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Phylogenetic Characterization and In Situ Detection of a *Cytophaga-Flexibacter-Bacteroides* Phylogroup Bacterium in *Tuber borchii* Vittad. Ectomycorrhizal Mycelium

ELENA BARBIERI,[1] LUCIA POTENZA,[1] ISMAELA ROSSI,[1] DAVIDE SISTI,[2] GIOVANNA GIOMARO,[2] SIMONA ROSSETTI,[3] CLAUDIA BEIMFOHR,[4] AND VILBERTO STOCCHI[1]*

"Giorgio Fornaini" Institute of Biochemistry[1] and Institute of Botany,[2] University of Urbino, 61029 Urbino, and Water Research Institute, CNR, 00198 Rome,[3] Italy, and Vermicon AG, 80992 Munich, Germany[4]

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Mycorrhizal ascomycetous fungi are obligate ectosymbionts that colonize the roots of gymnosperms and angiosperms. In this paper we describe a straightforward approach in which a combination of morphological and molecular methods was used to survey the presence of potentially endo- and epiphytic bacteria associated with the ascomycetous ectomycorrhizal fungus *Tuber borchii* Vittad. Universal eubacterial primers specific for the 5′ and 3′ ends of the 16S rRNA gene (16S rDNA) were used for PCR amplification, direct sequencing, and phylogenetic analyses. The 16S rDNA was amplified directly from four pure cultures of *T. borchii* Vittad. mycelium. A nearly full-length sequence of the gene coding for the prokaryotic small-subunit rRNA was obtained from each *T. borchii* mycelium studied. The 16S rDNA sequences were almost identical (98 to 99% similarity), and phylogenetic analysis placed them in a single unique rRNA branch belonging to the *Cytophaga-Flexibacter-Bacteroides* (CFB) phylogroup which had not been described previously. In situ detection of the CFB bacterium in the hyphal tissue of the fungus *T. borchii* was carried out by using 16S rRNA-targeted oligonucleotide probes for the eubacterial domain and the *Cytophaga-Flexibacter* phylum, as well as a probe specifically designed for the detection of this mycelium-associated bacterium. Fluorescent in situ hybridization showed that all three of the probes used bound to the mycelium tissue. This study provides the first direct visual evidence of a not-yet-cultured CFB bacterium associated with a mycorrhizal fungus of the genus *Tuber*.

The mycorrhizal ascomycetous fungi belonging to the genus *Tuber*, commonly called truffles, are obligate ectosymbionts that colonize the roots of gymnosperms and angiosperms (23, 39, 45). Ectomycorrhizal fungi are present in natural and agricultural ecosystems, provide health benefits to plants, and contribute to soil nutrient cycling. The symbiotic development of mycorrhizal fungi on plant roots has been reported to be influenced by bacteria present in the mycorrhizosphere (10, 16, 35, 47). Although various bacterial populations, such as fluorescent *Pseudomonas* strains and the spore-forming bacteria *Micrococcus* spp., *Moraxella* spp., *Corynebacterium* spp., and *Staphylococcus* spp., have been isolated from truffles (3, 14, 20), no molecular evidence has been found concerning the relationship between these organisms and their specific locations in the *Tuber* host tissue. There is also no information concerning the endo- and epiphytic bacteria which throughout life or during part of the life cycle may invade the tissues of the living fungus and cause unapparent and asymptomatic infections throughout the fungal life cycle and thus during production of the mycelium, contact with the host root, and development of the ectomycorrhizae and fruit bodies.

Since at least 95% of all soil bacteria have not been cultured (2, 25, 36), bacteria isolated from truffles could represent only a fraction of the entire natural bacterial community associated with truffles. For this reason in this study we attempted to use a combination of morphological and molecular 16S rRNA-based approaches to survey the presence of potential bacterial endo- and epiphytes associated with the ectomycorrhizal fungus *Tuber borchii* Vittad. These tools have been used previously to identify endophytic bacteria such as the *Burkholderia* endosymbiont of *Gigaspora margarita* (7) and members of the alpha and beta subclasses of the *Proteobacteria* detected as epibionts in ectomycorrhizae of *Fagus sylvatica*, *Lactarius vellereus*, and *Lactarius subdulcis* (33).

In order to achieve our goal, we used a model for in vitro ectomycorrhizal synthesis developed recently for biotechnological applications (43).

MATERIALS AND METHODS

Biological materials: mycelia, mycorrhizal roots, and bacteria. Four different mycelia (1BO [= ATCC 96540], 10RA, 17BO, and Z43) were isolated from fresh *T. borchii* fruit bodies collected in natural truffle grounds in central Italy. Dried samples of each specimen are preserved in the herbarium of the Mycology Center of Bologna (Bologna, Italy). The isolates were grown in the dark at 24°C with no agitation in modified Melin-Norkrans nutrient solution (MMN) (pH 6.6) by using the method of Molina (34). Each 100-ml flask contained 70 ml of medium inoculated with fungus cultured in potato dextrose agar plugs, as described by Saltarelli et al. (41). Ectomycorrhizae of *T. borchii* were obtained aseptically in vitro in a peat-vermiculite nutrient mixture from infection of *Tilia platyphyllos* Scop. with mycelium strains 1BO, 10RA, 17BO, and Z43 (43). *Pseudomonas fluorescens* B20 and *Bacillus subtilis* C15 were isolated on tryptic soy agar (Oxoid) from a *T. borchii* fruit body collected in central Italy (20).

Morphological observations. Extreme care was taken to avoid bacterial contamination: all solutions used in this study were filter sterilized, and sterile procedures were used during fixing and/or crushing. Microscopic observations were carried out with each culture of *T. borchii* mycelium and with the medium used. The mycelium strains were grown in parallel in potato dextrose agar plates on which sterile cover slides were placed. The hyphae growing on the cover slides were directly stained with 4′,6′-diamidino-2-phenylindole (DAPI) (0.01 μg/ml). Samples were viewed with a Zeiss Axioskope microscope (Carl Zeiss, Milan, Italy) with contrast phase and epifluorescence and equipped with UV, rhodamine, or fluorescein excitation filter sets.

DNA extraction. Mycelial genomic DNA was extracted from 1-month-old cultures of *T. borchii* 1BO, 10RA, 17BO, and Z43 by using the protocol described by Erland et al. (17). Ectomycorrhizal and plant DNA were extracted by using the method of Henrion et al. (24). Bacterial DNA was extracted directly from

* Corresponding author. Mailing address: "Giorgio Fornaini" Institute of Biochemistry, University of Urbino, Via Saffi, 2, 61029 Urbino (PU), Italy. Phone: 39 0722 305262. Fax: 39 0722 320188. E-mail: vstocchi@uniurb.it.

TABLE 1. Primers and probes used in the present study

Primer or probe	Type	Sequence (5′-3′)	Position[a]	Reference
Primers				
b-17BO-f		AAC CCT TTC ACG TGT G	451–465	This study
UP-Forward	Eubacteria	AGA GTT TGAT YM TGG C	8–24	48
UP-Reverse	Eubacteria	GYT ACC TTG TTA CGA CTT	1493–1513	48
Probes				
CF319	*Cytophaga-Flexibacter* cluster	TGG TCC GTG TCT CAG TAC	319–336	32
EUB338	Eubacteria	GCT GCC TCC CGT AGG AGT	338–355	1
STBb-654		GCC CAC ATC ATC TGT ACT	654–672	This study

[a] *E. coli* numbering (9).

colonies by using the standard phenol DNA extraction method (42). In order to eliminate the possible presence of contaminants from our templates, DNA from a different clone of the same mycelium strain, 1BO (= ATCC 96540), was extracted in the laboratory of P. Bonfante, University of Turin, Turin, Italy. Furthermore, to confirm the presence of the bacterium in other *T. borchii* mycelium strains, DNA was extracted from strain B2 (at the University of Urbino, Urbino, Italy), as well as strains A1 and A2 (at Centre INRA, Clermont Ferrand, France), and DNA from a strain of *Neurospora crassa* was used as a negative control.

PCR conditions. Before starting our study, we confirmed that the mycelium strains belonged to the species *T. borchii* Vittad. by using PCR strategies developed in previous work on species-specific identification (5, 6). Once the mycelia were identified as *T. borchii*, amplification of the 16S rRNA gene (rDNA) of bacteria potentially associated with the ectomycorrhizal fungus was performed in 25-μl (final volume) mixtures by using 100- to 200-ng portions of genomic DNA from mycelia, ectomycorrhizae, nonmycorrhizal roots, and bacteria. Universal eubacterial primers (UP-Forward and UP-Reverse) were used to amplify the 16S rDNA from all samples (Table 1). A specific primer was designed based on the sequence data obtained from mycelium strain 17BO (b-17BO-f [Table 1]). The specificity of primer b-17BO-f was checked with the Ribosomal Database Project (RDP) (31) sequence database, and no matches with other available sequences were found. Primer b-17BO-f was used in combination with UP-Reverse to specifically detect the endophytic bacterium in other *T. borchii* mycelium strains and in other samples. The PCR conditions were as follows: 30 cycles consisting of denaturation at 94°C for 45 s, annealing at 53°C for 45 s, and elongation at 72°C for 2 min. Amplified products were purified with a QIAquick PCR purification kit (Qiagen GmbH, Hilden, Germany) and then digested with *Taq*I, *Alu*I, and *Msp*I enzymes for restriction fragment length polymorphism analyses. Representative PCR products with identical restriction fragment length polymorphism patterns were chosen for direct sequencing with an ABI Prism cycle sequencing kit (dRhodamine terminator cycle sequencing kit with AmpliTaq DNA polymerase FS; Perkin-Elmer).

Phylogenetic analysis. The Check_Chimera program of the RDP was used to search for chimeric sequences. Closely related or phylogenetically relevant sequences were obtained from the RDP database and the DDBJ/EMBL/GenBank databases. Sequences were aligned on the basis of secondary structure by using the RDP data and the sequence alignment editor with SeqPup, version 0.5 (22). Sequences from the RDP database served as the alignment guidelines. Corrected pairwise distances were computed by using the Jukes-Cantor correction (26). A distance matrix was inferred with the DNADIST program, and phylogenetic trees were constructed from the evolutionary distance matrix by using FITCH and from the alignment by using the DNAPAR program for parsimony analysis and DNAML for maximum-likelihood analysis, as implemented in the PHYLIP software package, version 3.5c (18). Bootstrap analyses were based on 200 resamplings of the sequence alignment and were performed with DNABOOT as implemented in PHYLIP, version 3.5c. The TreeView program was used to plot the tree files (37).

Fluorescent in situ hybridization (FISH): oligonucleotide probes. The 16S rRNA sequence was added to the VERMICON 16S rRNA database containing about 12,000 16S rRNA sequences by using the ARB program package (http://www.biol.chemie.tu-muenchen.de/pub/ARB/). The ARB_EDIT tool was used for sequence alignment. Probe design was computed by using the appropriate tool in the ARB software package. The nucleotide sequence of the 16S rRNA-targeted oligonucleotide probe STBb-654, which is specific for the *Cytophaga-Flexibacter-Bacteroides* phylogroup (CFB) bacterium described here, is shown in Table 1. This probe was labeled at the 5′ end with Cy3 (MWG Biotech AG, Ebersberg, Germany). Probes specific for the *Cytophaga-Flexibacter* group (CF319) (32), labeled at the 5′ end with fluorescein, and for the bacterial domain (EUB338) (1), labeled at the 5′ end with fluorescein, were purchased from Amersham Pharmacia Biotech, Cologno Monzese MI, Italy. The probe specifically designed for detecting the CFB bacterium and probe CF319 were used with hyphal tissue homogenates fixed in ethanol and paraformaldehyde (2). All the probes were used with DAPI and/or the EUB338 probe.

In situ hybridization. Mycelia growing in MMN liquid medium were fixed in formaldehyde–70% ethanol–acetic acid (5:90:5), dehydrated in a graded aqueous ethanol series (70, 80, 95, and 100% ethanol), clarified in xylol, embedded in paraffin wax (56 to 58°C), and cut with a rotary microtome (Top Rotary M, S132 Pablish) (thickness, 8 to 10 μm). The sections were mounted on glass slides, and the paraffin was removed by immersion in xylene for 15 min. Thin sections used for in situ hybridization with fluorescent probes were rehydrated with a graded ethanol series by 5-min incubations in 98%, 80%, and 60% ethanol. The same specimens were homogenized by using a sterile glass pestle and 500 μl of sterile MMN liquid medium, washed with sterile phosphate-buffered saline, and fixed in ethanol and paraformaldehyde as described by Amann et al. (2); this was followed by DAPI staining. Homogenized samples consisted of hyphal fragments ranging from 7.3 to 33 μm long. Fixed samples of hyphal tissue homogenate were immobilized on glass slides by air drying and were dehydrated in 60, 80, and 98% (vol/vol) ethanol (3 min each). In situ hybridizations were performed as previously described by Amann et al. (2). The optimal hybridization stringency for probe STBb-654 was obtained by adding formamide to a final concentration of 35%. For combinations of probes with different optimal hybridization stringencies, two hybridizations were done successively; hybridization with the probe which required the higher formamide concentration (EUB338) was performed first, and this was followed by a second hybridization at the lower stringency with the other specific probes (STBb-654 or CF319). Each hybridization set included an unstained sample used as a control for autofluorescence.

Nucleotide sequence accession numbers. The 16S rDNA sequences of *T. borchii* mycelium bacterial endophytes b-17BO, b-Z43, b-10RA, and b-1BO have been deposited in the DDBJ/EMBL/GenBank databases under accession numbers AF070444, AF233292, AF233293, and AF233294, respectively.

RESULTS

Morphological observations. DAPI staining revealed several cytoplasmic structures, as well as nuclei, in the 15-day-old cultures of *T. borchii* mycelia analyzed (1BO [= ATCC 96540], 10RA, 17BO, and Z43). Although several cytoplasmic rounded organelle-like structures and ca. two nuclei for each septum were fluorescent, no external bacteria or bacterium-like organelles were observed.

PCR assays. Molecular data obtained by PCR, as well as FISH, provided consistent proof of a bacterial presence in the ectomycorrhizal mycelium of *T. borchii* Vittad. High-molecular-weight DNA was extracted from the *T. borchii* Vittad. mycelium strains, and 16S rDNAs were amplified with eubacterium-specific PCR primers. The resulting products were estimated to be approximately 1,500 bp (Fig. 1A). Negative controls with no template consistently gave no amplification products (Fig. 1). Consensus sequence data encompassing 1,457 bp were obtained from the individual strains and were submitted to the DDBJ/EMBL/GenBank databases as b-17BO, b-1BO, b-10RA, and b-Z43. These sequences differed on average by about 1%.

A specific primer (b-17BO-f) was designed based on the variable region (positions 451 to 465) of the b-17BO 16S rDNA sequence, in order to check for the occurrence of this bacterium in vitro by using the model recently developed for ectomycorrhizal synthesis of *T. borchii* mycelium and micropropagated *T. platyphyllos* Scop. plantlets. This model was developed

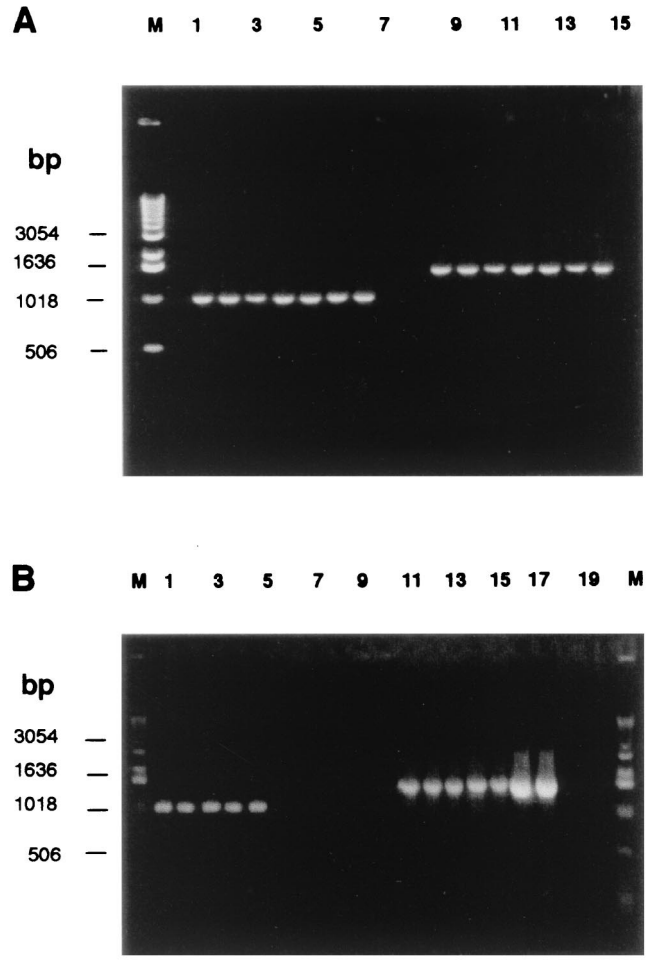

FIG. 1. PCR experiments. PCR assays were performed to check for the presence of the CFB bacterium in *T. borchii* Vittad. ectomycorrhizal mycelium. (A) Agarose (1%) gel electrophoresis of PCR products amplified with the b-17BO-f and UP-Reverse primers (lanes 1 to 8) or eubacterial primers UP-Forward and UP-Reverse (lanes 9 to 15). The templates used were *T. borchii* mycelium strains 1BO (= ATCC 96540) (lanes 1 and 9), 17BO (lanes 2 and 10), 10RA (lanes 3 and 11), Z43 (lanes 4 and 12), B2 (lanes 5 and 13), and A1 (lanes 6 and 14) and 1BO template extracted in the laboratory of P. Bonfante, University of Turin (lanes 7 and 15); no DNA was included in lanes 8 and 16. Lane M contained a fragment size marker (1-kb DNA ladder; GIBCO/BRL). (B) Agarose (1%) gel electrophoresis of PCR products amplified with the b-17BO-f and UP-Reverse primers (lanes 1 to 10) or eubacterial primers UP-Forward and UP-Reverse (lanes 11 to 20). The control templates used were ectomycorrhizae of *T. borchii* on *T. platyphyllos*, including ectomycorrhizae of 1BO (lanes 1 and 11), 17BO (lanes 2 and 12), 10RA (lanes 3 and 13), and Z43 (lanes 4 and 14). Mycelium strain 1BO was used as a positive control (lanes 5 and 15). Bacterial strains, including *P. fluorescens* C5 (lanes 6 and 16) and *B. subtilis* C15 (lanes 7 and 17), *N. crassa* (lanes 8 and 18), mycelial growth medium (lanes 9 and 19), and no DNA (lanes 10 and 20) were also used. Lanes M contained a fragment size marker (1-kb DNA ladder; GIBCO/BRL).

under controlled sterile conditions (43), in which exposure to bacterial infection or contamination was avoided and influence of other biotic and abiotic factors on the interactions between the fungus and the host was ruled out. Primer b-17BO-f was used in combination with the universal reverse primer in PCR assays performed with *T. borchii* mycelia and roots of *T. platyphyllos* Scop. infected with *T. borchii*. To assess primer specificity and to rule out the possibility that the amplified and sequenced DNA fragments were derived from contaminants, controls were tested with both universal and specific primer sets. Primer b-17BO-f gave a product of the expected length (1,043 bp) for all *T. borchii* mycelia tested and for roots infected with *T. borchii* (Fig. 1A). No amplification was obtained from nonmycorrhizal *T. platyphyllos* roots, *N. crassa*, *P. fluorescens*, *B. subtilis*, and the medium used for mycelium growth. PCR products of the expected length were obtained from the controls when the eubacterial primers were used. *T. platyphyllos* roots, *N. crassa*, and the medium gave no amplification products (Fig. 1B). The 1,043-bp band obtained from the four mycelial ectomycorrhizae and the other mycelial strains of *T. borchii* used as controls (B2, A1, and 1BO) was digested with the *Taq*I, *Alu*I, and *Msp*I restriction enzymes. Each enzyme provided the same patterns for mycelia and ectomycorrhiza samples (data not shown), suggesting high sequence similarity. This hypothesis was confirmed by the nearly identical sequences obtained (ca. 1% difference).

All of the sequences obtained were of bacterial origin due to the specificity of the UP-Forward primer; however, a search was performed by using the RDP mitochondrial database. This screening analysis revealed a range of similarity values between 13.5 and 14% for 1,281 nucleotides aligned with the mitochondrial rRNA gene sequences of some *Aspergillus* species (27). A pairwise comparison between the b-17BO sequence and a small-subunit mitochondrial sequence from a fruit body of *T. borchii* (44) revealed only about 15% similarity. This result ruled out the possibility of mycelial small-subunit mitochondrial gene amplification.

Phylogenetic analysis. In order to describe the relationship between the presumptive bacterial endo- or epiphyte and previously recognized bacterial taxa, extensive phylogenetic analyses using distance matrix, parsimony, and maximum-likelihood criteria were performed with nearly full-length sequences (length, approximately 1,500 bp). Table 2 shows the 16S rRNA

TABLE 2. Eubacterial 16S rDNA sequences from *T. borchii* Vittad. ectomycorrhizal fungi (mycelium strains 1BO, 17BO, 10RA, and Z43) and their close relatives

Taxon	Accession no.	Source or reference
b-17BO	AF070444	This study
b-10RA	AF233293	This study
b-1BO	AF233294	This study
b-1BO University of Turin		This study
b-Z43	AF233292	This study
Bacteroides fragilis	M11656	49
Burkholderia endosymbiont	X89727	7
CFB group strain PB90-2	BSA229236	13
CFB group strain A103	FSU85887	8
CFB group strain DF-3	U41355	46
CFB group strain XB45	BSA229237	13
Clone S-B5	AF029041	Phelps et al.[a]
Cytophaga flevensis	M58767	21
Cytophaga sp. strain BD2-2	AB015532	29
Cytophaga sp. uncultured strain Sva1038	UCY240979	40
Escherichia coli	J01695	9
Flavobacterium aquatile	M28236	21
Flavobacterium balustinum	M58771	21
Flavobacterium ferrugineum	FVBRRDD	21
Flexibacter canadensis	M62793	52
Flexibacter flexilis	M28056	52
Sphingobacterium heparinum	M11657	49
Sphingobacterium mizutaü	M58796	21
Sphingobacterium sp.	AB020206	Tsukamoto[b]
Sphingobacterium spiritivorum	M58778	21
Uncultured eubacterium WCHB1-69	AF050545	15
Uncultured eubacterium WCHB1-53	AF050539	15

[a] Phelps et al., DDBJ/EMBL/GenBank database.
[b] T. Tsukamoto, DDBJ/EMBL/GenBank database.

TABLE 3. Evolutionary distances among small-subunit rRNAs of various representatives of the CFB phylum

Organism	Evolutionary distance																								
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1. *Escherichia coli*																									
2. Endosymbiont	0.2059																								
3. b-17BO	0.3093	0.3202																							
4. b-10RA	0.3193	0.3316	0.0129																						
5. b-1BO	0.3171	0.3218	0.0129	0.0243																					
6. b-1BO University of Turin	0.3375	0.3565	0.0309	0.0293	0.0427																				
7. b-Z43	0.3209	0.3357	0.0169	0.0121	0.0285	0.0210																			
8. Strain PB90-2	0.2937	0.3246	0.1813	0.1895	0.1854	0.2043	0.1918																		
9. Strain XB45	0.2925	0.3209	0.1803	0.1885	0.1844	0.2032	0.1907	0.0080																	
10. *Flavobacterium*	0.3315	0.3165	0.1979	0.2042	0.1979	0.2237	0.2065	0.1656	0.1646																
11. *Cytophaga* strain BD-2	0.2949	0.3183	0.1752	0.1834	0.1752	0.2022	0.1876	0.1062	0.1062	0.1900															
12. *Cytophaga* strain Sva 1039	0.2949	0.3218	0.1771	0.1832	0.1781	0.2020	0.1875	0.1145	0.1164	0.1921	0.1099														
13. Uncultured strain WCHB1-53	0.2892	0.3149	0.1866	0.1949	0.1887	0.2119	0.1971	0.1165	0.1165	0.1850	0.1119	0.1372													
14. Uncultured strain WCHB1-69	0.2892	0.3237	0.1764	0.1845	0.1796	0.2024	0.1868	0.1403	0.1384	0.2061	0.1308	0.1509	0.1203												
15. Clone SB5	0.2879	0.3149	0.1639	0.1719	0.1649	0.1916	0.1762	0.0977	0.0958	0.1777	0.0895	0.1218	0.1210	0.1354											
16. *Sphingobacterium* sp.	0.3007	0.3118	0.1734	0.1795	0.1716	0.1981	0.1817	0.1772	0.1783	0.2042	0.1732	0.1864	0.1815	0.1744	0.1852										
17. *Sphingobacterium heparinum*	0.2995	0.3155	0.1664	0.1734	0.1666	0.1919	0.1756	0.1844	0.1854	0.2116	0.1803	0.1926	0.1784	0.1754	0.1862	0.0285									
18. *Sphingobacterium spiritivorum*	0.3113	0.3201	0.1736	0.1838	0.1789	0.2028	0.1840	0.1826	0.1805	0.2375	0.1795	0.1826	0.1880	0.1766	0.1814	0.1000	0.1093								
19. *Flexibacter canadensis*	0.2991	0.3100	0.1605	0.1696	0.1596	0.1883	0.1718	0.1796	0.1817	0.2126	0.1632	0.1755	0.1829	0.1593	0.1681	0.1025	0.1015	0.1087							
20. *Sphingobacterium mizutaü*	0.3063	0.3268	0.1675	0.1768	0.1770	0.1970	0.1770	0.1687	0.1749	0.2310	0.1770	0.1864	0.1823	0.1688	0.1800	0.1018	0.1018	0.0672	0.1200						
21. *Bacteroides fragilis*	0.3307	0.3512	0.2134	0.2231	0.2136	0.2396	0.2233	0.1749	0.1759	0.2372	0.1789	0.1933	0.1740	0.1924	0.1809	0.2166	0.1966	0.2315	0.2069	0.2248					
22. CFB group strain DF-3	0.3381	0.3444	0.2158	0.2276	0.2181	0.2454	0.2289	0.1720	0.1700	0.2093	0.1730	0.1832	0.1834	0.1823	0.1708	0.2147	0.2073	0.2164	0.1932	0.2110	0.1403				
23. *Cytophaga flevensis*	0.3199	0.3138	0.2008	0.2072	0.2019	0.2260	0.2085	0.1579	0.1589	0.0411	0.1855	0.1762	0.1774	0.1901	0.1793	0.2040	0.2170	0.2246	0.2038	0.2212	0.2253	0.2038			
24. *Flavobacterium aquatile*	0.3236	0.3232	0.2139	0.2204	0.2139	0.2382	0.2227	0.1585	0.1555	0.0537	0.1838	0.1777	0.1748	0.1998	0.1664	0.1969	0.2107	0.2311	0.2182	0.2228	0.2419	0.2178	0.0561		
25. *Flavobacterium balustinum*	0.3313	0.3167	0.2177	0.2266	0.2167	0.2460	0.2290	0.1818	0.1818	0.1659	0.1902	0.1966	0.1758	0.1864	0.1822	0.2036	0.2211	0.2126	0.2087	0.2162	0.2544	0.2248	0.1646	0.1553	
26. *Flavobacterium ferrugineum*	0.3119	0.3194	0.2175	0.2262	0.2199	0.2386	0.2275	0.2262	0.2262	0.2275	0.2132	0.2208	0.2342	0.2254	0.2429	0.2351	0.2328	0.2664	0.2281	0.2668	0.2516	0.2644	0.2239	0.2387	0.2438

[a] All positions used to calculate the distances meet the condition that a known nucleotide is present in all of the sequences analyzed (51). The alignment used was based on sequences from position 177 to position 1457 (*E. coli* numbering).

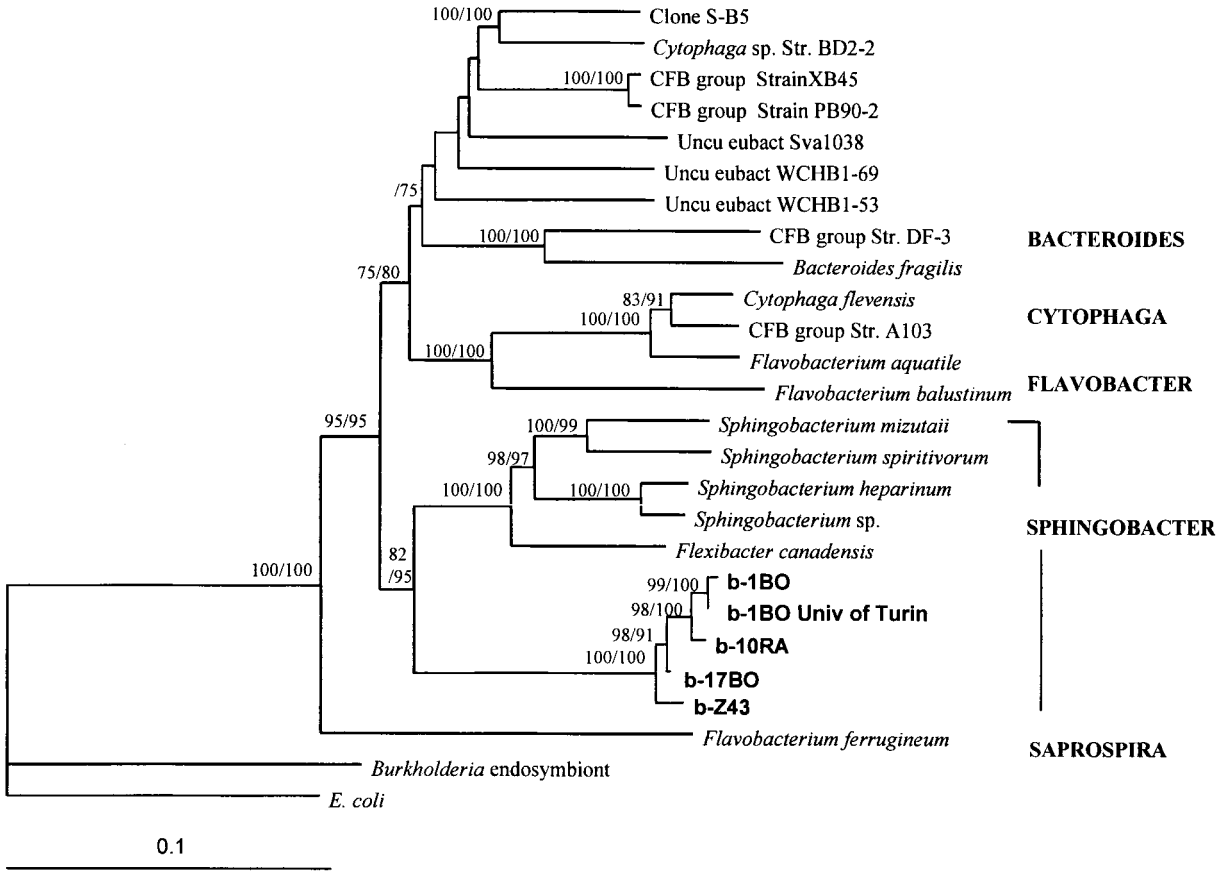

FIG. 2. Phylogenetic tree for representative 16S rRNA gene sequence from *T. borchii* mycelium based on nearly complete 16S rRNA sequences. The tree was derived from the evolutionary distances shown in Table 3. The two numbers at each branch node are bootstrap values based on 200 resamplings; the first number is the distance matrix value, and the second number is the parsimony bootstrap value. The sequence of the *Burkholderia* endosymbiont of *G. margarita*, an arbuscular mycorrhizal fungus, is included for comparison. Only values greater than 75 are shown. The scale bar represents a 10% difference in nucleotide sequences, as determined by measuring the lengths of the horizontal lines connecting two species. Uncu eubact, uncultivated eubacterium.

sequences used in the present study. Table 3 shows the evolutionary distances derived from nearly full-length 16S rRNA gene sequences found in different strains of *T. borchii* mycelia (b-17BO, b-1BO, b-Z43, b-10RA, and b-1BO University of Turin) and closely related eubacterial sequences obtained from the RDP and the DDBJ/EMBL/GenBank databases (8, 13, 15, 29, 46, 49; C. D. Phelps, L. Kerkhof, and L. Y. Young, DDBJ/EMBL/GenBank databases). Relevant sequences considered representative of the five most well-defined subgroups in the CFB phylum as suggested by Gherna and Woese (21) were also included, as were the 16S rDNAs of a *Burkholderia* strain (a bacterial endosymbiont of the endomycorrhizal fungus *G. margarita*, for comparison) and *Escherichia coli*, which served as an outgroup. Most of the sequences with high levels of similarity (>90%), as determined by the RDP sequence match program, either were partial sequences not useful for phylogenetic analyses (28) or were described as sequences of uncultivated soil bacteria which may not have been identified (19, 30). However, all methods of phylogenetic reconstruction, based on the sequences selected, unambiguously placed the mycelial bacterial sequences in a single new rRNA branch among the *Sphingobacter* subgroup of the CFB phylum defined by Gherna and Woese (21). The stability of this new branch was verified by bootstrap analysis, and a confidence level of 100% was obtained with all methods. A phylogenetic tree relating the five most well-defined subgroups in the CFB phylum derived from the evolutionary distances in Table 2 is shown in Fig. 2. Trees showing the same affiliation for the *T. borchii* CFB organism were obtained after complete exclusion or inclusion of both insertions and deletions and/or variable regions.

CFB bacterium in *T. borchii* hyphal tissue. The CFB bacterium in the hyphal tissue of the fungus *T. borchii* was analyzed by FISH. Mycelium homogenate was stained with DAPI and hybridized with three different 16S rRNA-targeted FISH probes (Table 1); the first probe, EUB338 (positions 338 to 355) (1), was specific for the eubacterial domain, the second probe, CF319 (positions 319 to 336) (2, 32), was specific for the *Cytophaga-Flexibacter* group, and the third probe, STBb-654 (positions 654 to 672), was specifically designed on the basis of the 16S rDNA sequence (b-17BO) of the bacterium detected by PCR in mycelium strain 17BO. Two sets of hybridization were performed with the 17BO mycelium strain; one set was performed with EUB338 and specific probe STBb-654, and one set was performed with EUB338 and CF319, followed by DAPI staining of the same sample. Each set included an unstained sample used as a control for autofluorescence.

In these experiments the same cell which hybridized with the eubacterial probe (EUB338) was fluorescent with CF319 or STBb-654, and DAPI staining confirmed (Fig. 4) the presence of the CFB bacterium in the 17BO mycelium strain. Double hybridization with the CF319 and EUB338 probes was also carried out with 1BO, 10RA, and Z43, and this analysis showed

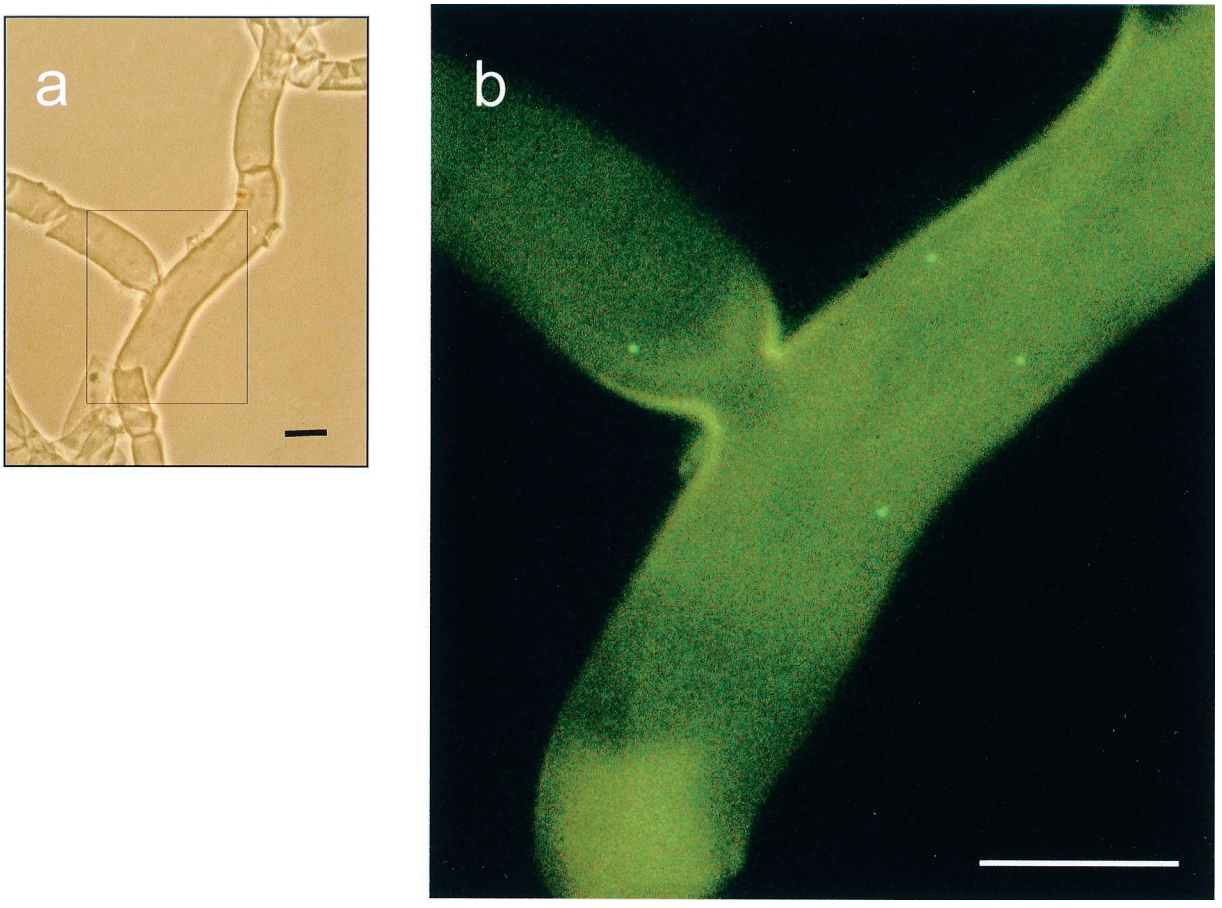

FIG. 3. Detection of the CFB bacterium associated with *T. borchii* Vittad. hyphal tissue (mycelial strain 17BO). (a) Phase-contrast micrograph of *T. borchii* hyphal tissue homogenate. (b) Same sample after hybridization with fluorescein-labeled eubacterial probe EUB338. Fluorescent CFB cells are visible. Scale bars, 5 μm.

the presence of fluorescent cells in all of the samples examined. In all of the FISH experiments no autofluorescence from the samples was observed. To clarify the location of the CFB bacterium in the ectomycorrhizal fungus, thin sections of *T. borchii* mycelium tissue were hybridized with EUB338, the general bacterial probe used for the homogenate. Few cells per septum hybridized with the general bacterial probe. In contrast, the difficulty of determining the exact position of the CFB bacterium with respect to the cytoplasm or the hyphal wall was evident, and it was difficult to determine where the bacterium was located since hybridization was successful in homogenate samples and in sections in which the hyphal wall was heterogeneously fragmented.

DISCUSSION

This paper describes molecular characterization of a CFB bacterium that is found in the mycorrhizal *T. borchii* mycelium and has not been cultured yet. PCR assays demonstrated that this uncultured CFB bacterium is present in all of the mycelia of *T. borchii* studied and in in vitro ectomycorrhizae. Simultaneous hybridization of the general eubacterial and specific probes with the hyphal tissues revealed rare, small (diameter, 0.3 to 0.5 μm) but viable CFB bacteria within the hyphae (Fig. 3 and 4).

Although several recent papers have described numerous bacteria, such as members of the genus *Pseudomonas*, the *Bacillaceae*, and the *Actinomycetes*, living among the hyphae of the fruiting bodies of truffles (3, 14, 20), no molecular characterizations of these bacteria or the microbe-host associations are available. In general, few data for uncultured bacteria in fungi have been presented (11, 12, 50), and only recently have a few phylogenetic studies identified an endophytic bacterium that is the endosymbiont of *G. margarita* and is a member of the genus *Burkholderia* (7) and members of the alpha and beta subclasses of the *Proteobacteria* detected in ectomycorrhizae of *F. sylvatica*, *L. vellereus*, and *L. subdulcis* (33).

To our knowledge, no member of the *Cytophagales* has been identified previously in ectomycorrhizal symbioses. Although uncultivated CFB bacteria have been detected in soil environments (30), few of these bacteria have been described as symbionts and commensals (25). The discovery of a CFB bacterium in the *T. borchii* ectomycorrhizal mycelium and molecular characterization of this organism represent a starting point for systematic molecular identification and functional studies of bacterium-fungus-plant symbioses. Concerning phylogenetic position, we found that the overall tree topology is consistent with other 16S rDNA phylogenetic analyses (4, 28, 38, 52), and for all analyses, the bootstrap values supporting the new cluster were significant for all of the criteria used (distance matrix, parsimony, maximum likelihood). However, since few environmental 16S rDNA sequences from soil bacteria are nearly full length, it is not possible to know if the CFB bacterium is closely related to other uncultivated soil bacteria. A decision about rank and a formal description must await the availability of

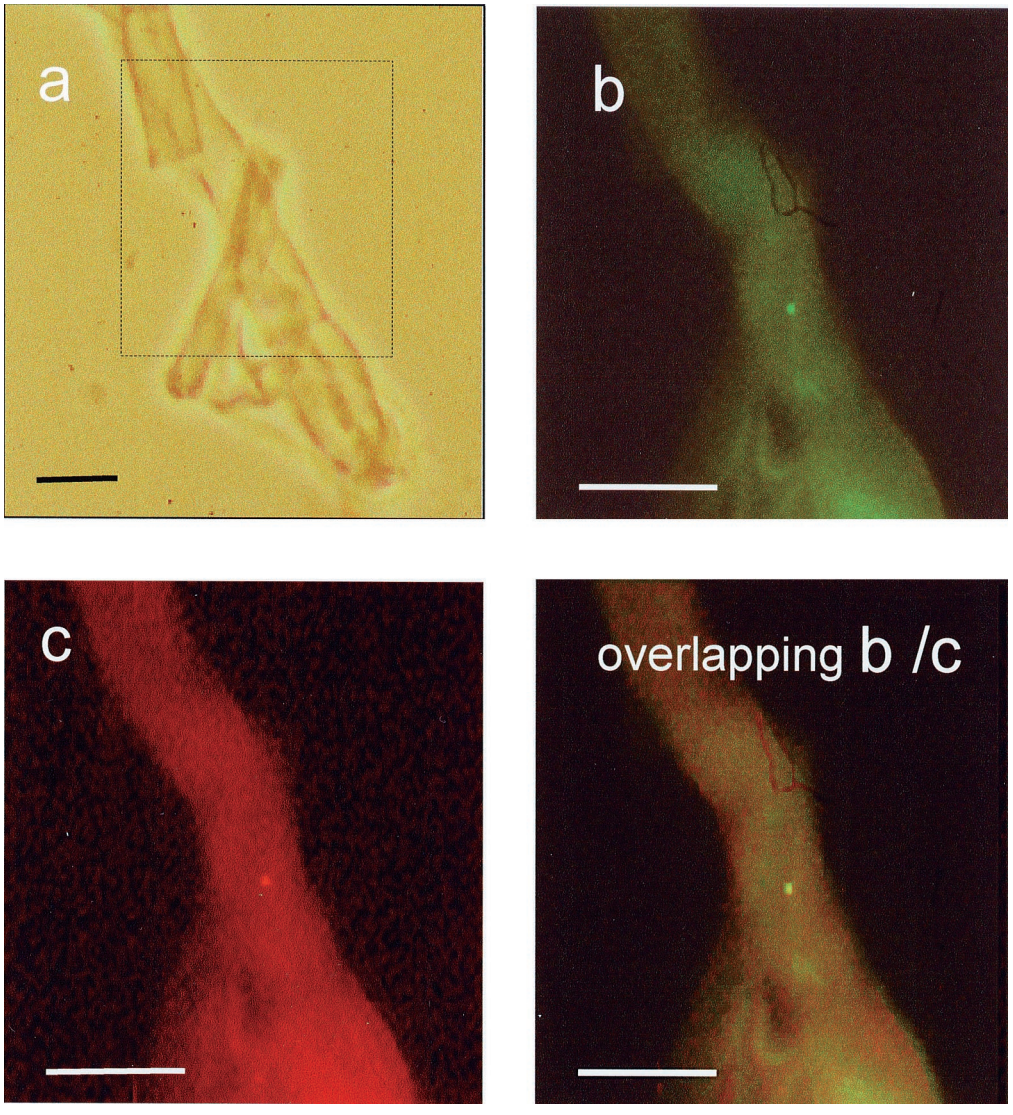

FIG. 4. (a) Phase-contrast micrograph of *T. borchii* hyphal tissue homogenate. (b) Detail of the same sample after hybridization with fluorescein-labeled eubacterial probe EUB338. (c) Detail of the same sample after hybridization with CY3-labeled probe specific for b-17BO. The panel on the lower right is an overlap of panels b and c showing the same cell hybridizing with both EUB338 and STBb-654 specific for the CFB bacterium. Scale bars, 5 μm.

more nearly complete sequences from environmental samples and phenotypic data.

The question of whether the new CFB bacterium is involved in the life cycle of the *T. borchii* truffle remains to be answered. PCR products obtained by using the specific b-17BO-f primer with templates from the mycelia and ectomycorrhizae and the probes used in the FISH experiment showed that this bacterium is a stable component of the *T. borchii* mycelium. This study provides the first direct evidence that a not-yet-cultured CFB bacterium is detectable in association with a mycorrhizal fungus of the genus *Tuber*.

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