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A Bacterium Belonging to the *Burkholderia cepacia* Complex Associated with *Pleurotus ostreatus*

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Pleurotus ostreatus **is a widely cultivated white-rot fungus. Owing to its considerable enzymatic versatility *P. ostreatus* has become the focus of increasing attention for its possible utility in biobleaching and bioremediation applications. Interactions between microorganisms can be an important factor in those processes. In this study, we describe the presence of a bacterial species associated with *P. ostreatus* strain G2. This bacterial species grew slowly (approximately 30 days) in the liquid and semi-solid media tested. When *P. ostreatus* was inoculated in solid media containing Tween 80 or Tween 20, bacterial microcolonies were detected proximal to the fungal colonies, and the relevant bacterium was identified via the analysis of a partial 16S rDNA sequence; it was determined to belong to the *Burkholderia cepacia* complex, but was not closely related to other fungus-isolated *Burkholderiaceae*. New specific primers were designed, and confirmed the presence of *in vitro P. ostreatus* cultures. This is the first time that a bacterial species belonging to the *B. cepacia* complex has been found associated with *P. ostreatus*.**

Keywords: mushrooms, *Pleurotus ostreatus*, associated bacterial species, bacterial fungi interactions, *Burkholderia cepacia* complex, 16S rDNA sequence

Pleurotus ostreatus is a white-rot edible mushroom, which is cultivated in several countries. It is known to be a cheap source of proteins (Ranzani and Sturion, 1998). Biographical studies have shown that the *Pleurotus* genus is among the more conspicuous fungi that induce wood decay in terrestrial ecosystems worldwide. Nevertheless, the *P. ostreatus* complex (a group of species related closely with *P. ostreatus*) is presently known to exist only in North America and Northern Eurasia (Vilgalys and Sun, 1994; Bao *et al.*, 2004).

White-rot fungi degrade lignin more extensively and rapidly than any other known organism. Due to its formidable enzymatic qualities, *P. ostreatus* can be employed in a variety of industrial processes, including biopulping and pulp bleaching (Sermanni, *et al.*, 1994). In addition, researchers focusing on bio-remediation processes (e.g. degradation of polycyclic aromatic hydrocarbons - PAHs) frequently utilize this fungus in their studies (Eichlerová *et al.*, 2000).

A number of bacteria that belong to different subdivisions of the Proteobacteria have been found in association with white-rot fungi. For example, Seigle-Murandi *et al.* (1996) previously isolated *Agrobacterium radiobacter* and atypical *Burkholderia* from *Phanerochaete chrysosporium,* and Coenye *et al.* (2001) later identified this atypical *Burkholderia* as *Burkholderia fungorum.* Also, Lim *et al.* (2003) isolated and described *B. sordidicola* from the mycelia of *Ph. sordida.* Other groups of fungi, principally the mycorrhizal fungi, are also associated with specific bacteria, and in some of them, intracellular relationships exist, as has been previously described by Jargeat *et al.* (2004), Levy *et al.* (2003), Bertaux *et al.* (2003) and de Boer *et al.* (2004).

Efforts to understand the relationship of *P. ostreatus* with other microorganisms may improve or expand its biotechnological applications. In this report, we provide evidence for the existence of a bacterial species which is closely associated with the *P. ostreatus* strain G2.

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Materials and Methods

Isolation of Pleurotus species

The *P. ostreatus* strain used in this study was obtained via aseptic removal of tissues from *P. ostreatus* carpophores harvested in a mushroom growing area in Mogi das Cruzes/ São Paulo State/ Brazil (23°31'S, 46°11'W), as previously described by Stamets and Chilton (1983). This strain has been designated *P. ostreatus* strain G2.

Isolation and culture conditions of bacterial species

Bacterial cultures were obtained from the strain G2 by grinding fungal mycelia in bacterial liquid media (described below), using a sterilized mortar and pestle. The liquid fraction was filtered through 800 and then 450 μm disk filters (Millipore, USA) and incubated at 28°C with agitation at 150 rpm (Tecnal, Brazil).

Different media were tested in this process, including: LB (Luria and Bertani), TSB (Tripticase Soy Broth), TST (TSB with 0.1% Tween 80), BHIT (7.4% brain-heart infusion with the addition of 0.1% Tween 80), PWM (PW modified by Araujo *et al.*, 2001) and the modified medium described by Sierra (1957) (1% peptone, 0.5% NaCl and 1.0% Tween 20). After 30 days, these samples were transferred to liquid media, semi-solid media (0.1% agar) or solid media (1.5% agar), and incubated for an additional 30 days, as described above. In another procedure the samples were milled, filtered, and directly inoculated in either semi-solid or solid media.

Co-culture system

In addition, an alternative system designated the co-culture system, was developed in this study. This system was developed on the basis of favoring the growth of bacteria rather than *P. ostreatus* mycelia. Therefore, the *P. ostreatus* strain G2 samples were inoculated in bacterial solid media containing Tween 80 or Tween 20. The initial inoculum was small blocks of agar (0.25 cm^2) that had been colonized with *P. ostreatus*. These blocks were inoculated upside-down, in order to ensure that the *P. ostreatus* mycelia remained in contact with the bacterial media. The presence of small bacterial colonies on the borders of the fungal colonies was evaluated and evaluated via nested 16S rDNA PCR.

DNA extraction

DNA extractions were conducted from the fungal hyphae and from the borders of the fungal colonies, in accordance with the protocol described by Raeder and Broda (1985).

DNA extraction from bacterial cultures was conducted after 20 minutes of centrifugation of liquid cultures at 14,000 × g, as previously described by Araujo *et al.* (2001).

PCR analysis

An 843 bp fragment of the 16S rDNA gene was PCR-amplified using the following parameters: the total reaction volume was 50 μl, and contained the primers SMY-F511 (5'-CTA TGT GCC AGC AGC CGC GGT A-3') and SMY-R1382 (5'-GGC GGT GTG TAC AAG ACC CGA G-3') at a concentration of 0.5 mM of each, *Taq* DNA polymerase 1.5 U (Gibco, USA), 0.2 mM of each dNTP, 10x Buffer (Gibco. USA), 2.0 mM $MgCl_2$ and 20 ng of DNA template. The PCR conditions were as follows: Initial denaturation at 94°C for 3 minutes, followed by 40 cycles of 20 seconds at 94°C, primer annealing for 40 seconds at 50°C, elongation for 60 seconds at 72°C, and a final elongation step for 5 minutes at 72°C. Amplification was then conducted using a Gene Amp-PCR System 9700 thermocycler (Perkin-Elmer, USA). The PCR products were analyzed on 1.2% agarose gel, stained with ethidium bromide, and visualized with a UV transilluminator, in accordance with standard protocols. The PCR products were then purified using a Sephaglas Band Prep Kit (Pharmacia Biotech, USA), and cloned into pGEM-T KIT (Promega, USA). The plasmids were purified using a GFX KIT (Amersham Pharmacia, USA) and were sequenced at GENOMIC Ltda (São Paulo, Brazil).

The nucleotide sequence obtained in this study was submitted to the GenBank database and assigned the accession number DQ163907.

Phylogenetic analysis

The obtained sequence was analyzed by a Basic Local Alignment Search Tool (BLAST) search against the NCBI database (National Center for Biotechnology Information website [http://www.ncbi.nlm.nih.gov]). The obtained sequence and selected sequences were then aligned using ClustalX software (Thompson *et al.*, 1997), and manually corrected using BIOEDIT software (Hall, 1999). The distance matrix and phylogenetic tree were calculated using the estimator of Jukes and Cantor (1969) and constructed via the neighbor-joining method (Saitou and Nei, 1987), respectively.

Primer design and nested PCR performance

In order to confirm the obtained sequence, new primers were designed to target *Pleurotus*-associated bacterial species and *B. cepacia*. For this purpose, the sequences obtained were aligned with the following sequences: *B. cepacia* X87275, *B. cepacia* AF097532, *Mycoplasma gallisepticum* M22441, *M. buccale*

AF125586 and *M. faucium* AF125590. These sequences were selected from the GenBank database (http://www.ncbi.nlm.nih.gov/). Alignment was then conducted using ClustalW 1.8 software (http://dot.imgen.bcm.tmc.edu:9331/multi-align/multi-align.html).

The new primers, BUR-F587 (5'-GGT TTG CTA AGA CCG ATG TG-3') and BUR-R1127 (5'-TTA GAG TGC TCT TGC GTA GC-3'), were employed in nested PCR reactions combined with 1378R (5'-CGG TGT GTA CAA GGC CCG GGA ACG-3') universal 16S rDNA primer. The first nested PCR reaction was conducted with the BUR-F587 and 1378R primers, as previously described. The reaction conditions were as follows: initial denaturation for 4 minutes at 94°C, followed by 10 cycles of 30 seconds at 94°C, 60 seconds at 65°C (decreasing 1°C per cycle), 60 seconds at 72°C, followed by 15 cycles of 30 seconds at 94°C, 60 seconds at 55°C, 60 seconds at 72°C, and a final extension of 5 minutes at 72°C. The second nested PCR reaction was conducted using the BUR-F587 and BUR-R1378 primers as described above. The following program was used for the second reaction: an initial denaturation step for 4 minutes at 94°C, 26 cycles of 30 seconds each at 94°C, 60 seconds at 64.5°C, and 60 seconds at 72°C, with a final extension step for 5 minutes at 72°C. The presence of fungal DNA was also confirmed via amplification of the fungal ITS region, as described by Rubini *et al.* (2005).

Results

Isolation and characterization of bacterial species

Bacterial growth was observed after 30 days in all tested liquid media. Assays with semi-solid media indicated weak bacterial growth after 25 days, as is shown in Fig.1a.

In solid media, bacterial growth was observed only when the *P. ostreatus* fungus was inoculated on bacterial solid media in the presence of Tween (Fig. 1b). In this case, structures, like microcolonies, immersed in the media were observed outside of the borders of the fungal colonies (Fig. 1c). These structures resembled bacterial colonies immersed on agar, as has been previously described by Miyata *et al.* (2000). This bacterial-fungi growth pattern was dubbed the co-culture system, and was associated with the fastest bacterial growth (around 15 to 20 days) among other attempted approaches. In order to ascertain whether the structures observed in the solid medium were microorganisms or artifacts, nested PCR was conducted, as described below.

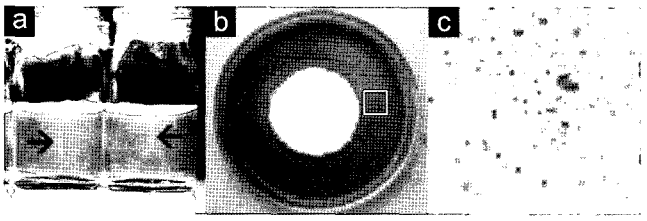

Fig. 1. Fastidious bacterial growth on different media; (a) Bacterial growth on TSB semi-solid medium, with indication of a submersed growth pellicle (arrow); (b) Co-culture system, *P. ostreatus* growing on BHIT; (c) Enlarged view from co-culture system (Fig. 1b, square area), bacterial microcolonies growing immersed in agar close to the fungal colony in BHIT agar medium.

16S rDNA similarity and phylogenetic analysis

The molecular analysis of the partial 16S rDNA sequence obtained from the bacterial growth in liquid media and in the co-culture system suggested that this organism is related to a group of species defined as the *Burkholderia cepacia* complex (β-Proteobacteria), with substantial similarity to *Burkholderia cepacia* ATCC 53933 (AY741357, Identities = 842/844 99%, Jun 25th 2005).

As compared to different *Burkholderia* species, our phylogenetic analysis also confirmed that this bacterial species is a member of the *Burkholderia cepacia* complex (Fig. 2).

Detection of bacterial species via nested PCR

The first nested PCR reaction with BUR-F587 and 1378R primers generated an 811 bp fragment, which was either not observed, or weakly observed, on agarose gel electrophoresis. The second reaction with BUR-F587 and BUR-R1127 generated a strong band of 562 bp, as had been predicted by sequence analysis.

The sequencing of the nested PCR products confirmed the partial sequence obtained in the first assay (data not shown). In addition, the application of nested PCR revealed the presence of bacterial species in different samples. In one instance, bacterial DNA was detected in the co-culture system (Fig. 3). In this case, samples from two different regions of the Petri dish were tested using two nested PCR reactions. The first region (I) included only the white halo and no visible mycelia (Fig. 3a I), but the second region (II) hyphae (Fig. 3a II) contained visible *P. ostreatus* hyphae. Using PCR targeting fungal ITS, no fungal DNA was detected in region I outside of the fungal colony (Fig. 3b 2), but fungal DNA was amplified in region II within the fungal colony (Fig. 3b 5). By way of contrast, the nested PCR for 16S rDNA generated positive results in both regions (Fig. 3b 1 and 4). This approach also verified the occurrence of bacterial microcolonies near the border of the *P. ostreatus* colony growing on the co-culture system.

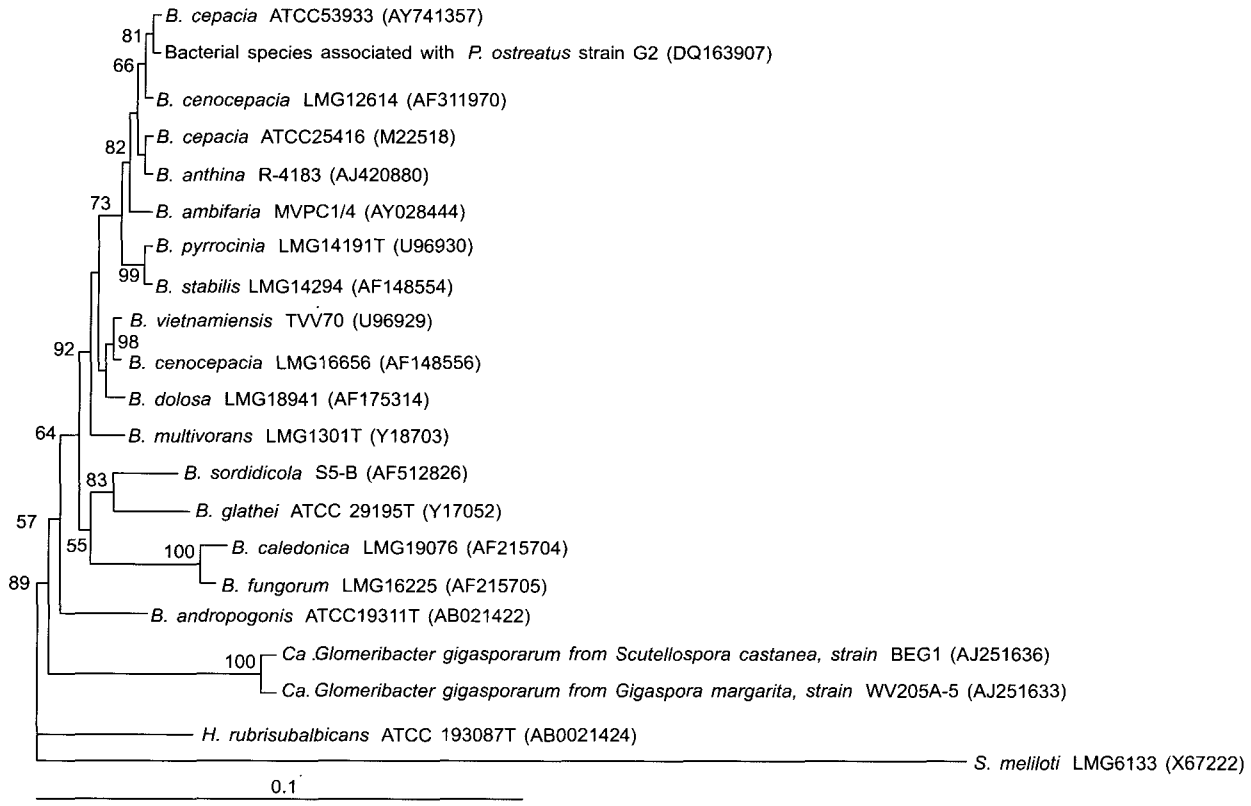

Fig. 2. Phylogram showing the relationship of the bacterial species associated with *P. ostreatus* (DQ163907) to reference members of the β-proteobacteria phylum, on the basis of 16S rDNA sequence analysis. Sequences from *Candidatus Glomeribacter gigasporarum* (AJ251635) an endocellular bacterium species of the mycorrhizal fungus *Gigaspora margarita* and with *B. fungorum* (AF215705) and *B. sordidicola* (AF512826) isolated from *Phanerochaete* were included in the analysis. Bootstrap values (1000 runs) are given in the nodes. A *Sinorhizobium meliloti* LMG 6133 (X67222) sequence was used as an outgroup.

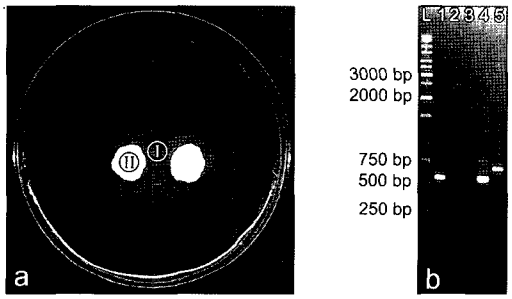

Fig. 3. DNA amplification of bacterial 16S rDNA and fungal ITS; (a) *P. ostreatus* growing in a co-culture system on Sierra modified medium. The circles indicate regions from which the samples were obtained. (b) Agarose gel electrophoresis showing nested PCR amplification. Lanes: L) ladder 1) bacterial 16S rDNA from region I; 2) fungal ITS DNA from region I; 3) empty; 4) bacterial 16S rDNA from region II 5) fungal ITS DNA from region II.

Discussion

The primary objective of this study was to confirm the presence of associated bacterial species in *P. ostreatus* strain G2, as has already been described with other white-rot fungi, including *P. chrysosporium* (Seigle-Murandi *et al.*, 1996; Coenye *et al.*, 2001) and *P. sordida* (Lim *et al.*, 2003). Although *B. fungorum* and *B. sordidicola* grow readily on TSB, the isolated bacterial species grew relatively slowly (approximately 30 days) in this medium, as well in other tested media. However, no growth was detected in the solid media in cases in which the bacterial species alone was inoculated.

By way of contrast, bacterial development was observed to improve when grown in a co-culture with *P. ostreatus* (Fig. 1b). This indicates that the growth of the bacterial species may be reliant on certain metabolites generated by the fungus, and suggests that a close relationship exists between these organisms.

As mentioned, this co-cultivation system was designed to inhibit *P. ostreatus* mycelia and to promote bacterial development. The main basis for this strategy was predicated on the fact that *P. ostreatus* strain G2 normally grows in fungi media (e.g. MEA or PDA) for a maximum of 6 days after inoculation in a 10 cm diameter Petri dish. As a

fastidious organism, the development of the associated bacteria requires at least 15 days. Thus, the inhibition of fungal mycelia growth is an important factor in protocols based on bacterial visualization.

16S rDNA analysis revealed that this bacterial species was closely related to the *B. cepacia* complex group. In addition, the new primers designed for nested PCR targeting *B. cepacia* (Fig. 3) confirmed that the isolated bacterial species was related to this cluster. Vandamme *et al.* (2003) consider this group contains at least nine genomic species, which share a high degree of 16S rDNA (98-100%) similarity. These results imply that 16S rDNA analyses are insufficient for the identification of the bacterial species. Furthermore, microbiological data, including growth rates, appear to indicate that the bacterial species isolated in this study is different from the other previously described species within this complex.

Candidatus Glomeribacter gigasporarum is a homogeneous bacterial population which is closely related to the *Burkholderia* genus detected from *Gigaspora margarita* fungus DNA (Bianciotto *et al.*, 2003). Therefore, this uncultivable bacterium was incorporated in our 16S rDNA analyses. The results of a phylogenetic analysis established that *Candidatus* Glomeribacter gigasporarum is not related to the fastidious *P. ostreatus* bacteria, and was probably derived from an independent lineage.

Occurrences of intracellular bacterial species have previously been demonstrated clearly in *G. margarita* (Bianciotto and Bonfante, 2002; Bianciotto *et al.*, 2003) and *G. decipiens* (Levy *et al.*, 2003). Among the basidiomycetes, Bertaux *et al.* (2003) has shown that *Paenibacillus* spp. colonize the hyphae of *Laccaria bicolor* S238N (mycorrhizal fungi). Our previous research has also revealed some intracellular bacteria-like body structures in the hyphae of *P. ostreatus* strain G2 (Yara *et al.*, 1999), which suggests that this bacterial species is capable of establishing an endosymbiotic relationship with *P. ostreatus*.

This communication is, to the best of our knowledge, the first report regarding the isolation of bacterial species grown in association with *P. ostreatus* hyphae, a major fungus in food production, as well as in bioremediation processes.

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