RESEARCH ARTICLE

Microbial community analysis in the termite gut and fungus comb of Odontotermes formosanus: the implication of Bacillus as mutualists

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Abstract

The microbial communities harbored in the gut and fungus comb of the fungus-growing termite Odontotermes formosanus were analyzed by both culture-dependent and culture-independent methods to better understand the community structure of their microflora. The microorganisms detected by denaturing gradient gel electrophoresis (DGGE), clonal selection, and culture-dependent methods were hypothesized to contribute to cellulose-hemicellulose hydrolysis, gut fermentation, nutrient production, the breakdown of the fungus comb and the initiation of the growth of the symbiotic fungus Termitomyces. The predominant bacterial cultivars isolated by the cultural approach belonged to the genus Bacillus (Phylum Firmicutes). Apart from their function in lignocellulosic degradation, the Bacillus isolates suppressed the growth of the microfungus Trichoderma harzianum (genus Hypocrea), which grew voraciously on the fungus comb in the absence of termites but grew in harmony with the symbiotic fungus Termitomyces. The in vitro studies suggested that the Bacillus sp. may function as mutualists in the termite-gut–fungus-comb microbial ecosystem.

Introduction

Termites are a group of eusocial insects that harbor a consortium of aerobic, anaerobic, and microaerophilic bacteria that are responsible for the degradation of cellulose and hemicellulose and benefit their host organism (Scharf & Tartar, 2008; Bignell, 2011). Various groups of insects have developed symbiotic relationships with fungi; notable examples in three different insect orders include termites, ants, and ambrosia beetles. Interactions associated with termites and fungi have been reported in both lower and higher termites (Cornelius et al., 2002; Aanen et al., 2007; Nobre et al., 2011). In the higher termites, the fungus-growing termites (Subfamily Macrotermitinae, Family Termitidae) have evolved symbiotically with the fungus Termitomyces. (Aanen et al., 2007).

The macrotermitine termites are distributed throughout the tropical and subtropical areas of Asia and Africa and have over 330 species belonging to 12 Genera (Aanen et al., 2007). The mutualistic system of the macrotermitine termite Odontotermes formosanus Shiraki (Order Isoptera), which is found in Southern China and southeast Asian countries (Shinzato et al., 2007), comprises the fungus Termitomyces, a fungus comb and the termite castes consisting of the reproductives, sterile workers, soldiers, and immature individuals (Eggleton, 2011). The fecal material of these termites, consisting of digested plant materials, is fashioned into a small ventilated structure called the fungus comb, or the ‘fungus garden’, which acts as a substrate for the growth of the symbiotic fungus Termitomyces (Rouland-Lefèvre et al., 2006). Through this relationship, the termite acquires nitrogenous compounds from Termitomyces, and in return, the fungus is provided with a suitable growth substrate by the worker termites (Rouland-Lefèvre et al., 2006). The worker termites also play a significant role in culturing Termitomyces and transferring the regurgitated substrate materials to other termites and nonforagers by trophallaxis (Huang et al., 2008; Eggleton, 2011).
Alternatively, the microbes residing in the termite gut demonstrate various types of relationships, ranging from pathogenicity to obligate mutualism (Rosengaus et al., 2011). The termites have accordingly developed several defensive strategies against pathogens and parasites (Mueller et al., 2005). Although worker termites can control infection by pathogens or parasites by secreting antimicrobial substances in defensive glandular secretions, feces, and body exudates (Rosengaus et al., 2004, 2011), it has been reported that microorganisms harbored by these social insects can either act as symbionts or as antagonists to parasitic microorganisms (Currie & Stuart, 2001; Currie, 2004; Nobre et al., 2011).

The fungus combs/fungus gardens of macrotermite termites and leaf-cutting ants are vulnerable to attack by garden parasites, i.e. fungal ‘weeds’, which are competitive, saprophytic, and pathogenic (Batra & Batra, 1979; Wood & Thomas, 1989; Currie, 2004; Mueller et al., 2005; Van Bael et al., 2009). Bipartite, tripartite, and quadripartite associations are well defined in leaf-cutter ants (the genera *Atta* and *Acromyrmex*, Myrmicinae) to protect their fungus gardens from these microfungi (Currie et al., 2003; Currie, 2004; Poulsen et al., 2007; Van Bael et al., 2009). Some actinomycete mutualists isolated from the leaf-cutter ants inhibit the growth of a microfungus, *Escovopsis* (Family Hypocreaceae) that parasitizes their preferred fungal cultivar *Leucocprinus gongylaphorus* (Family Leptotaceae) (Currie, 2004; Sen et al., 2009). A similar phenomenon has been observed in *Dendroctonus frontalis* beetles (Family Coleoptera), where the actinomycete cultivars are able to protect their symbiotic fungus, an *Entomocorticium* sp. (Family Peniophoraceae), against the antagonistic microfungus *Ophiostoma minus* (Family Ophiostomataceae) (Scott et al., 2010). In macrotermite termites, the common microfungi that infect the fungus comb in the absence of termites belong to the genera *Aspergillus*, *Penicillium*, and *Hypocrea* (Trichoderma spp.), and they affect the growth of the symbiotic fungus *Termiotomycetes* (Wood & Thomas, 1989; Gullan & Cranston, 2010). However, the interactions of mutualists against these microfungi have not been explored in macrotermite termites.

*Bacillus* species have been detected in the gut of soil termites and other invertebrates (König, 2006). Also, multiple species of *Bacillus* are reported to be readily cultured from bulk and rhizospheric soil (McSpadden Gardener, 2004). Many of them function as antagonists against various fungal and nematode pathogens of plants by secreting various kinds of antibiotics (Chaurasia et al., 2005; Swain et al., 2008). Apart from that, *Bacillus* populations may function as mutualists by enhancing the plant’s health by stimulating the plant host or microbial symbionts (McSpadden Gardener, 2004). Therefore, it is a possible that the *Bacillus* species inhabiting the gut and fungus comb could probably function as mutualists in the subterranean nests of macrotermite termites.

Although the microbial diversity in the fungus comb and gut microbiota have been extensively studied in these termites (Shinzato et al., 2005, 2007), the role of bacterial and fungal cultivars in suppressing the growth of microfungi and the mechanism for controlling the structure of the microbial communities are not well defined. Therefore, the objective of this study is to analyze the microbial community structure in the gut and fungus comb of *O. formosanus* using both culture-dependent and culture-independent approaches and also to determine the role of *Bacillus* cultivars in functioning as a mutualist and their role in lignocellulosic degradation. This is the first study to report the function of bacterial cultivars in macrotermite termites.

**Materials and methods**

**Termites and fungus combs**

Worker termites and fungus combs of *O. formosanus* were collected from subterranean nests near the National Museum of Natural Science (24.156112° N latitude and 120.666275° E longitude), Taichung, Taiwan, in June, 2009. The fungus combs were carefully removed from the subterranean nest, and the worker termites were separated from the fungus combs, surface sterilized with 70% alcohol, and used for DNA extraction. The fungus combs were aseptically collected in polypropylene tubes and frozen at −80 °C until use.

**Isolation of cultivable bacteria from the termite gut and fungus comb**

Twenty worker termites were surface sterilized with 70% ethanol and degutted using sterile forceps according to Long et al. (2010). The guts were homogenized, and dilution series (up to a dilution of 10⁻¹²) were spread-plated on Luria–Bertani agar (MDBio, Inc.) and King’s B medium (peptone, glycerol, K₂HPO₄, MgSO₄ and agar) to isolate bacteria. To obtain anaerobic cultures, the dilution series (up to 10⁻¹²) of the homogenized termite gut and fungus comb were spread-plated on PYG medium (peptone, yeast and glucose) in an anaerobic chamber (COY Laboratory Products Inc.).

**Culture of Termiotomycetes and microfungi**

Two different methods were used for isolating the microfungal communities present in the fungus garden of *O. formosanus*. First, portions of the fungus comb were
homogenized and serially diluted (up to $10^{-12}$) and plated onto potato dextrose agar (MDBio, Inc.). In the second method, a fragment of the fungus comb was placed in a Petri dish containing sterile moist cotton under sterile conditions to create a humid environment, or wet chamber; after incubation for 2 days (Roderigues et al., 2008), the fungus comb was serially diluted as before. The fungal isolates were identified by internal transcribed spacer (ITS) primer sequencing (ITS1 forward primer: TCCGTAGGTAACCTGCGG and ITS4 reverse primer: TCCTCCGCTTATGGATATGC) (White et al., 1990). Termitomyces were isolated using a modified soil fungus medium containing antibiotics (Thomas, 1985).

**DNA extraction**

Fifty worker termites were degutted using fine-tipped sterile forceps, and total microbial DNA was extracted from their intestines using the Easy Tissue and Cell Genomic DNA Purification Kit, (Genemark) according to the manufacturer’s instructions. Initial attempts to isolate DNA from the fungus comb proved to be unsuccessful using either the PowerSoil™ DNA Isolation Kit (Mo Bio Laboratories) or the UltraClean™ fecal DNA Isolation Kit (Mo Bio Laboratories) according to the manufacturer’s instructions, probably due to excess humic acid contamination. A modified method described by Saghai-Maroof et al. (1984) was used in the isolation of total DNA from the fungus comb. In this method, 0.25 g of fungus comb was weighed and ground using a precooled mortar and pestle by adding autoclaved refined glass powder (prepared manually), 10 mL of cetyl trimethyl ammonium bromide (CTAB) extraction buffer containing 0.1 M Tris (pH 7.5), 1% cetyl trimethyl ammonium bromide, 0.7 M NaCl, 10 mM ethylenediaminetetraacetic acid (EDTA), 1% β-mercaptoethanol, and 0.3 g mL$^{-1}$ proteinase K (which was added immediately prior to use) and incubated at 65 °C for 30 min. After cooling on ice, an equal volume of chloroform/isoamyl alcohol (24 : 1) was added, and the tubes were shaken and centrifuged at 18 000 g for 15 min. The aqueous phase was carefully collected, and an equal volume of ice-cold isopropanol was added. If the DNA precipitate appeared colored (because of humic acid contamination), the above steps were repeated using phenol/chloroform/isoamyl alcohol (25 : 24 : 1) until a white, translucent DNA was obtained.

**PCR amplification using bacterial primers, denaturing gradient gel electrophoresis (DGGE) and cloning**

The concentration and purity of total DNA isolated from the termite gut and fungus comb were measured using a NanoDrop®-ND-1000 spectrophotometer (NanoDrop Technologies Inc.). The DNA was then purified using the QIAquick (Qiagen) kit to remove the humic acid contaminants. To identify the bacterial species present in the termite gut and fungus comb, PCR amplifications and DGGE were initially attempted using universal Eub 968GCF (AACGCGAAGAACCTTACGCCCCGGGCCGGCGG CCCCAGGGCAGGGGGGGGGACGAGG; the GC clamp is in bold interface) and Univ 1392R (ACGGGCGGTGTGTTACGACG; the GC clamp is in bold typeface) and RP534 (ATTACCAGGGCTGCTGG) (Kopečný et al., 2004) was also used for PCR–DGGE. DGGE was performed according to the protocol described by Hayashi et al. (2007) using the Dcode system (Bio-Rad Laboratories). The prominent DGGE bands were excised using a sharp, sterile scalpel under UV illumination. DNA was extracted from the gel by electroelution using an electroelutor (Genepure ELR9280). The supernatant containing DNA was purified using the QIAquick (Qiagen) kit. The aliquots of the purified supernatant (0.5 μL) were reamplified with the sample PCR mixture using touchdown conditions. The amplicons were ligated into the ‘YT and A’ cloning vector (Yeastern Biotech Co. Ltd., Taiwan) and transformed into chemically competent Escherichia coli DH5α. The colonies were plated onto Luria–Bertani plates containing ampicillin (50 μg mL$^{-1}$), 7 μL of 20% isopropl thio-β-D-galactoside (IPTG), and 40 μL of 2% X-gal by blue and white colony selection (Sambrook & Russell, 2001). The white colonies were selected, and colony PCR was performed using the yeast primers M13 F and M13 R (Long et al., 2010). The positive transformants were selected for sequencing.

**PCR amplification using bacterial primers and clonal selection**

The total DNA obtained from the termite gut and the fungus comb were amplified using the Eub 968F (AACGCGAAGAACCTTAC) and Univ 1392R (ACGGGCGGTGTGTTACG) primers (Nielsen et al., 1999), and the PCR amplicons were ligated into the ‘YT and A’ cloning vector. Transformation and blue and white colony selection were performed as previously described.

**Nested PCR and DGGE**

To identify all of the fungal species present in the termite gut and fungus comb, direct PCR amplifications were initially attempted using universal ITS1/ITS4 primers.
(Sheffield et al., 1989; White et al., 1990). However, DGGE bands were not detected, despite various experimental conditions. Therefore, nested PCR–DGGE was performed following the protocol of Guedegbe et al. (2009) using a GC-clamp primer in the second PCR.

**Phylogenetic analysis**

Sequencing of the clones was performed by the Genedragon service (Genedragon, Taiwan) using Seqman (DNAsStar). The 16S rRNA gene and 18S rDNA gene sequences were compared with the closest sequences deposited in the GenBank (NCBI) public database using the BLASTN software (http://blast.ncbi.nlm.nih.gov/Blast.cgi). To assign a definite taxonomic rank, sequences showing maximum identity and with the similar region and sequence length were selected to construct a phylogenetic tree. All of the sequences were aligned using the CLUSTALW software (European Bioinformatics Institute; http://www.ebi.ac.uk). Phylogenetic analyses and distance analyses (Jukes–Cantor) were conducted with the MEGA software version 4.1 (Tamura et al., 2007) with the sequence data using the neighbor-joining method, and the bootstrap support was assessed using 500 replicates.

**Interaction assays in vitro**

We examined the interactions between the bacterial symbionts (Bacillus, Ochrobactrum and Rhizobium) and the microfungi Trichoderma harzianum according to Sen et al. (2009). The second bioassay was performed to study whether the Bacillus cultivars (antagonistic to T. harzianum) inhibited or promoted the growth of Termitomyces. In the third bioassay, the mode of interaction of Termitomyces with T. harzianum was studied as per the protocol of Poulsen et al., 2007. As Termitomyces possesses slow growth rate compared with T. harzianum, a 1-month-old culture of Termitomyces was used for the above experiments and all the bioassays were performed in duplicates.

**Enzyme assays of Bacillus species isolated from the termite gut and fungus comb**

To investigate the functional aspects of the Bacillus species, the isolates obtained from the termite gut and fungus comb were qualitatively tested for a number of enzyme activities including lignin peroxidase, laccase, endoglucanase, xylanase, pectinase, protease, and 1, 3-β-glucanase. The lignin degradation was qualitatively determined using Remazol Brilliant Blue R (RBBR; 0.04%) (Kuhnigk & König, 1997) for laccase activity, and the drop-test method was used for lignin peroxidase activity using 0.01% guaiacol as the substrate (Okino et al., 2000). The proteolytic activity of the microbes was determined by the skim-milk agar method (Downes & Ito, 2001). The plate assay for xylanase was performed by growing the microbes in the medium containing xylan, and endoglucanase activity was assessed using carboxymethyl cellulose (CMC) as a substrate using the protocol of Skipper et al. (1985). Bacillus sp. was tested for polygalacturonase activity by measuring the degradation of the heteropolysaccharide pectin using a ruthenium red staining solution (0.05%) after incubation for 2 days at 37 °C (McKay, 1988). The 1, 3-β-glucanase activity was determined according to Nakanishi et al. (1976).

**Results**

**Bacterial community structure analysis by DGGE**

The bacterial composition determined from the total DNA extracts of the termite gut and fungus comb was analyzed by DGGE using universal primers and gene-targeted primers for cellulose degradation (FP338c1 and RP534). The representative DGGE bands were excised from the gel, cloned, sequenced, and numbered (check Supporting Information, Fig. S1 for universal primer DGGE and Fig. S2 corresponding to Table 1, for cellulolytic gene–targeted PCR–DGGE). The PCR product of universal bacterial primers was about 350 bp long, and only few bands could be detected after several attempts. On using the specific cellulolytic bacteria-specific primers, the V2–V3 fragments obtained from PCR amplification ranged from 180 to 200 bp long. A BLAST analysis revealed that the DGGE pattern of the bacteria in the termite gut differed from the fungus comb. Uncultured bacterial sequences were similar to known symbionts in macrotermite termites including Macrotermes gilvus from Thailand (Hongoh et al., 2006), Macrotermes michaelseni (Sjöestedt) (Mackenzie et al., 2007), and O. formosanus from the Ryuku archipelago (Shinzato et al., 2007). The DGGE results of the comb and gut indicated that the dominant uncultivated bacterial sequences were affiliated with Bacteriodetes followed by the phyla Firmicutes and Proteobacteria. No clones were affiliated with the phylum Spirochetes, and only Planctomyces were visualized in the fungus comb samples. Most of the Bacteriodetes detected from the DGGE patterns were similar to isolates from the alimentary canals of insects and mammals.

The sequences of 38 clones obtained by clonal selection using the EUB 968F and Univ1392R primers from the gut and fungus comb were deposited in GenBank with accession numbers JN049416–JN049447, JN081868. Based on the results (Table S1), 13...
phylogenotypes were identified with ≥ 97% identity. By BLAST searching, all were categorized into three groups in the domain bacteria and were as follows: Proteobacteria, Firmicutes, and uncultured bacteria. All of the uncultured Clostridiales clones showed resemblance to clones from fungus-growing termites from the Asian continents.

**Cultivable bacteria**

Based on the two media used for bacterial culture, the dominant groups of aerobic and anaerobic isolates belonged to the phylum Firmicutes (Table 2). The majority of the isolates in this phylum were members of the order Bacillales. By anaerobic culturing, a Lactococcus sp. (Lactococcus garvieae GU299084) was isolated from the gut, and *Clostridium* spp. (Clostridium bifurmentans HQ013322 & HQ123326) were obtained from the fungus comb.

**Phylogenetic analysis of the Bacillus strains**

The *Bacillus* isolates associated with the termite gut and comb (GenBank accession numbers JN000910-JN000915, JN000919-JN000923, JN000926, JN000927, and JN000929) were analyzed by constructing a phylogenetic tree using partial 16S rRNA gene sequences of similar lengths in the neighbor-joining method (Fig. 1). The termite gut *Bacillus* formed a separate cluster with the fungus-comb *Bacillus*, indicating that they may not be horizontally transmitted. The gut *Bacillus* was similar to other soil *Bacillus*, whereas fungus comb *Bacillus* showed more similarity to uncultured *Bacillus* based on their clustering. The use of *Bacillus*-specific primers also confirmed that our isolates (BFC and MGB) belonged to the genus *Bacillus* (Wu et al., 2006).

**Fungal community structure (culture-independent methods)**

Uncultured *Termitomyces* species detected in the fungus comb using a nested PCR–DGGE approach (see Fig. S3 and Table S2) and by BLAST analysis were similar to uncultured *Agaricales* clones obtained from Yunnan, China (Qian & Wen, unpublished data from GenBank). The uncultured fungal clones (designated 'fung') from the fungus comb samples were deposited in GenBank under the accession numbers JN000916-JN000918 (Table S2).

Repeated attempts at nested PCR for the identification of fungal species in termite gut samples failed. As a result, clonal library selection was performed with the termite gut samples using ITS1 and ITS 4 primers (Sheffield et al., 1989; White et al., 1990), and the clones (designated Ter) were sequenced and deposited in GenBank under the accession numbers JN000930-JN000932 (Table S2). Phylogenetic tree construction using the neighbor-joining method with ITS sequences was based on the *Termitomyces* species obtained from *Odontotermes* spp. native to the Asian and African subcontinents (Fig. 2) and clones from the fungus comb (obtained by nested PCR–DGGE) and the termite gut (by clonal library selection). It was shown that all of the clones except Ter 8 clustered with uncultured *Agaricales* from Yunnan and *Termitomyces* sp. from Kura. *Termitomyces* (Ter8) resembled the macrotermite termite *Macrotermes annandalei* from Khaokitchagoot, Thailand (Taprab et al., 2005). This species was detected in the fungus comb of *O. formosanus*, theoretically indicating that its spores were vertically transmitted. The similarity of the fungal clones associated with the termite gut and the fungus comb confirmed that...
the Termitomyces spores were horizontally transmitted from the termite gut to the fungus comb (via the worker termites). The use of Termitomyces-specific primers confirmed that the uncultured Agaricales clones belonged to the genus Termitomyces (Aanen et al., 2007).

Isolation of other microfungi

The presence of microfungi other than Termitomyces was detected by culture-dependent methods. Yeast species were isolated from the termite gut and fungus comb by direct serial dilution, whereas other filamentous fungi were selected by the wet-plate method (Table 3). Several fungi from the fungus garden were members of the class Sordariomycetes in the division Ascomycota (EU725821, FN666093, FJ799943, and DQ310778). Pestalotiopsis maculans and Xylaria, both in the order Xylariales, were also detected growing on the fungus comb. The Xylaria sp. appeared as a club-shaped stromal structure on the fungus comb that grew in the absence of termites. T. harzianum (AY857235) and Trichoderma viride (FN666093) (both in the genus Hypocrea) also grew vigorously on the fungus comb within a week, thus invading the entire fungus comb in the absence of termites (Fig. 4a). From our observations in vivo, it was noted that termites failed to survive on the fungus comb infected with Trichoderma species in a petri plate (data not shown).

Interaction studies

This study was carried out to assess the interactions of termite-associated bacterial cultivars with the cultivated fungus Termitomyces and the garden ‘weed’ fungus T. harzianum (Table 4). From the plate assays, it was observed that Termitomyces showed a chemotrophic

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**Table 2. Bacterial strains isolated from the gut and fungus comb of Odontotermes formosanus by culture-dependent methods**

<table>
<thead>
<tr>
<th>Bacterial strains</th>
<th>Source</th>
<th>Nearest neighbour in Genbank</th>
<th>% identity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Proteobacteria</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uncultured bacterium</td>
<td>Fungus comb</td>
<td>DQ340954</td>
<td>100</td>
</tr>
<tr>
<td>Uncultured bacterium</td>
<td>Termite gut</td>
<td>GQ476395</td>
<td>100</td>
</tr>
<tr>
<td><em>Ochrobactrum</em> sp.</td>
<td>Fungus comb/termite gut</td>
<td>HM066231</td>
<td>99</td>
</tr>
<tr>
<td><em>Rhizobium</em> species</td>
<td>Fungus comb</td>
<td>HM233995</td>
<td>94</td>
</tr>
<tr>
<td><em>Shigella</em> sp. (JN00925)</td>
<td>Termite gut</td>
<td>JF833739</td>
<td>100</td>
</tr>
<tr>
<td>E. coli (JN00924)</td>
<td>Termite gut/Fungus comb</td>
<td>HM097755</td>
<td>99</td>
</tr>
<tr>
<td>E. coli (JN00928)</td>
<td>Termite gut</td>
<td>JN180970</td>
<td>100</td>
</tr>
<tr>
<td>γ proteobacteria (JN00928)</td>
<td>Termite gut</td>
<td>HQ012019</td>
<td>99</td>
</tr>
<tr>
<td><strong>Firmicutes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Bacillus</em> sp. (JN00910-JN00913)</td>
<td>Fungus comb</td>
<td>AB244530</td>
<td>99, 100</td>
</tr>
<tr>
<td><em>Bacillus</em> sp. (JN00914)</td>
<td>Fungus comb</td>
<td>JF418154</td>
<td>100</td>
</tr>
<tr>
<td><em>Bacillus thuringiensis</em> (JN00915)</td>
<td>Fungus comb</td>
<td>HQ710547</td>
<td>99</td>
</tr>
<tr>
<td><em>Bacillus</em> sp. (JN00919, JN00921, JN00922, JN00926 &amp; JN00927)</td>
<td>Termite gut</td>
<td>FM180506</td>
<td>99, 100</td>
</tr>
<tr>
<td><em>Bacillus</em> sp. (JN00920, JN00923, JN00929)</td>
<td>Termite gut</td>
<td>JF753532</td>
<td>99 &amp; 100</td>
</tr>
<tr>
<td><em>Lactococcus garrvea</em></td>
<td>Termite gut</td>
<td>GU299084</td>
<td>100</td>
</tr>
<tr>
<td><em>Clostridium bifermentans</em></td>
<td>Fungus comb</td>
<td>HQ013222</td>
<td>100</td>
</tr>
<tr>
<td><em>Clostridium bifermentans</em></td>
<td>Fungus comb</td>
<td>HQ123326</td>
<td>98</td>
</tr>
</tbody>
</table>
growth toward the Bacillus strain (BFC1) (Fig. 3b; inset figure). Microscopic studies revealed that the Bacillus sp. colonized around the mycelial filaments of Termitomyces but did not inhibit their growth (Fig. 3b). The mycelia of Termitomyces appeared to be healthy and rich with cytoplasmic contents after interacting with Bacillus. 

In our study, the termite-associated Bacillus sp. from the gut (MGB) and the fungus comb (BFC) of O. formosanus were able to suppress the in vitro growth of the invasive T. harzianum (Fig. 4d). Furthermore, light microscopy studies with a co-culture of Bacillus and T. harzianum (Fig. 4e) showed that the mycelial tips of T. harzianum were rounded, indicating appressorium formation and suggesting the moderation of attack. However, in the control T. harzianum, the ends appeared to be normal (Fig. 4c), which indicates that the morphology of Trichoderma was rapidly altered in the presence of Bacillus sp. Biomass studies with the antagonistic Bacillus species and T. harzianum grown in potato dextrose broth (PDB) showed no decrease in biomass compared with the control containing only T. harzianum (Fig. 4c) after incubation for 48 h.

Trichoderma harzianum grew rapidly over a 1-month-old Termitomyces isolate (T-983, 99% similarity to AB073529) in 2 days (Fig. 3a). An Ochrobactrum sp. (HM056231) and a Rhizobium sp. (HM233995) isolated from the fungus comb were used as control strains to monitor antagonistic activity against T. harzianum. Apparently, Ochrobactrum and Rhizobium strains did not inhibit T. harzianum (Fig. 4b), suggesting that the termite-associated Bacillus sp. may play a major role in suppressing the overgrowth of Trichoderma in the fungus comb.

**Functional analysis of the Bacillus species**

The termite-associated Bacillus species was also assessed qualitatively for lignocellulosic degradation capability (Table 5). We observed that most of the Bacillus isolates from the termite gut were capable of degrading hemicelluloses such as xylan and carboxymethyl cellulose. The lignin-degradation activity of the Bacillus isolates was monitored by detecting lignin peroxidase and laccase enzyme activities. Lignin peroxidase activity was detected after 48 h of incubation, whereas laccase activity could not be detected.

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**Table 3.** Fungal isolates obtained from the termite gut and fungus comb by culture-dependent approaches

<table>
<thead>
<tr>
<th>Fungal isolate</th>
<th>Source</th>
<th>Accession number of nearest neighbor</th>
<th>% identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Candida orthopsilosis</td>
<td>Fungus comb</td>
<td>FN812686</td>
<td>99</td>
</tr>
<tr>
<td>Candida parapsilosis</td>
<td>Fungus comb</td>
<td>AY055855</td>
<td>100</td>
</tr>
<tr>
<td>Candida inconspicua</td>
<td>Fungus comb</td>
<td>EF152417</td>
<td>98</td>
</tr>
<tr>
<td>Pichia guilliermondii</td>
<td>Fungus comb/Termite gut</td>
<td>EU784644</td>
<td>99</td>
</tr>
<tr>
<td>Debaryomyces hansenii</td>
<td>Fungus comb/Termite gut</td>
<td>DQ534404</td>
<td>98</td>
</tr>
<tr>
<td>Endothia spp.</td>
<td>Fungus comb</td>
<td>EU812126</td>
<td>99</td>
</tr>
<tr>
<td>Pestalotiopsis maculans</td>
<td>Fungus comb</td>
<td>EU725821</td>
<td>99</td>
</tr>
<tr>
<td>Trichoderma viride</td>
<td>Fungus comb</td>
<td>FJ799943</td>
<td>97</td>
</tr>
<tr>
<td>Sordariomycetes spp.</td>
<td>Fungus comb</td>
<td>DQ310778</td>
<td>97</td>
</tr>
<tr>
<td>Ascomycetes spp.</td>
<td>Fungus comb</td>
<td>AY857235</td>
<td>99</td>
</tr>
<tr>
<td>Trichoderma harzianum</td>
<td>Fungus comb</td>
<td>GU324757</td>
<td>99</td>
</tr>
<tr>
<td>Xylaria spp.</td>
<td>Fungus comb</td>
<td>FJ799943</td>
<td>97</td>
</tr>
</tbody>
</table>

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not be detected after 48 h either by the drop test or using Remazol brilliant blue. All the *Bacillus* isolates in the gut and the comb exhibited protease activity. The isolates also exhibited 1, 3-β-glucanase activity, indicating their ability to degrade fungal cell walls.

**Discussion**

The termite gut and the fungus comb harbor a wide variety of novel genera and species (Katoh *et al.*, 2002; Shinzato *et al.*, 2005; Long *et al.*, 2010; Ohkuma & Brune, 2011). Owing to limitations in the traditional cultivation-dependent methods of identifying members of the insect gut microbiome and soil microorganisms, culture-independent methods were used, namely, gene-targeted PCR followed by DGGE (Hill *et al.*, 2000; Shi *et al.*, 2010). The DGGE patterns were analyzed using universal and bacterial primers specific for the V2–V3 region of the 16S rRNA gene (positions 339–539 in *E. coli* DNA; Kopecˇny´ et al., 2004). The gene-specific bacterial primers are generally used to detect cellulolytic bacteria belonging to the genera *Bacteriodetes*, *Eubacterium*, *Clostridium*, *Bifidobacterium*, *Ruminococcus*, *Faecalibacterium* (*Fusobacterium*), *Peptococcus*, *Lactobacillus*, and *Peptostreptococcus*. Surprisingly, *Termitomyces* sp. was also detected using these bacterial primers (see Table 1 and Fig. S2). The *Termitomyces* clone (FC4 in Table 1) is closely affiliated to the sequences from geographically related Kura (98% identity to AB051892) (an island in the eastern part of the China Sea, near Taiwan), proving that this result is reliable. The majority of the cellulolytic bacteria are more abundant in the alimentary canal of the insects than in the fungus comb, indicating that cellulolytic degradation took place in the termite gut rather than in the fungus comb. Previously, the bacterial community structure of the gut of *O. formosanus* from Iriomote Island was studied by restriction fragment length polymorphism (RFLP) (Shinzato *et al.*, 2007). Our DGGE and clonal selection studies indicated that the bacterial species and the *Termitomyces* sp. from the termite gut and fungus comb were consistent within macrotermitine termites (Katoh *et al.*, 2002; Shinzato *et al.*, 2005, 2007). However, there is no direct evidence that the gut microbes were co-evolved with the termites and were transferred by vertical transmission. It can only be proved theoretically as several bacteria and fungi in our study showed similarity to the symbionts in the other fungus-growing termites.

*Odontotermes formosanus*, being a higher termite, lack the flagellated protists that assist the cellulolytic degradation in lower termites. Therefore, the breakdown of cellulose and hemicelluloses is performed by gut microbes, and host cellulase genes present in the termite (Kuhnigk & König, 1997; Yang *et al.*, 2004; Huang *et al.*, 2008). König (2006) explained the function of various termite gut microbes involved in the different stages of cellulose, hemicellulose, and lignin degradation, which can be

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### Table 4. Petri dish bioassay experiments to check the mode of the interaction of the cultivar strains

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Cultivar strains</th>
<th>Mode of interaction</th>
<th>Fungal strain</th>
</tr>
</thead>
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<tr>
<td>I bioassay</td>
<td><em>Bacillus</em></td>
<td>Antagonistic to</td>
<td><em>T. harzianum</em> (intruder strain)</td>
</tr>
<tr>
<td></td>
<td><em>Ochrobactrum</em></td>
<td>Invaded by</td>
<td><em>T. harzianum</em> (intruder strain)</td>
</tr>
<tr>
<td></td>
<td><em>Rhizobium</em></td>
<td>Invaded by</td>
<td><em>T. harzianum</em> (intruder strain)</td>
</tr>
<tr>
<td>II bioassay</td>
<td><em>Bacillus</em></td>
<td>Symbiotic to</td>
<td><em>Termitomyces</em> (cultivar strain)</td>
</tr>
<tr>
<td>III bioassay</td>
<td><em>Termitomyces</em></td>
<td>Invaded by</td>
<td><em>T. harzianum</em> (intruder strain)</td>
</tr>
</tbody>
</table>

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**Fig. 3.** Interaction of *Termitomyces* with *Bacillus* (BFC1) and *Trichoderma harzianum* (Th) (a) Interaction of 2-day-old *T. harzianum* (Th) with 1-month-old *Termitomyces* (Ter). (b) The inset figure shows the interaction of *Bacillus* species (BFC1) with *Termitomyces* on a PDA plate. The square in the inset picture is an image viewed under light microscopy at 40× magnification. Yellow arrows indicate their interaction points.
associated with our results. The predominance of coccoi d lactic acid bacteria reported in the hindguts of lower termites such as *Mastotermes darwiniensis* (Family *Mastotermitidae*) and *Cryptotermes primus* (Family *Mastotermitidae*) and even in higher termites such as *Nasutitermes arborum* (Family *Nasutitermitinae*), *Thoracotermes macrorys* (Family *Termitidae*) and *Anoplotermes pacificus* (Family *Apicotermitidae*) (Brune, 2006; Brune & Ohkuma, 2011) can facilitate the production of lactate from glucose under anaerobic conditions. However, this is the first study to show the presence of coccoi d lactic acid bacteria (GU299084) in the gut of macrotermitine termites. The sulfate-reducing *Desulfovibrio* sp. have also been isolated from other termites and have known to reduce oxygen in the presence of hydrogen (Brune & Ohkuma, 2011). Thus, the termite gut of *O. formosanus* acts as a bioreactor containing aerobic, facultative, and strictly anaerobic bacteria that are capable of breaking 

Fig. 4. Interaction studies of the bacteria and fungi isolated from the fungus comb (a) Invasive growth of *Trichoderma harzianum* (green spores) on the fungus comb in the absence of termites. (b) Interaction of *Ochrobactrum* (Och) and *Rhizobium* (Rhi) with *T. harzianum* (Th). No antagonism was exhibited by these strains against *Trichoderma*. (c) Light microscopy views of *T. harzianum* at 40× magnification. (d) Interaction of *Bacillus* species from gut (MGB1/MGB10) and fungus comb (BFC1) with *T. harzianum* (Th). Intermediate levels of antagonism were exhibited by MGB1/MGB10 (0.65 cm) and BFC1 (0.70 cm). (e) Light microscopy views of *T. harzianum* inhibited by BFC1 isolate at 40× magnification. Yellow arrows indicate the swollen mycelial ends of *T. harzianum*.

Table 5. MGB denotes the *Bacillus* species that were isolated from the termite gut, and BFC refers to the *Bacillus* species isolated from the fungus comb by culture-dependent methods; ‘+’ denotes enzyme activity, ‘−’ denotes no enzyme activity and ‘ND’ indicates that enzyme activity was not clearly defined.

<table>
<thead>
<tr>
<th>Isolate name</th>
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<th>Protease</th>
<th>Xylanase</th>
<th>CMCase</th>
<th>Lignin peroxidase</th>
<th>Laccase</th>
<th>1,3-β-glucanase</th>
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<td>+</td>
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<tr>
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<td>+</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
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<td>+</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
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<td>+</td>
<td>+</td>
<td>ND</td>
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<td>+</td>
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<tr>
<td>BFC3</td>
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<td>+</td>
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<td>BFC5</td>
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<td>−</td>
<td>+</td>
<td>ND</td>
<td>+</td>
</tr>
</tbody>
</table>

from glucose under anaerobic conditions. However, this is the first study to show the presence of coccoi d lactic acid bacteria (GU299084) in the gut of macrotermitine termites. The sulfate-reducing *Desulfovibrio* sp. have also been isolated from other termites and have known to reduce oxygen in the presence of hydrogen (Brune & Ohkuma, 2011). Thus, the termite gut of *O. formosanus* acts as a bioreactor containing aerobic, facultative, and strictly anaerobic bacteria that are capable of breaking
down cellulose and hemicellulose into glucose and converting glucose into lactate or acetate, CO$_2$ and H$_2$ (Scharf & Tartar, 2008; Shi et al., 2010; Brune & Ohkuma, 2011). Apart from their role in cellulolytic degradation and fermentation, their nitrogen-fixing ability remains unclear. *Bacterioedetes* were predominantly found in the gut of *O. formosanus* (Shinzato et al., 2007), and there is a possibility that these microbes increase the nitrogen supply by recycling host uric acid wastes (Brune, 2006). It has been speculated that in macrotermite termites, the symbiotic fungus *Termitomyces* is the major contributor to termite nutrition and nitrogen fixation, rather than the bacterial symbionts (Brune, 2006; Brune & Ohkuma, 2011).

Only few clones were obtained by clonal selection probably because the extraction efficiencies varied among different microorganisms. One of the reasons is that some of the bacterial cells were easily lysed than others (Hill et al., 2000). Secondly, the termite gut and fungus comb were rich in humic acid, and removal of humic acid by the purification step might have reduced a significant fraction of nucleic acid (Moran et al., 1993). The purification step was vital for termite gut and fungus comb DNA samples, as humics interfered with PCR amplification. No actinobacterial clones were found in the termite gut likely because actinobacteria were present in the salivary glands of termites or found on the surface of termites, as in the galleries of *D. frontalis* beetles and their laterocervical plates and on the forelegs of ants (Apterostigma spp.) (Currie et al., 2003; Currie, 2004; Scott et al., 2010). Similar results were reported for the termite gut and the fungus combs of *Odontotermes yumanensis* (Family *Macrotermitinae*) from China (Long et al., 2010), contrary to those for the soil-feeding termite *Cubitermes niokoloensis* (Family *Termitidae*) (Ohkuma & Brune, 2011). Previously, only very few actinobacteria were isolated in individual *O. formosanus* termites from Iriomote island (Shinzato et al., 2007; Bignell, 2011). From our studies, no visible actinobacteria were also detected by culture-based methods, probably because Chitin-specific medium was required to screen actinomycetes.

Considering the fungal symbionts, approximately 40 different lineages of *Termitomyces* sp. have been identified in the fungus gardens of Asian and African continents (Aanen et al., 2007). It has been reported that a greater number of *Termitomyces* lineages were observed in *Odontotermes* sp. than in other macrotermite termites (Osiemo et al., 2010). Various culture-independent methods such as terminal restriction fragment length polymorphism (T-RFLP), arbitrarily primed polymerase chain reaction (AP-PCR), suicide polymerase restriction endonuclease (SuPER)-PCR, and DGGE have been used to detect the presence of *Termitomyces* and non-*Termitomyces* fungi in the fungus gardens of *Odontotermes* spp. and *Macrotermes gilvus* (Katoh et al., 2002; Moriya et al., 2005; Guedegbe et al., 2009). Using the SuPER method, only yeast species such as *Pichia caribbica* were detected in *Odontotermes* spp. from Thailand and *Candida* spp. in *Macrotermes subhyalinus* from Africa (Guedegbe et al., 2009). Based on our results and previous reports, the genus *Candida* is dominant among the yeast species in the fungus combs of both ants (*Candida famata*, *Candida colliculosa*, and *Candida homilentoma*) (Carreiro et al., 1997) and *O. formosanus* (*Candida orthopsilosis*, *Candida parasilosis*, and *Candida inconspicua*). These yeast species have been reported to be present in the paunch region of both lower and higher termites and are able to degrade hemicelluloses (Schäfer et al., 1996). Other microfungi, such as *T. harzianum*, were observed to be the most prominent and fast-growing fungi detected in the fungus gardens of leaf-cutter ants (Roderigues et al., 2008) and are parasitic to edible mushrooms such as *Agaricus bisporus* (Castle et al., 1998) and *Termitomyces*, based on our results. Another fungi of the genus *Hypocreta*, *T. viride*, has been reported as a fungus garden parasite in leaf-cutter ants but was proven not to be directly harmful to the ants (Currie & Stuart, 2001); their role in the fungus gardens of termites is still unknown. Members of the *Sordariomyces* and other *Ascomycetes* have also been previously isolated from the fungus combs of *O. formosanus* in the absence of termites (Shinzato et al., 2005). *Xylaria* sp. develop fruiting bodies through the soil from termite-abandoned dead and decaying fungus combs (Batra & Batra, 1979; Wood & Thomas, 1989). These species have also been found on the abandoned termite nests of *O. formosanus* from Taiwan (Ju & Hsieh, 2007). In our findings, none of these microfungi were detected by nested PCR–DGGE. Thus, we propose that *Termitomyces* exists as a monoculture (Shinzato et al., 2005) in the fungus comb and termite gut.
known to be present in the gut of termites from previous studies (König, 2006). In our work, Bacillus sp. in fungus combs were detected only by culture-dependent methods and not by clonal selection or by DGGE, which was likely because they existed as spores and required a higher temperature to extract their DNA by rupturing the spore cap or probably because of their small sampling size. However, the termite gut Bacillus of O. formosanus was detected by culture-dependent and culture-independent method using universal bacterial primers (Fig. S1 and Table 2). Also, the Bacillus from our studies were found to be divergent from the Bacillus sequences (deposited in GenBank), which belonged to lower termite Zootermopsis augusticollis (Wenzel et al., 2002) and higher soil and wood-feeding termites (Thongaram et al., 2003) (data not shown). Apart from the Bacillus in other termites, this is the first study to report the occurrence of Bacillus in the termite gut and fungus comb of macrotermitine termites. It is essential to know the functioning of termite-associated Bacillus as a mutualist because of their relative abundance in the termite–fungus-comb system. As the termite gut Bacillus was directly isolated from the termite and because of their function in the degradation of lignocellulose, they may be regarded as mutualists. The efficient utilization of the lignocelluloses (in fungus comb) by the well-coordinated cooperation of termites and fungi has been explained in Ohkuma et al. (2001). Until now, no study has mentioned the contribution of any bacterial species in the degradation of the lignocellulosic materials in the fungus comb of macrotermite termites. As the fungus comb Bacillus could degrade lignocelluloses by enzymatic plate assays, they could probably cooperate along with Termitomyces in the degradation of lignin as well as breakdown hemicellulosic substances in association with the host cellulase genes (Yang et al., 2004). Our bioassay studies also showed that Bacillus is beneficial for the growth of Termitomyces and suppressed the growth of the microfungi such as T. harzianum. The Bacillus–Termitomyces association could be advantageous in initiating the lignin breakdown, therefore indicating that the fungus comb Bacillus may also function as a mutualist.

Earlier reports have stated the fungus comb of fungus-growing termites were prone to attack by unwanted invasive bacteria and fungi and that self-grooming and weeding by the termites may remove or minimize the occurrence of these microbes from the fungus comb (Batra & Batra, 1979; Rosengaus et al., 2011). Furthermore, the growth of other fungi may be restricted because of inhibition by termite secretions and fungus garden physiochemical conditions such as the temperature, antibiotic, and chemical composition of the comb (Wood & Thomas, 1989). Reactive and prophylactic defense mechanisms similar to the application of pesticides and weeding have been observed in leaf-cutter ants (Currie & Stuart, 2001; Rosengaus et al., 2011). In vitro studies were performed to analyze the ecological interactions of various microbes in the gut and fungus comb of O. formosanus, and a hypothetical model was postulated from our observations (Fig. 5). Trichoderma sp. are carried in by the worker termites along with the foraged plant materials and exist as ungerminated spores until they are removed from the nest or abandoned by the macrotermite termites (Wood & Thomas, 1989). We speculate that the T. harzianum conidia were not detected in the termite gut by either culture-dependent or culture-independent methods because the conidia were probably deactivated in the gut (Batra & Batra, 1979; Wood & Thomas, 1989; Yanagawa & Shimizu, 2007). Trichoderma harzianum did not outgrow or invade the fungus comb in the presence of termites, which was likely because they were present only in certain regions of the fungus comb and because these isolates could only be visualized by the wet-plate method and not by direct serial dilution of the fungus comb. This apparent distribution of T. harzianum in the comb could be due to the regulation of its growth by the secretion of certain fungicides by the termites or perhaps by certain fungistatic compounds present in the fecal matter of termites (Rosengaus et al., 2011). Trichoderma grew faster at a higher temperature and humidity in the fungus comb under artificial conditions, but these microclimatic conditions were fatal to the termites (Chi-Yung Lai, unpublished results). We assume that under the typical in vivo conditions (temperature and humidity) of the nest environment, Trichoderma is suppressed by the Bacillus sp. present in the gut and fungus comb probably by the secretion of antibiotics (Chaurasia et al., 2005). It is not known whether the antagonist T. harzianum coevolved along with O. formosanus termites similarly to the garden parasites Escovopsis with ants (Currie et al., 2003) and Xylaria with

Fig. 5. Hypothetical model for the interactions among microbes residing in the termite gut and fungus comb of Odontotermes formosanus.
termites. As this study was based on a single termite colony, it is not known whether the same Bacillus would inhibit the Termitomyces belonging to other species. The similar experiments need to be repeated with other Termitomyces species in future. Also, we need to assess the impact of Bacillus on the host fitness.

In conclusion, this study provides an insight of the bacterial and fungal microbes inhabiting the fungus-growing termite system of O. formosanus with prime focus on the role of Bacillus and their probability of functioning as a mutualist. In vivo studies remain to be performed to confirm the role of Bacillus cultivars in an active termite fungus-comb system, and their exact mechanism of inhibition need to be elucidated.

Acknowledgements

The authors wish to thank Mr Cho for the field sampling work and for the identification of the termites, Dr L.C. Chen for his ideas on Termitomyces and their cultivation methods, and Mr Asif Hameed and Mrs Mariyam Shahnaz for their valuable suggestions on the phylogenetic tree construction and deposition of the sequences in GenBank. The authors would also like to sincerely acknowledge the reviewers (anonymous) for their valuable suggestions and comments for the betterment of this paper. This work was financially supported by IPMB Summit Team Project, Academia Sinica, Taiwan and the Ministry of Education, Taiwan R.O.C under the ATU plan.

References


Allanheld, Montclair, NJ.


Additional Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. DGGE profile of the termite gut (A) and fungus comb (B) of O. formosanus using universal primers EUB 968F with GC clamp and Univ1392R.

Fig. S2. DGGE profiles of the termite gut and fungus comb using cellulolytic bacterial specific primers FP 338c1 and RP534. The DGGE bands are designated as ‘OF’ for the termite gut and ‘FC’ for the fungus comb. The information of the corresponding DGGE bands is mentioned in Table 1.

Fig. S3. DGGE profiles of the nested-PCR-amplified ITS sequences from the fungus comb. The DGGE bands are designated as ‘fung’. The details corresponding to the DGGE bands are mentioned in Table S2.

Table S1. Bacterial strains isolated from the gut and fungus comb of O. formosanus by clonal selection.

Table S2. Fungal clones obtained from the fungus comb (fung) and termite gut (Ter) of O. formosanus using ITS1 and ITS4 primers.

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