Bacteria associated with truffle-fruiting bodies contribute to truffle aroma

Article *in* Environmental Microbiology · June 2014

DOI: 10.1111/1462-2920.12521 · Source: PubMed

6 authors, including:

Richard Splivallo
Goethe-Universität Frankfurt am Main
58 PUBLICATIONS **2,932** CITATIONS
SEE PROFILE

Aurélie Deveau
French National Institute for Agriculture, Food, and Environment (INRAE)
69 PUBLICATIONS **4,610** CITATIONS
SEE PROFILE

Pascale Frey-Klett
French National Institute for Agriculture, Food, and Environment (INRAE)
93 PUBLICATIONS **9,119** CITATIONS
SEE PROFILE

Petr Karlovsky
Georg-August-Universität Göttingen
273 PUBLICATIONS **8,130** CITATIONS
SEE PROFILE

environmental microbiology

Environmental Microbiology (2014) doi:10.1111/1462-2920.12521

Bacteria associated with truffle-fruiting bodies contribute to truffle aroma

Richard Splivallo,[1,2]* Aurélie Deveau,[3] Nayuf Valdez,[1] Nina Kirchhoff,[1] Pascale Frey-Klett[3] and Petr Karlovsky[1]

[1]*Molecular Phytopathology and Mycotoxin Research, Georg-August University of Goettingen, Goettingen, Germany.*

[2]*Institute for Molecular Biosciences, Biozentrum/Campus Riedberg, Frankfurt am Main, Germany.*

[3]*INRA, UMR 1136 INRA Université de Lorraine 'Interactions Arbres/Micro-organismes', Champenoux, France.*

Summary

Truffles, symbiotic fungi renown for the captivating aroma of their fruiting bodies, are colonized by a complex bacterial community of unknown function. We characterized the bacterial community of the white truffle *Tuber borchii* and tested the involvement of its microbiome in the production of sulphur-containing volatiles. We found that sulphur-containing volatiles such as thiophene derivatives, characteristic of *T. borchii* fruiting bodies, resulted from the biotransformation of non-volatile precursor(s) into volatile compounds by bacteria. The bacterial community of *T. borchii* was dominated by α- and β-*Proteobacteria*. Interestingly, all bacteria phyla/classes tested in this study were able to produce thiophene volatiles from *T. borchii* fruiting body extract, irrespective of their isolation source (truffle or other sources). This indicates that the ability to produce thiophene volatiles might be widespread among bacteria and possibly linked to primary metabolism. Treatment of fruiting bodies with antibacterial agents fully suppressed the production of thiophene volatiles while fungicides had no inhibitory effect. This suggests that during the sexual stage of truffles, thiophene volatiles are exclusively synthesized by bacteria and not by the truffle. At this stage, the origin of thiophenes precursor in *T. borchii* remains elusive and the involvement of yeasts or other bacteria cannot be excluded.

Received 24 April, 2014; revised 26 May, 2014; accepted 28 May, 2014. *For correspondence. E-mail richard.splivallo@a3.epfl.ch; Tel. +49 69 798 42 193; Fax +49 69 798 29 527. Authors' contributions: RS and AD drafted the manuscript with input from all other co-authors. RS designed the experiments, performed the statistics and also performed the bioassays with truffle mycelium/fruiting bodies/bacteria/antibacterial agents. N.V. isolated bacteria and performed the bacterial sequence analysis. N.K., P.K. and R.S. performed the volatile profiling and volatile data analysis on fruiting bodies. A.D., P.F.-K. and R.S. designed the FISH analysis, and R.S. and A.D. analysed the FISH data. All authors read and approved the final manuscript.

Introduction

Truffles are symbiotic fungi that develop underground in association with plant roots, forming ectomycorrhizas (Mello *et al.*, 2006). Ectomycorrhizal associations, dominant in boreal and temperate forests, are of high ecological relevance since they improve plant nutrition and health (Read, 1991; Buscot *et al.*, 2000; Martin *et al.*, 2001). About 180 truffle species (*Tuber spp*) associate with angiosperm and gymnosperms and naturally occur in Europe, North-America and Asia (Bonito *et al.*, 2010).

The genome sequencing of the Périgord black truffle *Tuber melanosporum* expanded the status of truffles from a food delicacy to a scientific model valuable for the study of complex symbiotic interactions (Martin *et al.*, 2010). Truffles ectomycorrhizas and fruiting bodies harbour a diverse microbial community including bacteria, yeasts and filamentous fungi (Barbieri *et al.*, 2005; 2007; Buzzini *et al.*, 2005; Pacioni *et al.*, 2007). Of these microbes, only bacteria have been extensively studied in truffles. Complex bacterial communities have been reported to establish in different truffle species: *T. aestivum* (Gryndler *et al.*, 2013), *T. magnatum* (Barbieri *et al.*, 2007), *T. melanosporum* (Antony-Babu *et al.*, 2013) and *T. borchii* (Sbrana *et al.*, 2000; Barbieri *et al.*, 2005). Bacteria colonize both the external (peridium) and internal part (gleba) of truffles and seem to be selected from the soil communities during the early stage of truffle formation (Antony-Babu *et al.*, 2013). Although bacterial communities differ depending on truffle species analysed, a core microbiome composed of *α-Proteobacteria* from the family of *Bradyrhizobiaceae* seems common to all species studied so far (Barbieri *et al.*, 2005; 2007; Antony-Babu *et al.*, 2013). Factors responsible for the selection of these bacteria remain mysterious. However, these bacteria could have a role in the development, growth and nutrition of truffle-fruiting bodies (Sbrana *et al.*, 2000; 2002; Barbieri *et al.*, 2007; 2010; Antony-Babu *et al.*, 2013; Pavić *et al.*, 2013).

Truffle-fruiting bodies emit intense aromas (Splivallo and Maier, 2011; Splivallo *et al.*, 2011). Sulphur-containing volatiles have a central role in truffle aroma because they serve as attractants to mammals and contribute to truffle aroma sensed by humans (Talou *et al*., 1990; Culleré *et al*., 2010; Splivallo and Maier, 2011; Splivallo *et al*., 2011). The origin of sulphur-containing volatiles in truffles is unclear as they might be derived from the truffle fungus itself but also from the microbial community inhabiting truffle-fruiting bodies (Buzzini *et al*., 2005; Splivallo and Maier, 2011; Splivallo *et al*., 2011). Analysis of the Black truffle's genome *T. melanosporum*, however, suggested that Black truffle may produce its volatiles without the involvement of bacteria (Martin *et al*., 2010; Maxmen, 2010). Whether other truffle species can produce their volatiles by themselves or require the participation of associated bacteria remain unknown.

Here, we investigated the role of truffle-associated bacteria in the formation of the aroma of a white truffle species, *Tuber borchii*. *T. borchii* is a cultivated truffle species naturally occurring in Europe that has been recently introduced in New Zealand (Bonito *et al*., 2010). *T. borchii* has been for long used in laboratories as a model organism to study truffles because of the relative faster growth of its mycelium compared with other truffle species. *T. borchii*-fruiting bodies emit some volatiles [thiophene derivatives such as 3-methylthiophene, hereafter referred to as **(1)**, and 3-methyl-4,5-dihydrothiophene, referred to as **(2)**], which are species-specific and might be partially responsible for their characteristic aroma (Bellesia *et al*., 2001; Mauriello *et al*., 2004; Zeppa *et al*., 2004; Splivallo *et al*., 2007). The concentrations of thiophene volatiles in *T. borchii* have been reported to occur solely in fully mature (71–100% maturity) truffles (Zeppa *et al*., 2004) and to increase upon storage at room temperature (Bellesia *et al*., 2001), questioning the role of truffle-associated bacteria in the production of the aroma. To test this hypothesis, we analysed the evolution of the composition of the bacterial communities along storage as well as the production of thiophene derivatives. Then we tested the ability of bacterial isolates from *T. borchii* to produce thiophene volatiles. Overall, our results demonstrated that thiophene volatiles characteristic of *T. borchii*-fruiting bodies were produced by the microbiome inhabiting truffle-fruiting bodies.

Results

Composition of T. borchii *bacterial communities evolves during storage*

As a first step, we characterized the composition of bacterial communities associated to *T. borchii* during storage. Six *T. borchii* truffles were subsampled after 0, 2, 4 and 6 days of storage at room temperature. The bacterial communities were characterized and quantified by fluorescent *in situ* hybridization (FISH) using the eubacterial universal probe EUB338 as well as probes specifically targeting *Firmicutes* (LGC354A), *Actinobacteria* (HGC69a), *Bacteroidetes* (CF319), α- (ALF1b), β- (BET42a), γ-*Proteobacteria* (GAM42a) and *Enterobacteriales* (EntB and EntD). Because bacterial distribution within fruiting bodies can be highly non-homogenous (Antony-Babu *et al*., 2013), quantification of the bacterial communities was done by homogenizing gleba samples through grinding. *T. borchii* bacterial community composition was comparable with the one previously described in *T. borchii* (Barbieri *et al*., 2007), with an overall majority of *Proteobacteria* (Fig. 1) among which α- and β-*Proteobacteria* were dominant. γ-*Proteobacteria,* including *Enterobacteriales,* and bacteria belonging to *Bacteroidetes* were also detected but at a much lower level. No *Firmicutes* or *Actinobacteria* could be detected at day 0 (Fig. 1). The overall bacterial community evolved upon storage with a shift of community composition with time. The quantity of bacteria first tended to increase at 2 days and was then reduced at day 6. A similar reduction in community size over time was observed in the α- and the β-*Proteobacteria.* The *Bacteroidetes*, the *Enterobacteriales*, the *Actinobacteria* and the γ-*Proteobacteria* populations remained quite low and stable during storage. Last, the *Firmicutes* population size slightly increased during the last days of storage.

Additionally to address bacterial cell distribution within undisrupted truffle tissues, thin sections of *T. borchii* were hybridized with FISH probes against α- and β-*Proteobacteria*, the two main bacterial groups identified in ground samples. α- and β-*Proteobacteria* could be observed in both the peridium and the gleba by confocal microscopy imaging (Fig. 2). Dense colonies of α- and β-*Proteobacteria* were observed in the peridium. However, this colonization was patchy with vast area without visible bacterial cells (Fig. 2A and B). Bacterial cells were present in between fungal cells but not inside the fungal cells in both gleba and peridium. Colonies of α-*Proteobacteria* did not appear to contain bacteria from other phyla in the peridium as illustrated by the complete overlay of *Eubacteria* and alpha probes (Fig. 2A). In contrast, β-*Proteobacteria* were found in mixed population with other bacteria (Fig. 2B) as demonstrated by the absence of overlay between eubacterial probe (FITC, green) and β-*Proteobacteria* (cy3, red). In the gleba, α-*Proteobacteria* were found as isolated cells as well as dense colonies (Fig. 2C) while β-*Proteobacteria* were more evenly distributed (Fig. 2D).

Bacterial community composition differ between peridium and gleba

Antony-Babu and colleagues (2013) recently demonstrated that bacterial communities from *Tuber melanosporum* strongly differed between the peridium and the gleba. To determine if a similar pattern was also present

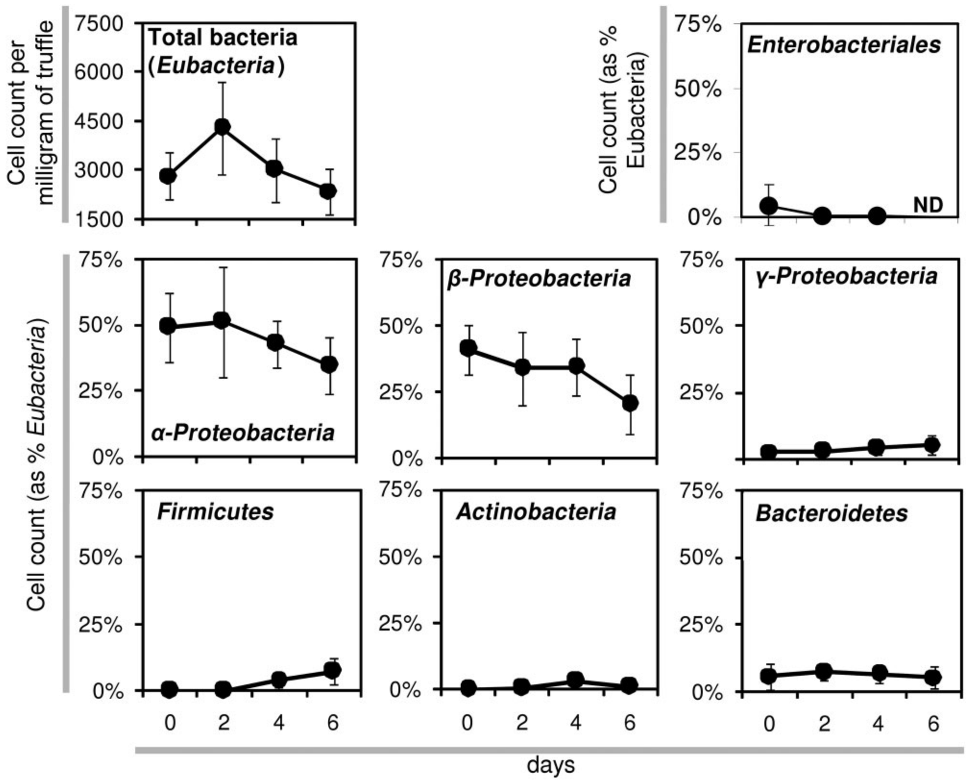

Fig. 1. Change in the bacterial population inside *T. borchii* fruiting bodies as a function of storage time. The panels show bacterial population dynamics (±SE) of all bacteria [*Eubacteria* expressed as bacterial cell count per milligram of truffle-fruiting body (dry weight)] and specific classes/groups (expressed as percentage of the total *Eubacteria*). The community was dominated by α-and β-*Proteobacteria*. No statistical difference was detected among storage days within *Eubacteria* or bacterial classes/groups ($n = 6$ truffle-fruiting bodies, for all ANOVA: $0.03 < F < 1.62$; $0.22 < P < 0.99$). *Enterobacteriales* data from FISH probe EntD (probe EntB gave comparable results – not shown).

in *T. borchii*, we compared by FISH bacterial community composition in gleba and peridium samples at day 0. Only the population size of *Bacteroidetes* significantly differed between gleba and peridium. The population size of the latter phylum was five times higher in the peridium compared with the gleba (gleba: 137 ± 122; peridium: 697 ± 101; unit: cell count/mg dry weight; *P*-value: 0.026, Mann–Whitney *U*-test). No statistical difference between peridium and gleba was observed for the *Firmicutes* (gleba: 0 ± 0; peridium: 10 ± 10), *Actinobacteria* (gleba: 0 ± 0; peridium: 45 ± 45), α-*Proteobacteria* (gleba: 1370 ± 499; peridium: 947 ± 365), β-*Proteobacteria* (gleba: 1356 ± 453; peridium: 1336 ± 483), γ-*Proteobacteria* (gleba: 115 ± 82; peridium: 137 ± 43) and *Enterobacteriales* (gleba: 240 ± 210; peridium: 6 ± 3).

Concentration of thiophene volatiles in T. borchii *gleba correlates with the bacterial abundance*

Concentrations of thiophene volatiles have been reported to increase with the storage of truffle-fruiting bodies at room temperature (Bellesia *et al*., 2001), suggesting a potential role of bacteria in the production of these volatile compounds. We hypothesized that the concentrations of thiophene volatiles correlated with bacterial community composition and abundance within truffle-fruiting bodies. To test this hypothesis, thiophenes produced from the same gleba samples characterized by FISH were quantified by solid-phase microextraction–gas chromatography/mass spectrometry (SPME-GC/MS). Thiophene volatiles **(1)** and **(2)** were emitted all along the time course. The total density of *Eubacteria* were significantly correlated with the levels of thiophene volatile **(2)** only [volatile **(1)**: Pearson $R^2 = 0.008$, $P = 0.686$; volatile **(2)**: $R^2 = 0.220$, $P = 0.021$). A significant correlation for volatiles **(1)** and/or **(2)** was also observed for the dominant α-*Proteobacteria* and a minor group representing *Bacteroidetes* (for α-*Proteobacteria*, volatile **(1)**: $R^2 = 0.171$, $P = 0.045$; volatile **(2)**: $R^2 = 0.172$, $P = 0.044$; for *Bacteroidetes*, volatile **(1)**: $R^2 = 0.002$, $P = 0.821$; volatile **(2)**: $R^2 = 0.303$, $P = 0.005$).

Bacteria isolated from fruiting bodies, but not T. borchii *mycelia, are able to generate thiophene volatiles from* T. borchii*-fruiting bodies*

The correlation between the evolution of bacterial communities during storage and the concentration of

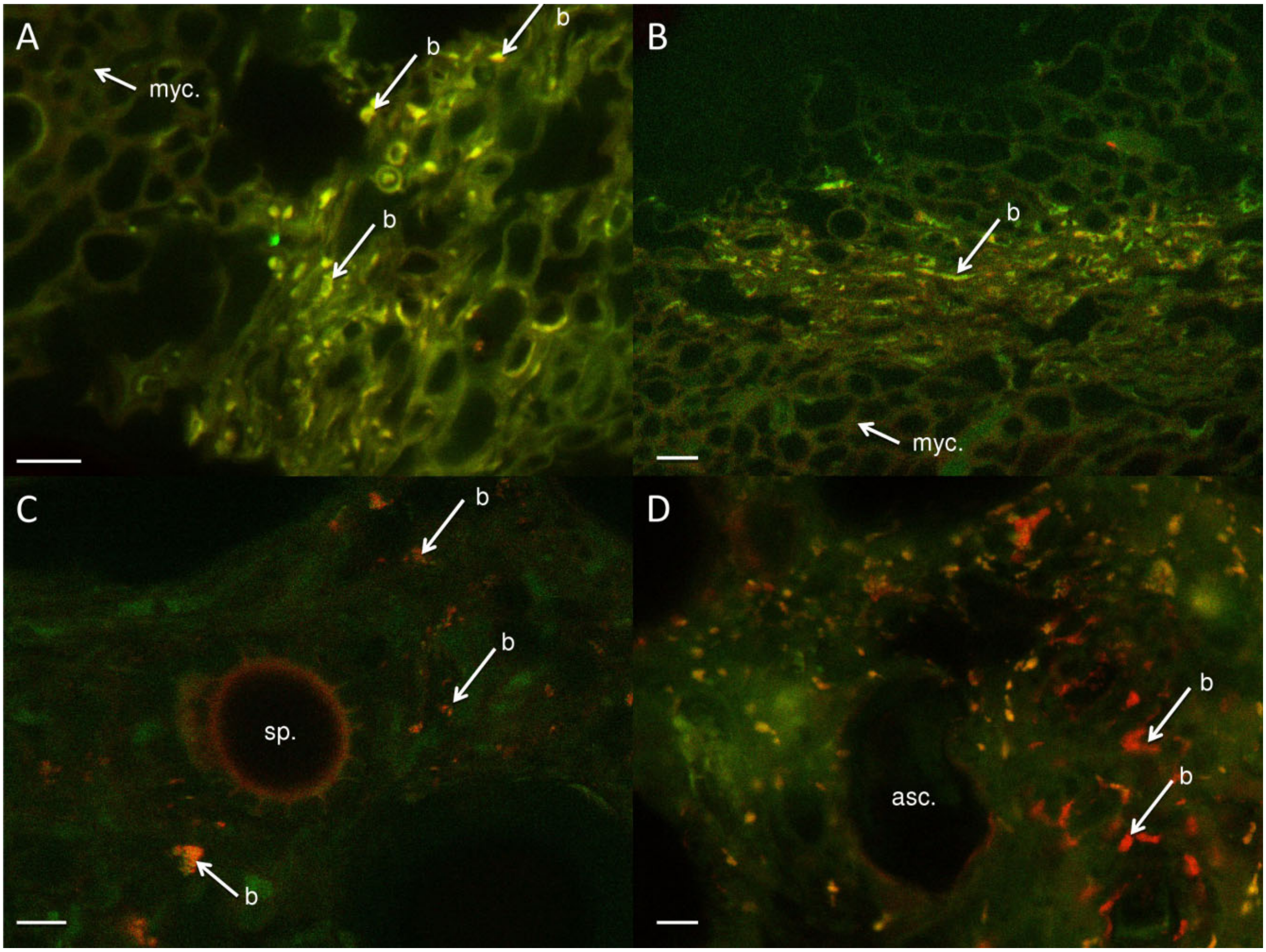

Fig. 2. Localization of α- and β-*Proteobacteria* in thin sections of *T. borchii* peridium and gleba.
A,B. Hybridization of 30 μm sections of *T. borchii* of peridium.
C,D. Hybridization of gleba (C,D) with the universal eubacteria Eub338 mix probe coupled to FITC (A, B, C) or cy3 (D) and with α-*Proteobacteria* specific probe coupled to cy3 (A, C) or β-*Proteobacteria* specific probe BET42A coupled to cy3 (B) or FITC (D) as observed by confocal microscopy.
White bars represent 10 μm. asc., ascii; b, bacterial cluster; myc., fungal cell; sp., spore. Each picture is representative of observations.

thiophene volatiles led us to hypothesize that some bacteria could be involved in the production of these compounds. To test this hypothesis, we tested the ability of bacteria isolated from *T. borchii*-fruiting bodies to produce thiophene compounds. Bacteria were isolated from soil adhering to the peridium and from the gleba of *T. borchii*-fruiting bodies on tryptic soy agar (TSA 3%) plates. Based on colony PCR and 16S rRNA sequences, the isolated strains belonged to β- and γ-*Proteobacteria*, *Bacteroidetes*, *Firmicutes* and *Actinobacteria* (Table 1). We were not able to isolate strains from the α-*Proteobacteria* phylum despite their high abundance in the fruiting body. Since those bacteria could also be involved in *T. borchii* aroma, we tested two *Rhizobiales* strains obtained from the German Collection of Microorganisms and Cell Cultures (Table 1).

A bioassay approach was used to test the ability of single bacterial strains to transform ethyl acetate extracts of truffle-fruiting bodies into thiophene volatiles (Supporting Information Fig. S2). Surprisingly, thiophene volatiles **(1)** and **(2)** were produced by all bacterial isolates but only in the presence of fruiting body extract (Fig. 3A and B). The amount of thiophene compounds produced varied between bacterial strains, *Proteobacteria* and more especially α-, β-*Proteobacteria* and *Enterobacteriales* among the γ-*Proteobacteria* being the most efficient producers (Fig. 3B). Surprisingly thiophene volatiles **(1)** and **(2)** were not only produced by bacteria isolated from *T. borchii*-fruiting bodies, but also by bacterial isolated from other sources (i.e. soil, plants, Table 1). The quantities of thiophene volatiles **(1)** and **(2)** released in our bioassays were similar to ~10 times lower to those detected from

Table 1. Bacteria tested in this study for their ability to produce thiophene volatiles. List of bacteria isolated from *T. borchii* (from TartufLangue, Cuneo, Italy, and collected 03/2008) and elsewhere.

Isolate reference in the present manuscript	Isolated from	Gleba or peridium/ adhering soil	GenBank Accession Number 16S rRNA	Bacterial phylum/class or order	Genus
A	*T. borchii*	Gleba	KC618433	*Actinobacteria*	*Microbacterium*
B	*T. borchii*	Gleba	KC618434	*Bacteroidetes/Flavobacteria*	*Chryseobacterium*
C	*T. borchii*	Peridium/adhering soil	KC618435	*Bacteroidetes/Sphingobacteria*	*Sphingobacterium*
D	*T. borchii*	Gleba	KC618436	*Bacteroidetes/ Sphingobacteria*	*Sphingobacterium*
E	*T. borchii*	Peridium/adhering soil	KC618437	*Firmicutes/Bacillales*	*Brochothrix*
F	*T. borchii*	Peridium/adhering soil	KC618438	*Firmicutes/Bacillales*	*Brochothrix*
G	*T. borchii*	Peridium/adhering soil	KC618439	*β-proteobacteria/Burkholderiales*	*Comamonas*
H	*T. borchii*	Gleba	KC618440	*β-proteobacteria/Burkholderiales*	*Comamonas*
I	*T. borchii*	Peridium/adhering soil	KC618441	*γ-proteobacteria/Enterobacteriales*	*Serratia*
J (*Serratia plymuthica* IC14[b])	Soil (melon plantation)	–	–	*γ-proteobacteria/Enterobacteriales*	*Serratia*
K (*Serratia marcescens* MG1[b])	Cucumber (fruit)	–	–	*γ-proteobacteria/Enterobacteriales*	*Serratia*
L (*Serratia proteamaculans* B5a[b])	Salmon	–	–	*γ-proteobacteria/Enterobacteriales*	*Serratia*
M	*T. borchii*	Peridium/adhering soil	KC618442	*γ-proteobacteria/Pseudomonadales*	*Pseudomonas*
N	*T. borchii*	Gleba	KC618443	*γ-proteobacteria/Pseudomonadales*	*Pseudomonas*
O	*T. borchii*	Peridium/adhering soil	KC618444	*γ-proteobacteria/Xanthomonadales*	*Stenotrophomonas*
P	*T. borchii*	Gleba	KC618445	*γ-proteobacteria/Xanthomonadales*	*Stenotrophomonas*
Q (Ensifer adhaerens strain LFG19a; DSMZ ref. 18131[a])	Not determined	–	–	*α-proteobacteria/Rhizobiales*	*Ensifer*
R (Bosea thiooxidans strain BI-42; DSMZ ref. 9653[a])	Agricultural Soil	–	AJ250796	*α-proteobacteria/Rhizobiales*	*Bosea*

a. Available from the German Collection of Microorganisms and Cell Cultures (www.dsmz.de).
b. Isolates of *Serratia* tested in an earlier report for their plant-growth-promoting properties (Blom *et al.*, 2011) (kindly provided by Dr. Laure Weisskopf, University of Zürich, Switzerland).

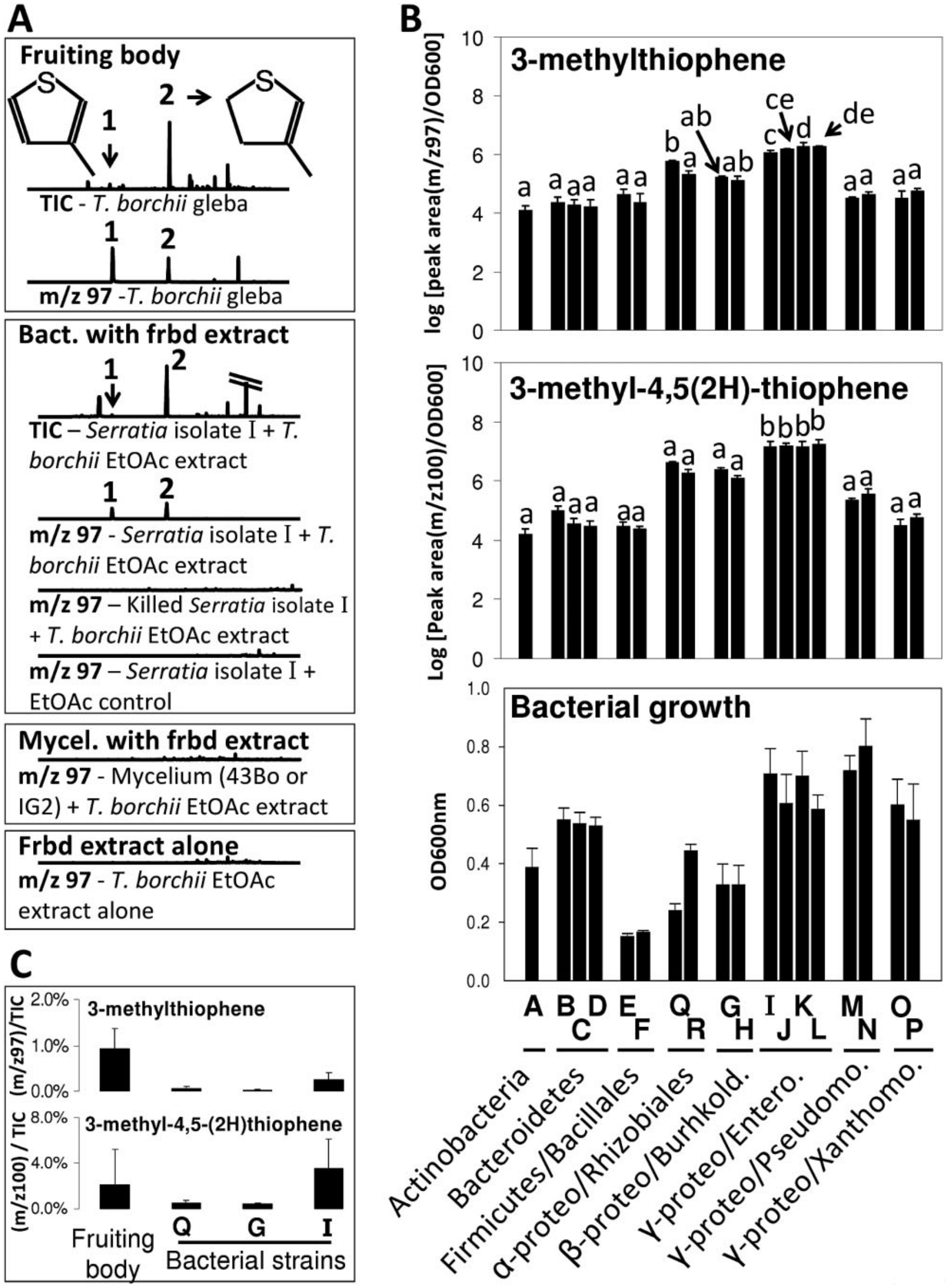

Fig. 3. Ability of bacteria and truffle mycelium to produce thiophene volatiles.
A. Chromatograms [total ion current (TIC) and extracted m/z 97] illustrating that bacteria but not truffle mycelium have the ability to transform truffle extract into two volatiles characteristic of *T. borchii*-fruiting bodies; volatile **(1)** is 3-methylthiophene and volatile **(2)** is 3-methyl-4,5(2H)thiophene. Chromatograms are shown with normalized intensities.
B. Levels (±SE) of thiophene volatiles **(1)** and **(2)** (log scale normalized to growth) and corresponding growth (±SE) (OD600) of different bacterial classes/groups ($n = 3$) (isolates are coded from A to R – See Table 1). Different letters denote values that differ significantly from each other (Fischer LSD post-hoc test $P < 0.05$) – ANOVA for 3-methylthiophene: $F = 15.94$, $P = 0.000000$; ANOVA for 3-methyl-4,5(2H)thiophene: $F = 6.26$, $P < 0.001$].
C. Quantities of thiophenes (1) and (2) released per SPME vial by *T. borchii* gleba ($n = 6$) and in the bioassays with bacteria ($n = 3$ per strain) described in Supporting Infomation Fig. S1. To make the values comparable between bioassays and gleba, thiophene quantities (extracted masses m/z 97 and m/z100) have been normalized to the TIC of the chromatograms.

T. borchii-fruiting bodies (Fig. 3C). Killed bacteria by autoclaving did not produce thiophene volatiles indicating that active metabolism was required (Fig. 3A).

To test whether the fungus was also able to produce thiophene volatiles by itself, volatile profiles of *in vitro* pure culture of *T. borchii* strain 43Bo isolated in 1996 were analysed. No thiophene volatiles were produced by *T. borchii* mycelial cultures *in vitro* (Fig. 3A). To make sure that the inability of *T. borchii* mycelial culture to produce thiophene volatiles was not a consequence of prolonged storage in pure culture of strain 43Bo, mycelium was freshly isolated from a fruiting body producing thiophene volatiles. The newly isolated strain IG2 was not able to produce any thiophene derivatives, confirming the previous results. Altogether these data indicate that bacteria but not truffle mycelium are able to biotransform the non-volatile precursors of *T. borchii* gleba into volatile thiophenes.

The precursor of thiophene volatiles is specific of the sexual stage of T. borchii *and of no other truffle species*

In a second step, we tested whether bacteria could produce thiophene volatiles when grown on *T. borchii* mycelium. Most surprisingly, even the most efficient thiophene-producer under our assay's conditions, an *Enterobacteriales* of the *Serratia* genus (Strain I, Table 1), failed to produce the thiophene volatiles typical of *T. borchii*-fruiting bodies when grown on sterilized *T. borchii* mycelium (strain 43Bo) that had been pre-grown in pure culture. Live mycelial cultures of *T. borchii* strain 43Bo also failed to produce thiophene volatiles on malt extract with or without addition of L-methionine, which had been suggested to be the precursor of thiophene volatiles in *T. borchii* (Zeppa *et al*., 2004). Methionine, however, did induce in live mycelial cultures (strain 43Bo) the synthesis of numerous sulphur (non-thiophene) volatiles such as dimethyl sulphide and dimethyl disulphide (data not shown). These results suggest that mycelium of *T. borchii* does not produce the precursors of thiophenes *in vitro.* Furthermore, the *Serratia* isolate I (Table 1) produced the thiophene volatiles **(1)** and **(2)** on *T. borchii* gleba but not when grown on the gleba of other truffle species, including the white truffle *T. magnatum* or the black truffles *T. aestivum*, *T. mesentericum* and *T. brumale*.

Overall, these results indicate that thiophene production is linked to the sexual stage (fruiting body) of *T. borchii*. They also demonstrate that the aroma precursor of thiophene volatiles is/are unique to *T. borchii*.

Antibacterial but not antifungal agents inhibit the production of thiophene volatiles in T. borchii-*fruiting bodies*

To further investigate which organisms are responsible for the production of specific truffle-fruiting body volatiles, we used antimicrobial agents targeting either fungi or bacteria. A homogenate of *T. borchii*-fruiting bodies was treated with aqueous solutions containing either the broad-spectrum antibacterial agent streptomycin, or one of the antifungal agents clotrimazole or amphotericin B, or pure water as a control. The concentrations of volatiles **(1)** and **(2)** were measured at the beginning of the experiment and after 48 h (Fig. 4). Production of thiophene volatiles **(1)** and **(2)** was only suppressed by the bactericide streptomycin (Fig. 4). The concentration of thiophenes **(1)** and **(2)** in samples treated with the fungicides (48 h, Fig. 4) increased to the same level as in the water control (Fig. 4), indicating that fungal metabolism was not necessary for the production of thiophene volatiles. Furthermore, the concentration of thiophene volatiles **(1)** and **(2)** induced by the different treatments correlated with the bacterial population density in the gleba samples (cultivable fraction of bacteria – Fig. 4).

Bacteria fed with methionine produce a volatile related in structure to the thiophenes characteristic of T. borchii*-fruiting bodies*

Interaction of fungi with bacteria might activate fungal biosynthetic pathways that are otherwise suppressed in pure mycelial cultures (Scherlach and Hertweck, 2009; Schroeckh *et al*., 2009). We therefore co-cultured our *Serratia* bacterial isolate I (Table 1) with mycelia of white and black truffles, with or without an excess of L-methionine. Regardless of L-methionine addition, neither volatile **(1)** and **(2)** was detected. Surprisingly, another thiophene volatile, 2-methyltetrahydrothiophen-3-one [designated **(3)**], was detected in the presence of excess methionine not only from co-cultures of white or black truffle mycelia with *Serratia* but also from the single bacterial cultures without mycelium (Fig. 5). Cultures of truffle mycelia supplemented with extra methionine failed to produce volatile **(3)** when *Serratia* was absent (Fig. 5), confirming our hypothesis that the formation of thiophene derivatives by truffle-fruiting bodies depends on bacteria generating sulfur-containing heterocycles from linear precursor(s) such as L-methionine.

Discussion

Some fungi are hotspot for bacteria (Warmink *et al*., 2009). Truffle-fruiting bodies do not derogate to this observation and are intensively colonized by bacteria that can reach densities up to 10^8 cells per gram of truffle (Barbieri *et al*., 2005; 2007; Antony-Babu *et al*., 2013). Similar to what has been previously described (Barbieri *et al*., 2005), samples of *T. borchii* analysed here were intensely colonized by bacteria. In line with previous reports about *T. borchii* (Barbieri *et al*., 2005),

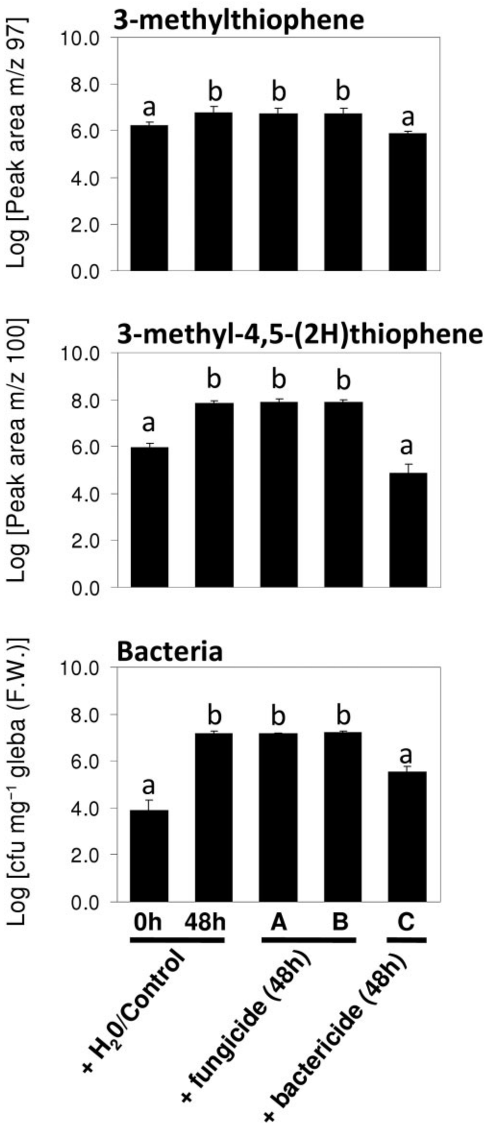

Fig. 4. Effect of antimicrobial treatments on truffle volatiles. Mean levels (±SD – log scale) of thiophene volatiles **(1)** and **(2)** and fraction of cultivable bacteria in fruiting body homogenate of *T. borchii* upon treatment with:
A. H_20/control or aqueous solutions of the antifungal agents clotrimazole
B. Amphotericin B.
C. Antibacterial agent streptomycin
n = 9 replicates per treatment. Compared with the initial concentrations of thiophene volatiles released at the beginning of the experiment (H20/Control, 0h), synthesis of thiophene volatiles was only blocked by the antibacterial agent streptomycin (C). The increase in thiophene concentrations observed in the samples treated with the antifungal agents (A, B) for 48 h were equivalent to the H2O/Control 48 h, demonstrating that truffle fungi are not involved in the production of thiophene volatiles. Different letters indicate significant differences among treatments ($P < 0.05$, ANOVA and Fischer LSD *post-hoc* test).

T. magnatum (Barbieri *et al*., 2007) and *T. melanosporum* (Antony-Babu *et al*., 2013), the bacterial community was dominated by α-*Proteobacteria.* These similarities between studies and between truffles species suggest that truffle-fruiting bodies, regardless the species, provide a specific habitat for some bacteria. This core microbiome of truffle-fruiting bodies might be supplemented with additional species depending on the fungal species, the maturation stage or the environment as suggested by the divergence observed for some classes. For example the abundance of β-*Proteobacteria* (Fig. 1) was much higher here compared with earlier reports on white truffles *T. borchii* (Barbieri *et al*., 2005) and *T. magnatum* (Barbieri *et al*., 2007).

α-*Proteobacteria* are very versatile in adapting to diverse environments ranging from the oceans (volcanoes on the ocean floor, surface waters) to soil (Ettema and Andersson, 2009). They also have an intriguing ability to interact with plants in either pathogenic or non-pathogenic mutualist/commensal relations (i.e. *Rhizobium*, *Azospirillum*). In fungi, the α-*Proteobacteria* phylum is

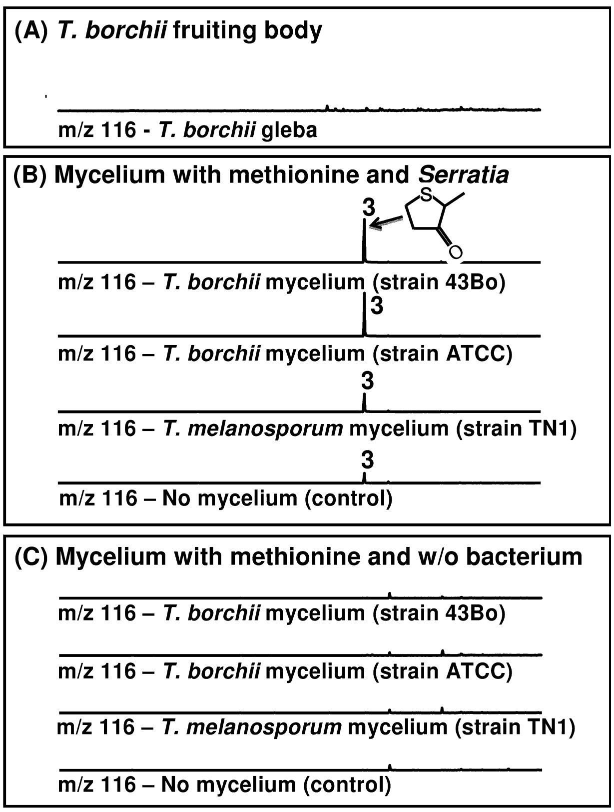

Fig. 5. Methionine induces thiophene derivative 2-methyltetrahydrothiophen-3-one in *Serratia*.
A. Volatile **(3)** was not detected from *T. borchii* fruiting bodies.
B. It was detected whenever the bacterium was present.
C. It was not detected when the bacterium was absent.
Chromatograms (extracted mass m/z 116) are shown with normalized intensities.

dominant not only in truffles (Barbieri *et al*., 2005; 2007; Antony-Babu *et al*., 2013), but also in the ectomycorrhizal fungus *Laccaria bicolor* (Bertaux *et al*., 2005), suggesting the existence of some adaptative or competitive mechanism over other bacterial phyla. What creates the competitive advantage for α-*Proteobacteria* in truffles is unknown, but theoretically it might be driven by an increased ability to compete for specific resources (niche effect) or to degrade/produce toxic secondary metabolites (Hibbing *et al*., 2010). A study on the genome of 92 α-*Proteobacteria* revealed major genetic differences between those whose lifestyles were associated to plants (symbiotic and non-symbiotic) compared with those that were not plant-associated (Pini *et al*., 2011). By similarity, genetic adaptation might also exist in α-*Proteobacteria* associated to fungi and might possibly result from co-evolution between bacteria and their fungal hosts. In the specific case of the Périgord truffle *T. melanosporum*, the abundance of α-*Proteobacteria* was shown to increase in the truffle gleba over the period from a collection season (Antony-Babu *et al*., 2013). Taken together with the information that nitrogen fixation actively takes place in truffle-fruiting bodies of *T. magnatum* (Barbieri *et al*., 2010), and that some members of the α-*Proteobacteria* are well known for their ability to fix nitrogen, this suggests that the dominance of the latter bacterial phylum in truffles might be driven by special nutritional requirements. Another possibility might be that α-*Proteobacteria* might be better at degrading antibacterial agents (maybe the precursor of thiophene derivatives) than other bacterial phyla. Both theories, only speculative at this stage, will need to be tested experimentally.

In addition, our work suggests the existence of a structuration of the bacterial communities within the fruiting bodies of *T. borchii*. As observed in *T. melanosporum* (Antony-Babu *et al*., 2013), the composition of bacterial communities in the peridium diverged from the one of the gleba with an enrichment of bacteria belonging to the *Bacteroidetes* phylum in the peridium of both truffle species. Interestingly, our data also suggest that bacterial taxa could behave differently within the truffle with α-*Proteobacteria* tending to form 'pure' colonies, while β-*Proteobacteria* being present in mixed communities. These patterns suggest the existence of regulated network of interactions between bacteria in the truffle-fruiting bodies. Mechanisms regulating these networks and their consequence on the community function remain to be explored.

If the presence of bacteria in truffle-fruiting bodies is known since decades, the nature of the interactions occurring between bacterial communities and truffle fungi remain unclear. Do bacteria only consume truffle nutrients or do mutualistic interactions occur between the fungal host and the bacterial community? One of our aims was to investigate if thiophene derivatives, volatiles characteristic of *T. borchii's* aroma, were actually derived from the truffle fungus itself or from the microbiome inhabiting truffle-fruiting bodies. By following an approach similar to Koch's postulate in microbiology, we demonstrated that the bacterial community inhabiting *T. borchii*-fruiting bodies was responsible for the production of thiophene derivatives **(1)** and **(2)** characteristic of the fruiting body aroma of the latter species. Such intimate interactions between bacteria and fungi have been documented earlier, for example in the case of the rice fungal pathogen *Rhizopus* which toxins are derived from endobacteria of the *Burkholderia* genus (Partida-Martinez and Hertweck, 2005). Unlike in the *Rhizopus*/*Burkholderia* case, the interaction in truffles is not limited to a single bacterial genus, and indeed all bacteria tested in this study were able to produce thiophene volatiles from *T. borchii*-fruiting body extract, irrespective of whether they had been isolated from truffles or from other sources (i.e. plant, soil). This indicates that the ability to produce thiophene volatiles is widespread among bacteria and might possibly be linked to primary metabolism. This also raises the question of the ecological role of thiophene volatiles in nature.

Methionine was suggested to be the precursor of thiophene volatiles in *T. borchii* (Zeppa *et al*., 2004). Methionine did induce in our assays with *Serratia* volatile **(3),** a thiophene of related structure to volatiles **(1)** and **(2)**, but not the latter two volatiles. There is no data in literature on the pathways or the genes leading to the synthesis of thiophene volatiles **(1)** and **(2).** However, data exist on the synthesis of volatile **(3)**, both in yeast (Howell *et al*., 2005) and in bacteria (Nawrath *et al*., 2010). In the yeast *Saccharomyces cerevisiae*, volatile **(3)** might be derived from the cyclization and reorganization of 4-mercapto-4-methylpentan-2-one (4-MMP), itself released from the cleavage from a cystein moiety (Cys-4-MMP). Volatile **(3)** is furthermore strongly induced by the deletion of YAL012W, a putative carbon-sulfur lyase, suggesting an enzymatic biosynthesis route in yeast (Howell *et al*., 2005). A BLAST search of YAL012W against 3271 expressed sequence tags of *T. borchii* (Lazzari *et al*., 2007) did not reveal any match in the white truffle; this suggests that the latter gene might either not be expressed or might be absent in *T. borchii.* In the *bacterium Chitinophaga Fx7914,* volatile **(3)** is derived from two primary metabolites, homocysteine and pyruvate (Nawrath *et al*., 2010). By analogy to the intermediates and pathways of the latter two examples, thiophene volatiles **(1)** and **(2)** might be derived in truffles from primary metabolites synthesized or transformed by truffles, but also by yeasts (Buzzini *et al*., 2005) and bacteria.s

The production of volatiles **(1)** and **(2)** by bacteria from *T. borchii* gleba (or gleba extract) but not from other truffle species supports the inference that *T. borchii* is involved in the synthesis of at least one intermediate leading to thiophene volatiles **(1)** and **(2)**. Additionally, the upregulation in sulfur metabolism in the fruiting bodies of *T. borchii* (Zeppa *et al.*, 2010) and *T. melanosporum* (Martin *et al.*, 2010; Splivallo and Maier, 2011; Splivallo *et al.*, 2011) provides indirect evidence that truffles are actively involved in the production of sulfur metabolites during their sexual stage. Indeed the upregulation in homocysteine synthase (*tbhos*) and putative sulfate transporter (*tbsul1*) in mature *T. borchii*-fruiting bodies suggests that these genes might be indirectly involved in the synthesis of volatiles (Zeppa *et al.*, 2010).

Overall, this strongly suggests that thiophene volatiles are derived from bacteria and further suggests that the non-volatile precursors of thiophene compounds might be produced by the intimate interactions of truffles, yeasts and bacteria. In contrast, other non-thiophene volatiles might be completely synthesized by the fungus itself. The complex blend of 20 to 50 molecules creating the aroma of *T. borchii* would thus originate from the mixed activity of the fungus and its microbiome.

Our results demonstrate that bacteria are central players in the production of thiophene volatiles **(1)** and **(2)** in *T. borchii.* The formation of other volatiles or the contribution of bacteria to aroma formation in other truffle species remains to be investigated. Similarly, the full biosynthetic pathway leading to thiophene volatiles and the exact contribution of yeasts and truffle remains to be elucidated. Our results open new horizons in the biotechnological production of fungal aromas suggesting that microbes might play a central role in the production of key fungal odorants (Splivallo and Maier, 2011). They also raise new ecological and mechanistic questions about the multitrophic interactions among truffles, their plant symbionts, mammals, insects and the bacterial community associated with truffle-fruiting bodies. By participating in the elaboration of sulphur volatile compounds that attract mammals, bacteria could indirectly participate in the dissemination of truffle spores and thus play a key role in the life cycle of the fungus.

Experimental procedures

Truffle-fruiting bodies

Truffle-fruiting bodies of *T. borchii*, *T. magnatum*, *T. aestivum*, *T. mesentericum* and *T. brumale* were purchased (Supporting Information Table S1) and were identified based on spore morphology (Ceruti *et al.*, 2003).

Truffle mycelial cultures

Mycelia of *T. borchii* isolated more than 15 years ago (strain 1Bo = ATCC 96540 and 43Bo) (Bonuso *et al.*, 2009) as well as a strain (strain IG2 – GenBank Accession KF414978) freshly isolated from a *T. borchii*-fruiting body which produced volatiles **(1)** and **(2)**) and *T. melanosporum* (strain TN1) were grown as described earlier (Splivallo *et al.*, 2012). For the time series and feeding experiment with methionine, mycelial cultures and culture-negative controls (no mycelium) in malt extract broth were homogenized with a blender and transferred to SPME vials (aliquots of 4.5 ml per vial). Vials were supplemented with 0.5 ml of an aqueous solution containing either L-methionine (final concentration in SPME vials 5 mM) or water (control). Samples were either incubated in the dark or under 16 h photoperiods (to reflect natural conditions in the soil) at 23°C. Volatiles were measured by SPME-GC/MS after 1, 2, 3, and 5 days of incubation as described hereafter.

Selection of bacterial strains

Bacteria were isolated from the surface (peridium and adhering soil) and the inner part (gleba) of six fresh mature *T. borchii*-fruiting bodies (purchased in 2008 from TartufLangue, Cuneo, Italy). Unwashed truffles were shortly vortexed in a sterile 0.85% NaCl solution, and a ~300 mg piece of gleba was subsequently excised and homogenized in 0.85% NaCl. Bacteria were isolated from the NaCl solutions by dilution plating on TSA 3% (Barbieri *et al.*, 2005). A total of 29 strains were selected based on their colony morphology and colour (not shown). The full 16S rRNA was amplified by colony PCR using the universal primers 'UP-Forward' and 'UP-Reverse' (Barbieri *et al.*, 2000; 2005). Sequencing was performed from both ends by Macrogen Europe (Amsterdam, the Netherlands).

The ability of bacteria to produce thiophene volatiles was tested on the following isolates: (i) A sub-selection of 13 bacterial isolates from our 29 strains was done reflecting the typical bacterial diversity in *T. borchii* (Barbieri *et al.*, 2005) and included two strains of β- *Proteobacteria*, five strains of γ-*Proteobacteria*, three strains of the *Bacteroidetes*, two strains of the *Firmicutes* and one strain of the *Actinobacteria* (GenBank accession numbers are listed in Table 1). (ii) Five bacterial strains isolated from sources different from truffle-fruiting bodies (i.e. plant, soil) were also tested for their ability to produce thiophene volatiles. They included two strains of α-*Proteobacteria* and three strains of *Serratia* (*Enterobacteriales*) (Table 1). All bacterial isolates were stored at −70°C in 1:1 glycerol (85%) : tryptic soy broth (TSB). Before bioassays were performed, bacterial glycerol stocks were plated on TSA (3%) to check for purity.

Analysis of aroma profiles by SPME-GC/MS

Aroma of truffle gleba (300 mg), bacterial and mycelial cultures were profiled by SPME-GC/MS as described earlier (Splivallo *et al.*, 2012). The occurrence of thiophene volatiles 3-methylthiophene **(1)** and 3-methyl-4,5-dihydrothiophene **(2)** was confirmed in *T. borchii* samples collected independently in Piedmont ($n = 20$ samples collected in 2006, 2008 – See Supporting Information Table S1). The identity of all volatiles listed in this study was confirmed through their MS fragmentation patterns, Kovats indices and when available with synthetic standards (i.e. 3-methylthiophene and see also Supporting Information Table S2).

Bioassays with fruiting body ethyl acetate extract

To test the ability of bacteria isolated from *T. borchii*-fruiting bodies and of the other bacterial isolates from soil and plants (Table 1), as well as the ability of truffle mycelium to generate thiophene volatiles, 490 g *T. borchii*-fruiting bodies were freeze dried, homogenized in a mortar and extracted overnight with 2 × 400 ml hexane followed by 2 × 400 ml ethyl acetate. The ethyl acetate fractions (800 ml) were pooled, concentrated till dryness in a vacuum evaporator at 50°C and resuspended in 450 ml malt extract broth (1%, pH 7.0). A negative control containing only dried ethyl acetate was prepared in the same way. The malt extract solution was sterile filtered (0.22 μm) and transferred (900 μl) to sterile SPME vials. SPME vials were either inoculated with (i) 100 μl of actively growing bacterial cultures (single strains in TSB 30 g l^{-1}), with (ii) TSB only (negative control for the bacterial cultures) or with (iii) a pellet of 100 μl of *Serratia* Isolate I previously killed by autoclaving (another negative control for the bacterial cultures to make sure that active bacterial growth was required to produce thiophene volatiles) – see Supporting Information Fig. S1 for experimental design – or with (iv) *T. borchii* mycelium strain 43Bo or IG2, or with (v) malt extract broth (control for the mycelial cultures described in *Truffle mycelial cultures*). SPME vials containing bacterial cultures (and the malt extract control) were incubated under gentle vertical shaking at 15°C, reflecting spring soil temperatures typical of Italian regions where *T. borchii* truffles are collected. Furthermore, emission of thiophene volatiles and bacterial growth were determined after 25 h of incubation, which corresponds to the late exponential growth phase for our *Serratia* isolate I strain and the maximum emission of thiophene volatiles **(1)** and **(2)**. SPME vials containing homogenized truffle mycelium were incubated for 25 h in the dark at 23°C (optimal growth condition for *T. borchii*) before generating volatile fingerprints.

Determination of bacterial growth

Bacterial growth was measured at 600 nm (OD600) after blank subtraction (100 μl of bacterial culture or malt extract broth per well) in an Epoch 96-well microtiter plate spectrophotometer (BioTeck Instruments, VT, USA).

Bioassays with dried fruiting body and mycelium homogenates

Truffle-fruiting bodies (1–3 g per species) of *T. borchii*, *T. magnatum*, *T. aestivum* (syn. *T. uncinatum*), *T. mesentericum*, *T. brumale* (Supporting Information Table S1) or mycelial cultures of *T. borchii* (strains 1Bo and 43Bo) (Bonuso *et al*., 2009), and *T. melanosporum* (strain TN1) and negative culture controls (prepared as described in *Truffle mycelial cultures*), were freeze dried and each sample was homogenized separately to a fine powder in a mortar. The homogenates were sterilized with 10 ml CH_2Cl_2 (overnight incubation), after which CH_2Cl_2 was removed from the homogenates in a two-step process (vacuum evaporation at 50°C for 6 h followed by heating to 80°C for 3 h). A quantity of 50 mg of sterile fruiting body homogenate or mycelium homogenate was transferred to a sterilized 20 ml SPME vial, which was then inoculated with either 100 μl bacterial glycerol stock (1:1 bacterium in TSB : 85% glycerol), or with 100 μl 1:1 TSB : 85% glycerol as control or with truffle mycelium following the same scheme described in *Bioassays with fruiting body ethyl acetate extract*. The detailed scheme of the growth conditions and volatile sampling is described in Supporting Information Fig. S2.

Bioassays with mixed cultures truffle mycelium/bacteria

A quantity of 2.0 g of truffle mycelial cultures (*T. borchii* strains 1Bo and 43Bo, and *T. melanosporum* strain TN1) and culture-negative controls (without mycelium), prepared as described earlier (Splivallo *et al*., 2012), were transferred to 20 ml SPME vials and inoculated with 100 μl of *Serratia sp.* isolate I (identified as *Serratia sp.* based on 16S rRNA sequencing) glycerol stock (1:1 bacterium grown in TSB:85% glycerol) or with 100 μl 1:1 TSB : 85% glycerol as control. Samples in SPME vials were either incubated as such or supplemented with excess L-methionine (3 mg per SPME vials supplied in 100 μl H_2O) in order to check if excess methionine induced the production of thiophene volatiles. Samples were incubated for 48 h at 23°C in the dark before generating the aroma fingerprinting by SPME-GC/MS.

Treatment of fruiting bodies with antibacterial and antifungal agents

Two *T. borchii* truffles (~40 g in total) were homogenized in a mortar until obtaining a paste which was then transferred to 2.0 ml Eppendorf tubes containing each 300 mg truffle homogenate. Sterile water was added to the samples: 1.0 ml per tube of either pure water (control) or water containing the broad-spectrum antibacterial agent streptomycin (400 μg ml^{-1}) or one of the antifungal agents clotrimazole (400 μg ml^{-1}) or amphotericin B (250 μg ml^{-1}). Tubes were closed and incubated at 20°C on a rotary shaker (160 r.p.m.). After 48 h incubation, samples were centrifuged (2 min at 12 000 g), the aqueous phase was removed from the gleba homogenate, and the latter was then transferred to 20 ml SPME vials to quantify thiophene volatiles **(1)** and **(2)**. Additionally, volatile fingerprinting was performed on 300 mg gleba homogenate at the beginning of the experiment (0 h) to measure the initial quantities of thiophene volatiles **(1)** and **(2)**.

Fluorescence in situ hybridization (FISH)

Six fruiting bodies of *T. borchii* were washed with tap water, dried and cut using a sterile scalpel to generate four subsamples of 500 mg each per truffle-fruiting body. Samples were stored in 2.0 ml Eppendorf tubes and allowed to age at room temperature for 0 (samples processed immediately), 2, 4 and 6 days. Each subsample was divided in two parts, one 300 mg sample for volatile fingerprinting by SPME-GC/MS and one 200 mg sample which was fixed for subsequent FISH analysis. Fixation was performed in 2.0 ml Eppendorf tubes by incubating samples overnight at 4°C in 1200 μl of a 2.25% paraformaldehyde solution in phosphate saline buffer (PBS) – PBS = 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, 0.24 g of KH_2PO_4 in 100 ml distilled H_20, pH adjusted to 7.4.

Samples were washed with 3 × 1.0 ml ice-cold PBS after fixation and stored in 1:1 EtOH96% : PBS at −20°C until FISH analysis. FISH was performed on fruiting body homogenates (gleba samples at days 0, 2, 4 and 6 and peridium samples at day 0) or on thin sections of gleba and peridium samples (30 μm) as described for *T. magnatum* (Barbieri *et al*., 2007) with the probes listed in Supporting Information Table S3. Each sample was co-hybridized with the universal probe mix Eub338 coupled to FITC to quantify bacterial population and a phylum specific probe coupled to cy3 (Supporting Information Table S3). Signals representing all bacterial cells and specific phyla were quantified visually on homogenized samples after image acquisition under an epifluorescence microscope (BX41, Olympus). Each homogenized sample was mounted and hybridized independently two times and three images were acquired for each replicate (6 images in total). The mean bacterial cell number was normalized to the sample biomass and expressed as [cell count/mg dry weight of gleba]. Fluorescent signals representing *Firmicutes*, α-, β-, γ-*Proteobacteria*, *Bacteroidetes* and *Enterobacteriales* (Supporting Information Table S3) were counted directly under the fluorescence microscope and expressed as the percentage of *Eubacteria* (mean of $n \geq 3$ observation from the same sample). Values expressed as %*Eubacteria* were then transformed to [cell count / mg dry weight of gleba] after multiplication by the total bacterial cell count in the sample.

Thin sections of *T. borchii*-fruiting bodies (30 μm) were hybridized with the probes specific for α- and β-*Proteobacteria* listed in Supporting Information Table S3 and observed with a Radiance 2100 Rainbow confocal microscope (Bio-Rad).

Data processing

All bioassays in SPME vials performed to check the capacity of bacteria or truffle mycelia to produce thiophene volatiles comprised at least three independent replicates. Treatment of *T. borchii* fruiting body with antimicrobial agents was performed on four replicates per treatment. Six *T. borchii*-fruiting bodies were used for FISH (subsamples at 0, 2, 4 and 6 days of aging).

Volatiles fingerprints were processed as described earlier (Splivallo *et al*., 2012). Specific m/z typical of thiophene volatiles were furthermore extracted from the total ion chromatograms for data visualization and statistics (m/z 97 occurs both in compounds **(1)** and **(2),** while m/z 100 occurs in **(2)** only and m/z 106 in 2-methyltetrahydrothiophen-3-one **(3)** only – MS fragmentation patterns are shown in Supporting Information Fig. S3.

Acknowledgements

We would like to thank the following people for their support and for material provided: Prof. Ivo Feussner and Dr. Cornelia Herfurt for access to the GC/MS and Dr. Laure Weisskopf for providing the *Serratia* strains MG1, B5a and IC14 (Supporting Information Table S1); Prof. Alessandra Zambonelli for *T. borchii* mycelia strains 1Bo and 43Bo, and Dr. Gérard Chevalier for *T. melanosporum* mycelium strain TN1. Financial support was provided by the Deutsche Forschungsgemeinschaft – DFG (http://www.dfg.de, DFG Grants SPL1191/2-1 and SPL1191/4-1 to RS). AD lab is supported by the Laboratory of Excellence ARBRE (ANR-11-LABX-0002-01).

Conflict of interest statement

RS declares that a patent has been filed regarding the production of truffle aroma using truffle-associated microbes (Splivallo and Maier, 2011). The other author(s) declare that they have no competing interests.

References

Antony-Babu, S., Deveau, A., Van Nostrand, J.D., Zhou, J., Le Tacon, F., Robin, C., *et al*. (2013) Black truffle-associated bacterial communities during the development and maturation of Tuber melanosporum ascocarps and putative functional roles. *Environ Microbiol*. doi:10.1111/1462-2920.12294.

Barbieri, E., Potenza, L., Rossi, I., Sisti, D., Giomaro, G., Rossetti, S., *et al*. (2000) Phylogenetic characterization and in situ detection of a Cytophaga-Flexibacter-Bacteroides phylogroup bacterium in Tuber borchii vittad. Ectomycorrhizal mycelium. *Appl Environ Microbiol* **66:** 5035–5042.

Barbieri, E., Bertini, L., Rossi, I., Ceccaroli, P., Saltarelli, R., Guidi, C., *et al*. (2005) New evidence for bacterial diversity in the ascoma of the ectomycorrhizal fungus Tuber borchii Vittad. *FEMS Microbiol Lett* **247:** 23–35.

Barbieri, E., Guidi, C., Bertaux, J., Frey-Klett, P., Garbaye, J., Ceccaroli, P., *et al*. (2007) Occurrence and diversity of bacterial communities in *Tuber magnatum* during truffle maturation. *Environ Microbiol* **9:** 2234–2246.

Barbieri, E., Ceccaroli, P., Saltarelli, R., Guidi, C., Potenza, L., Basaglia, M., *et al*. (2010) New evidence for nitrogen fixation within the Italian white truffle Tuber magnatum. *Fungal Biol* **114:** 936–942.

Bellesia, F., Pinetti, A., Tirillini, B., and Bianchi, A. (2001) Temperature-dependent evolution of volatile organic compounds in *Tuber borchii* from Italy. *Flavour Fragrance J* **16:** 1–6.

Bertaux, J., Schmid, M., Hutzler, P., Hartmann, A., Garbaye, J., and Frey-Klett, P. (2005) Occurrence and distribution of endobacteria in the plant-associated mycelium of the ectomycorrhizal fungus Laccaria bicolor S238N. *Environ Microbiol* **7:** 1786–1795.

Blom, D., Fabbri, C., Connor, E.C., Schiestl, F.P., Klauser, D.R., Boller, T., *et al*. (2011) Production of plant growth modulating volatiles is widespread among rhizosphere bacteria and strongly depends on culture conditions. *Environ Microbiol* **13:** 3047–3058.

Bonito, G.M., Gryganskyi, A.P., Trappe, J.M., and Vilgalys, R. (2010) A global meta-analysis of Tuber ITS rDNA sequences: species diversity, host associations and long-distance dispersal. *Mol Ecol* **19:** 4994–5008.

Bonuso, E., Zambonelli, A., Bergemann, S.E., Iotti, M., and Garbelotto, M. (2009) Multilocus phylogenetic and coalescent analyses identify two cryptic species in the Italian bianchetto truffle, Tuber borchii Vittad. *Conserv Genet* **11:** 1453–1466.

Buscot, F., Munch, J.C., Charcosset, J.Y., Gardes, M., Nehls, U., and Hampp, R. (2000) Recent advances in exploring physiology and biodiversity of ectomycorrhizas highlight the functioning of these symbioses in ecosystems. *FEMS Microbiol Rev* **24:** 601–614.

Buzzini, P., Gasparetti, C., Turchetti, B., Cramarossa, M.R., Vaughan-Martini, A., Martini, A., *et al.* (2005) Production of volatile organic compounds (VOCs) by yeasts isolated from the ascocarps of black (Tuber melanosporum Vitt.) and white (Tuber magnatum Pico) truffles. *Arch Microbiol* **184:** 187–193.

Ceruti, A., Fontana, A., and Nosenzo, C. (2003) *Le specie europee del genere Tuber: Una revisione storica* (Torino: Museo regionale di scienze naturali di Torino).

Culleré, L., Ferreira, V., Chevret, B., Venturini, M.E., Sánchez-Gimeno, A.C., and Blanco, D. (2010) Characterisation of aroma active compounds in black truffles (Tuber melanosporum) and summer truffles (Tuber aestivum) by gas chromatography–olfactometry. *Food Chem* **122:** 300–306.

Ettema, T.J.G., and Andersson, S.G.E. (2009) The alpha-proteobacteria: the Darwin finches of the bacterial world. *Biol Lett* **5:** 429–432.

Gryndler, M., Soukupová, L., Hršelová, H., Gryndlerová, H., Borovička, J., Streiblová, E., and Jansa, J. (2013) A quest for indigenous truffle helper prokaryotes. *Environ Microbiol Rep* **5:** 346–352.

Hibbing, M.E., Fuqua, C., Parsek, M.R., and Peterson, S.B. (2010) Bacterial competition: surviving and thriving in the microbial jungle. *Nat Rev Microbiol* **8:** 15–25.

Howell, K.S., Klein, M., Swiegers, J.H., Hayasaka, Y., Elsey, G.M., Fleet, G.H., *et al*. (2005) Genetic determinants of volatile-thiol release by Saccharomyces cerevisiae during wine fermentation. *Appl Environ Microbiol* **71:** 5420–5426.

Lazzari, B., Caprera, A., Cosentino, C., Stella, A., Milanesi, L., and Viotti, A. (2007) ESTuber db: an online database for Tuber borchii EST sequences. *BMC Bioinformatics* **8** (Suppl. 1)**:** S13.

Martin, F., Duplessis, S., Ditengou, F., Lagrange, H., Voiblet, C., and Lapeyrie, F. (2001) Developmental cross talking in the ectomycorrhizal symbiosis: signals and communication genes. *New Phytol* **151:** 145–154.

Martin, F., Kohler, A., Murat, C., Balestrini, R., Coutinho, P.M., Jaillon, O., *et al*. (2010) Perigord black truffle genome uncovers evolutionary origins and mechanisms of symbiosis. *Nature* **464:** 1033–1038.

Mauriello, G., Marino, R., DAuria, M., Cerone, G., and Rana, G.L. (2004) Determination of volatile organic compounds from truffles via SPME-GC-MS. *J Chromatogr Sci* **42:** 299–305.

Maxmen, A. (2010) Truffle's savoury secret revealed. *Nat News*. doi:10.1038/news.2010.156.

Mello, A., Murat, C., and Bonfante, P. (2006) Truffles: much more than a prized and local fungal delicacy. *FEMS Microbiol Lett* **260:** 1–8.

Nawrath, T., Gerth, K., Müller, R., and Schulz, S. (2010) The Biosynthesis of the Aroma Volatile 2-Methyltetrahydrothiophen-3-one in the Bacterium Chitinophaga Fx7914. *Chembiochem* **11:** 1914–1919.

Pacioni, G., Leonardi, M., Aimola, P., Ragnelli, A.M., Rubini, A., and Paolocci, F. (2007) Isolation and characterization of some mycelia inhabiting Tuber ascomata. *Mycol Res* **111:** 1450–1460.

Partida-Martinez, L.P., and Hertweck, C. (2005) Pathogenic fungus harbours endosymbiotic bacteria for toxin production. *Nature* **437:** 884–888.

Pavić, A., Stanković, S., Saljnikov, E., Krüger, D., Buscot, F., Tarkka, M., and Marjanović, Ž. (2013) Actinobacteria may influence white truffle (Tuber magnatum Pico) nutrition, ascocarp degradation and interactions with other soil fungi. *Fungal Ecol* **6:** 527–538.

Pini, F., Galardini, M., Bazzicalupo, M., and Mengoni, A. (2011) Plant-bacteria association and symbiosis: are there common genomic traits in alphaproteobacteria? *Genes* **2:** 1017–1032.

Read, D.J. (1991) Mycorrhizas in ecosystems. *Experientia* **47:** 376–391.

Sbrana, C., Bagnoli, G., Bedini, S., Filippi, C., Giovannetti, M., and Nuti, M.P. (2000) Adhesion to hyphal matrix and antifungal activity of Pseudomonas strains isolated from Tuber borchii ascocarps. *Can J Microbiol* **46:** 259–268.

Sbrana, C., Agnolucci, M., Bedini, S., Lepera, A., Toffanin, A., Giovannetti, M., and Nuti, M.P. (2002) Diversity of culturable bacterial populations associated to Tuber borchii ectomycorrhizas and their activity on T. borchii mycelial growth. *FEMS Microbiol Lett* **211:** 195–201.

Scherlach, K., and Hertweck, C. (2009) Triggering cryptic natural product biosynthesis in microorganisms. *Org Biomol Chem* **7:** 1753–1760.

Schroeckh, V., Scherlach, K., Nützmann, H.-W., Shelest, E., Schmidt-Heck, W., Schuemann, J., *et al*. (2009) Intimate bacterial-fungal interaction triggers biosynthesis of archetypal polyketides in Aspergillus nidulans. *Proc Natl Acad Sci USA* **106:** 14558–14563.

Splivallo, R., and Maier, C. (2011) Production of natural truffle flavours from truffle mycelium (in French). Patent publication nb PCT/IB2010/052913.

Splivallo, R., Bossi, S., Maffei, M., and Bonfante, P. (2007) Discrimination of truffle-fruiting body versus mycelial aromas by stir bar sorptive extraction. *Phytochemistry* **68:** 2584–2598.

Splivallo, R., Ottonello, S., Mello, A., and Karlovsky, P. (2011) Truffle volatiles: from chemical ecology to aroma biosynthesis. *New Phytol* **189:** 688–699.

Splivallo, R., Valdez, N., Kirchhoff, N., Ona, M.C., Schmidt, J.-P., Feussner, I., and Karlovsky, P. (2012) Intraspecific genotypic variability determines concentrations of key truffle volatiles. *New Phytol* **194:** 823–835.

Talou, T., Gaset, A., Delmas, M., Kulifaj, M., and Montant, C. (1990) Dimethyl sulphide: the secret for black truffle hunting by animals? *Mycol Res* **94:** 277–278.

Warmink, J.A., Nazir, R., and van Elsas, J.D. (2009) Universal and species-specific bacterial 'fungiphiles' in themycospheres of different basidiomycetous fungi. *Environ Microbiol* **11:** 300–312.

Zeppa, S., Gioacchini, A.M., Guidi, C., Guescini, M., Pierleoni, R., Zambonelli, A., and Stocchi, V. (2004) Determination of specific volatile organic compounds synthesised duringTuber borchii fruit body development by

solid-phase microextraction and gas chromatography/ mass spectrometry. *Rapid Commun Mass Spectrom* **18:** 199–205.
Zeppa, S., Marchionni, C., Saltarelli, R., Guidi, C., Ceccaroli, P., Pierleoni, R., *et al*. (2010) Sulfate metabolism in Tuber borchii: characterization of a putative sulfate transporter and the homocysteine synthase genes. *Curr Genet* **56:** 109–119.

Supporting information

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

Supporting Information Fig. S1. Schematic representation of the bioassay used to test the ability of bacteria to transform truffle-fruiting body extract into thiophene volatiles.
Supporting Information Fig. S2. Schematic representation of the bioassay used to test the ability of bacteria to transform truffle-fruiting body homogenate into thiophene volatiles.
Supporting Information Fig. S3. MS fragmentation pattern of thiophene volatiles.
Supporting Information Table S1. Countries/regions of origin and species of truffle-fruiting bodies used in this study.
Supporting Information Table S2. Volatiles listed in this study and mode of identification.
Supporting Information Table S3. List of FISH probes used in this study.