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Competitive PCR for Quantitation of a *Cytophaga-Flexibacter-Bacteroides* Phylum Bacterium Associated with the *Tuber borchii* Vittad. Mycelium

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An uncultured bacterium associated with the ectomycorrhizal fungus *Tuber borchii* Vittad. was identified as a novel member of the *Cytophaga-Flexibacter-Bacteroides* group. Utilizing a quantitative PCR targeting the 16S rRNA gene, we relatively quantified this bacterium in the host. The estimated number of bacteria was found to be approximately 10^6 cells per 30-day-old *T. borchii* mycelium culture. This represents the first molecular attempt to enumerate an uncultured bacterium associated with a mycorrhizal fungus.

A recent straightforward molecular study aimed at surveying potential endo- and epiphytic bacteria within the mycelium of the ectomycorrhizal fungus *Tuber borchii* Vittad. revealed the presence of a novel uncultured bacterium associated with the hyphae of this fungus (1). The phylogenetic analysis unequivocally placed the bacterial sequences in a single new rRNA branch in the *Sphingobacterium* subgroup of the *Cytophaga-Flexibacter-Bacteroides* (CFB) phylum (6, 18). A few CFB cells per septum of mycelium were detected by the fluorescent in situ hybridization technique, with both general and specific oligonucleotide probes (1).

The occurrence of this novel bacterium associated with the *T. borchii* fungus will shed light on the relative importance of this bacterial species as a new, significant partner in fungus-plant symbiosis. Thus, in order to estimate the abundance of this strain within the fungal host tissue, we used a competitive PCR (cPCR) technique (3, 7, 10, 11, 15, 22). This represents the first molecular attempt to enumerate an uncultured bacterium associated with a mycorrhizal fungus.

Mycelia and bacterial strain. Four mycelium strains of *T. borchii* Vittad. (1BO [ATCC 96540], 10RA, 17BO, and Z43), in which the presence of the CFB bacterium (1) had been revealed, were used in this study. Mycelial cultures were grown in MNN liquid medium (21) and analyzed after 30 days. The 16S rRNA gene (rDNA) from the associated CFB bacterium was amplified by PCR following the procedures described by Barbieri et al. (1). In addition, *Sphingobacterium heparinum* ATCC 13125 was chosen from among the most closely related culturable strains to determine its rRNA operon copy number.

Ergosterol assay. Ergosterol is a component of the fungal membrane and provides a reliable indication of metabolically active fungal biomass (4). The assay for ergosterol was carried out on 30-day-old cultures of the four *T. borchii* mycelium strains utilized in this study, as described by Zeppa et al. (21).

Construction of the QPCR competitor. The 16S rDNA amplification product from the mycelium strain 17BO was cloned into the TA cloning vector pGEM (Promega) and sequenced. The sequence obtained was identical (1,450 of 1,450 nucleotides) to the b-17BO 16S rDNA sequence (GenBank no. AF070444). The recombinant DNA plasmid *p*b-17BO was cleaved with *Age*I (cleavage sites, positions 1243 and 1422, 5′-3′), generating a 179-bp DNA fragment and the competitor *p*b-17BO-c, a new plasmid which has a deletion but has the same priming sequences as the DNA target. After ligation, the 179-bp deletion was confirmed by sequencing. An internal primer, SH-878f (5′ CGA TGA TAC GCG AGG 3′; *Escherichia coli* positions 878 to 893) (2) was designed based on the 16S rDNA sequences of clones b-17BO, b-Z43, b-10RA, and b-1BO of *S. heparinum*, available in GenBank/EMBL/DDBJ. The primer SH-878f was used in combination with a universal primer reverse primer (1). The quantitative PCR (QPCR) procedure consisted of 22 cycles (unless specified otherwise): denaturation at 94°C for 45 s, annealing at 58°C for 45 s, and elongation at 72°C for 2 min. The specificity of the primer was checked by cloning and sequencing the amplification product obtained from the *T. borchii* mycelium strains (1BO, 10RA, 17BO, and Z43). The sequences obtained confirmed that only the CFB bacterium from the samples was amplified.

We determined the amplification kinetics of the target sequences and the competitor over a range of 35 cycles. PCR products were quantified as a function of the cycle number by measuring the ethidium bromide-stained DNA with the Gel Doc 2000 Quantity One software program (Bio-Rad), in which the pixel densities of the bands were transformed into pixel intensity ratios (14).

Secondly, a calibration curve was constructed by amplifying a range of masses of the 16S rDNA target from a CFB bacterium which is present in the four *T. borchii* mycelium strains used in this study, in the presence of a constant mass (or number of molecules) of competitor *p*b-17BO-c. The two fragments obtained in cPCR were resolved by electrophoresis using a 1.5% agarose gel containing ethidium bromide (1 μg/ml). The intensities of the ethidium bromide staining of the 540- and 361-bp PCR bands were then plotted as a function of the

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$\log_{10}$ of the known competitor copy number. The point of equivalence was that at which the intensities from the competitor and the target were equal and represented the relative number of copies of the 16S rDNA in the *T. borchii* mycelium strains.

rRNA operon copy number determination for *S. heparinum*. Since the CFB bacterium is not yet culturable, *S. heparinum* (strain ATCC 13125), the most closely related culturable bacterium, was chosen to relatively quantify the *rrn* copies of the CFB bacterial cell in the fungal tissue. Genomic DNA from *S. heparinum* was digested with three different restriction enzymes (*Sac*I, *Pst*I, and *Pvu*II; Promega Inc.) noncutting for 16S rDNA. rRNA operon copy numbers were determined by Southern blotting analysis of gel-separated restriction digests using a labeled DNA probe complementary to nearly the full length of the 16S rRNA gene of *S. heparinum* (positions 8 to 1456). Hybridization was carried out at 65°C and washing was done under stringent conditions, as described by Sambrook et al. (16). Genomic DNA from *E. coli* was used as a positive control.

QPCR competitor. The target sequence is not present in pure cultures of the microorganism. For this reason, the target used to construct the calibration curve had to be a cloned version of the CFB bacterium 16S rDNA within the *T. borchii* mycelium. Fungal strain 17BO was the first to be analyzed, and *p*b-17BO was the clone designed. The competitor *p*b-17BO-c, obtained by deletion of the *Age*I restriction site, was 179 bp smaller than the original clone. Both *p*b-17BO and *p*b-17BO-c were amplified by using the QPCR condition previously described and quantified as a function of the cycle number. The competitive control (*p*b-17BO-c) amplifies with equal efficiency and achieves a plateau simultaneously with the target DNA (*p*b-17BO) in a linear range of DNA masses (or molecules). From these results, it was possible to apply *p*b-17BO-c as an internal standard for the relative quantification of the bacterial DNA within *T. borchii* mycelium strains. Figure 1 shows a progressive competition between variable quantities of *p*b-17BO (from 100 to 10^7 molecules) and a fixed amount of *p*b-17BO-c (10^4 molecules). Three experiments were carried out to confirm test reproducibility; the point of equivalence at the intersection of the two curves corresponded to a mean of 1.2 [1.078; 1.332] $\times$ 10^4 molecules of *p*b-17BO (95% confidence interval).

Copies of the CFB bacterium 16S rDNA in *T. borchii* mycelium. To determine the quantitative frame of QPCR amplification of the 16S rDNA targets from the mycelium strains 1BO, 17BO, 10RA, and Z43 and the competitor (*p*b-17BO-c), their kinetics were compared by a PCR cycle test. The cPCR amplification efficiencies of target and competitor were calculated from the slope of the curves of amplification, between cycles 18 and 28, following the linear regression shown in Fig. 2, (efficiency [eff] = $10^{\alpha} - 1$, where α is the slope of the regression line). The efficiency of *p*b-17BO-c was slightly higher than that of the 17BO target ($\text{eff}_{\text{target}} = 0.441$; $\text{eff}_{\text{competitor}} = 0.464$). However, they reached saturation at the same number of cycles (35 cycles), and the efficiency ratio of target and competitor, between cycles 18 and 28, showed a constant value of 1.052.

This slight difference in efficiency between target and competitor may be due to the presence of minimal traces of inhibitory compounds (3) such as fungal polysaccharides, which could interfere with the PCR, or to the low density of the bacterial cells within the mycelial host tissue. Moreover, the differences in *rrn* copy number between a lab culture and environmental bacterial cells may affect the PCR amplification of target 16S rDNA (5). The amplification rates of the other samples, 1BO, Z43, and 10RA, were nearly identical, and their amplification efficiencies were comparable to those of 17BO (data not shown).

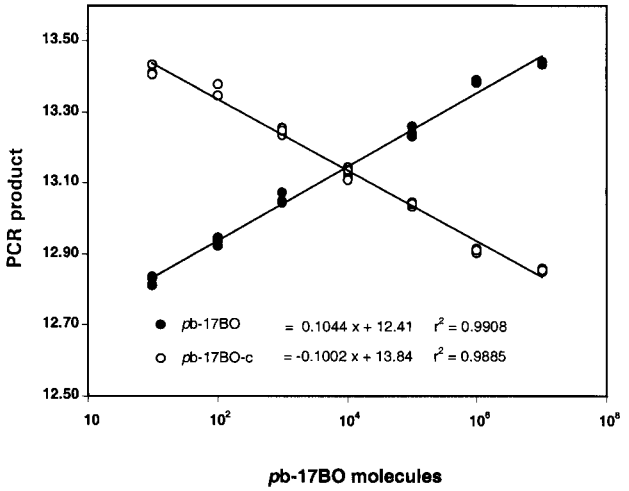

FIG. 1. QPCR of *p*b-17BO and competitor. Increasing numbers of molecules (from 100 to 10^7) of *p*b-17BO target (●) were coamplified with a fixed amount (10^4 molecules) of competitor, *p*b-17BO-c (○). The intensity value incorporated the band area for each PCR band visualized in the agarose gel was plotted as a function of the number of *p*b-17BO molecules. The intersection of the two curves shows the point of equivalence. The determined value of $(1.2 \pm 0.122) \times 10^4$ molecules for the input *p*b-17BO sequence corresponds with the actual input of 1.2×10^4 molecules of the competitor *p*b-17BO-c. Three independent QPCRs were carried out, and some points in the figure are hidden.

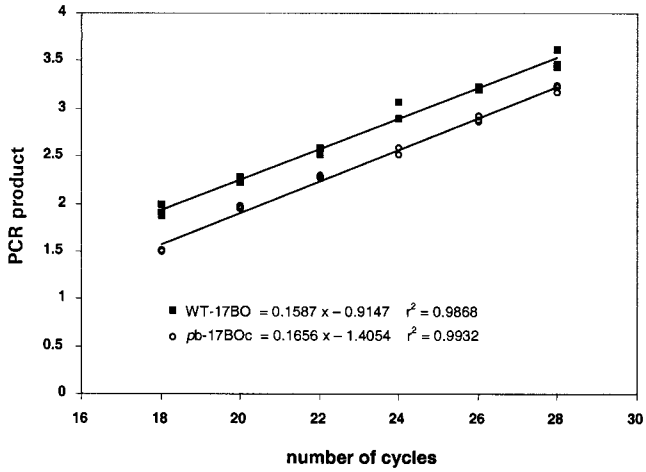

FIG. 2. Amplification efficiency of the 16S rDNA from the 17BO *T. borchii* mycelium (■) and the competitor *p*b-17BOc (○). Band intensities were calculated as described for Fig. 1. Efficiency was calculated from the slope of each regression.

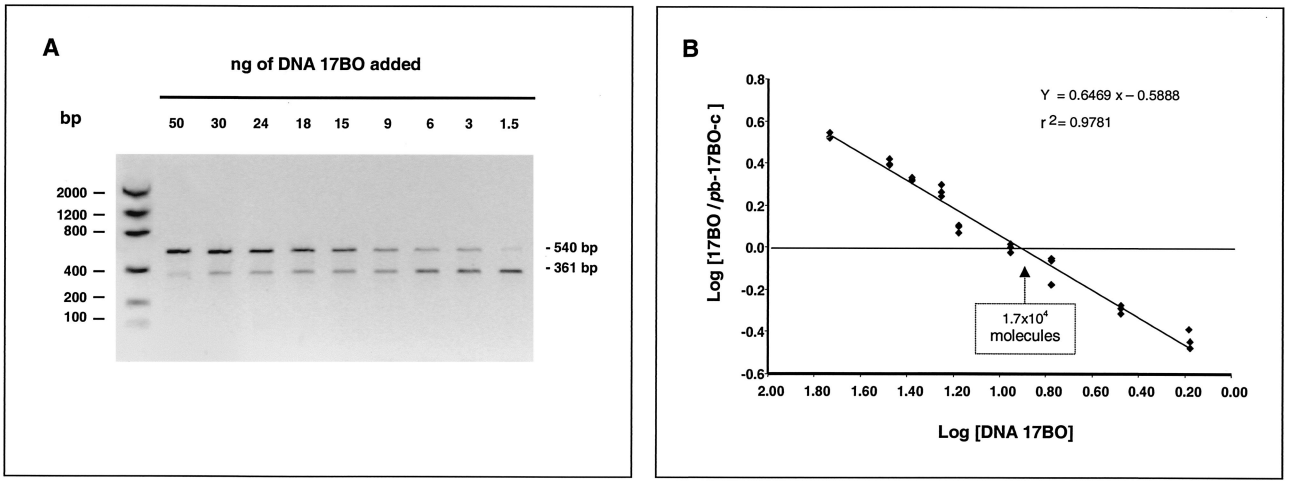

FIG. 3. cPCR for 16S rRNA gene from the uncultured CFB bacterium in the host *T. borchii* mycelium. DNAs extracted from *T. borchii* mycelium (strain 17BO) with masses ranging from 50 to 1.5 ng were coamplified with a fixed amount of competitor, 0.025 pg (1.20×10^4 molecules). The relative intensities of the PCR bands, corresponding to the target and the competitor of the agarose gel (A), were used for the determination of the competition equivalence point (B). Three determinations are plotted.

rRNA operon copy number determination for *S. heparinum*. To determine the rRNA operon copy number in *S. heparinum* strain ATCC 13125, its 16S rRNA sequence (GenBank accession no. M11657.1) (19) was examined. These analyses revealed that the 16S rRNA gene did not contain any *Sac*I, *Pst*I, or *Pvu*II sites. Digestion of *S. heparinum* chromosomal DNA with each of the noncutting restriction enzymes and subsequent Southern hybridization of the 16S rRNA gene results in three bands in each lane (data not shown). *E. coli* genomic DNA containing seven rRNA operon copies (http://rrndb.cme.msu.edu) (8) was used as a control. These results confirmed that *S. heparinum* strain ATCC 13125 has three copies of the rRNA operon.

QPCR of the 16S rDNA in *T. borchii* mycelium strains. After construction of the competitor and determination of the internal control concentration, a QPCR was carried out by coamplifying a range of masses of the target DNA from the *T. borchii* mycelia, strain 17BO, ranging from 1.5 to 50 ng in the presence of a constant mass of competitor (0.025 pg, corresponding to 1.20×10^4 molecules of *p*b-17BO). As shown in Fig. 3, the logarithm of the intensity ratios of the 16S rDNA amplification products from the mycelium strain 17BO to that of the competitor was plotted as a function of the initial number of target DNA molecules. The Zachar equation (20) was applied: $\log(Nn_1/Nn_2) = \log(No_1/No_2) + n \log(eff_1/eff_2)$, where Nn_1 and Nn_2 are the PCR products (intensity of ethidium bromide staining), No_1 and No_2 are the initial number of molecules or mass (nanograms) of target and competitor templates, respectively, n is the PCR cycle number, and eff_1 and eff_2 are the efficiencies of template amplifications ($10^{\alpha} - 1$, as reported above). This equation is valid assuming that the efficiency of amplification of both target and competitor remains constant for each cycle in the amplification reaction (14, 20, 22). QPCR assays were carried out by amplifying the range of masses of the other *T. borchii* mycelium strains (1BO, Z43, and 10RA) using the same procedure and concentration as for the 17BO strain (Table 1).

The molecular identification of the CFB bacterium within *T. borchii* mycelium clearly placed this strain among the *Sphingobacter* subgroup of the CFB phylum; however, attempts to isolate and grow this bacterium from the mycelium have been unsuccessful. Thus, a molecular approach has been applied for the enumeration of this strain. Through a cPCR, we could relatively estimate its *rrn* copy number within *T. borchii* mycelium tissue. Since the *rrn* copy number of *S. heparinum*, the most closely related strain, was found to be three (three operons), it is likely that the CFB is present at a level of 10^6 cells per 30-day-old *T. borchii* mycelial culture. Moreover, in order to better relate the number of CFB cells to the fungal biomass, the content of free ergosterol in 30-day-old mycelium strains is reported in Table 1.

The number of CFB bacteria estimated by QPCR includes

TABLE 1. Relationship between CFB cells and *T. borchii* mycelium strains[a]

T. borchii strain	Dry wt (mg)	Amt of total DNA (ng)	Ergosterol concn (μg/mg)	No_1 in QPCR (ng)[b]	No. of CFB cells	
					1 *rrn* copy (10^7)	3 *rrn* copies (10^6)
17BO	9 ± 3.2	7,450.8 ± 1,490	3.214 ± 0.013	8.13 [7.98; 8.28]	1.56 ± 0.37	5.19 ± 1.25
10RA	13 ± 4.1	9,233.6 ± 2,585.2	3.569 ± 0.018	12.50 [12.29; 15.17]	1.98 ± 0.39	6.60 ± 1.31
1BO	14 ± 4.9	9,747.0 ± 2,241.5	3.862 ± 0.013	10.26 [10.15; 10.37]	2.01 ± 0.30	6.71 ± 1.01
Z43	8 ± 2.6	7,069.2 ± 1,767.3	2.890 ± 0.017	8.08 [7.68; 8.48]	1.15 ± 0.67	3.81 ± 2.28

[a] Data are means ± standard deviations.
[b] Quantity of DNA from *T. borchii* mycelium extrapolated by the Zachar equation at the equivalence point obtained by QPCR (mean [95% interval of confidence]).

live as well as dead bacterial cells. However, the detection of these bacteria within the hyphae of *T. borchii* by in situ hybridization experiments using eubacterial and specific 16S rRNA-based probes (1) was possible due to the of presence of mature rRNA in live cells, a condition sine qua non for the hybridization assays. Thus, we consider that 10^6 cells per 30-day-old mycelium culture of *T. borchii* is representative of a probable live population.

Since the fungus *T. borchii* is utilized as a competent fungus for in vitro ectomycorrhizal synthesis and expression studies (9, 13, 17), the presence of prokaryotic sequences or genes within DNA extracted from *T. borchii* mycelium strains might be considered for further biological investigations or biotechnological applications.

The biological function of this CFB bacterium in the fungal-bacterial interaction is still unknown, but since similar bacterial sequences have also been found in *Tuber aestivum* and *Tuber uncinatum* mycelium species (12), we cannot exclude coevolutionary events.

Molecular studies are in progress for a better understanding of this fungal-bacterial interaction.

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