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Identification and characterization of a new xylanase from Gram-positive bacteria isolated from termite gut (*Reticulitermes santonensis*)

Christel Mattéotti [a,*], Julien Bauwens [b], Catherine Brasseur [c], Cédric Tarayre [d], Philippe Thonart [d], Jacqueline Destain [d], Frédéric Francis [b], Eric Haubruge [b], Edwin De Pauw [c], Daniel Portetelle [a], Micheline Vandenbol [a]

[a] *Unité de Biologie Animale et Microbienne, Gembloux Agro-Bio Tech, Université de Liège, B_5030 Gembloux, Belgium*
[b] *Unité d'Entomologie Fonctionnelle et Evolutive, Gembloux Agro-Bio Tech, Université de Liège, B_5030 Gembloux, Belgium*
[c] *Laboratoire de Spectrométrie de Masse, Faculté des Sciences, Université de Liège, B_4000 Liège, Belgium*
[d] *Unité de Bio-Industries, Gembloux Agro-Bio Tech, Université de Liège, B_5030 Gembloux, Belgium*

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ABSTRACT

Termites are world champions at digesting lignocellulosic compounds, thanks to cooperation between their own enzymes and exogenous enzymes from microorganisms. Prokaryotic cells are responsible for a large part of this lignocellulolytic activity. Bacterial enzyme activities have been demonstrated in the higher and the lower termite gut. From five clones of Gram-positive bacteria isolated and identified in a previous work, we constructed a genomic DNA library and performed functional screening for alpha-amylase, beta-glucosidase, and xylanase activities. One candidate, Xyl8B8, showed xylanase activity. Sequence analysis of the genomic insert revealed five complete ORFs on the cloned DNA (5746 bp). Among the encoded proteins were a putative endo-1,4-beta-xylanase (XylB8) belonging to glycoside hydrolase family 11 (GH11). On the basis of sequence analyses, genomic DNA organization, and phylogenetic analysis, the insert was shown to come from an actinobacterium. The mature xylanase (mXylB8) was expressed in *Escherichia coli* and purified by affinity chromatography and detected by zymogram analysis after renaturing. It showed maximal xylanase activity in sodium acetate buffer, pH 5.0 at 55 °C. Its activity was increased by reducing agents and decreased by Cu^{2+}, some detergents, and chelating agents. Its substrate specificity appeared limited to xylan.

Introduction

Termites are among the best decomposers of lignocellulosic plant materials in nature, partly thanks to an astonishing biodiversity in their guts [1]. The termite gut is in itself an incredible ecosystem, whose wealth has been shown in numerous studies. Lower termites harbor eukaryotes and prokaryotes showing different distributions among the gut compartments. Eukaryotes are represented by fungi [2], notably yeasts [3], and flagellate protozoa [4]. Prokaryotes are largely represented by members of the phyla *Actinobacteria*, *Firmicutes*, *Bacteroidetes*, *Probacteria*, and *Spirochaetes* [5–7], of Termite Group 1 (TG1)[1] (the so-called "endomicrobia"), a distinct lineage of unculturable intracellular bacteria of flagellate protozoa [8,9], and of the Archaea (e.g. *Methanobrevibacter* [10]).

The capacity of termites to depolymerize lignocellulosic compounds relies on the synergic action of enzymes of various microorganisms and of the termite itself [11–15]. Lower termites produce numerous endogenous enzymes (such as beta-glucosidases, exoglucanases, endoglucanases, chitinases) secreted in particular by the salivary glands and foregut [13,16,17]. Exogenous enzymes are produced by various bacteria and protozoa. In *Reticulitermes* and *Coptotermes*, several studies have demonstrated the production of lignocellulolytic enzymes by flagellate protists located in the gut [13,18]. Bacteria produce numerous enzymatic activities in termite guts. In higher termites, Warnecke et al. [19] have identified by metagenomic and functional analysis several tens of bacterial genes for cellulose and hemicellulose hydrolysis. In the gut of *Reticulitermes flavipes*, Tartar et al. [16] have revealed numerous and diverse enzymatic activities by transcriptomic analysis. Another team, focusing on different higher and lower termites, has isolated yeasts and bacteria possessing hemicellulosic activities [3]. From the lower termites *Neotermes* and *Reticulitermes*, diverse cultivable

* Corresponding author. Address: Unité de Biologie Animale et Microbienne, Gembloux Agro-Bio Tech, Université de Liège, 6 Avenue Maréchal Juin, 5030 Gembloux, Belgium. Fax: +32 81611555.
E-mail addresses: christel.matteotti@ulg.ac.be, microbio.gembloux@ulg.ac.be (C. Mattéotti).

[1] *Abbreviations used:* TG1, Termite Group 1; ORF xylB8, xylanase-encoding open reading frame; SP, signal peptide; SDS–PAGE, sulfate–polyacrylamide gel electrophoresis; IPTG, isopropyl-β-D-thiogalactoside; CMC, carboxymethylcellulose sodium salt; GH11, glycoside hydrolase family 11; XylB8, endo-1,4-beta-xylanase; mXylB8, mature xylanase; BSA, bovine serum albumin; RT, room temperature; CBM2a, carbohydrate-binding module type 2.

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Gram-positive and Gram-negative bacteria have been isolated and found to possess enzymatic activities such as beta-glucosidase, cellobiohydrolase, beta-xylosidase, carboxymethylcellulase [15,20]. We have previously identified several bacterial glycosidases by functional screening of the gut microbiota of *Reticulitermes santonensis* termites [21,22]. All of these bacterial enzymes may participate in depolymerizing the polysaccharide chains composing wood and plants (such as cellulose and hemicelluloses) in the termite gut.

Our aim here is to discover new bacterial enzymes possessing alpha-amylase, beta-glucosidase, or xylanase activities, involved in depolymerizing polysaccharides such as starch, cellulose, and hemicellulose, with a view to better understanding digestion mechanisms in the termite gut. For this we have focused on five clones of Gram-positive bacteria previously isolated from *R. santonensis* termite guts [21].

Materials and methods

Cultures of bacteria and agar plate tests

Gram-positive bacteria were isolated and purified from *R. santonensis* termite guts in a previous study [21]. Among them, five clones displaying different features were selected for the present study: TOPO-2YTclone2A, TOPO-colonie13C, TOPO-YPDclone5A, TOPO-YPDclone7A, and TOPO-cloneB. The first two were grown in 2YT and the last three in YPD medium at 37 °C for 24 h. The bacterial cultures were deposited on agar medium containing an appropriate substrate. Alpha-amylase activity was detected after incubation for 24 h at 37 °C on 2YT agar containing 0.5% soluble starch (Sigma–Aldrich, St. Louis, Mo) and the medium was covered with Gram's iodine solution (Sigma–Aldrich) for detection of α-amylase-producing colonies, identifiable by the absence of dark blue stain (due to the starch–iodine complex) in the zone surrounding them. Xylanase activity was detected on 2YT agar containing 0.1% chromogenic substrate AZCL-xylan (from birchwood) (Megazyme, Wicklow, Ireland). Colonies possessing this activity were identified by the blue color produced. Beta-glucosidase activity was detected on 2YT agar containing 0.5% esculin and 0.1% ammonium iron(III) citrate (Sigma–Aldrich) after incubation at 37 °C for 24 h. Bacteria with β-glucosidase activity hydrolyze the substrate esculin to glucose and esculetin, which combines with ferric ions to yield a dark-brown color in the agar around the bacteria. Commercial enzymes were used as positive controls (xylanase from *Trichoderma viride*, alpha-amylase from *Bacillus subtilis*, and beta-glucosidase from almonds (Sigma–Aldrich).

Construction of a genomic DNA library and functional screening on agar plates

Cultures in 2YT and YPD medium of the five selected bacteria were centrifuged at 6000 rpm for 10 min. Each pellet of bacteria was mixed with lysis buffer 1 (10 mM Tris–HCl pH 8.0, 1 mM EDTA, 2% Triton-X 100, 20 mg/ml lysozyme from chicken egg white (Sigma–Aldrich)) and incubated at 37 °C for 1 h. Genomic DNA was purified with the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions (method described for pretreatment of Gram-positive bacteria). The genomic DNAs of the five strains were mixed in equal parts and partially digested with *Sau*3AI. The restriction products were purified by gravity on a CHROMA SPIN TE-1000 column (Clontech, Mountain View, CA) to remove small fragments of less than 1 kb. The digested DNA fragments were ligated into the *Bam*HI-linearized vector pHT01 (MoBiTec, Göttingen, Germany) and the ligation products were introduced into competent ElectroMAX DH10B T1 phage-resistant cells (Invitrogen, Carlsbad, California). The quality of the library was determined on randomly selected clones. The plasmids were isolated and then digested with *Xho*I. The digestion products were analyzed by agarose gel electrophoresis (90% DNA inserts averaging 4–10 kb in size). The various colonies were pooled in 2YT medium containing 100 μg/ml ampicillin and incubated at 37 °C for 30 min. The pool was then diluted to 3750 cfu/ml and 400 μl of the diluted pool was spread onto 2YT agar plates (square Petri dish 12 × 12 cm, Greiner bio-one, Frickenhausen, Germany) containing the indicated substrate. The plasmids of positive clones were isolated and digested with *Hind*III or *Eco*RI or *Xho*I and the digestion products analyzed by agarose gel electrophoresis. The positive clone Xyl8B8 was analyzed by DNA sequencing (GATC Biotech, Konstanz, Germany).

Sequence and phylogenetic analysis

The BLASTX and BLASTP programs were used to compare, respectively, the determined nucleotide and deduced amino acid sequences with those of Genbank (http://www.ncbi.nlm.nih.gov/BLAST/). The putative signal peptide cleavage sites were predicted with the SignalIP 3.0 server (http://www.cbs.dtu.dk/services/SignalP/). Theoretical isoelectric points and molecular weights were computed with Compute pI/Mw tool (http://web.expasy.org/compute_pi/). Conserved regions and active site signatures were identified with Prosite (http://prosite.expasy.org/). Pairwise sequence alignments were done with EMBOSS Matcher (http://www.ebi.ac.uk/Tools/psa/emboss_matcher/). Phylogenetic trees were made with the neighbor-joining and bootstrap methods in MEGA5 [23].

Cloning of xylanase XylB8 and agar plate assays with AZCL-xylan

The xylanase gene *xylB8* was amplified by PCR from the pHT01-Xyl8B8 plasmid. The primers used were pHT01-ORF1for and pHT01-ORF1rev (Table1) containing a *Bam*HI site. The 1.2-kb PCR product was purified from an agarose gel with the QIAquick Gel Extraction Kit (Qiagen), digested with *Bam*HI (Roche, Basel, Switzerland), and cloned into the *Bam*HI-linearized vector pHT01. The recombinant plasmid pHT01-XylB8 was introduced into OmniMAX 2 T1 phage-resistant chemically comptetent *Escherichia coli* cells (Invitrogen). It was then isolated and its nucleotide sequence checked by DNA sequencing.

The complete xylanase-encoding open reading frame (ORF *xylB8*) and a truncated version lacking the sequence coding for

Table 1
Primers used in this study.

Primer	Orientation	Sequence (5′–3′)[a]	Nucleotides (b)
pHT01-ORF1for	Forward	cgcGGATCCATGAGGAGGAAGCACGTCCA	29
pHT01-ORF1rev	Reverse	cgcGGATCCTCGAAGGTGAGTCCGACGTA	29
pET30b-XylB8forNdeI	Forward	gggaattcCATATGAAGATCCGCAGCCGAAGA	32
pET30b-XylB8forNdeI-SP_{del}	Forward	gggaattcCATATGGACACGGTCATCACCTCGAAC	35
pET30b-XylB8revBamHI	Reverse	cgcGGATCCTCATCA**GTGGTGGTGGTGGTGGTG**GTTGGCCGCACAGCTGAACGT	54

[a] Restriction sites are underlined and the histidine tag sequence appears in bold.