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Cellulase gene expression profiles in termites according to habitat and diet

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ABSTRACT

To investigate the expression profiles of cellulase genes in termites due to differences in habitat and diet, we collected one species (*Microtermes pakistanicus*) from dry wood, one species (*Macrotermes gilvus*) from rotten damp wood and one species (*Microtermes* sp.) from cow dung. Total RNA was extracted from the hind guts containing paunch and from other tissues from each collected species and used for the suppression subtractive hybridization to enrich cellulase genes. The resulting EST libraries were sequenced, the cellulase genes were identified by BLAST search, and their phylogenetic relationships were determined. Depending on habitat and diet, termites exhibited different expression profiles of cellulase genes. Endo-β-1,4-glucanase (glycosyl hydrolase family 9, GHF 9) was the predominant cellulase expressed in *M. pakistanicus*, which consume dry wood. In contrast, *M. gilvus*, consuming rotten damp wood, utilized cellobiohydrolase (GHF 7) as the major cellulase. Additionally, β-glucosidase, a cellobiase (GHF 1), was found to be the major cellulase in *Microtermes* sp., which consume cow dung. Based on these findings and the supposition that raw cellulose materials are likely preprocessed by cellulolytic microorganisms in damp wood and cow dung, we hypothesized that termites consuming these preprocessed cellulose materials (i.e., cellobiase) have adapted to produce more exocellulases than endocellulases. This notion was supported by the fact that endo-β-1,4-glucanase (i.e., endocellulase) was predominantly expressed only in *M. pakistanicus*, which consume the raw cellulose of dry wood. An analysis of the catalytic domains of the cloned cellulases suggested that these cellulases possess enzymatic activity.

Introduction

The cellulases (EC 3.2.1.4) are a group of enzymes that catalyze cellulolysis, which is the hydrolytic reaction of cellulose and related polysaccharides, the most abundant sources of biomass on the planet. Cellulases are mainly produced by bacteria, fungi, and protozoans. Symbiotic gut bacteria in the ruminating organs of herbivores produce cellulases that allow their host to digest and utilize the cellulose in plant diets. Cellulases are also produced by a few arthropod species, such as some termites. There are various types of cellulases, among which three (endocellulase, exocellulase and cellobiase) are the most important in cellulolysis. Endocellulase (EC 3.2.1.4) randomly breaks down internal glycosidic bonds, thereby generating new chain ends. Exocellulase (EC 3.2.1.91) or cellobiohydrolase then cleaves di- or tetrasaccharide units from the exposed ends of the chains generated by endocellulase, producing cellotetraose or cellobiose. Finally, cellobiase (EC 3.2.1.21) or β -glucosidase hydrolyses the cellotetraose or cellobiose into di- or monosaccharides.

Termites can be classified into two clades, lower and higher termites. The evolutionarily lower termites harbor symbiotic protozoa and a diverse community of bacteria and archaea, whereas most of the higher termites harbor bacteria and archaea or a small number of protists, such as amoeba and flagellates (Bignell et al., 1979). Some higher termites ingest fungi to aid in the digestion of celluloses, resulting in the growth of these fungi in their feces (Martin, 1992). Other higher termites consuming cellulose-rich diets harbor many spirochaetes and Fibrobacteres in their guts. Although the habitats and diets of lower termites are generally restricted to wood, the higher termites, constituting most (>80%) termite species, thrive in various habitats and consume a variety of diets, including wood, dry grass, plant litter and herbivore dung. The successful adaptations of higher termites to such varied

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Table 1
Primer set used for 5′- & 3′-RACE.

Gene	Name	Sequence (5′- to 3′-)
3′ & 5′ RACE		
Endo-β-1,4-glucanase (GHF9)	MpEG 5′-GSP	CCATCGGGAAGCCGAACTTCACAA
	MpEG	AATCTCC
	5′-NeGSP	GCATCATAGTATCCGCCTGTCAG
Cellobiohydrolase (GHF7)	MgCBH 5′-GSP	GTGGTGTGTATGCTGTTGCATATTTG
	MgCBH	TTGGC
	5′-NeGSP	GATATCCATTTCTGTGCAGCATGC
	MgCBH 3′-GSP	GTTAAGAGAGGACCATGTGCAACA
	MgCBH	TCCTC
	3′-NeGSP	GATGTTGAAAGCCAACACCCAGATTC
β-glucosidase (GHF1)	MsBG 5′-GSP	CAACACCCTATTTGGCCATCCTCCCA
	MsBG	GATC
	5′-NeGSP	GGGGCAGATCCCAGTGATAC
	MsBG 3′-GSP	CTGACACAGGAATGGCTCCATCAATC
	MsBG	AACG
	3′-NeGSP	CGGCACACACTGTGATCCTTGCC

food resources are likely due to a diverse array of symbiotic intestinal prokaryotes dwelling in the hindgut paunch of termites, which provide important groups of cellulases (i.e., endo-β-1,4-glucanse, cellobiohydrolase, and β-glucosidase) (Matsui et al., 2009).

Owing to the high efficiency of their hindgut bioreactors, higher termites are a promising reservoir of cellulases with biotechnological potential for industrial production of biofuels from plant lignocelluloses, the major component of biomass (Scharf and Boucias, 2010). To date, most associated studies have focused on biochemical characterization, functional expression and genetic engineering of endogenous endoglucanases from higher termites. However, identification and characterization of other cellulase groups, including cellobiohydrolase and β-glucosidase, are also crucial as effective cellulolysis of diverse natural substances requires all of the important groups of cellulases.

In this study, under the assumption that termites have evolved to exploit specific groups of cellulases as their main enzyme, depending on habitat and food resources, we searched for endogenous cellulase genes from three species of higher termites with different habitats and diets. To this end, total RNA was extracted from the hindguts of termites and used in suppression subtractive hybridization (SSH) to enrich cellulose genes. Then, the most predominant cellulase gene in each termite species was identified and characterized.

Materials and methods

Termite collection

Microtermes pakistanicus (a dry wood-dwelling termite) and *Macrotermes gilvus* (a rotten damp wood-dwelling termite) were collected from Mondulkiri in Cambodia in August 2012. *Microtermes* sp. (a cow dung-dwelling termite) was collected from the Southern Central Cardamom Protected Forest in Cambodia in February 2011. Worker termites were used in the experiments, and soldier termites were used for identification purposes. Termite specimens were stored in 100% ethanol at −20 °C until use.

Construction of EST libraries

Hindguts containing paunch were dissected from live worker termites under a microscope and immediately placed in TRI reagent (MRC, Cincinnati, OH). Total RNA was extracted from approximately 20 hindguts and 10 other tissues (abdominal tissues without alimentary tracts) using 200 μl TRI reagent, following the manufacturer's protocol. Double-stranded cDNA from each sample ('testers' from hind guts vs. 'drivers' from other tissues) was synthesized from the total RNA using the Super SMART PCR cDNA synthesis kit (Clontech, Palo Alto, CA). The termite hindgut-specific cDNA was selected by SSH using the PCR-select cDNA Subtraction Kit (Clontech), according to the manufacturer's instructions. PCR-amplified fragments were ligated directly into the pGEM-T Easy Vector (Promega, Madison, WI) in order to construct the subtractive cDNA library specific to the termite hindgut. A total of 369, 188, and 1030 clones (Macrogen, Seoul, Republic of Korea) from *M. pakistanicus*, *M. gilvus* and *Microtermes* sp., respectively, were

Table 2
The list of EST library.

Contig number	EST no.	Best-matched protein	Species	GenBank	E-value
Microtermes pakistanicus					
Term12_02-E08-F	7	Endo-β-1,4-glucanase (Glycosyl hydrolase family 9)	*Coptotermes formosanus*	BAB40697.1	8.00E−116
CL23Contig1	1	Putative endo-β-1,4-glucanase (Glycosyl hydrolase family 9)	*Odontotermes formosanus*	BAD12010.1	4.00E−73
Term12_03-E09-F	1	Cellulase [Endo-β-1,4-glucanase (Glycosyl hydrolase family 9)]	*Coptotermes acinaciformis*	AAK12339.1	8.00E−47
Term12_04-E08-F	1	β-glucosidase (Glycosyl hydrolase family 1)	*Coptotermes formosanus*	ADB23476.1	6.00E−61
Macrotermes gilvus					
Term8_02-H10-F	6	Cellulase (Glycosyl hydrolase family 7)	*Pseudotrichonympha grassii*	BAB69425.1	5.00E−119
Term8_03-O9	2	Putative glycosyl hydrolase family 5	*Cryptocercus punctulatus*	BAF57460.1	2.00E−49
Term8-E11F	1	Putative glycosyl hydrolase family7	Protist of *Neotermes koshuensis*	BAF57378.1	4.00E−17
Term8-E02F	1	Putative glycosyl hydrolase family45	Protist of *Hodotermopsis sjoestedti*	BAF57355.1	2.00E−102
Term8-A04F	1	Endoglucanase (Glycosyl hydrolase family 5)	*Clostridium clariflavum*	AEV70372.1	2.00E−30
Microtermes sp.					
CL2Contig3blast	58	β-glucosidase (Glycosyl hydrolase family 1)	*Coptotermes formosanus*	ADB23476.1	1.00E−38
CL50Contig1blast	42	β-glucosidase (Glycosyl hydrolase family 1)	*Nasutitermes takasagoensis*	BAI50023.1	2.00E−61
206_06-D12-F	1	β-glucosidase (Glycosyl hydrolase family 1)	*Reticulitermes flavipes*	ADK12988.1	2.00E−58
206_05-G02-F	1	Glycosyl hydrolase family 3 protein	*Laccaria bicolor*	XP_001878554.1	8.00E−50