RESEARCH ARTICLE

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

Gincy Marina Mathew[1], Yu-Ming Ju[2], Chi-Yung Lai [3], Dony Chacko Mathew[1] & Chieh Chen Huang[1,4]

[1]Department of Life Sciences, National Chung Hsing University, Taichung, Taiwan; [2]Institute of Plant and Microbial Biology, Academia Sinica, Taipei, Taiwan; [3]Department of Biology, National Changhua University of Education, Changhua, Taiwan; and [4]Agricultural Biotechnology Center, National Chung Hsing University, Taichung, Taiwan

Correspondence: Chieh Chen Huang, Department of Life Sciences, National Chung Hsing University, Taichung, Taiwan. Tel.: +886 4 22840416#404; fax:+886 4 22874740; e-mail:cchuang@dragon.nchu.edu.tw

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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).

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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 macrotermitine 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 *Leucocoprinus gongylophorus* (Family *Lepiotaceae*) (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 macrotermitine 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 *Termitomyces* (Wood & Thomas, 1989; Gullan & Cranston, 2010). However, the interactions of mutualists against these microfungi have not been explored in macrotermitine 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 rhizopheric 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 macrotermitine 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 macrotermitine 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, K_2HPO_4, $MgSO_4$ 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 *Termitomyces* 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: TCCGTAGGTGAACCTGCGG and ITS4 reverse primer: TCCTCCGCTTATTGATATGC) (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 prechilled 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 (AACGCGAAGAACCTTACCGCCCGGGGCGCG CCCCGGGCGGGGCGGGGGCACGGGGG; the GC clamp is in bold interface) and Univ 1392R (ACGGGCGGTGT GTAC) primers (Nielsen *et al.*, 1999).

A primer set specific for bacterial cellulose degrading genes FP338c1 (CGCCCGCCGCGCCCCGCGCCCGGCC CGCCGCCGCCGCCGCATCCTACGGGAGGCAGCAG; the GC clamp is indicated in bold typeface) and RP534 (ATTACCGCGGCTGCTGG) (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% isopropyl 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 (ACGGGCGGTGTGTAC) 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 (DNAstar). 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* possess 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 carboxy methyl 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 macrotermitine 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. [Correction added after online publication 7 December: JN086818 changed to JN081868]. Based on the results (Table S1), 13

Table 1. DGGE profiles of PCR-amplified partial 16S rRNA gene bacterial sequences from the termite gut (OF) and fungus comb (FC) using the bacterial primer set FP338cl and RP534. Check Fig. S2 for the corresponding DGGE image

Band name	Phylum	Closest neighbor	Accession number	% identity
OFI	*Bacteriodetes*	Uncultured *Bacteriodales* bacterium	AB234422	95
OF2	Unidentified bacterium	Uncultured bacterium	AF371636	97
OF3	*Bacteriodetes*	Uncultured *Bacteriodales*	AB234384	96
OF4	*Firmicutes*	Uncultured *Clostridium* sp.	GU255481	100
OF5	*Bacteriodetes/Chlorobi*	Uncultured bacterium	AB288898	92
OF6	*Bacteriodetes*	Uncultured bacterium	AY791235	94
OF7	*Bacteriodetes/Chlorobi*	Uncultured bacterium	AB288916	98
OF8	*Proteobacteria*	*Enterobacteriales* bacterium	FJ650514	96
OF9	*Firmicutes*	Uncultured bacterium	AB288910	99
FC1	*Bacteriodetes*	Uncultured *Bacteriodales*	AB234435	94
FC2	*Firmicutes*	Uncultured bacterium	EF404556	94
FC3	*Planctomycetes*	Uncultured bacterium	DQ307719	93
FC4	*Basidiomycota* (Fungi)	*Termitomyces* sp.	AB051892	98
FC5	*Proteobacteria*	Uncultured bacterium	FJ457978	98
FC6	(*Bacteriodetes/Firmicutes*)	Uncultured bacterium	DQ795983	100

phylotypes were identified with $\geq$ 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 bifermentans* 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 macrotermitine termite *Macrotermes annandalei* from Khao Kitchagoot, 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

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

Bacterial strains	Source	Nearest neighbour in Genbank	% identity
Proteobacteria			
Uncultured bacterium	Fungus comb	DQ340954	100
Uncultured bacterium	Termite gut	GQ476395	100
Ochrobactrum sp.	Fungus comb/termite gut	HM056231	99
Rhizobium species	Fungus comb	HM233995	94
Shigella sp. (JN000925)	Termite gut	JF833739	100
E.coli	Termite gut/Fungus comb	HM209775	99
E.coli (JN000924)	Termite gut	JN180970	100
γ *proteobacteria* (JN000928)	Termite gut	HQ012019	99
Firmicutes			
Bacillus sp. (JN000910-JN000913)	Fungus comb	AB244530	99, 100
Bacillus sp. (JN000914)	Fungus comb	JF418154	100
Bacillus thuringiensis (JN000915)	Fungus comb	HQ710547	99
Bacillus sp. (JN000919,JN000921, JN000922,JN000926 & JN000927)	Termite gut	FM180506	99, 100
Bacillus sp. (JN000920,JN000923, JN000929)	Termite gut	JF753532	99 & 100
Lactococcus garviea	Termite gut	GU299084	100
Clostridium bifermentans	Fungus comb	HQ013322	100
Clostridium bifermentans	Fungus comb	HQ123326	98

The new accession numbers for *Bacillus* species are in parentheses.

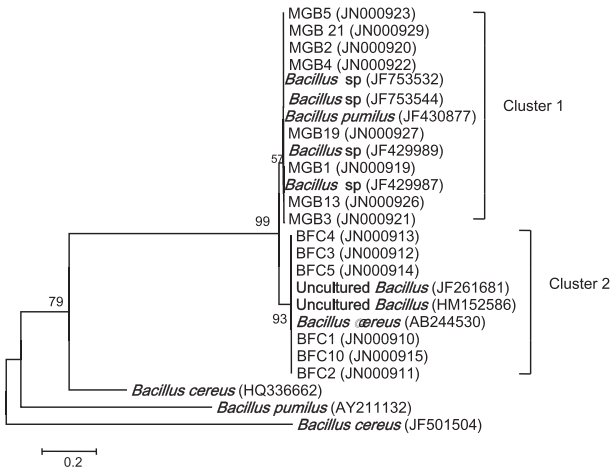

Fig. 1. Neighbor-joining phylogenetic tree of partial bacterial 16S rRNA gene sequences (495 nucleotides). Bootstrap values above 50% are shown. Accession numbers of the *Bacillus* species (in bold) from GenBank are shown in parentheses. The sequences from the GenBank used for the phylogenetic tree construction mainly belong to soil *Bacillus*. The culture-dependent *Bacillus* species from the gut and fungus comb are designated as 'MGB' and 'BFC', respectively, with accession numbers given in parentheses.

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

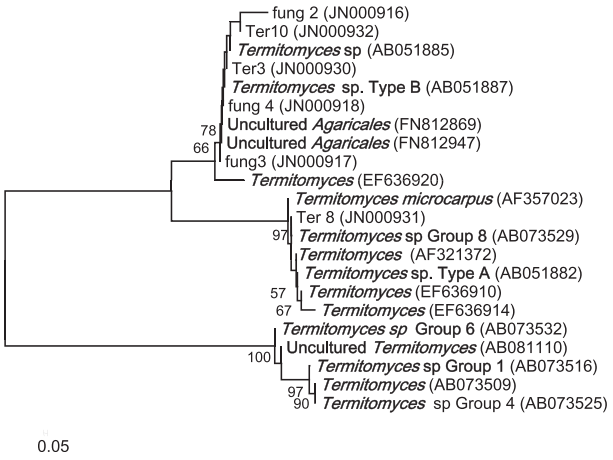

Fig. 2. Neighbor-joining phylogenetic tree of partial ITS sequences from the termite gut (Ter) and fungus comb (fung) of *Odontotermes formosanus* using ITS1 and ITS4 primers (approximately 600 nucleotides). Bootstrap values above 50% are shown. Other selected *Termitomyces* species from Asian and African continents (in bold) and their accession numbers are shown in parentheses. The DGGE bands of *Termitomyces* from fungus combs are designated fung2, fung3 and fung4. Ter denotes the clones that were obtained from the clonal library of the termite gut. The accession numbers of the fungal clones are given in parentheses.

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

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

Fungal isolate	Source	Accession number of nearest neighbor	% identity
Yeast			
Candida orthopsilosis	Fungus comb	FN812686	99
Candida parapsilosis	Fungus comb	AY055855	100
Candida inconspicua	Termite gut/fungus comb	EF152417	98
Pichia guilliermondii	Fungus comb/Termite gut	EU784644	99
Debaryomyces hansenii	Fungus comb/Termite gut	DQ534404	98
Filamentous fungi			
Endothia spp.	Fungus comb	EU812126	99
Pestalotiopsis maculans	Fungus comb	EU725821	99
Trichoderma viride	Fungus comb	FN666093	99
Sordariomycetes spp.	Fungus comb	FJ799943	97
Ascomycetes spp.	Fungus comb	DQ310778	97
Trichoderma harzianum	Fungus comb	AY857235	99
Xylaria spp.	Fungus comb	GU324757	99

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

Experiment	Cultivar strains	Mode of interaction	Fungal strain
I bioassay	*Bacillus*	Antagonistic to	*T. harizanum* (intruder strain)
	Ochrobactrum	Invaded by	*T. harizanum* (intruder strain)
	Rhizobium	Invaded by	*T. harizanum* (intruder strain)
II bioassay	*Bacillus*	Symbiotic to	*Termitomyces* (cultivar strain)
III bioassay	*Termitomyces*	Invaded by	*T. harzianum* (intruder strain)

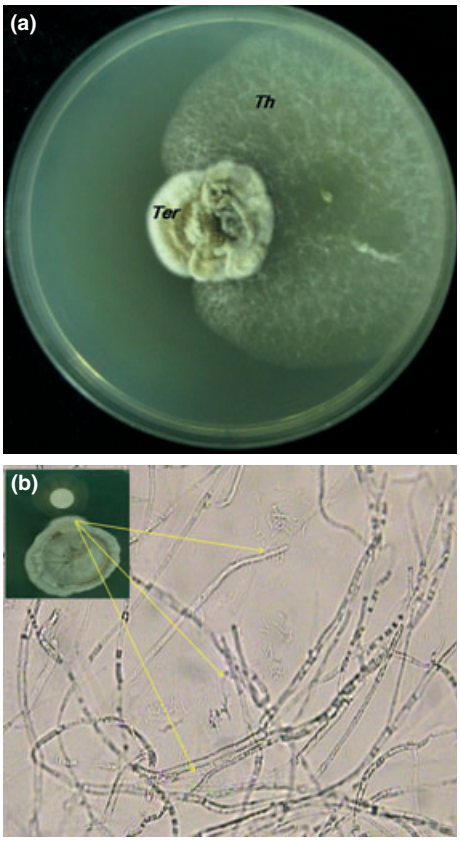

Fig. 3. Interaction of *Termitomyces* with *Bacillus* (BFC1) and *Trichoderma harzianum* (Th) (a) Interaction of 2-day-old *T. harzianum* (Th) with 1-month-old *Termitomyces* (Ter). (b) The inset figure shows the interaction of *Bacillus* species (BFC1) with *Termitomyces* on a PDA plate. The square in the inset picture is an image viewed under light microscopy at 40× magnification. Yellow arrows indicate their interaction points.

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; Kopečný *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

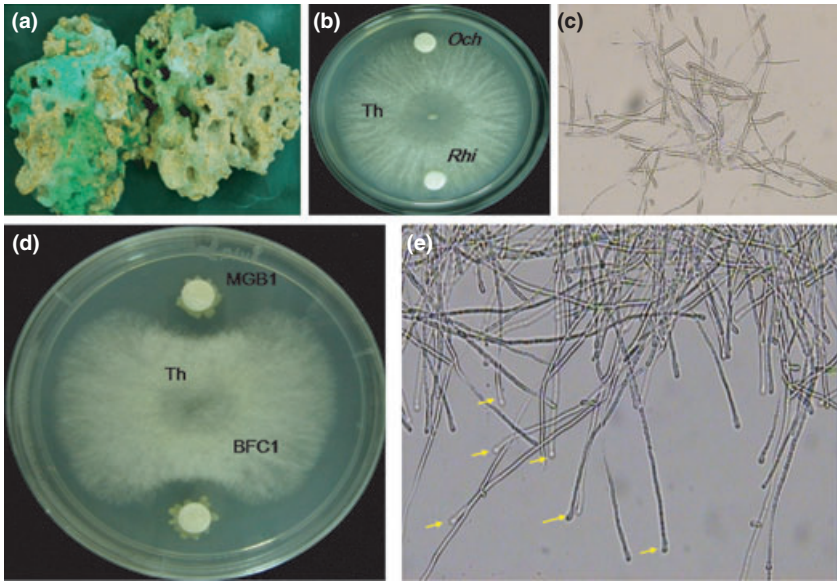

Fig. 4. Interaction studies of the bacteria and fungi isolated from the fungus comb (a) Invasive growth of *Trichoderma harzianum* (green spores) on the fungus comb in the absence of termites. (b) Interaction of *Ochrobactrum* (Och) and *Rhizobium* (Rhi) with *T. harzianum* (Th). No antagonism was exhibited 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

Isolate name	Pectinase	Protease	Xylanase	CMCase	Lignin peroxidase	Laccase	1,3-β -glucanase
MGB1/10	−	+	+	ND	+	ND	+
MGB2	+	+	ND	ND	+	ND	+
MGB3	+	+	+	+	+	ND	+
MGB4	+	+	+	+	+	ND	+
MGB5	+	+	+	+	+	ND	+
MGB13	+	+	+	+	+	ND	+
MGB19	+	+	+	+	+	ND	+
MGB21	+	+	+	+	+	ND	+
BFC1	−	+	−	−	+	ND	+
BFC2	−	+	ND	−	+	ND	+
BFC3	−	+	−	−	+	ND	+
BFC4	−	+	−	−	+	ND	+
BFC5	−	+	−	−	+	ND	+
BFC10	+	+	−	−	+	ND	+

associated with our results. The predominance of coccoid 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 macrothorax* (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 coccoid 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. *Bacteriodetes* 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 macrotermitine 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 humic acids 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 yunnanensis* (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* spp. than in other macrotermitine 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 *Hypocrea*, *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 *Sordariomycetes* 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.

From our studies and previous reports, *Bacillus* species were predominant in the termite gut, with titers up to 10^7 mL^{-1} (Wenzel *et al.*, 2002), whereas *Clostridiales* dominated the fungus comb of *Odontotermes* sp. (Long *et al.*, 2010). They may contribute to cellulose and hemicellulosic degradation (Schäfer *et al.*, 1996; Brune & Ohkuma, 2011). Earlier reports have thoroughly documented that *Bacillus* sp. isolated from the guts of lower and higher termites were capable of lignin and biphenyl degradation (Bugg *et al.*, 2010). *Bacillus* sp. isolated from the termite gut and fungus comb were different based on our phylogenetic studies, indicating that these species were acquired by the worker termites, possibly from different environments, by their food habits.

The exact locations of the *Bacillus* cultivars on the surface of worker termites are still unknown, but they are

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 macrotermitine 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

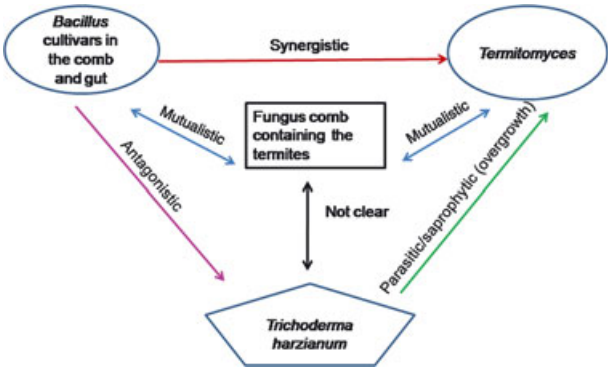

Fig. 5. Hypothetical model for the interactions among microbes residing in the termite gut and fungus comb of *Odontotermes formosanus*.

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 macrotermitine 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

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.

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References

Aanen DK, Ros VID, de Fine Licht HH, Mitchell J, de Beer ZW, Slippers B, Rouland-Lefèvre C & Boomsma JJ (2007) Patterns of interaction specificity of fungus-growing termites and *Termitomyces* symbionts in South Africa. *BMC Evol Biol* **7**: 115–126.

Batra LR & Batra SWT (1979) Termite fungus mutualism. *The Insect–Fungus Symbiosis* (Batra LR, ed), pp. 117–163. Allanheld, Montclair, NJ.

Bignell DE (2011) Morphology, physiology, biochemistry and functional design of the termite gut. *Biology of Termites: A Modern Synthesis* (Bignell DE, Roisin Y & Lo N, eds), pp. 375–412. Springer-Verlag, Berlin.

Brune A (2006) Symbiotic associations between termites and prokaryotes. *The Prokaryotes: A Handbook on the Biology of Bacteria: Symbiotic Associations, Biotechnology, Applied Microbiology*, Vol. 1 (Dworkin M, Falkow S, Rosenberg E, Schleifer K-H & Stackebrandt E, eds), pp. 439–474. Springer-Verlag, Berlin.

Brune A & Ohkuma M (2011) Role of termite gut microbiota in symbiotic digestion. *Biology of Termites: A Modern Synthesis* (Bignell DE, Roisin Y & Lo N, eds), pp. 439–475. Springer-Verlag, Berlin.

Bugg TDH, Ahmad M, Hardiman EM & Singh R (2010) The emerging role for bacteria in lignin degradation and bio-product formation. *Curr Opin Biotechnol* **22**: 1–7.

Carreiro SC, Pagnocea FC, Beuno OC, Bacci M Jr, Hebling MJA & Silva OA (1997) Yeast associated with the leaf-cutting ant *Atta sexdens rubropilosa* Forel, 1908. *Antonie Van Leeuwenhoek* **71**: 243–248.

Castle A, Speranzini D, Rghei N, Alm G, Rinker D & Bissett J (1998) Morphological and molecular identification of *Trichoderma* isolates on North American mushroom farms. *Appl Environ Microbiol* **64**: 133–137.

Chaurasia B, Pandey A, Palni LMS, Trivedi P, Kumar B & Colvin N (2005) Diffusible and volatile compounds produced by an antagonistic *Bacillus subtilis* strain cause structural deformations in pathogenic fungi *in vitro*. *Microbiol Res* **160**: 75–81.

Cornelius ML, Daigle DJ, Connick WJ, Tellez M, Williams KS & Lovisa MP (2002) Interactions between Formosan subterranean termites (Isoptera: Rhinotermitidae) and wood decaying fungi. *Proceedings of 4th International Conference on Urban Pests* (Jones SC, Zhai J & Robinson WH, eds), pp. 319–324. Pocahontas Press, Blacksburg, VA.

Currie CR (2004) Ants, agriculture and antibiotics. *Symbiosis: Mechanisms and Model Systems Series: Cellular Origin, Life in Extreme Habitats and Astrobiology*, Vol. 4 (Seckbach J, ed), pp. 685–699. Springer-Verlag, Berlin.

Currie CR & Stuart AE (2001) Weeding and grooming of pathogens in agriculture by ants. *Proc R Soc Lond B* **268**: 1033–1039.

Currie CR, Wong B, Stuart AE, Schultz TR, Rehner SA, Mueller UG, Sung GH, Spatafora JW & Straus NA (2003) Ancient tripartite coevolution in the attine ant–microbe symbiosis. *Science* **299**: 386–389.

Downes FP & Ito K (Eds) (2001) *Compendium of Methods for the Microbiological Examination of Foods*, 4th edn. American Public Health Association, Washington, DC.

Eggleton P (2011) An introduction to termites: biology, taxonomy and functional morphology. *Biology of Termites: A Modern Synthesis* (Bignell DE, Roisin Y & Lo N, eds), pp. 1–26. Springer-Verlag, Berlin.

Guedegbe HJ, Miambi E, Pando A, Roman J, Houngnandan P & Rouland-Lefevre C (2009) Occurrence of fungi in the combs of fungus-growing termites (Isoptera: Termitidae, Macrotermitinae). *Mycol Res* **113**: 1039–1045.

Gullan PJ & Cranston PS (2010) *The Insects: An Outline of Entomology*, 4th edn. Wiley-Blackwell, Oxford.

Hayashi A, Aoyagi H, Yoshimura T & Tanaka H (2007) Development of novel method for screening microorganisms using symbiotic association between insect (*Coptotermes formosanus* Shiraki) and intestinal microorganisms. *J Biosci Bioeng* **103**: 358–367.

Hill GT, Mitkowski NA, Aldrich-Wolfe L, Emele LR, Jurkonie DD, Ficke A, Maldonado-Ramirez S, Lynch ST & Nelson EB (2000) Methods for assessing the composition and diversity of soil microbial communities. *Appl Soil Ecol* **15**: 25–36.

Hongoh Y, Ekpornprasit L, Inoue T, Moriya S, Trakulnaleamsai S, Ohkuma M, Noparatnaraporn N & Kudo T (2006) Intracolony variation of bacterial gut microbiota among castes and ages in the fungus-growing termite *Macrotermes gilvus*. *Mol Ecol* **15**: 505–516.

Huang QY, Wang WP, Mo RY & Lei CL (2008) Studies on feeding and trophallaxis in subterranean termite *Odontotermes formosanus* using Rubidium chloride. *Entomol Exp Appl* **129**: 210–215.

Ju YM & Hsieh HM (2007) *Xylaria* species associated with nests of *Odontotermes formosanus* in Taiwan. *Mycologia* **99**: 936–957.

Katoh H, Miura T, Maekawa K, Shinzato N & MatsumotoT (2002) Genetic variation of symbiotic fungi cultivated by the Macrotermitine termite *Odontotermes formosanus* (Isoptera: Termitidae) in the Ryukyu Archipelago. *J Mol Ecol* **11**: 1565–1572.

König H (2006) *Bacillus* species in the intestine of termites and other soil invertebrates. *J Appl Microbiol* **101**: 620–627.

Kopečný J, Hajer J & Mrázek J (2004) Detection of cellulolytic bacteria from the human colon. *Folia Microbiol* **49**: 175–177.

Kuhnigk T & König H (1997) Degradation of dimeric lignin model compounds by aerobic bacteria isolated from the hindgut of xylophagous termites. *J Basic Microbiol* **37**: 205–211.

Long YH, Xie L, Liu N, Yan X, Li MH, Fan MZ & Wang Q (2010) Comparison of gut-associated and nest associated microbial communities of a fungus-growing termite (*Odontotermes yunnanensis*). *Insect Sci* **17**: 265–276.

Mackenzie LM, Muigai AT, Osir EO, Lwande W, Keller M, Toledo G & Bogo HI (2007) Bacterial diversity in intestinal tract of fungus-cultivating termite *Macrotermes michaelseni* (Sjöestedt). *Afr J Biotechnol* **6**: 658–667.

McKay AM (1988) A plate assay method for the detection of fungal polygalacturonase secretion. *FEMS Microbiol Lett* **56**: 355–358.

McSpadden Gardener BB (2004) Ecology of *Bacillus* and *Paenibacillus* spp. in agricultural systems. *Phytopathology* **94**: 1252–1258.

Moran MA, Torsvik VL, Torsvik T & Hodson RE (1993) Direct extraction and purification of rRNA for ecological studies. *Appl Environ Microbiol* **59**: 915–918.

Moriya S, Inoue T, Ohkuma M, Yaovapa T, Johjima T, Suwanarit P, Sangwanit U, Vonkaluang C, Noparatnaraporn N & Kudo T (2005) Fungal community analysis of fungus gardens in termite nests. *Microbes Environ* **20**: 243–252.

Mueller UG, Gerardo NM, Aanen DK, Six DL & Schultz TR (2005) The evolution of agriculture in insects. *Annu Rev Ecol Evol Syst* **36**: 563–595.

Nakanishi I, Kimura K, Suzuki T, Ishikawa M, Banno I, Sakane T & Harada T (1976) Demonstration of curdlan-type polysaccharide and some other β-1,3-glucan in microorganisms with aniline blue. *J Gen Appl Microbiol* **22**: 1–11.

Nielsen AT, Liu WT, Filipe C, Grady L Jr, Molin S & Stahl DA (1999) Identification of novel group of bacteria in sludge from a deteriorated biological phosphorous removal reactor. *Appl Environ Microbiol* **65**: 1251–1258.

Nobre T, Rouland-Lefèvre C & Aanen DK (2011) Comparative biology of fungus cultivation in termites and ants. *Biology of Termites: A Modern Synthesis* (Bignell DE, Roisin Y & Lo N, eds), pp. 193–210. Springer-Verlag, Berlin.

Ohkuma M & Brune A (2011) Diversity, structure and evolution of the termite gut microbial community. *Biology of Termites: A Modern Synthesis* (Bignell DE, Roisin Y & Lo N, eds), pp. 413–438. Springer-Verlag, Berlin.

Ohkuma M, Maeda Y, Johjima T & Kudo T (2001) Lignin degradation and roles of white rot fungi; study on an efficient symbiotic system in fungus-growing termites and its application to bioremediation. RIKEN Rev Vol. 42, Focused on Eco-molecular Science Research.

Okino LK, Machado KMG, Fabris C & Bonon VLR (2000) Ligninolytic activity of tropical rainforest basidiomycetes. *World J Microbiol Biotechnol* **16**: 889–893.

Osiemo ZB, Marten A, Kaib M, Gitonga LM, Boga HI & Brandl R (2010) Open relationships in castles of clay: high diversity and low host specificity of *Termitomyces* fungi associated with fungus-growing termites in Africa. *Insect Soc* **57**: 351–363.

Poulsen M, Erhardt DP, Molinaro DJ, Lin TL & Currie CR (2007) Antagonistic bacterial interactions help shape host-symbiont dynamics within fungus-growing ant-microbe mutualism. *PLoS ONE* **9**: e960.

Roderigues A, Bacci M Jr, Mueller UG, Ortiz A & Panocc FC (2008) Microfungal "weeds" in the leafcutter ant symbiosis. *Microb Ecol* **56**: 604–614.

Rosengaus RB, Traniello JFA, Lefebvre ML & Maxmen AB (2004) Fungistatic activity of the sternal gland secretion of the dampwood termite *Zootermopsis angusticollis*. *Insect Soc* **51**: 1–4.

Rosengaus RB, Traniello JFA & Bulmer MS (2011) Ecology, behavior and evolution of disease resistance in termites. *Biology of Termites: A Modern Synthesis* (Bignell DE, Roisin Y & Lo N, eds), pp. 165–190. Springer-Verlag, Berlin.

Rouland-Lefèvre C, Inoue T & Johjima T (2006) Termite/*Termitomyces* interactions. *Soil Biology: Intestinal Microorganisms of Soil Invertebrates*, Vol. 6 (König H & Varma A, eds), pp. 335–347. Springer-Verlag, Berlin.

Saghai-Maroof MA, Soliman KM, Jorgensen RA & Allard RW (1984) Fungal DNA isolation. *P Natl Acad Sci USA* **81**: 8014–8018.

Sambrook J & Russell DW (2001) Screening bacterial colonies using X-gal and IPTG: α-complementation. *Molecular Cloning, Vol. 1* (Argentine J, Irwin N, Janssen KA, Curtis S & Zierler M, eds), pp. 1.123–1.125. Cold Spring Harbor Laboratory Press, Spring Harbor, NY.

Schäfer A, Konard R, Kuhnigk T, Kämpfer P, Hertel H & König H (1996) Hemicellulose-degrading bacteria and yeast in the termite gut. *J Appl Bacteriol* **80**: 471–478.

Scharf ME & Tartar A (2008) Termite digestomes as sources for novel lignocellulases. *Biofuels Bioprod Bioref* **2**: 540–552.

Scott JJ, Oh DC, Yuceer MC, Klepzig KD, Clardy J & Currie CR (2010) Bacterial protection of beetle-fungus mutualism. *Science* **32**: 63.

Sen R, Ishak HD, Estrada D, Dowd SE, Hong E & Mueller UG (2009) Generalized antifungal activity and 454-screening of *Pseudonocardia* and *Amycolatopsis* bacteria in nests of fungus-growing ants. *P Natl Acad Sci USA* **106**: 17805–17810.

Sheffield VC, Cox DR, Lerman LS & Myers RM (1989) Attachment of a 40-base-pair G+C rich sequence (GC–clamp) to genomic DNA fragments by the polymerase chain reaction results in improved detection of single-base changes. *P Natl Acad Sci USA* **86**: 232–236.

Shi W, Syrenne R, Sun JZ & Yuan JS (2010) Molecular approaches to study the insect gut symbiotic microbiota at the 'omics' age. *Insect Sci* **17**: 199–219.

Shinzato N, Muramatsu M, Matsui T & Watanabe Y (2005) Termite regulated monoculture in the fungus combs of macrotermitine termite *Odontotermes formosanus*. *Zool Sci* **22**: 917–922.

Shinzato N, Muramatsu M, Matsui T & Watanabe Y (2007) Phylogenetic analysis of the gut bacterial microflora of the fungus-growing termite *Odontotermes formosanus*. *Biosci Biotechnol Biochem* **71**: 906–915.

Skipper N, Sutherland NM, Davies RM, Kilburn D, Miller R, Warren A & Wong R (1985) Secretion of a bacterial cellulase by yeast. *Science* **230**: 958–960.

Swain MR, Ray RC & Nautiyal CS (2008) Biocontrol efficacy of *Bacillus subtilis* strains isolated from cow dung against post harvest yam (*Dioscorea rotundata* L.) pathogens. *Curr Microbiol* **57**: 407–411.

Tamura K, Dudley J, Nei M & Kumar S (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol* **24**: 1596–1599.

Taprab Y, Ohkuma M, Johjima T, Maeda Y, Moriya S, Trakulnaleamsai S, Noparatnaraporn N, Ohkuma M & Kudo T (2005) Symbiotic fungi produce laccases potentially involved in phenol degradation in fungus combs of fungus-growing termites in Thailand. *Appl Environ Microbiol* **71**: 7696–7704.

Thomas RJ (1985) Selective medium for isolation of *Termitomyces* from the termite nests. *Trans Br Mycol Soc* **84**: 519–526.

Thongaram T, Kosono S, Ohkuma M, Hongoh Y, Kitada M, Yoshinaka T, Trakulnaleamsai S, Noparatnaraporn N & Kudo T (2003) Gut of higher termites as a niche for alkaliphiles as shown by culture-based and culture-independent studies. *Microbes Environ* **18**: 152–159.

Van Bael SA, Fernández-Marín H, Valencia MC, Rojas EI, Wcislo WT & Herre EA (2009) Two fungal symbioses collide: endophytic fungi are not welcome in leaf-cutting ant gardens. *Proc R Soc B* **276**: 2419–2426.

Wenzel M, Schönig M, Berchtold M, Kämpfer P & König H (2002) Aerobic and facultatively anaerobic cellulolytic bacteria from the gut of the termite *Zootermopsis angusticollis*. *J Appl Microbiol* **92**: 32–40.

White TJ, Burns T, Lee S & Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR Protocols: A Guide to Methods and Applications* (Innes MA, Gelfand DH, Sninsky JJ & White TJ, eds), pp. 315–322. Academic Press, New York, NY.

Wood TG & Thomas RJ (1989) The mutualistic association between Macrotermitinae and *Termitomyces*. *Insect–Fungus Interactions* (Wilding N, Collins NM, Hammond PM & Webber JF, eds), pp. 69–92. Academic Press, New York, NY.

Wu X-Y, Walker MJ, Hornitzky M & Chin J (2006) Development of a group-specific PCR combined with ARDRA for the identification of *Bacillus* species of environmental significance. *J Microbiol Meth* **64**: 107–119.

Yanagawa A & Shimizu S (2007) Resistance of the termite, *Coptotermes formosanus* Shiraki to *Metarhizium anisopliae* due to grooming. *Biocontrol* **52**: 75–85.

Yang T-C, Mo J-C & Cheng J-A (2004) Purification and some properties of cellulase from *Odontotermes formosanus* (Isoptera: Termitidae). *Entomol Sin* **11**: 1–10.

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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