably, although the structure of the gleba-associated bacterial communities evolved along the maturation process according to the first axis (F1), these communities were always separated from those of the peridium and the soil compartments. The peridium-associated bacterial communities clustered together, but appeared more connected to the ascocarpic soil samples, particularly at the beginning of maturation. The soil samples were clustered together regardless of their sampling time.

Taxonomic assignments confirmed the dominance of the same phyla as those observed in November samples, but also highlighted significant differences among the compartments and the maturation stages considered (Fig. 3). Notably, the peridium-associated bacterial communities from young ascocarps (October, stage 6a) were very similar to those of the soils and could not be statistically separated for all phyla. After this date, significant

Fig. 2. Multivariate analysis of bacterial communities associated to *T. melanosporum*.
A. Multivariate analysis of bacterial communities associated to ectomycorrhizae, ascocarps and associated soils collected in November 2010. The relative distribution of the major phyla was determined on ascocarp, ectomycorrhiza and soil samples. Principal component axes 1 and 2 explain most of the variance in the data cumulatively (F1 = 51.5% and F2 = 25%). Samples are presented as follow: black triangles, biological replicates from soil related samples (bulk soil surrounding ectomycorrhizae and ascocarps, ectomycorrhizospheric and ascocarpic adhering soils); grey dots, biological replicates from ectomycorrhizae; white dots, biological replicates from peridium; black dots, biological replicates from gleba.
B. Multivariate analysis of bacterial communities associated to the ascocarp along maturation. The relative distribution of the major phyla was determined on ascocarps (gleba and peridium), ascocarpic and bulk soil samples collected from October 2010 to January 2011. Principal component axes 1 and 2 explain most of the variance in the data cumulatively (F1 = 49.4% and F2 = 16.7%). Samples are presented as follow: black triangles, biological replicates from soil related samples (ascocarpic adhering and bulk soils); white dots, biological replicates from peridium; black dots, biological replicates from gleba.

Table 2. List of the ten most observed genera in the different compartments sampled in November 2010. Average percentages are given into brackets. Each value is the mean value of three biological replicates. Genera which were found to be significantly more present in one compartment than an other according to a one-way ANOVA (compartment origin, Fisher Test $P < 0.05$) are highlighted with bold letters.

Bulk soil surrounding ectomycorrhizae	Adherent soil to ectomycorrhizae	Ectomycorrhizae	Gleba	Peridium	Adherent soil to the ascocarp	Bulk soil surrounding ascocarps
Terrimonas (5.7)	***Terrimonas* (5.6)**	***Terrimonas* (3.7)**	***Variovorax* (1.6)**	***Pedobacter* (6.7)**	***Terrimonas* (6.3)**	***Terrimonas* (6.6)**
Microscilla (2.7)	***Microscilla* (4.4)**	***Streptomyces* (3.5)**	*Bradyrhizobium* (1.1)	***Bradyrhizobium* (3.1)**	***Microscilla* (3.3)**	***Microscilla* (3.7)**
Flavobacterium (2.1)	*Streptomyces* (2.2)	***Bradyrhizobium* (2.9)**	*Mitsuaria* (0.9)	*Terrimonas* (2. 4)	***Flavobacterium* (2.3)**	***Flavobacterium* (3.0)**
***Pedosphaera* (2.1)**	***Flavobacterium* (2.1)**	***Thermoleophilum* (2.6)**	*Sphingobacterium* (0.4)	*Acidovorax* (2.4)	*Rubritalea* (1.6)	*Candidatus Koribacter* (1.7)
***Streptomyces* (1.5)**	*Pedosphaera* (2.0)	*Glechoma* (2.4)	*Pedobacter* (0.4)	**Variovorax (2.3)**	*Candidatus Koribacter* (1.3)	*Pedosphaera* (1.6)
***Candidatus Koribacter* (1.4)**	*Cytophaga* (1.7)	*Pseudonocardia* (1.2)	*Myroides* (0.2)	*Dyadobacter* (1.7)	*Pedosphaera* (1.1)	*Cytophaga* (1.3)
***Bacillus* (1.2)**	*Mycobacterium* (1.5)	*Acidovorax* (1.0)	*Pseudomonas* (0.2)	*Flavobacterium* (1.2)	*Cytophaga* (1.1)	*Verrucomicrobium* (1.0)
***Mycobacterium* (1.1)**	*Veillonella* (0.8)	*Mycobacterium* (1.0)	*Flavobacterium* (0.2)	*Rhizobium* (1.1)	*Mycobacterium* (1.0)	*Bradyrhizobium* (0.9)
***Acidobacterium* (1.1)**	*Finegoldia* (0.7)	*Microscillia* (1.0)	*Acidovorax* (0.1)	***Sphingobacterium* (1.1)**	*Chitinophaga* (1.0)	*Mycobacterium* (0.9)

differences were observed between the ascocarpic compartments and the soil samples (Fig. 3). At the phylum level, three main groups were identified, based on their ecological habitat and the evolution of their relative distribution along the truffle maturation, although the bacterial communities were dominated by Proteobacteria (up to 72% of the 16S rRNA sequences). The first group corresponded to phyla poorly represented or even absent along the study in the ascocarp but highly represented in the soil such as *Acidobacteria*, *Actinobacteria* and *Planctomycetes*. Notably, the relative abundance of these phyla increased significantly from October to January in the soil compartments. A second group, corresponding to phyla such as *Betaproteobacteria* and *Verrucomicrobia*, showed a significant decrease of abundance in the ascocarp (gleba and peridium) from October to January and, in contrast, a relative stability in the soil compartments. A last group, which corresponded to phyla such as *Bacteroidetes*, *Firmicutes* and *Alphaproteobacteria* showed a significant increase of abundance in the ascocarp from October to January. In addition to the differences observed at the phylum level, a detailed analysis showed that the different ascocarpic compartments were significantly differentiated according to the relative distribution of the 16S rRNA sequences in classes, orders, families and genera (Table S3). The percentage of OTUs attributed to unassigned bacteria varied between 19% and 85% depending on the compartment and the stage considered (Fig. 3).

A specific comparison of the gleba and peridium compartments along the maturation stages revealed that some phyla such as *Bacteroidetes*, *Firmicutes* and *Alphaproteobacteria* did not evolve in the same way in the two ascocarpic compartments. *Bacteroidetes*, particularly the *Pedobacter* genus, appeared to increase significantly in the peridium and to decrease in the gleba along maturation. *Firmicutes* increased significantly in the gleba of fully mature ascocarps (January), while it remained stable in the peridium. *Alphaproteobacteria*, mainly identified as representatives of the *Bradyrhizobium* genus, increased significantly along time in the gleba to become the dominant phylum (from $30.4 \pm 7.2\%$ in October to $56.6 \pm 10.8\%$ in January; $P < 0.001$), while it remained stable and less dominant in the peridium. In contrast, *Betaproteobacteria* from the *Variovorax* genus strongly decreased in both compartments (15.2 ± 3.6 in October, 0.6 ± 0.2 in January; $P < 0.001$).

Fluorescent in situ hybridization (FISH) confirms the dominant bacterial phyla within mature ascocarps

To confirm the presence of living bacteria within the ascocarps and their taxonomic identity, sections from the peridum and the gleba of three mature truffles collected in

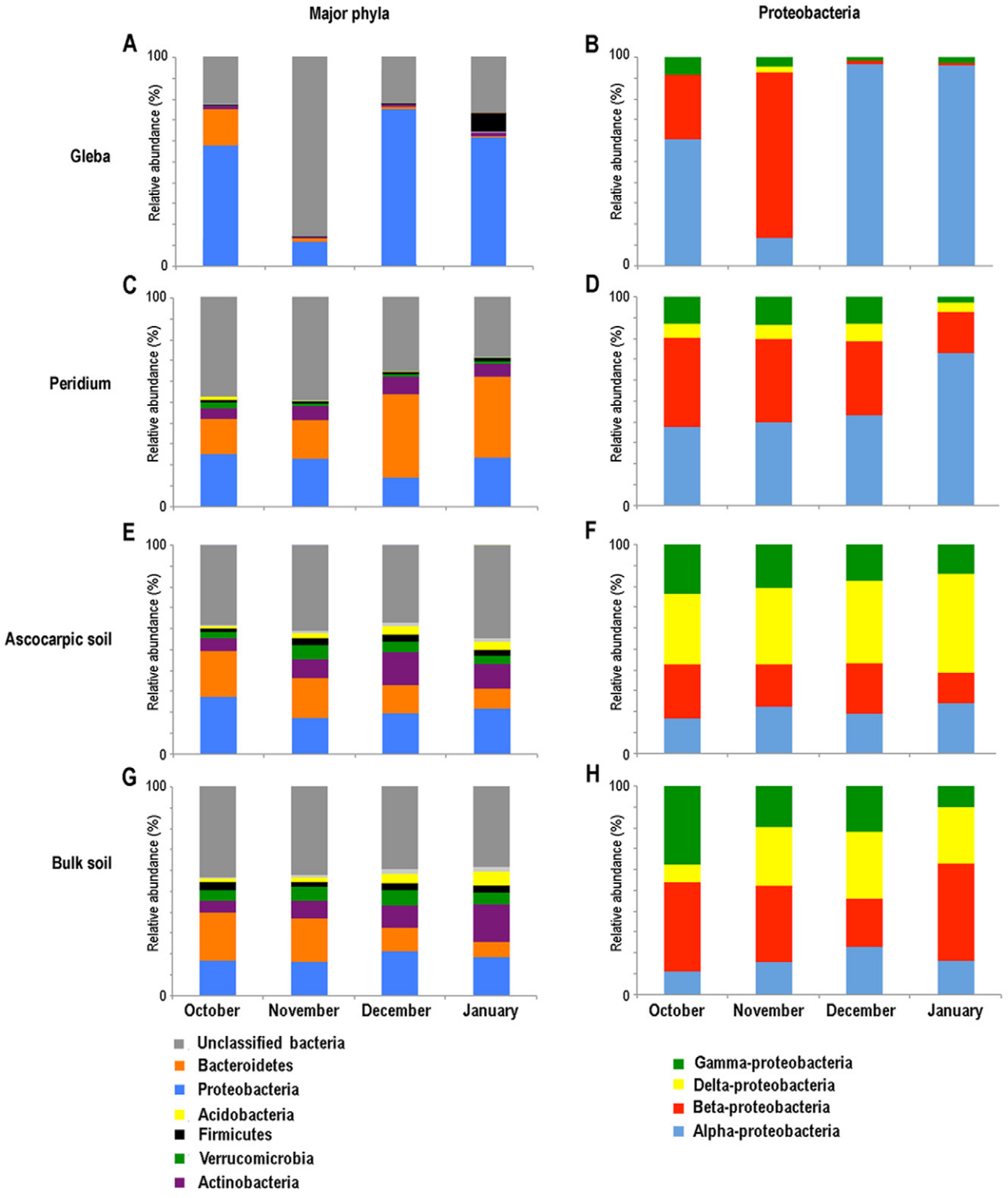

Fig. 3. Evolution of the relative distribution of the major phyla (A, C, E, G) and proteobacteria classes (B, D, F, H) in the different compartments considered (gleba, peridium, ascocarpic adhering soil and bulk soil) along truffle maturation (from October 2010 to January 2011).

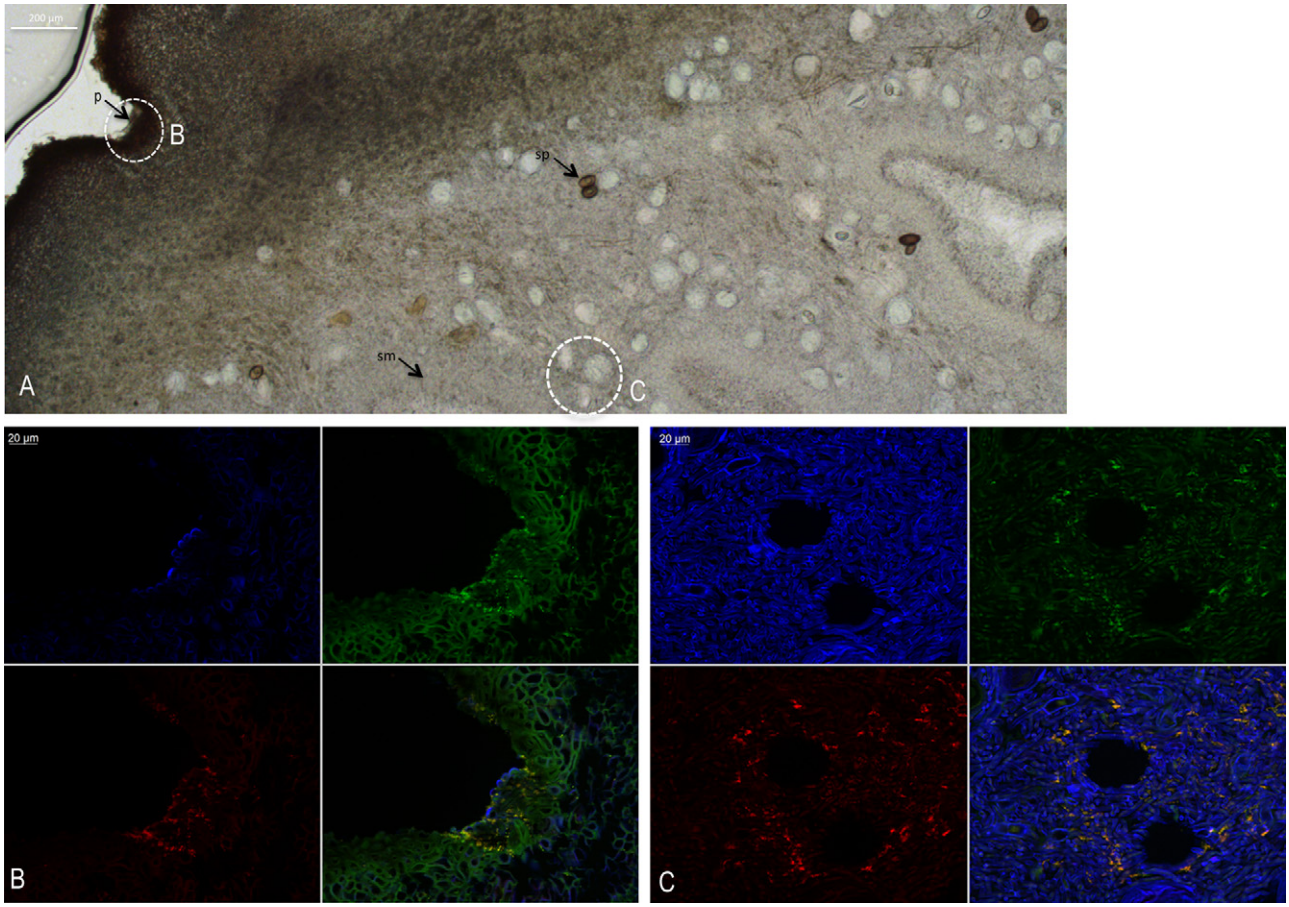

Fig. 4. *In situ* hybridization of mature truffle sections with group-specific 16S rRNA oligonucleotide probes.
A. Brightfield view of a truffle section; p, peridium; as, ascus; sm, maternal mycelium; sp, moved ascospore.
B. Close up on a portion of the peridium. Upper left: calcofluor staining of fungal hyphae, upper right, hybridization with the universal eubacteria Eub338mix-FITC probe, down left: hybridization with the probe ALF1a/ALF968mix specific for alpha-proteobacteria, down right: overlay of three channels.
C. Close up on a portion of the gleba. Upper left: calcofluor staining of fungal hyphae, upper right, hybridization with the universal eubacteria Eub338mix-FITC probe, down left: hybridization with the probe ALF1a/ALF968mix specific for alpha-proteobacteria, down right: overlay of three channels.

January 2011 were hybridized with labelled bacteria- and group-specific 16S rRNA oligonucleotide probes. Distinguishable bacteria cells could be visualised on the surface of the ascocarps, in between cells of the peridium and within the gleba using eubacteria universal FISH probes (Fig. 4). In the gleba, bacterial cells could be seen in the maternal tissues and surrounding the ascii but none were detected within the ascii themselves. Notably, bacterial colonization of the inner tissues of the ascocarps appeared to be very patchy. Some examples of ascocarp tissues: (i) free of bacterial cells, (ii) slightly colonized by individual cells or (iii) massively colonized are presented in Fig. S2.

The same truffle sections were hybridized with dedicated probes to *Alpha-, Betaproteobacteria, Bacteroidetes and Firmicutes*: they revealed a dominance of alpha-proteobacteria that were widely distributed in the truffle tissues (Fig. 4), supporting the pyrosequencing conclusions. Alphaproteobacteria were often found to form massive colonies between hyphal cells (Fig. S2). Bacterial cells belonging to *Betaproteobacteria*, *Bacteroidetes* and *Firmicutes* were also observed, but appeared to be more locally distributed (Table S4, Fig. S2).

Functional attributes of ascocarp-associated bacterial communities

To obtain insights into the potential functional attributes of the bacterial communities inhabiting the black truffles and their potential roles in the maturation of the ascocarp, functional gene arrays were used. DNA extracts from the peridium of mature truffles and ascocarpic soils collected in January 2011 were hybridized to GeoChip 3.0 (He *et al.*, 2010). A total of 5574 and 14 198 probes were

Table 3. Average percent of probes hybridizing with DNA from peridium or ascocarpic soil classified by functional categories. Each data is the average value of two biological replicates.

Processes	Ascocarp	Ascocarpic soil
Antibiotic resistance	4.3	4.2
Carbon cycling	11.5	10.9
Energy process	1.2	1.3
Metal resistance	12.7	12.9
Nitrogen cycling	7.6	7.3
Organic remediation	23.4	25.7
Phosphorous utilization	1.8	1.6
Soil benefit	3.9	3.8
Soil born pathogen	0.7	0.7
Stress response	22.7	22.5
Sulphur	3.0	3.0
Virulence	5.1	4.1
Other	2.1	2.0

hybridized by DNA from ascocarpic and soil compartments respectively. This result shows the higher diversity of the soil bacterial communities compared with those of the ascocarp, confirming 454-pyrosequencing results. However, the relative distribution of gene functions was very similar between the soil and ascocarpic compartments despite a lower number of probes providing signal with the ascocarpic samples (Table 3). A detailed analysis highlighted a set of 541 probes with at least a fivefold increase in probe intensity in the ascocarpic compartment compared with the soil (Table S5). These genes were found to contribute towards nutrient cycling such as carbon, nitrogen, phosphorous and sulphur, as well as organic compound remediation. Notably, nitrogenase encoding genes assigned to uncultured bacteria, as well as genes involved in sulphur metabolism were detected in the ascocarp. The presence of NifH genes in the ascocarp was confirmed by a PCR approach on the same DNA using primer sets described by Widmer and colleagues (1999) (data not shown).

Because of the dominance of *Actinobacteria* and *Bacteroidetes* phyla and *Bradyrhizobium* genus in the pyrosequencing data obtained from the peridium of mature ascocarps, probes affiliated to these phyla and genera were extracted from the GeoChip data and analysed to identify potential functional abilities of these ascocarpic bacterial groups. A total of 468, 128 and 20 probes specifically assigned to *Actinobacteria*, *Bacteroidetes* and *Bradyrhizobium* hybridized with DNA extracted from the peridium respectively. According to the GeoChip characteristics, probes corresponding to genes linked to antibiotic and metal resistances, oxygen stress, phosphate limitation and nitrogen reduction were shared by these three bacterial groups. In contrast, genes associated with carbon degradation and fixation, and phosphorous limitation only gave a signal for probes related to Bacteroidetes and Actinobacteria (Fig. S3). Probes related to nitrogen ammonification and fixation were positive for Actinobacteria only. Sulphur oxidation abilities were present in bacteria from the *Bradyrhizobium* genus, but not in Actinobacteria and Bacteroidetes colonizing peridium.

Discussion

Although truffles are cultivated since decades with more and more efficiency, their life cycle and especially the conditions stimulating ascocarp development remain unknown. Recently, the presence of specific bacterial communities in and outside the ascocarp of several truffle species was reported and their potential role in the development of the ascocarp discussed (Sbrana *et al*., 2000; 2002; Barbieri *et al*., 2005; 2007; 2010; Splivallo *et al*., 2007; Gryndler *et al*., 2013; Mello *et al*., 2013), but nothing was known regarding the natural bacterial communities colonizing the species *T. melanosporum*. Concurrently, our knowledge of the interactions between bacteria and fungi and the reciprocal impact of such interactions on the physiology of both partners has considerably increased (Frey-Klett *et al*., 2011; Scherlach *et al*., 2013), encouraging to infer that bacterial communities could interfere positively or negatively with the life cycle of *T. melanosporum*.

Studies performed so far, based on clone libraries, fingerprinting methods or isolation of cultivable strains, provided a relatively low resolution, compared with the opportunities offered by the recent high throughput technologies. Here, we used a polyphasic approach combining a broad range of complementary molecular tools to obtain the most comprehensive view of the structure of the bacterial communities associated to *T. melanosporum* along its life cycle and ascocarp maturation. Similar patterns were obtained regardless of the methods used, confirming the data robustness. However, TTGE did not permit to detect variations in the ascocarp-associated bacterial communities during maturation as precisely as pyrosequencing because these variations occurred mainly in low represented taxa. Consequently, 454 pyrosequencing appears to be more suitable than TTGE to analyse the truffle-associated bacterial communities. A relatively high number of unassigned sequences were obtained (37% in average) compared with other analyses performed on forest soils or ectomycorrhizospheres (10–30%; Uroz *et al*., 2010; 2012). These sequences were manually checked to control that they do not correspond to chimera, plastidic or mitochondrial sequences. Either they correspond to unknown bacteria yet to be identified, or they are the result of unknown artefacts. Nevertheless, *in situ* hybridization provided a similar picture of the composition of the bacterial communities of the truffle than those assigned by pyrosequencing demonstrating

that 454 data provide a reliable picture of the truffle-associated bacterial communities.

Similarly to other truffle species ascocarps (Sbrana *et al*., 2000; 2002; Barbieri *et al*., 2007), inner and outer parts of *T. melanosporum* ascocarps were deeply colonized by bacteria. Bacterial communities from the ascocarp were separated from those of the bulk soil, suggesting that the *T. melanosporum* ascocarps create a niche that selects specific bacterial communities. This selective hypothesis is also supported by the significant decrease of the bacterial diversity observed in the ascocarp compared to the bulk soil (Warmink *et al*., 2009). In contrast to the bulk soil that harboured a high diversity and was colonized by phyla such as *Proteobacteria, Bacteroidetes, Verrucomicrobia, Actinobacteria, Firmicutes* and *Acidobacteria*, as previously observed in other studies (Gryndler *et al*., 2013; Mello *et al*., 2013), the ascocarp was dominated by alphaproteobacteria related to the *Bradyrhizobium* genus. These latter accounted for up to 56% of the 16S rRNA sequences, versus less than 1% in bulk soil (Fig. 3). The dominance of *Alphaproteobacteria* within *T. melanosporum* was confirmed by FISH analyses (Fig. 4). To our knowledge, this is the first report of the presence of bacteria belonging to the *Bradyrhizobium* genus in *T. melanosporum* ascocarps. Interestingly, presence and predominance of the *Bradyrhizobium* genus was also reported in several other truffle species such as in *T. borchii* (Barbieri *et al*., 2005), *T. magnatum* (Barbieri *et al*., 2007) and *T. aestivum* (C. Murat and A. Deveau, unpublished). The fact that *Bradyrhizobium* was consistently found at all stages of the maturation process and in different truffle species suggests that the selection of this genus results from deterministic events. The biochemical composition of the ascocarps and the building of an efficient barrier preventing soil exchanges may be involved in this process. Although the bacterial communities of *T. melanosporum* share similarities with those of other truffle species through the massive colonization by *Bradyrhizobiaceae*, it also distinguishes from other truffle species by the very weak colonization of the ascocarps by representative of the *Pseudomonas* genus. Numerous *Pseudomonas* strains have been isolated from ascocarps of *T. borchii* (Bedini *et al*., 1999; Sbrana *et al*., 2000) and from *T. magnatum* (Barbieri *et al*., 2007), and some were identified as potential helper bacteria of *T. borchii* (Sbrana *et al*., 2000). Consistently with the observation of Mello and colleagues (2013) that pseudomonads tended to be excluded from *T. melanosporum* brûlés, the sequences assigned to *Pseudomonas* genus represented less than 0.5% of the sequences in our soil and ascocarpic samples collected in October and November. Pseudomonads were even absent from mature truffles. From our data, *Pseudomonas* seems therefore to be a rare genus in truffle orchards of Rollainville. This could be due to unfavourable conditions to the presence of pseudomonads in the soil reservoir that would prevent a further colonization of *T. melanosporum*. It is possible that *T. melanosporum* does not provide a suitable habitat for this group of bacteria.

Selection of specific bacterial communities by the ectomycorrhizosphere (Frey *et al*., 1997; Uroz *et al*., 2007; 2012; Izumi and Finlay, 2010) and the mycosphere (i.e. the hyphal network underneath fungal fruiting bodies; Boersma *et al*., 2009) has been reported for many ectomycorrhizal fungi. However, surprisingly few studies have investigated the bacterial communities of fruiting bodies of ectomycorrhizal fungi (Rangel-Castro *et al*., 2002). It is not known whether ectomycorrhizal fungi are providing a single habitat throughout their life cycle or the different organs – mycorrhizae, fruiting bodies, hyphal networks – select for different bacterial communities. This question is technically difficult to answer because it requires to be able to sample the different organs at the same time and in the same point. The truffle orchard of Rollainville provided a unique opportunity to answer this question, thanks to the very high colonization level of trees roots by *T. melanosporum*. Comparison performed on samples collected in November 2010 revealed that the ascocarp-associated bacterial communities were distinct from those of the ectomycorrhizosphere, suggesting that the fungus provided at least two different habitats. Such differences may be attributed to their biochemical composition. *T. melanosporum* ascocarps accumulate carbohydrates, proteins and lipids (Harki *et al*., 2006), while ectomycorhizae are rather transfer structures of more simple metabolites like simple sugars, amino acids, organic acids or fatty acids between the host and the fungus. A comparison of the bacterial communities at the phylum level, based on the data clustering from Bray–Curtis similarity matrices (MG-RAST) (data not shown), demonstrated that the ascocarp bacterial communities were more related to those of the surrounding bulk soil than to those of the ectomycorrhizosphere. It suggests that the soil constitutes a reservoir of biodiversity, from which specific bacterial communities are differentially recruited in the ascocarp and in the ectomycorrhizosphere.

The next question we asked is whether the structure and composition of the bacterial communities of the ascocarp remained stable during the truffle maturation process. Barbieri and colleagues (2007) observed that the composition of the bacterial communities was stable along the development of *T. magnatum* ascocarp, using a cloning-sequencing procedure. In contrast, we showed that the ascocarp-associated bacterial communities evolved during the ascocarp maturation. The higher resolution of 454-pyrosequencing compared with cloning-

sequencing may explain this difference. It is also possible that bacterial communities from *T. magnatum* and *T. melanosporum* behave differently during the maturation of the ascocarp because the process of development of white and black truffles is very different. A relative turnover seems to exist in the gleba of *T. melanosporum*, apart from the predominance of *Alphaproteobacteria*. As an example, *Bacteroidetes*-related sequences present in the early stage were replaced by *Firmicutes* in the fully mature stage. These changes could be explained by the biochemical composition modifications of the truffle tissues, which happen during truffle maturation. In immature truffles, the major constituents are proteins (Harki *et al*., 2006). Throughout the maturation process, the glucose and mannose content increase and rhamnose is detected at complete maturation (Harki *et al*., 2006). In addition, sulphur metabolism gets activated in the late stages of maturation (A. Kohler and F. Martin, pers. comm.). Interestingly, community composition evolved differently in the peridium and in the gleba. In contrast to the gleba for which bacterial communities were very distinct from the soil's community since early maturation stages, bacterial communities colonizing the peridium were very close to the one's of the soil in October samples. Then, peridium-associated bacterial communities also evolved with maturation, but in a different way than the gleba's and the soil's ones. For example, populations of *Bacteroidetes* significantly increased in the peridium during the maturation, while they decreased in the soil and gleba compartments. This differentiation of the ascocarp-associated bacterial communities between the peridium and the gleba may be due to the mechanism of development of the ascocarp itself. Colonization of the ascocarpic tissue by soil bacteria probably occurs before the differentiation of the peridium when the primordium is directly in contact with the soil. Then, the bacteria are trapped in the gleba and partly protected from soil exchanges by the warted peridium. In contrast, the peridium remains in contact with the soil all along the development process of the ascocarp and breaks in its surface appear regularly with the size augmentation of the ascocarp.

Presence of bacteria in the ascocarps raises questions on their functional role in the life cycle of the fungus: are these bacterial communities selected by *T. melanosporum* because of their functional abilities to complement the fungus during its development? Or are they just profiteers taking advantage of the nutrients provided by the fungus? In the present study, functional potentials related to nitrogen fixation, cellulose/chitin degradation and sulphur metabolism were detected. Interestingly, nitrogen fixation function was also previously reported in *T. magnatum* (Barbieri *et al*., 2010). These authors not only detected the functional *nif* genes, but also showed that nitrogen fixation by *Bradyrhizobium* cells occurred within truffle ascocarps. However, no demonstration of transfer of fixed nitrogen to the fungal partner has been done yet. In our study, the functional *nif* probes giving a positive signal were not related to *Bradyrhizobium*, suggesting that other bacteria may be involved in this process and that the *Bradyrhizobia* present in the ascocarp may not have the ability to fix nitrogen. This would not be surprising since *nif* genes are harboured by a small subset of *Bradyrhizobium* strains (Itakura *et al*., 2009). However, it is also possible that *Bradyrhizobium* strains colonizing the truffle are too divergent from the ones used to design *Bradyrhizobium* nif probes of the GeoChips. Therefore, further analyses will be necessary to completely decipher which bacterial species colonizing *T. melanosporum* possess nitrogen fixation abilities. Concerning cellulose and chitin degradation, the presence of bacterial communities harbouring such function is not completely surprising. Indeed, part of these bacteria such as *Bacteroidetes* and *Actinomycetes* may contribute to the release of the ascospores (Gazzanelli *et al*., 1999). GeoChip analyses also revealed the presence of numerous bacterial genes linked to sulphur metabolism and related to *Bradyrhizobium*. Mature truffles are known to produce aromatic volatile compounds (VOCs) containing sulphur, responsible for the so much appreciated aroma of truffles (Splivallo *et al*., 2011). Presence of such bacterial communities suggests that they may participate in the VOC refining (Splivallo *et al*., 2007) or degrade them. Some *Bradyrhizobium* strains have been shown to perform sulphur oxidation and aromatic degradation (Okubo *et al*., 2012). Degradation of fungal VOCs was recently demonstrated for *Agaricus bisporus*-associated bacteria (Noble *et al*., 2009). Thus, it could be interesting to test whether these bacteria could be, for a part, responsible for the short life span of the famous Black truffle aroma.

Conclusion

Our study based on a polyphasic approach presents the first comprehensive view of the structure and functional potential of the *T. melanosporum*-associated bacterial communities and their evolution along the maturation of Black truffles. *Tuber melanosporum* ascocarps provide a habitat for specific bacterial communities which are selected from the surrounding soil and evolve during the ascocarp maturation. Additional studies are required to determine whether the picture of the *T. melanosporum*-associated bacterial communities we obtained from truffles collected in a single orchard is similar, whatever the environment considered or site dependent. Nevertheless, our results open new questions regarding the ecology and

the role of bacterial communities selected by truffle ascocarps. Are these communities only 'fungiphiles', taking advantage of truffle resources? Or do they participate in the life cycle and maturation process of the truffle? Our first GeoChip results suggest that the ascocarp-associated bacteria could participate in both: development, maturation and even aroma of the Black truffle. A culture-dependent approach is required to determine whether the taxa specific of the ascocarpic habitat participate in nitrogen fixation, cellulose/chitin degradation and aroma development in *T. melanosporum*.

Experimental procedures

Sampling site

All samples were collected under a single hazel tree (*Corylus avellana)* labelled 'A11' in the truffle orchard of Rollainville (48°21′N, 5°44′E; Lorraine region, France) during the truffle maturation process from October 2010 to January 2011. The truffle orchard consists of an open woodland of hazel and oak trees inoculated with *T. melanosporum* and *T. aestivum* and planted in 1991 (Murat *et al*., 2013) on a brown calcisoil with a silt clay texture and an alkaline pH of 7.97. The first Black truffle was harvested in November 2005.

Sampling method and storage

Two distinct sample types were collected (Table S2). The first type of samples collected from October 2010 to January 2011 corresponded to the ascocarp-associated samples with a distinction of the fruiting body (ascocarp), the ascocarpic soil (soil adhering to the ascocarps, AS) and the surrounding bulk soil (non-adhering ascocarpic soil obtained by shaking the ascocarps, ASBS) (Fig. S4A). The second type of samples, collected in November 2010 in the same time than the ascocarpic samples, corresponded to the ectomycorrhizal-associated samples with a distinction of the *T. melanosporum* ectomycorrhizal root tips (EcA), the ectomycorrhizospheric soil (soil adhering to the ectomycorrhizal root tip, AEcS) and the surrounding bulk soil (MBS). All the sample types were collected from four independent locations around the tree labelled 'A11' and spaced about 2 m apart (Fig. S4B). For each sampling date and sample type, three to six samples were collected (Table 1).

The samples were stored on ice during transport to the laboratory. On arrival at the laboratory, the *T. melanosporum* ectomycorrhizal root tips were collected under a microscope in a laminar flow cabinet. Ectomycorrhizae were characterized both by morphotyping and using specific molecular probes as described in (Paolocci *et al*., 1999). One to five grams and 1–2 g of non-adhering bulk soils surrounding ascocarps and ectomycorrhizae were collected by gently shaking the samples respectively. Soil adhering to ascocarps (0.5–1 g) and ectomycorrhizae (100–300 mg) were obtained using needles and forceps. After collection of bulk and adhering soils, ascocarps were brushed under water to remove soil particles and dried with absorbent paper. Then, the ascocarps were aseptically split open using sterile forceps. Scalpels were not used to break open the ascocarps to avoid external contamination during slicing. Pieces of the peridium (P) and the inside of the ascocarps (gleba; G) were collected and stored separately. Maturation degrees were determined using stereomicroscope and light transmission microscope according to G. Pacioni (pers. comm.) and Le Tacon and colleagues (2013). In this study, truffles ranged from stage 6a (early maturation, October) to 6c (fully mature, January). A complete description of the stages is given in Table S2B.

All samples were stored in –80°C directly without addition of any preservative additives, except for samples collected for *in situ hybridization* which were processed as described below. All these samplings gave a total of 116 samples.

Community DNA extraction

Because of the different type of samples considered in the study (truffle, mycorrhizal tips and soil) and the molecular technique used, three different extraction methods were necessary to isolate genomic DNA from the samples with convenient quality (Antony-Babu *et al*., 2013). Soil samples were processed using Fast-DNA Spin kit (Q-Biogene, Heidelberg, Germany) for temporal temperature gradient-gel electrophoresis (TTGE) and 16S rRNA gene amplicon pyrosequencing analyses. Ectomycorrhizal and ascocarpic samples were isolated using DNeasy Plant Mini Kit (Qiagen SA, Courtaboeuf, France). In both cases, we followed manufacturer's guidelines. The efficacy of the DNA extraction was confirmed by 16S rRNA gene PCR amplification using two different primer sets; pA (AGAGT TTGAT CCTGG CTCAG; Edwards *et al*., 1989) with 907r (CCGTC AATTC ATTTG AGTTT; Lane *et al*., 1985) and 799f (AACMG GATTA GATAC CCKG; Lane, 1991) with 1429r (GGYTA CCTTG TTACG ACTT; Chelius and Triplett, 2001). A positive PCR result confirmed the presence of bacterial DNA and certified the quality of the sample. The primer pair 799f/1429r was used because it enables differentiation of bacterial 16S rRNA gene product to that of eukaryotic mitochondria.

Lastly, the quality requirement of genomic DNA necessary to perform GeoChip microarrays analyses (OD 260 nM/230 nm > 1.7) required the development of a new extraction method based on a succession of pre-lysis and pre-elution wash steps combined with a PowerSoil DNA isolation kit (MO-BIO Laboratories Inc., Carlsbad, CA, USA). The method is fully described in Antony-Babu and colleagues (2013).

TTGE community fingerprint analyses

TTGE fingerprinting analyses were performed on all the 116 samples (Table 1) in order to compare all them together and to determine if clusters can be obtained according to the habitat or the sampling time. GC-clamped PCR amplicons of an approximately 450 bp fragment of the 16S rRNA gene between positions 954 and 1369 of the *Escherichia coli* SSU-rRNA sequence were amplified using GC-954f (CGCC GGGGG CGCGC CCCGG GCGGG GCGGG GGCAC GGGGG G GCACA AGCGG TGGAG CATGT GG) and 1369r (GCCCG GGAAC GTATT CACCG). The primers covered the V6 to V8 regions of the 16S rRNA gene. PCR amplifications were carried out under the following conditions: 94°C for

10 min, 10 touchdown cycles of 30 s at 94°C (denaturation), annealing temperature starting at 57°C for 1 min and per cycle decrement of 0.5°C followed by 72°C for 90 s (extension). The touchdown cycle was followed by 30 cycles of 94°C for 30 s, 52°C for 1 min and 72°C for 90 s. The reactions concluded with final extension for 10 min at 72°C and held at 4°C until use. Two independent PCR amplifications were done per sample and were analysed separately.

A 30 ml 6% (wt/vol) acrylamide gel was prepared in 1.5× TAE with 7M urea, 0.2% glycerol (for flexibility), 30 µl tetramethyl-ethylenediamine and 300 µl of 10% ammonium per sulphate. After the gels were cast in the sandwich plates, the wells were briefly washed with buffer and fixed to a DCode Universal Mutation Detection System (Bio-Rad Laboratories, München, Germany). After quantification of the TTGE PCR products on agarose gel, using the low DNA mass ladder (Invitrogen, Saint-Aubin, France), equal concentrations of each sample were loaded on the gel. The products were separated during the run in 1X TAE buffer (40 mM Tris–acetate, 1 mM EDTA, pH 8.0) at 110 volts with a temperature gradient increasing from 38°C to 58°C with a temperature increment of 4°C per hour. After completion of the run, the gels were stained with SYBR Gold stain (1/10 000 final, Molecular Probes) and analysed on a GelDoc transilluminator (BioRad, BioRad Laboratories, München Germany) coupled to the QuantityOne software for band pattern analysis. Gel images were exported to TIFF format for data analysis using BIONUMERICS software (AppliedMaths, Sint-Martens-Latem, Belgium).

Barcoded pyrosequencing, DNA sequence processing and taxonomic analyses

For the 16S rRNA gene amplicon pyrosequencing analyses, between 3 and 6 replicates per condition and sampling date were considered, which corresponded to a total of 69 samples (Table 1). The samples were sorted into two libraries sequenced in the same 454 run: one for ascocarpic samples and other for ectomycorrhizal-associated samples. Both libraries consisted of samples covering different habitats through time. The ascocarpic library included ascocarpic inside-tissue (G), ascocarp peridium (P), ascocarpic adhering soil (AS) and bulk soil associated to the ascocarp (ASBS). The ectomycorrhizal-associated samples included ectomycrorrhizae (EcA), mycorrhizosphic-soil (AEcS) and bulk soil associated to the ectomycorrhizae (BS). The ascocarp library covered 4 months from October 2010 to January 2011 and ectomycorrhizal-associated library included samples from November 2010 only.

Amplicon libraries were generated using the primers 787r (xxxxx-ATTAGATACCYTGTAGTCC) (Nadkarni *et al*., 2002) and 1073F (xxxxx-ACGAGCTGACGACARCCATG) (On *et al*., 1998) to generate PCR 16S rRNA fragments of ca 250 bp, where xxxxx represents the sample identification barcoding key. PCR amplifications were carried out with the following conditions: 94°C for 10 min, 30 cycles of 30 s at 94°C (denaturation), annealing temperature starting at 48°C for 45 s followed by 72°C for 90 s (extension). The reactions concluded with final extension for 10 min at 72°C. PCR amplicons were purified using Qiagen PCR purification kit or Qiagen gel purification kit for samples showing multiple bands. Quality of the purified PCR amplifications were checked on 1% agarose gels using the low DNA mass ladder (Invitrogen). Quantification of the PCR products was performed using both gel and nanodrop. The PCR barcoded amplicons were pooled into respective libraries in equimolar concentrations. Amplicon pyrosequencing was performed on the GS-FLX 454 Titanium platform of Beckman Coulter Genomics (Danvers, MA, USA).

The sequences obtained were analysed using MOTHUR (Schloss *et al*., 2009). After demultiplexing and quality control (quality score = 30), the orientation of the sequences was double-checked using the alignment mode of MEGA4 suite (Tamura *et al*., 2007). The sequences were then classically processed using MOTHUR suite to determine: OTUs (97% sequence similarity), rarefaction analysis, richness estimates Chao1, Shannon-Weaver index. Rarefactions curves were generated using the R package (http://www.r-project.org). After quality trimming and denoising, sets of 16SrRNA sequences were adjusted randomly at 1671 (corresponding to the smallest sample). Phylogenetic affiliation was performed (80% sequence similarity) with the MG-RAST platform (Metagenome Rapid Annotation using Subsystem Technology; Meyer *et al*., 2008) and the RDP database (Ribosomal data project, Cole *et al*., 2009).

Fluorescence in situ *hybridization*

Half centimetre large pieces of ascocarpic tissue containing both peridium and gleba, collected in January 2011 (Table 1) and used for 454 pyrosequencing were fixed by immersing them in 3% paraformaldehyde-1X Phosphate Buffered Saline (130 mM NaCL, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4; pH 7.3) 3:1, for 4 h at 4°C (Bertaux *et al*., 2003). Fixed samples were embedded in a 6% agarose matrix and 25 µm sections were obtained with a Vibratome VT1200 (Leica, Mannheim, Germany). Sections were deposited on glass slides and proceeded for fluorescence *in situ* hybridization as described in Bertaux and colleagues (2005). Hybridizations were performed for 1 h and 30 min at 46°C with a formamide stringency of 35%, then the slides were washed at 48°C for 10 min. Probes used in this study are described in Table S6. Fungal cell walls were stained with calcofluor white (Sigma, Saint-Louis, MO, USA). The probes EUBI, EUB II and EUBIII and the probes ALF1b and ALF968 were used in equimolar mixtures, as each single probe of the mix is not sufficient to detect all eubacteria or allalpha-proteobacteria. Oligonucleotide probes labelled at the 5′ end with Cy3 or fluorescein-isothiocyanate (FITC) were purchased from Eurofins MWG (Germany). Images were taken using a NIKON-BIORAD Radiance 2100 Rainbow confocal scanning laser microscope and an Axio Observer inverted epifluorescence microscope equipped with an ApoTome capture device (Carl Zeiss MicroImaging, Munich, Germany).

GeoChip microarray analyses

Analyses were performed on genomic DNA extracted from the ascocarpic peridium, and ascocarpic adhering soil that were collected on January 2011 (Table 1). For each habitat considered, biological duplicates were analysed. The GeoChip microarray 3.0 (He *et al*., 2010) analyses were

carried out as previously described (Van Nostrand *et al*., 2009). Probes were considered positive if the signal to noise ratio was > 2.0 and the probes were detected in at least two biological replicates. The signal intensities of each remaining probe were then normalized and averaged across the two biological replicates. After clean up of the data, a total of 14 607 probes representing 383 functional genes pertaining to eubacteria were considered for further analyses. The data obtained were statistically analysed using ADE-4 and *R* statistical packages.

The microarray data presented here are available for download at http://ieg.ou.edu/4download/

Statistical analyses

The different relative values (%) used in this study were transformed by arcsin sqrt for statistical analyses. The habitat and sampling times effects on the structure of the bacterial communities were determined by ANOVA at a threshold level of $P = 0.05$ and Fisher test. All the statistical analyses were performed with XL stat 2011 (Addinsoft, Paris, France).

Nucleotide sequence accession numbers. The 454 sequences generated have been deposited on the Sequence Read Archive of the NCBI under the SUB291410 accession number.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Multivariate analysis of bacterial communities associated to Tuber melanosporum studied with TGGE.
A. Bacterial communities associated to T. melanosporum samples collected in November 2010. The relative distribution of the major phyla was determined on ascocarpic, ectomycorrhizae and soil samples. Principal component axes 1 and 2 explain most of the variance in the data cumulatively (F1 = 21.1% and F2 = 19.2%).
B. Bacterial communities associated to the ascocarp along maturation. The relative distribution of the major phyla was determined on ascocarps (gleba and peridium), ascocarpic adhering soil and bulk soil samples collected from October 2010 to January 2011. Principal component axes 1 and 2 explain most of the variance in the data cumulatively (F1 = 22.6% and F2 = 12.9%).
Fig. S2. In situ hybridization of sections from mature truffle collected in January 2011 with group-specific 16S rRNA oligonucleotide probes.
A. Partial colonization of the peridium by Bacteroidetes. Sample was hybridized with the universal eubacteria Eub338mix-FITC probe (green) and the Bacteroidetes-specific probe CF319-cy3 (red). White arrow points at bacteria from Bacteroidetes.
B. Colonization of the peridium by Beta-proteobacteria. Sample was hybridized with the universal eubacteria Eub338mix-FITC probe (green) and the Beta-proteobacteria-specific probe BET42a-cy3 (red). White arrow points at bacteria from Beta-proteobacteria.
C. Bacteria are present around ascii but not on spores. Sample was hybridized with the universal eubacteria Eub338mix-FITC probe (green) and the alpha-proteobacteria-specific probe ALF1a-cy3 (red) and the fungal cell walls were stained with calcofluor white. The white arrow points at bacterial cells. As: ascospore.
D. Massive colonization of gleba by alpha-proteobacteria. Sample was hybridized the alpha-proteobacteria-specific probe ALF1a-cy3 (red) and the fungal cell walls were stained with calcofluor white. White arrow point at interspaces between hyphae heavily colonized with bacteria. E. Dispersed colonization of gleba by beta-proteobacteria. Sample collected in January 2011 was hybridized with the Beta-proteobacteria-specific probe BET42a-cy3 (red) and the fungal cell walls were stained with calcofluor white. White arrow points at single bacterial cells in between fungal cells.
Fig. S3. Functional assignment of probes hybridized by DNA from Bradyrhizobium, Bacteroidetes and Actinobacteria colonizing mature truffle peridium according to GeoChip analysis. Data are expressed as the percentage of probes providing a significant signal for the two biological replicates in each functional category.
Fig. S4. Truffle life cycle and sample description.
A. Life cycle of Tuber melanosporum and the stages considered in sampling. Main stages are denoted by smaller case alphabets. a: Spore germinates, b: mycelium spreads, c: truffle forms, d: truffle grows, e: truffle maturation starts, f: ripening process starts, g: end of truffle maturation and h: truffle disintegrates and spore disperses. Sampling period for the ectomycorrhizal-associated and ascocarp-associated samples are presented by red and green asterisk, respectively.
B. Representation of aerial view of the tree. The numbers denote the sampling sites for ectomycorrhizae at November time point.
C. Ectomycorrhizal root tip with soil adhering to it (ectomycorrhizosphere soil), D. Surface of truffle with white external mycelium. E, F. Cross-section of a truffle (E: early stage, F: full mature).
Table S1. Comparison of the bacterial communities colonizing the truffle habitats at the phylum, class, order and genus levels in November 2010. The relative distribution of the sequences in the different taxonomic levels considered was analyzed with a one-factor ANOVA (and a Fisher Test, $p < 0.05$). Sample types with similar letters are not significantly different. The symbols '$>$' , '$=$' and '$\geq$' mean 'significantly more abundant', 'not significantly different' and 'not significantly different from the preceding sample type but significantly more abundant than the first preceding sample type with a single letter'. EcA : ectomycorrhizae, AEcS : adherent soil to ectomycorrhizae, BS : bulk soil surrounding the ectomycorrhizae, G : gleba, P : peridium, AS : ascocarpic soil, ASBS : bulk soil surrounding the ascocarps. Grey shading highlights significantly enriched communities in the ascocarp compared to the soil and/or the ectomycorrhizae.
Table S2. Sample description (A) and description of the ascocarp maturation stages (B).
Table S3. Lists of phyla, classes, orders, families and genera, which are significantly differently represented in the bulk soil and ascocarp according to a one-way ANOVA (sample, $p < 0.05$) in samples collected from October 2010 to January 2011. D3: October, D4: November, D5: December, D6: January. G: gleba, P: peridium, AS: ascocarpic soil, ASBS : bulk soil surrounding the ascocarps.
Table S4. Colonization of mature truffle collected in january as observed by FISH. Scoring was performed on 25μm sections of three independant ascocarps. Each section was

hybridized with universal eubacterial probe eub338-FITC and a phylum specific probe coupled to cy3 (alpha-proteobacteria: ALF968 and ALF1, beta-proteobacteria: BET42a, Bacteroidetes: CF319, Firmicutes: LGC354). For each specific probe, at least three different sections were observed on their entirety. A score was given for each probe as following: 4: massive colonization, more than 70% of cells hybridizing with eub338 universal eubacterial probe also hybridize with the specific probe; 3: dense colonization (20–50%); 2: low density (5–20%); 1: few independent cells (<5%); 0: no cells hybridized with the specific probe.

Table S5. List of probes with at least a 5-fold increase in probe intensity in the peridium compartment compared to the ascocarpic soil. Each value is the mean value of two replicates.

Table S6. Oligonucleotide FISH probes used in this study.

Table S7. Band pattern of TTGE gels. Each gel was imaged with GelDoc transilluminator and bands were detected using the QuantityOne software from BioRad. A total of 35 bands were identified in the different samples. '1'and '0' values correspond to presence or absence of the corresponding band, respectively. EcA: ectomycorrhizae, AEcS: mycorrhizospheric-soil, BS: bulk soil associated to the ectomycorrhizae, G: gleba, P: peridium, AS: ascocarpic adhering soil, ASBS: bulk soil associated to the ascocarp.