Microb Ecol (2014) 68:416–425
DOI 10.1007/s00248-014-0388-3

HOST MICROBE INTERACTIONS

Phylogenetic and Functional Analysis of Gut Microbiota of a Fungus-Growing Higher Termite: Bacteroidetes from Higher Termites Are a Rich Source of β-Glucosidase Genes

Meiling Zhang · Ning Liu · Changli Qian · Qianfu Wang · Qian Wang · Yanhua Long · Yongping Huang · Zhihua Zhou · Xing Yan

Received: 25 August 2013 / Accepted: 4 February 2014 / Published online: 2 March 2014

Abstract Fungus-growing termites, their symbiotic fungi, and microbiota inhibiting their intestinal tract comprise a highly efficient cellulose-hydrolyzing system; however, little is known about the role of gut microbiota in this system. Twelve fosmid clones with β-glucosidase activity were previously obtained by functionally screening a metagenomic library of a fungus-growing termite, *Macrotermes annandalei*. Ten contigs containing putative β-glucosidase genes (*bgl1–10*) were assembled by sequencing data of these fosmid clones. All these contigs were binned to Bacteroidetes, and all these β-glucosidase genes were phylogenetically closed to those from *Bacteroides* or *Dysgonomonas*. Six out of 10 β-glucosidase genes had predicted signal peptides, indicating a transmembrane capability of these enzymes to mediate cellulose hydrolysis within the gut of the termites. To confirm the activities of these β-glucosidase genes, three genes (*bgl5*, *bgl7*, and *bgl9*) were successfully expressed and purified. The optimal temperature and pH of these enzymes largely resembled the environment of the host's gut. The gut microbiota composition of the fungus-growing termite was also determined by 454 pyrosequencing, showing that Bacteroidetes was the most dominant phylum. The diversity and the enzyme properties of β-glucosidases revealed in this study suggested that Bacteroidetes as the major member in fungus-growing termites contributed to cello-oligomer degradation in cellulose-hydrolyzing process and represented a rich source for β-glucosidase genes.

Electronic supplementary material The online version of this article (doi:10.1007/s00248-014-0388-3) contains supplementary material, which is available to authorized users.

M. Zhang
School of Life Sciences, East China Normal University, Shanghai, China

C. Qian · Q. Wang · Z. Zhou · X. Yan
Key Laboratory of Synthetic Biology, Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China

N. Liu · Q. Wang · Y. Huang
Key Laboratory of Insect Development and Evolutionary Biology, Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China

N. Liu
Zhejiang Academy of Agricultural Sciences, Hangzhou, China

Y. Long
School of Life Science, Anhui Agricultural University, Hefei, Anhui, China

X. Yan (✉)
Key Laboratory of Synthetic Biology, Shanghai Institute of Plant Physiology and Ecology, Chinese Academy of Sciences, 300 Fenglin Road, 200032 Shanghai, China
e-mail: yanxing@sippe.ac.cn

Introduction

Termites comprise one of the most efficient lignocellulose conversion biosystems on earth, and it has been found that they do so with major help from their symbiotic microorganisms [1, 2]. In lower termites, protists play major roles in plant matter degradation, while in higher termites, bacteria are proved to be largely responsible [3]. Among higher termites, *Macrotermitinae* spp., abundant and influential in Asia and Africa, are the most distinctive, as they obligately cultivate a basidiomycete fungi of genus *Termitomyces* in their nests [4], earning the name of fungus-growing termites. The symbiotic relationship between fungus-growing termites and the *Termitomyces* fungi is known to be maintained by a most complex polyethism implemented by different castes and ages [5]. Briefly, old workers forage for plant matters and carry

them back to the nest, where young workers ingest the collected plant matters and produce largely undigested primary feces to construct a fungus comb (or a fungus garden) as consortium for growth of their ectosymbiotic fungi. As the fungus grows, in all forms of mycelium, fungus nodules (aggregate of asexual spores of *Termitomyces*), and seasonally flourished fruit bodies, young workers ingest fungus nodules while old workers feed on the aged part of fungus combs which is composed mainly of digested plant matters and mature mycelium. Meanwhile, as other higher termites, these termites also harbor a diverse milieu of gut bacteria whose compositions have been extensively studied [5–9].

In macrotermitine termites, the plant lignocellulose degradation is carried out within a multipartite symbiosis involving the termite host, the gut microbes, and the ectosymbiotic fungi *Termitomyces* [10, 11]. Endogenous cellulases from macrotermitine termite have been reported for both endoglucanase expressed in the salivary glands of the fungus grower *Odontotermes formosanus* [12] and β-glucosidases expressed primarily in the midgut of the fungus grower *Macrotermes barneyi* [13], suggesting that termite hosts themselves directly take part in the hydrolysis of cellulose. The symbiotic fungi could help the host to degrade lignin [14, 15], in addition to provision of cellulase and hemicellulase [16–18], while symbiotic roles of gut microbes residing in fungus-growing termite, especially in the process of lignocellulose degradation, remained largely undefined until several of recent works [19–21].

Gut microbiota of the fungus grower *Odontotermes yunnanensis* has been investigated by 454 pyrosequencing-based overall metagenomic analysis [20]. Carbohydrate-active enzymes (CAZymes) harbored by this microbiome, which are distributed in many protein families, are related to the hydrolysis of cellulose and hemicellulose [20]. This study suggested that the intestinal bacteria of fungus-growing termites mainly participate in the hydrolysis of cellulose and hemicellulose, but not in the degradation of lignin. Most of these CAZymes belonged to catalogs of debranching enzymes or oligosaccharide-processing enzymes; however, their properties, including substrates specificity, enzyme activity, optimal pH, and temperature, have not been characterized further. Functional screening of fosmid metagenome libraries of other fungus growers also indicated that hemicellulase, including arabinofuranosidase (debranching enzymes), xylosidase (oligosaccharide-processing enzyme), and xylanase, are present in the microbiome of the gut of fungus-growing termites, and characterization of these enzymes suggested that gut microbiota participated in hydrolysis of hemicellulose [19, 21].

In our previous study, a fosmid library was constructed from gut metagenome of old adult workers of the fungus-growing termite *Macrotermes annandalei*, and 12 clones with β-glucosidase (oligosaccharide-processing enzyme) activity were recovered from functional screening of 10,000 clones, which suggested that gut microbiota also took part in the hydrolysis of cellulose, especially in cello-oligomer degradation [19]. In this study, β-glucosidase genes were predicted from sequences of these fosmids and heterologously expressed and characterized, which provided more information about the role of gut microbiota in cellulose hydrolysis. The composition of gut microbiota was also investigated by 16S rRNA gene survey, and the correlations between the symbiotic bacteria and their hosts were attempted.

Materials and Methods

Chemicals and Reagents

Restriction enzymes and T4 DNA ligase used for cloning were obtained from Takara (Dalian, China). The Plasmid MiniPrep Kit for plasmid purification and DNA Gel Extraction Kit for DNA purification were obtained from Axygen (Hangzhou, China). All other reagents were of analytical grade and were purchased from Sangon (Shanghai, China).

Termite Collection and Gut Bacterial Genomic DNA Extraction

Termite sampling and DNA extraction have been done and described in the previous study [19], in which whole guts of about 2,500 old adult workers in a nest of *M. annandalei* were used for metagenomic DNA extraction, and the extracted DNA was used directly as the template for amplifying the V3 regions of the 16S rRNA genes.

Vectors and Strains

EPI300-T1^{R} (Epicentre) was used for fosmid library construction. The selected β-glucosidase gene was cloned into plasmid pET-22b(+), and the expression was carried out in *Escherichia coli* BL21 (Novagen).

Microbial Diversity Survey

In order to investigate the microbial composition in the given termite gut, V3 regions of bacterial 16S rRNA genes were amplified and subjected to 454 pyrosequencing. Original metagenomic DNA was used directly as the template for 16S rRNA gene amplification. The forward primer was 341f,5′-ACGTCACCTACGGGAGGCAGCAG-3′, and the reverse primer was 534r,5′-ACGTCAATTACCGCGGCTGC TGG-3′. The six bases underlined in the primers were unique barcode for distinguishing PCR product from other samples. Reaction conditions were described as the follows: 25 μl of PCR reaction mixture, which contained 1 U of Ex Taq

polymerase, 2.5 μl of the corresponding 10× amplification buffer, 200 mmol/l of each deoxynucleoside triphosphate (dNTP), and 20 pmol of each primer, and 10 ng of total genomic DNA. PCR reactions were performed using the following program: 2 min denaturation at 95 °C followed by 24 cycles of 1 min at 94 °C (denaturation), 1 min for annealing (0.5 °C reduced for every cycle from 65 to 56 °C followed by 5 cycles at 55 °C), and 1 min at 72 °C (elongation), with a final extension at 72 °C for 6 min. Then, the PCR product was subjected to 454 pyrosequencing (GS FLX sequencing system). All sequences were aligned using the NAST aligner [22] from the Greengenes [23] website (http://greengenes.lbl.gov/). All possible chimeras were detected with Bellerophon [24] (version 3) hosted at Greengenes and then excluded from subsequent analyses. All the aligned sequences were inputted to ABR software to calculate distance matrix, and then, the distance matrices were used to calculate operational taxonomic units (OTUs) (similarity cutoff value 97 %) using DOTUR software [25]. The phylogeny of each read was determined by RDP Classifier software, and 50 % confidence was chosen because of the short length of the reads (<250 bp) [26].

Assembly and Annotation of Sequencing Data of Fosmids

Fosmid DNAs of 12 β-glucosidase-positive clones were pooled and then sequenced by using 454 pyrosequencing (GS FLX sequencing system) as described previously [19]. The 454 reads were assembled into different contigs by Newbler software (http://www.454.com). MEGAN software (http://ab.inf.uni-turbingen.de/software/megan/) was employed to assign the taxonomy of ORFs in each contig [21, 27, 28]. If 50 % ORFs of a contig were all binned to the same phylum, this contigs could be assigned to this phylum [21]. The ORFs of contigs were predicted by FgenesB (Softberry, Goteborg, Sweden). β-Glucosidases were annotated from these ORFs by using BLASTX against the NCBI nonredundant protein database (http://www.ncbi.nlm.nih.gov). Annotation of the other conserved domains was performed using Pfam (http://pfam.sanger.ac.uk/). Predication of signal peptides of these β-glucosidases was performed by SignalP 4.1 in CBS (http://www.cbs.dtu.dk/services/SignalP/). Molecular mass and isoelectric point were predicted by ExPASy (http://www.expasy.ch/tools/protparam.html). β-Glucosidase genes annotated from the contigs were aligned with their nearest neighbors using MAFFT version 6.864 [29], and phylogenetic analysis was performed with MEGA 5.0. Distance was calculated based on the maximum likelihood criterion. The bootstrap confidence values were obtained based on 100 replicates. The sequences of β-glucosidase genes were deposited in GenBank, and the accession numbers are JN848956, JN848957, JN848959, and JN848961 to JN848967. The accession numbers of the contigs are KJ095701–KJ095710.

Expression of β-Glucosidase Genes and Purification

In order to express β-glucosidase, the β-glucosidase genes were amplified from the fosmid DNAs by 35 cycles of PCR using the primer (Table 1). The amplicon was cloned into a pET22b(+) expression vector and then transformed into *E. coli* BL21. The bacteria were cultured at 37 °C in LB medium containing ampicillin (50 μg ml^{-1}) to an optical density of 0.6, and gene expression was induced by the addition of 50 μM l^{-1} isopropyl-β-D-thiogalactopyranoside (IPTG) at 28 °C for 16 h. Cells were harvested by centrifugation, and the cell pellet was resuspended in 2.5 ml lysis buffer (50 mM l^{-1} NaH_2PO_4, 300 mM l^{-1} NaCl, 10 mM l^{-1} imidazole, pH 8.0) and then disrupted by ultrasonic. The recombinant protein was purified using a nickel-nitrilotriacetic acid (NTA) column (Qiagen). The protein concentration was determined with a DC Protein Assay Kit (Bio-Rad Laboratories), and the target protein was detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by staining with Coomassie Blue G-250.

β-Glucosidase Activity Assay

β-Glucosidase activity was assayed at the optimum temperature by incubating 0.4 μg of diluted enzyme with 2.5 mM l^{-1} *p*-nitrophenyl-β-D-glucoside (Sigma) in 50 mM l^{-1} Na-phosphate buffer (pH 6.5) for 5 min, in triplicate. The reaction was terminated by adding 200 μl of 1 M l^{-1} $NaCO_3$. The release of *p*-nitrophenol (*p*NP) was determined by measuring the absorbance at 405 nm using a Multiskan Spectrum spectrophotometer (Thermo Scientific, Finland). One unit of β-

Table 1 Primers used for cloning and expression of three β-glucosidase genes in this work

Gene	Location	Sequence
bgl5	Forward	5′ ATCCCATGGAGCCTAAGGACGAGGCT 3′[a]
	Reverse	5′ CTTCTCGAGGTAGTTGAATGTTACTGG 3′[b]
bgl7	Forward	5′ GAGACTCCATATGCAAAGCGATGTGGATA AG 3′[c]
	Reverse	5′ CCGCTCGAGTTACTCTGTCACAGAGA 3′[b]
bgl9	Forward	5′ GAGACTCCATATGCAAAAAGATACAGATA AGC 3′[c]
	Reverse	5′ CGGAATTCTTATTCTGTCACAGAAAA 3′[d]

Restriction sites were underlined in each primer

[a] Restriction sites of *Nco*I

[b] Restriction sites of *Xho*I

[c] Restriction sites of *Nde*I

[d] Restriction sites of *Eco*RI

glucosidase activity was defined as the amount of enzyme required to hydrolyze 1 μmol of *p*NP-β-D-glucoside per minute under the standard assay conditions stated above.

To determine the optimum temperature, the standard activity assay was performed between 20 and 75 °C in 50mM l^{-1} Na-phosphate buffer (pH7.5). The optimum pH of three β-glucosidases was measured at the optimum temperature in the pH range of 4.5–5 (50 mM l^{-1} Na-acetate buffer) and 5.5–8.5 (50 mM l^{-1} Na-phosphate buffer).

Substrate specificity of BGL5 was determined by replacing the *p*-nitrophenyl-β-D-glucoside with *p*NP-β-D-cellobioside, cellobiose, and salicin. For 5 min, 2.5 % (*w*/*v*) substrates were incubated with 0.4 μg purified protein at 30 °C, pH 6.5. The enzyme assay for *p*NPC and salicin was under the experimental conditions described above. The enzyme activity for cellobiose degradation was assayed according to the Glucose Assay Kit (GAGO20, Sigma) manual.

The kinetic characteristics of BGL5 were measured with *p*-nitrophenyl-β-D-glucoside at concentrations ranging from 0 to 10 mM l^{-1}, and K_m and V_{max} were calculated based on the Lineweaver-Burk method.

To determine the relationship of various metal ions and the enzyme activity, 50 mM l^{-1} TMEMD-HCl buffer containing 1 mM metal ions was incubated with the purified protein. The enzyme activity was assayed as described above.

Results

Bacterial Diversity

As the limitations existed when culture-based method was employed to study the intestinal bacteria, 454 pyrosequencing was used to characterize the bacterial composition in the termites' gut. At 97 % cutoff, 1,185 qualified 16S rRNA gene sequences were obtained and classified into 378 OTUs. These 16S rRNA genes were mainly distributed into nine phyla. The relative abundance of each phylum was calculated, and the results indicated that the sequences affiliated to Bacteroidetes constituted the most dominant phylum in this termite (73.2 %), followed by Firmicutes (18.1 %) (Fig. 1a).

Bacteroidetes was the most abundant phylum in termites' gut, and sequences affiliated with Bacteroidetes were further analyzed. There were 872 sequences affiliated to Bacteroidetes, including *Flavobacteria* (1.4 %), *Sphingobacteria* (0.1 %), *Bacteroidia* (51.6 %), and unclassified Bacteroidetes(46.9 %). Within *Bacteroidia*, *Dysgonomonas* was the most dominant genus observed in the termites gut, and the proportion of this genus in *Bacteroidia* was 25.3 % (Fig. 1b).

Taxonomy Binning of Fosmid Contigs Containing β-Glucosidase Genes

By Newbler software, 454 reads of 12 fosmid clones with β-glucosidase activity were assembled into 55 large contigs (>500 bp) and 10 contigs were predicted to encode β-glucosidase genes. In order to reveal taxonomy source of these β-glucosidase genes, MEGAN program was used to bin all the 10 contigs containing β-glucosidase genes. The results indicated that all the contigs of β-glucosidases in this study can be assigned to the phylum Bacteroidetes (*Bacteroidales*, *Dysgonomonas*, etc.) (Table 2), suggesting that this phylum was important in affording functional enzyme within termites gut.

Phylogenetic Analysis of β-Glucosidase Genes

Based on the sequence analysis, all these β-glucosidases belong to GH3 family. Phylogenetic analyses based on amino acids were performed, and the results were shown in Fig. 2. BGL5 formed deep-branched lineages compared to other members analyzed. BGL5 was closely related to one β-glucosidase isolated from *Bacteroides* sp. (56 % amino acid identity). BGL2, BGL6, BGL7, BGL8, and BGL9 showed a high similarity to the hypothetical protein from the genus *Dysgonomonas* (amino acid identity varied from 81 to 100 %). BGL1, BGL3, BGL4, and BGL10 had a high homology to the enzymes originated from the genus *Bacteroides* (amino acid identity varied from 58 to 77 %).

Functional Characterization of β-Glucosidases

According to SignalP prediction, 6 out of 10 β-glucosidase genes possess the N-terminal signal peptides indicating a transmembrane capability of the enzymes (Fig. 3), suggesting that these enzymes could function extracellularly to take part in cellulose hydrolysis. The structures of these genes were predicted, and the results indicated that in addition to GH3 domains, eight of them also possessed Fn3-like domain at C-terminal (Fig. 3).

All of these genes were tried to be expressed in *E. coli*; however, only three of them (*bgl5*, *bgl7*, and *bgl9*) were successfully expressed. *bgl5* is composed of 2,214 bp, encoding a protein of 738 amino acids, with a predicted molecular mass of 79.4 kDa and a predicted isoelectric point of 5.43. *bgl7* encoded 798 amino acids, with a predicted molecular mass of 85.9 kDa and a predicted isoelectric point of 5.51.*bgl9* encoded 797 amino acids, and the predicted molecular mass and predicted isoelectric point were 86.2 kDa and 5.99, respectively.

All these genes were cloned in the pET22b(+) expression vectors and expressed in *E. coli* BL21. The protein was detected in both the supernatant and precipitation after IPTG induction. With an N-terminal 6× His tag, BGL5, BGL7, and

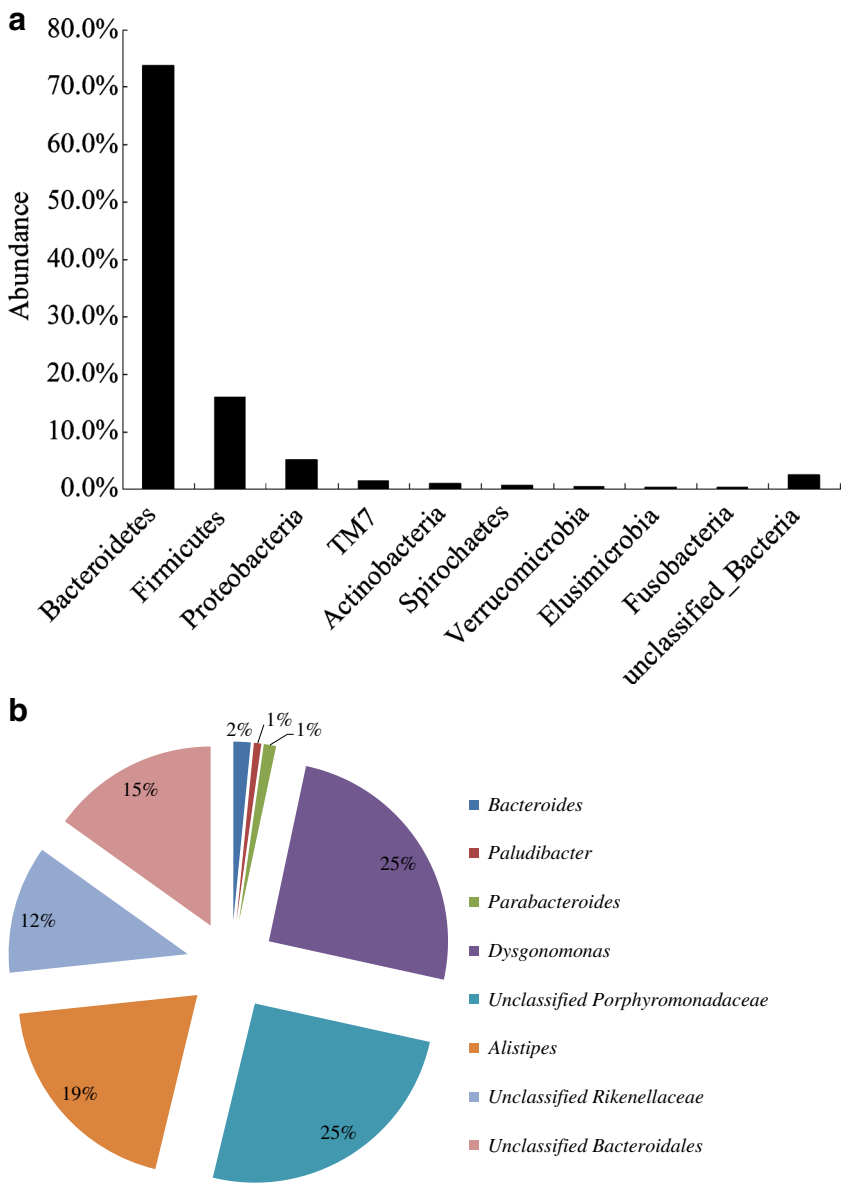

Fig. 1 Bacterial abundance in termites' gut. **a** Pyrosequencing of bacterial 16S rRNA gene V3 region in termites' gut was performed, and the sequence was divided into different groups based on distance matrix. **b** The distribution and proportion of genus in *Bacteroidales*

BGL9 were successfully purified by an elution buffer containing 250 mM l^{-1} imidazole (Supplementary Fig. 1). The molecular mass estimated by SDS-PAGE analysis agreed with the predicated values. The optimum pH of these enzymes was

Table 2 Taxonomic binning of contigs harbored β-glucosidases in this study

Enzyme	Contig number	Contig size (bp)	Accession number	Taxonomic assignment
BGL1	Contig 00003	2,250	KJ095701	Bacteroidales
BGL2	Contig 00005	1,692	KJ095702	Dysgonomonas
BGL3	Contig 00010	3,261	KJ095703	Bacteroidales
BGL4	Contig 00023	2,307	KJ095705	Bacteroidales
BGL5	Contig 00100	2,214	KJ095704	Bacteroidetes
BGL6	Contig 00008	2,313	KJ095706	Dysgonomonas
BGL7	Contig 00103	2,394	KJ095707	Bacteroidales
BGL8	Contig 00106	2,292	KJ095708	Dysgonomonas
BGL9	Contig 00110	2,391	KJ095709	Dysgonomonas
BGL10	Contig 00113	2,256	KJ095710	Bacteroidetes

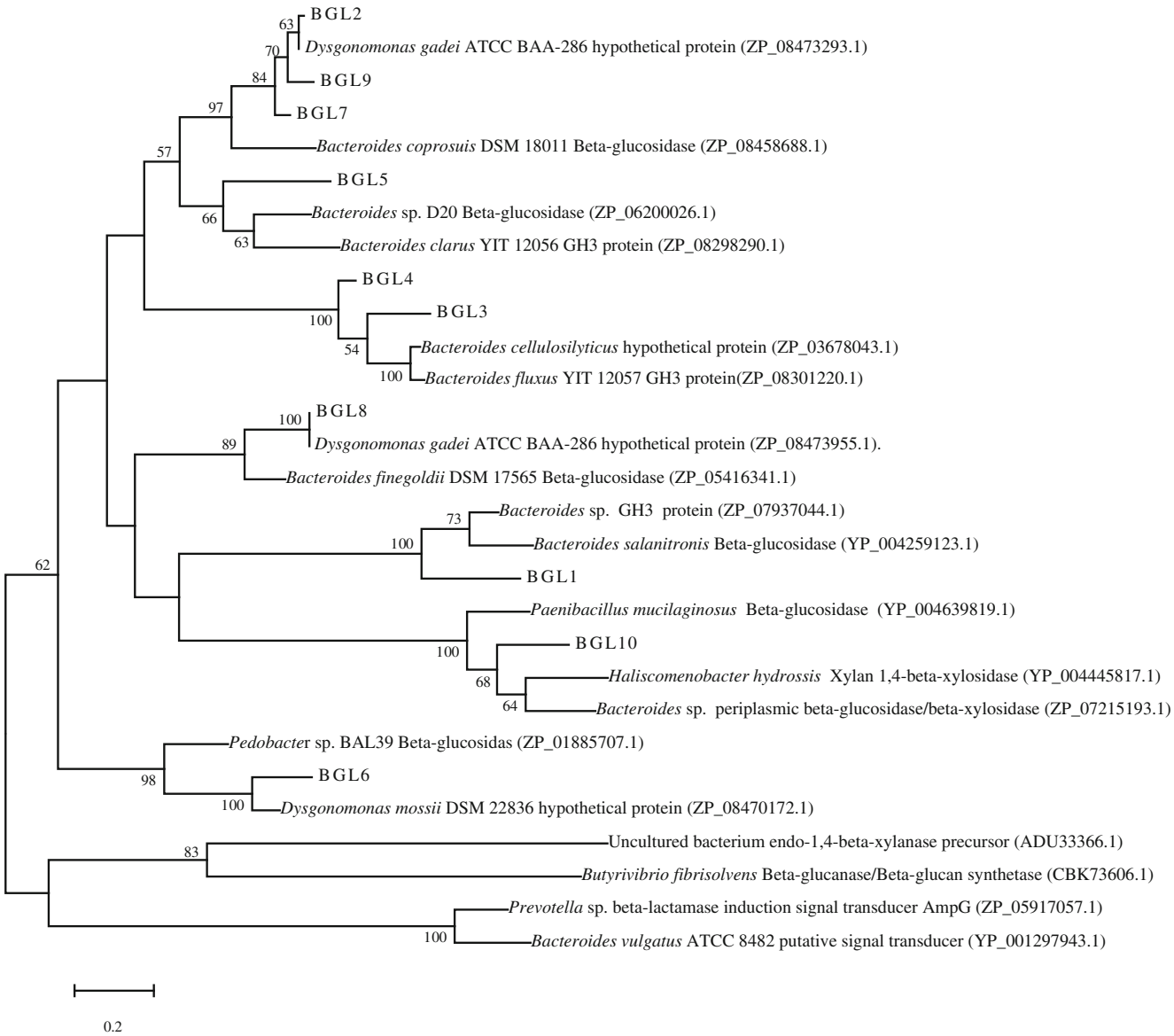

Fig. 2 Phylogenetic analysis of β-glucosidase detected in the termites' gut. The protein sequence of BGL5 was aligned with the reference sequences retrieved from GenBank. The phylogenetic analysis was inferred by maximum likelihood analysis using amino acid sequence. The reference sequences are marked with source strains and GenBank accession numbers are in *parentheses*

6.5, and the optimum temperature of BGL5 and BGL9 was 30–35 °C. The optimum temperature of the other enzyme BGL7 was 50 °C. The enzyme activity was detected with *p*-nitrophenyl-β-D-glucoside as the substrate under the optimum pH and temperature of each enzyme, and the results indicated that BGL5 showed the strongest catalytic activity (Table 3).

Considering the similar structure and higher enzyme activity, the substrate specificity of BGL5 was further characterized. BGL5 hydrolyzed *p*-nitrophenyl-β-D-glucoside (*p*NPG) with an activity of 42.3±2.0 U mg^{-1}, whereas it hydrolyzed *p*-nitrophenyl cellobioside (*p*NPC) and cellobiose at a rate of 16.85±0.53 and 5.26±0.37 U mg^{-1}, which were only 38 and 12 % of the rate to hydrolyze *p*NPG, respectively. Furthermore, it showed a very weak activity (1.21± 0.18 U mg^{-1}) on salicin. These results indicated that BGL5 was a specific β-glucosidase and its substrate range was very limited. The V_{max} and K_m of BGL5 were determined based on a Lineweaver-Burk plot, which were 0.13 μmol min^{-1} mg^{-1} protein and 1.99 mg ml^{-1}, respectively.

The effects of different metal ions and chemical reagents on BGL5 were also detected. It was shown that the enzymatic activity was significantly enhanced by Mg^{2+}, Co^{2+}, Zn^{2+}, Fe^{3+}, and Ni^{2+}. BGL5 was inhibited by Ag^{+}, Cu^{2+}, and EDTA which reduced its enzymatic activity to 4.21, 18.11, and 72.81 %, respectively, of that without addition these items in the reaction (Fig. 4).

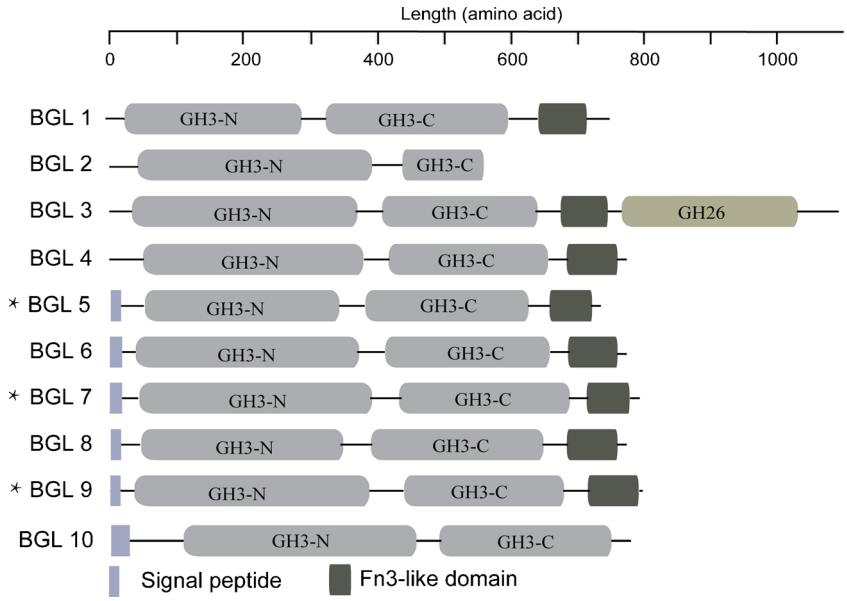

Fig. 3 Domain architecture of the β-glucosidase genes. *GH* glycoside hydrolase. The *asterisk* indicated the successfully purified proteins

Discussion

The microbial communities harbored in the gut of fungus-growing termites have been effectively studied, and the dominance of Bacteroidetes and Firmicutes revealed here in *M. annandalei* largely resembles that revealed in other investigated fungus growers, including *Macrotermes* [5, 30], *Odontotermes* [8, 11, 20], and *Pseudocanthotermes* [21], and this differs obviously to the predominance of Spirochaetes in the revealed wood feeders [7, 31, 32], further indicating a diet-driven phylum-level divergence between gut microbiota in the fungus-growing and wood-feeding higher termites.

Compared to the well-established contributions of the symbiotic fungi for their hosts' plant material decomposition [15, 17], the exploration of the function of intestinal bacteria in cellulose hydrolysis for their host appears particularly necessary and impendent. Here, extending the previous work [19], we further sequenced these β-glucosidase-positive clones and got 10 β-glucosidases genes. However, as expressing the target genes in a foreign host is the first step of functional screening, codon bias, missing substrates, and the inability to recognize foreign regulatory elements are several possibilities to limit the entirely identification of the potential genes or gene clusters [33], and more β-glucosidase genes may exist in termites' guts. Taxonomy analysis showed that all the contigs were assigned to the phylum Bacteroidetes, and phylogenetic analysis indicated that most of the β-glucosidases showed a high identity to those from *Dysgonomonas* spp. or *Bacteroides* spp. 454 pyrosequencing of 16S rRNA gene indicated that Bacteroidetes was predominant in the gut microbiota. Members of the phylum Bacteroidetes employ a highly specialized system for polysaccharide utilization which involves a core set of polysaccharide-binding proteins, outer membrane transporters, and glycolytic enzymes to cleave large polysaccharides into fragments [34]. It has been suggested that Bacteroidetes play an important role in the cellulose or hemicellulose degradation in human gut or rumen [34–36]. Our study suggested that Bacteroidetes possess β-glucosidases genes, which suggested that in termites' gut, Bacteroidetes as the dominant member played an important role in the last step of cellulose degradation.

There are 113 sequences (9.5 %) affiliated to *Dysgonomonas* spp. in this work. *Dysgonomonas* has been isolated from human sources and soil and showed positive reaction for β-glucosidases [33, 37–42]. However, the role of this genus in termite symbiotic system still remains elusive. In this study, 12 positive clones with β-glucosidase activity were screened from the termite gut fosmid library with 10,000 clones, and the hit rate was about 0.12 %. The hit rates for β-glucosidase of this fosmid library were modest compared with those of other metagenome libraries constructed from termite gut, biogas digest, and soils from wetland [21, 43].

Being a higher termite, *M. annandalei* lacks the flagellated protists, so the cellulolytic degradation process mainly

Table 3 Functional analysis of three β-glucosidase genes derived from the gut microbiota of a fungus-growing termite (*M. annandalei*)

Enzyme	Optimal pH	Optimal temperature (°C)	Enzyme activity (U mg^{-1})
BGL5	6.5	30–35	42.3±2.0
BGL7	6.5	50	30.6±3.9
BGL9	6.5	35	14.6±0.1

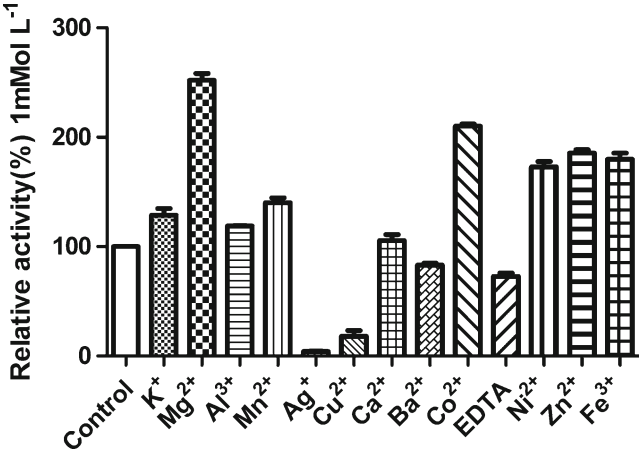

Fig. 4 Effect of different metal ions on BGL5 activity. PBS was used as the control. All the experiments were conducted in triplicate, and the relative activity was shown in this figure

depended on the gut microbes, symbiotic fungi, and host cellulases. β-Glucosidase activity in termites' midgut (including the host tissue and the content) was higher than that in a fungus-growing termite hindgut or in the fungus nodules [44]. The expressed sequence tag (EST) analysis indicated that the symbiotic fungi in *Macrotermes* spp. were more active in the hydrolysis of backbones of cellulose, hemicellulose, and pectins than in the hydrolysis of oligomers [16]. Specifically, the proportion of β-glucosidase was much lower than that of endoglucanase and exoglucanase [16], indicating a potential limit of the symbiotic fungi in processing downstream cellobiose and cello-oligosaccharides, while the diverse and function of β-glucosidase genes revealed in this study indicate that gut microbiota may work cooperatively with the *Termitomyces* fungi by further dissimilating fungi-processed plant derivatives, including cellobiose and cellodextrin, for host's plant matter degradation. This hypothesis is in accordance with our recent metagenomic overview of the degradative capacities of gut microbiome of another fungus-growing termite *O. yunnanensis* [20]. These findings will greatly broaden our understanding of the degradative symbiosis within fungus-growing termites.

Among the three major types of cellulases, β-glucosidases can hydrolyze cellobiose or cello-oligosaccharides to produce glucose, thus catalyzing the last and most critical step in cellulose degradation [45]. Cellobiose as an intermediate is known to exert a feedback inhibition on upstream cellulases; the timely removal of cellobiose by β-glucosidases is known to be able to accelerate the whole process of cellulose hydrolysis by reducing this feedback inhibition effect [46, 47]. The relative rates of hydrolysis of various substrates by the BGL5 were measured. β-Glucosidase mainly hydrolyze β-1,4-glucosidic bonds in cellobiose, short-chain cello-oligosaccharides and aryl-β-D-glucosides. Compared to other bacterial β-glucosidases retrieved by the metagenomic analyses from other environments, our enzymes had a relatively higher efficiency to hydrolyze *p*NPG [48, 49]. Eight out of the 10 β-glucosidases genes identified in this fungus-growing termite turn to include an Fn3-like domain at C-terminal, while the three enzymes expressed in our study all harbored the Fn3-like domain. It has been considered that this domain is helpful to improve the cellulose hydrolysis efficiency [50]; however, the function of Fn3-like domain in these enzymes has not been explored in this study.

This study revealed the diversity and enzyme property of β-glucosidase genes presented in the intestinal bacteria of fungus-growing termites, which highlighted the importance of intestinal bacteria in host's cellulose degradation and their potential as a good resource for mining novel β-glucosidase genes. Meanwhile, the successful recovery of a batch of enzymes from fungus-growing termite gut metagenome features the advantages of metagenomics in its application in biotechnology [21, 32, 49, 51].

Acknowledgments This work was supported by grants of the National Natural Science Foundation of China (31070098). We appreciate Professor Shubiao Wu from the University of New England for his critical reading and kind suggestions. We also give our thanks to Prashanth Singanallur and Lauren Christine Radlinski from the University of Illinois at Urbana-Champaign for proofreading. We appreciated Dr. Haokui Zhou from Department of Microbiology, The Chinese University of Hongkong and Lei zhang from Logic Informatics Co.,Ltd., for their kind help in data analysis.

Conflict of Interest The authors have no conflict of interest to declare.

References

1. Breznak JA (1994) Role of microorganisms in the digestion of lignocellulose by termites. Annu Rev Entomol 39:453–487
2. Abo-Khatwa AN (1989) Termitomyces: a new source of potent cellulases. JKing Abulaziz Univ Science 1:51–59
3. Breznak JA (1982) Intestinal microbiota of termites and other xylophagous insects. Annu Rev Microbiol 36:323–343
4. Wood TG, Thomas RJ (1989) The mutualistic association between Macrotermitinae and Termitomyces. In: Wilding N, Collins NM, Hammond PM, Webber JF (eds) Insect-fungus interaction. Academic, London, pp 69–92
5. 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(2):505–516
6. Mackenzie LM, Muigai AT, Osir EO, Lwande W, Keller M, Yoledo G, Boga HI (2007) Bacterial diversity in the intestinal tract of the fungus-cultivating termite *Macrotermes michaelseni* (Sjostedt). African Journal of Biotechnology 6(6):658–667
7. Hongoh Y, Deevong P, Inoue T, Moriya S, Trakulnaleamsai S, Ohkuma M, Vongkaluang C, Noparatnaraporn N, Kudo T (2005) Intra- and interspecific comparisons of bacterial diversity and community structure support coevolution of gut microbiota and termite host. Appl Environ Microbiol 71(11):6590–6599
8. 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(4):906–915

9. Long YH, Xie L, Liu N, Yan X, Li MH, Fan MZ, Wang QA (2010) Comparison of gut-associated and nest-associated microbial communities of a fungus-growing termite (*Odontotermes yunnanensis*). Insect Sci 17(3):265–276
10. Rouland-Lefevre C, Inoue T, Johjima T (2006) Termitomyces/termite interactions. In: Konig H, Varma A (eds) Intestinal microorganisms of soil invertebrates. Springer, Berlin, pp 335–350
11. Mathew GM, Ju YM, Lai CY, Mathew DC, Huang CC (2012) Microbial community analysis in the termite gut and fungus comb of *Odontotermes formosanus*: the implication of *Bacillus* as mutualists. FEMS Microbiol Ecol 79(2):504–517
12. Tokuda G, Lo N, Watanabe H, Arakawa G, Matsumoto T, Noda H (2004) Major alteration of the expression site of endogenous cellulases in members of an apical termite lineage. Molecular Ecology 13(10):3219–3228
13. Wu Y, Chi S, Yun C, Shen Y, Tokuda G, Ni J (2012) Molecular cloning and characterization of an endogenous digestive beta-glucosidase from the midgut of the fungus-growing termite *Macrotermes barneyi*. Insect Mol Biol 21(6):604–614
14. Hyodo F, Inoue T, Azuma JI, Tayasu I, Tabe T (2000) Role of the mutualistic fungus in lignin degradation in the fungus-growing termite *Macrotermes gilvus* (Isoptera; Macrotermitinae). Soil Biol Biochem 32:653–658
15. Hyodo F, Tayasu I, Inoue T, Azuma JI, Kudo T, Abe T (2003) Differential role of symbiotic fungi in lignin degradation and food provision for fungus-growing termites (Macrotermitinae: Isoptera). Functional Ecology 17:186–193
16. Johjima T, Taprab Y, Noparatnaraporn N, Kudo T, Ohkuma M (2006) Large-scale identification of transcripts expressed in a symbiotic fungus (*Termitomyces*) during plant biomass degradation. Appl Microbiol Biotechnol 73(1):195–203
17. Martin M, Martin J (1978) Cellulose digestion in the midgut of the fungus-growing termite *Macrotermes natalensis*: the role of acquired digestive enzyme. Science 199:1453–1455
18. Matoub M, Rouland C (1995) Purification and properties of the xylanases from the termite *Macrotermes bellicosus* and its symbiotic fungus *Termitomyces* sp. Comp Biochem Physiol B Biochem Mol Biol 112(4):629–635
19. Liu N, Yan X, Zhang M, Xie L, Wang Q, Huang Y, Zhou X, Wang S, Zhou Z (2011) Microbiome of fungus-growing termites: a new reservoir for lignocellulase genes. Appl Environ Microbiol 77(1): 48–56
20. Liu N, Zhang L, Zhou H, Zhang M, Yan X, Wang Q, Long Y, Xie L, Wang S, Huang Y, Zhou Z (2013) Metagenomic insights into metabolic capacities of the gut microbiota in a fungus-cultivating termite (*Odontotermes yunnanensis*). PLoS One 8(7):e69184
21. Bastien G, Arnal G, Bozonnet A, Laguerre S, Ferreir F, GFaure R, Henrissat B, Lefevre G, Robe P, Bouchez O, Noirot C (2013) Mining for hemicellulases in the fungus-growing termite *Pseudacanthotermes militaris* using functional metagenomics. Biotechnol Biofuels 6:78. doi:10.1186/1754-6834-1186-1178
22. DeSantis TZ, Jr., Hugenholtz P, Keller K, Brodie EL, Larsen N, Piceno YM, Phan R, Andersen GL (2006) NAST: a multiple sequence alignment server for comparative analysis of 16S rRNA genes. Nucleic Acids Res 34(Web Server issue):W394-399
23. DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, Huber T, Dalevi D, Hu P, Andersen GL (2006) Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Appl Environ Microbiol 72(7):5069–5072
24. Huber T, Faulkner G, Hugenholtz P (2004) Bellerophon: a program to detect chimeric sequences in multiple sequence alignments. Bioinformatics 20(14):2317–2319
25. Schloss PD, Handelsman J (2005) Introducing DOTUR, a computer program for defining operational taxonomic units and estimating species richness. Appl Environ Microbiol 71(3): 1501–1506
26. Wang Q, Garrity GM, Tiedje JM, Cole JR (2007) Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol 73(16):5261–5267
27. Huson DH, Auch AF, Qi J, Schuster SC (2007) Megan analysis of metagenomic data. Genome Res 17:377–386
28. Mitra S, Klar B, Huson DH (2009) Visual and statistical comparison of metagenomes. Bioinformatics 25:1849
29. Katoh K, Toh H (2008) Recent developments in the MAFFT multiple sequence alignment program. Brief Bioinformatics 9: 286–298
30. Mackenzie LM, Muigai AT, Osir EO, Lwande W, Keller M, Toledo G, Hi B (2007) Bacterial diversity in the intestinal tract of the fungus-cultivating termite *Macrotermes michaelseni* (Sjostedt). African Journal of Biotechnology 6:658–667
31. Kohler T, Dietrich C, Scheffrahn RH, Brune A (2012) High-resolution analysis of gut environment and bacterial microbiota reveals functional compartmentation of the gut in wood-feeding higher termites (*Nasutitermes* spp.). Appl Environ Microbiol 78(13):4691–4701
32. Warnecke F, Luginbuhl P, Ivanova N, Ghassemian M, Richardson TH, Stege JT, Cayouette M, McHardy AC, Djordjevic G, Aboushadi N, Sorek R, Tringe SG, Podar M, Martin HG, Kunin V, Dalevi D, Madejska J, Kirton E, Platt D, Szeto E, Salamov A, Barry K, Mikhailova N, Kyrpides NC, Matson EG, Ottesen EA, Zhang X, Hernandez M, Murillo C, Acosta LG, Rigoutsos I, Tamayo G, Green BD, Chang C, Rubin EM, Mathur EJ, Robertson DE, Hugenholtz P, Leadbetter JR (2007) Metagenomic and functional analysis of hindgut microbiota of a wood-feeding higher termite. Nature 450(7169): 560–565
33. Lawson PA, Falsen E, Inganas E, Weyant RS, Collins MD (2002) *Dysgonomonas mossii* sp. nov., from human sources. Syst Appl Microbiol 25(2):194–197
34. Martens EC, Koropatkin NM, Smith TJ, Gordon JI (2009) Complex glycan catabolism by the human gut microbiota: the Bacteroidetes Sus-like paradigm. J Biol Chem 284(37):24673–24677
35. Dodd D, Mackie RI, Cann IK (2011) Xylan degradation, a metabolic property shared by rumen and human colonic Bacteroidetes. Mol Microbiol 79(2):292–304
36. Mackenzie AK, Pope PB, Pedersen HL, Gupta R, Morrison M, Willats WG, Eijsink VG (2012) Two SusD-like proteins encoded within a polysaccharide utilization locus of an uncultured ruminant Bacteroidetes phylotype bind strongly to cellulose. Appl Environ Microbiol 78(16):5935–5937
37. Carpenter KJ, Keeling PJ (2007) Morphology and phylogenetic position of *Eucomonympha imla* (Parabasalia: Hypermastigida). J Eukaryot Microbiol 54(4):325–332
38. Hansen PS, Jensen TG, Gahrn-Hansen B (2005) Dysgonomonas capnocytophagoides bacteraemia in a neutropenic patient treated for acute myeloid leukaemia. APMIS 113(3):229–231
39. Hironaga M, Yamane K, Inaba M, Haga Y, Arakawa Y (2008) Characterization and antimicrobial susceptibility of *Dysgonomonas capnocytophagoides* isolated from human blood sample. Jpn J Infect Dis 61(3):212–213
40. Hofstad T, Olsen I, Eribe ER, Falsen E, Collins MD, Lawson PA (2000) *Dysgonomonas* gen. nov. to accommodate *Dysgonomonas gadei* sp. nov., an organism isolated from a human gall bladder, and *Dysgonomonas capnocytophagoides* (formerly CDC group DF-3). Int J Syst Evol Microbiol 50(6):2189–2195
41. Lawson PA, Carlson P, Wernersson S, Moore ER, Falsen E (2010) *Dysgonomonas hofstadii* sp. nov., isolated from a human clinical source. Anaerobe 16(2):161–164
42. Matsumoto T, Kawakami Y, Oana K, Honda T, Yamauchi K, Okimura Y, Shiohara M, Kasuga E (2006) First isolation of *Dysgonomonas mossii* from intestinal juice of a patient with pancreatic cancer. Arch Med Res 37(7):914–916

43. Collins MD, Lawson PA, Labrenz M, Tindall BJ, Weiss N, Hirsch P (2002) *Nesterenkonia lacusekhoensis* sp. nov., isolated from hypersaline Ekho Lake, East Antarctica, and emended description of the genus Nesterenkonia. Int J Syst Evol Microbiol 52(Pt 4):1145–1150
44. Martin MM, Matrtin JS (1979) The distribution and origins of the cellulolytic enzymes of the higher termite, *Macrotermes natalensis*. Physiological Zoology 52(1):11–21
45. Bhatia Y, Mishra S, Bisaria VS (2002) Microbial beta-glucosidases: cloning, properties, and applications. Crit Rev Biotechnol 22:375–407
46. Suto M, Tomita F (2001) Induction and catabolite repression mechanisms of cellulase in fungi. J Biosci Bioeng 92(4):305–311
47. Percival Zhang YH, Himmel ME, Mielenz JR (2006) Outlook for cellulase improvement: screening and selection strategies. Biotechnol Adv 24(5):452–481
48. Feng Y, Duan CJ, Liu L, Tang JL, Feng JX (2009) Properties of a metagenome-derived beta-glucosidase from the contents of rabbit cecum. Biosci Biotechnol Biochem 73(7):1470–1473
49. Jiang C, Ma G, Li S, Hu T, Che Z, Shen P, Yan B, Wu B (2009) Characterization of a novel beta-glucosidase-like activity from a soil metagenome. J Microbiol 47(5):542–548
50. Kataeva IA, Seidel RD 3rd, Shah A, West LT, Li XL, Ljungdahl LG (2002) The fibronectin type 3-like repeat from the *Clostridium thermocellum* cellobiohydrolase CbhA promotes hydrolysis of cellulose by modifying its surface. Appl Environ Microbiol 68(9):4292–4300
51. Brulc JM, Antonopoulos DA, Miller ME, Wilson MK, Yannarell AC, Dinsdale EA, Edwards RE, Frank ED, Emerson JB, Wacklin P, Coutinho PM, Henrissat B, Nelson KE, White BA (2009) Gene-centric metagenomics of the fiber-adherent bovine rumen microbiome reveals forage specific glycoside hydrolases. Proc Natl Acad Sci U S A 106(6):1948–1953