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Diverse genes of cellulase homologues of glycosyl hydrolase family 45 from the symbiotic protists in the hindgut of the termite *Reticulitermes speratus*

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Abstract Diverse genes encoding cellulase homologues belonging to glycosyl hydrolase family 45 were identified from the symbiotic protists in the hindgut of the termite *Reticulitermes speratus* through the use of consensus PCR and the screening of a cDNA library. Fifteen full-length cDNA clones were isolated and sequenced, which encoded polypeptides consisting of 218–221 amino acid residues showing up to 63% identity to known family 45 cellulases. The cellulase sequences of the termite symbiotic protists were phylogenetically monophyletic, showing more than 75% amino acid identity with each other. These enzymes consist of a single catalytic domain, lacking the ancillary domains found in most microbial cellulases. By whole-cell in situ hybridization using oligonucleotide probes specific for regions conserved in some of the sequences, the origin of the genes was identified as symbiotic hypermastigote protists. The presence of diverse cellulase homologues suggests that symbiotic protists of termites may be rich reservoirs of novel cellulase sequences.

Key words Cellulase · Protist · Early-branching eukaryote · Symbiosis · Termite

Introduction

Termites maintain anaerobic microbial communities in their guts, which are very small but highly structured microenvironments (Brune and Friedrich 2000). The symbiotic relationship between xylophagous termites and the flagellated anaerobic protists in their hindgut is a well-known example of symbiosis. Early studies have indicated that the presence of the symbiotic protists is critical to termite survival on a diet of sound wood or cellulose and that many of the protists are cellulolytic, producing acetate as an energy source and precursor for biosynthesis in the termite (Breznak and Brune 1994, and references therein). Recently, cellulases of termite origin (endogenous cellulases), which are secreted from the salivary glands in the case of lower termites, have been identified and characterized (Watanabe et al. 1997, 1998). Although a substantial amount of cellulose ingested by termites can be degraded by the endogenous cellulase, the cellulose not hydrolyzed in the anterior portion of the gut then travels to the hindgut, where it can be endocytosed and fermented by the symbiotic protists (Watanabe et al. 1998). The existence of this dual system in lower termites explains their ability to assimilate wood glucan to an extent greater than 90% (Breznak and Brune 1994).

Cellulases of protist origin, however, have never been analyzed at the molecular level to our knowledge until now, because pure cultures have been very difficult to establish and only a limited number of species have been axenically cultured (Yamin 1978, 1980a; Odelson and Breznak 1985a; Berchtold et al. 1995). Consequently, there have been relatively few biochemical investigations (Yamin and Trager 1979; Yamin 1980a, 1980b; Odelson and Breznak 1985b). Many studies on the symbiotic protists have been restricted to their morphology. Recently, culture-independent approaches involving PCR amplification of a certain gene and identification of the origin of the gene by in situ hybridization have been developed to investigate the molecular phylogeny of the symbiotic protists (Berchtold and König 1995; Moriya et al. 1998; Ohkuma et al. 1998, 2000). The symbiotic protists belonging to the orders Hypermastidida, Trichomonadida, and Oxymonadida have been found to represent early branching eukaryotes.

In this article, based on a culture-independent approach, we describe the characterization of the sequences of diverse genes of cellulase homologues, which belong to glycosyl

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hydrolase family 45 (GHF45), from symbiotic protists in the hindgut of the termite *Reticulitermes speratus*. *R. speratus* is one of the most extensively investigated termites in terms of both its cellulolytic and xylanolytic systems and the molecular phylogeny of its symbiotic protists (Inoue et al. 1997; Moriya et al. 1998; Ohkuma et al. 1998, 2000; Watanabe et al. 1997, 1998; Yamaoka and Nagatani 1975, 1977).

Materials and methods

PCR with consensus primers and cloning of the PCR product

A pair of degenerate oligonucleotide primers was designed: F45F (5′-ACIMGITAYTGGGAYTGYTG-3′) and F45R (5′-AAIRYICCIAVICCICCICCIGG-3′). These primers corresponded to the consensus amino acid segments (TRYWDCC, and PGGG[V/L]G[I/A/L][Y/F], respectively) of most members of GHF45, and served to amplify a fragment approximately 380 bp in size coding for the conserved catalytic domain of the GHF45 cellulases. As the template for PCR, we used DNA extracted from the whole population of a full-length cDNA library of the symbiotic protists in the hindgut of *R. speratus*. The cDNA library was constructed by the method of Carninci and Hayashizaki (1999) using mRNA extracted from the mixed population of the hindgut protists without the gut tissue as described previously (Moriya et al. 1998). PCR was performed using Ex-Taq DNA polymerase (Takara Shuzo, Kyoto, Japan) according to the manufacturer's directions; the reaction conditions were 35 cycles of 94°C for 30 s, 45°C for 45 s, and 72°C for 2 min. The PCR product of the appropriate size was purified and cloned into pGEM-T (Promega, Madison, WI, USA).

Screening of the cDNA library

The insert DNA fragments carrying the PCR products corresponding to GHF45 cellulase genes (clones 1, 2, 5, 8, 9, and 25) were excised by digestion with *Sac*II and *Spe*I (Takara Shuzo). Equimolar amounts of the insert DNA fragments were mixed and labeled with digoxigenin using a DIG DNA labeling kit (Boehringer Mannheim, Mannheim, Germany) and then were used as probes for plaque hybridization. Recombinant lambda phages in the cDNA library were transferred from plaques onto Hybond N^+ membranes (Amersham, Buckinghamshire, UK). Hybridization and detection of positive clones were performed using a DIG nucleic acid detection kit (Boehringer Mannheim) according to the manufacturer's directions. The temperature for hybridization was 37°C. Hybridization-positive clones were purified and recovered as plasmid DNAs as described previously (Carninci and Hayashizaki 1999).

DNA sequencing and phylogenetic analysis

DNA sequences were determined using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer Japan, Tokyo, Japan) and an automatic sequence analyzer (ABI model 377). The sequence data were aligned using the CLUSTAL W package (Tompson et al. 1994) and checked manually. Construction of a neighbor-joining phylogenetic tree and bootstrap analysis were performed using the PHYLIP ver. 3.5c package (Felsenstein 1989). The nucleotide sequence data determined in this study will appear in the databases under accession numbers AB045159-AB045164 for the six PCR product clones and AB045165-AB045179 for the 15 full-length cDNA clones.

In situ hybridization

Oligonucleotide probes, F45-D9 (5′-GCTGCTGCAGCA ATTTCAGG-3′) and F45-D3 (5′-ACMCCRTGGCAAG TRAGTGA-3′, where M and R represent A or C and A or G, respectively), which were labeled with fluorescein isothiocyanate (FITC) at the 5′-end, were used for in situ identification of the origin of the cellulase homologue genes. Fixation of the protist cells and whole-cell hybridization were performed as described previously (Ohkuma et al. 1998, 2000). After hybridization, the specimen was immunologically stained with alkaline phosphatase-conjugated anti-FITC antibodies (Boehringer Mannheim) as described previously (Moriya et al. 1998).

Results and discussion

Clones of the PCR product

Cellulases and other glycosyl hydrolases have been classified into more than 66 families based on protein sequence relatedness (Henrissat and Bairoch 1993, 1996). The presence of some amino acid regions conserved in an intrafamily manner but not in an interfamily manner has made it possible to design degenerate oligonucleotide primers for PCR amplification of the genes encoding cellulase homologues (Sheppard et al. 1994). In our attempts to amplify cellulase genes, the combination of the degenerate primers employed specific for glycosyl hydrolase family 45 (GHF45) was found to be effective for amplification of fragments of the expected size (approximately 380 bp) from the cDNA prepared from mRNA extracted from the hindgut protists of *R. speratus*. The PCR products were cloned, and 38 clones were categorized into six groups on the basis of nucleotide sequence identity (clones showing more than 98.8% identity were grouped together). These groups were represented by clones 1, 2, 5, 8, 9, and 25, respectively, and these groups consisted of 6, 13, 13, 2, 1, and 3 clones, respectively. The group represented by clone 1 and that represented by clone 2 showed relatively high nucleotide sequence identity (more than 96%), whereas the other groups showed less than 87% identity. The deduced amino acid sequences of the PCR product clones showed significant sequence similarity with members of GHF45 (see following).

Full-length clones

A mixture of the DNAs of the six representative clones was used in screening to detect full-length cDNA. In the hybridization under low-stringency conditions, positive signals from approximately 0.2% of the plaques in the cDNA library were observed, suggesting that the GHF45 cellulase homologues were moderately expressed genes. Thirty-three positive clones were isolated and the partial nucleotide sequences of these were determined. The clones showing more than 99% nucleotide sequence identity were grouped together to obtain 15 groups. Four groups, represented by clones 2-54, 2-6, 7-25, and 6-32, consisted of multiple clones (6, 4, 4, and 3 clones, respectively), and five groups, represented by clones 1-14, 1-16, 4-44, 7-50, and 8-44, consisted of 2 clones each. The other 6 clones (6-47, 7-10, 8-16, 8-38, 45-6, and B1-1) were unique sequences. The complete nucleotide sequence was determined in the case of each of these 15 representative clones.

An open reading frame encoding a polypeptide consisting of 218 to 221 amino acid residues was found in the case of each clone. It is unlikely that the coding region would extend upstream from the first ATG in the cDNAs because there are in-frame stop codons within 21 bp upstream of the ATG in 4 of the 15 representative clones. In all the 15 clones, the first 12 to 15 amino acid residues was a stretch of hydrophobic amino acids, seemingly a signal sequence. These observations suggested that the isolated cDNA clones probably encode the entire sequences of the GHF45 cellulase homologues.

The sequences of the PCR product clones were compared with those of the cDNA clones. The deduced amino acid sequences of PCR product clones 5, 8, and 25 were identical to those of cDNA clones 2-6, 8-44, and 45-6, respectively, and the percent nucleotide sequence identity was found to be 97.3%, 99.4%, and 97.9%, respectively. The sequences of the PCR product clones in the other three groups were not identical to any of the cDNA clones. However, the deduced amino acid sequences of PCR product clones 1, 2, and 9 showed relatively high amino acid sequence identity compared to the sequence of cDNA clone 4-44, that is, 96.4% in each instance, although the percent nucleotide sequence identities was less than 89% in each instance.

To identify the origin of the sequences, whole-cell in situ hybridization experiments were performed. Two probes, one corresponding to the conserved nucleotide sequence of clones 2-6 and 4-44 (D9 probe; see Fig. 1) and the other corresponding to that of clones 7-25, 7-10, B1-1, and 1-16 (D3 probe; data not shown), gave significant signals from the cells of the large hypermastigote protists *Trichonympha agilis* and *Teranympha mirabilis* in both cases. Very weak signals could be detected for the other protist species. Without the addition of the probe, no significant signal was observed from either of these protists (data not shown), indicating that the detected signal was attributable to the probes. The results suggested that these genes for the cellulase homologues originated from either of these protist species. The use of sequence-specific probes may be necessary to distinguish the origin of the cellulase homologues in assays of these two protist species.

Fig. 1. Whole-cell in situ hybridization with the F45-D9 probe specific for the clones 2-6 and 4-44. The photograph is a phase-contrast image showing stained symbiotic protists in the gut. Two large hypermastigote protists, *Teranympha mirabilis (Tm)* and *Trichonympha agilis (Ta)*, were detected. *Bar* 100 μm

Comparisons of amino acid sequences

The amino acid sequences identified in this study clearly belonged to GHF45 as determined on the basis of sequence similarity. In particular, they shared significant similarity with the catalytic domain of members of GHF45. Figure 2 shows the sequence alignment of representative members of GHF45, Table 1 shows the percent amino acid identity among members of GHF45, and Fig. 3A shows the phylogenetic relationship among members of this family. In the phylogenetic tree (Fig. 3A), all the sequences from the symbiotic protists formed a monophyletic lineage, which was supported statistically (bootstrap value, 98%). The deduced amino acid sequences of the GHF45 cellulase homologues from the symbiotic protists showed 57.4% to 63.2% identity to three sequences from *Humicola* (*H. insolens* EGV [Davies et al. 1993] and *H. grisea* egl3 and egl4 [Takashima et al. 1999]), and showed 53.4%–56.2% identity to *Fusarium oxysporum* Kfam1 (Sheppard et al. 1994), 40.5%–42.9% identity to *Ustilago maydis* EGI (Schauwecker et al. 1995) and 35.6%–38.5% identity to *Pseudomonas fluorescens* EGB (Gilbert et al. 1990). Comparing the deduced amino acid sequences of GHF45 cellulase homologues from the symbiotic protists, they showed more than 75% identity with each other. The monophylety of the cellulase homologue sequences of the termite symbiotic protists suggests their recent diversification within the protistan taxa.

The phylogenetic relationship among the GHF45 cellulase homologues from the symbiotic protists as inferred from the deduced amino acid sequences is shown in Fig. 3B. Three sequence clusters were found in the phylogenetic tree. The first group consisted of 7-25, 7-10, B1-1, 1-16, and 1-14. The sequences within this group showed more than 90% amino acid identity; especially, the sequences of the clones 7-10 and B1-1 showed 98.6% identity. The second group consisted of 8-44, 7-50, and 6-47 sharing 93.2%–

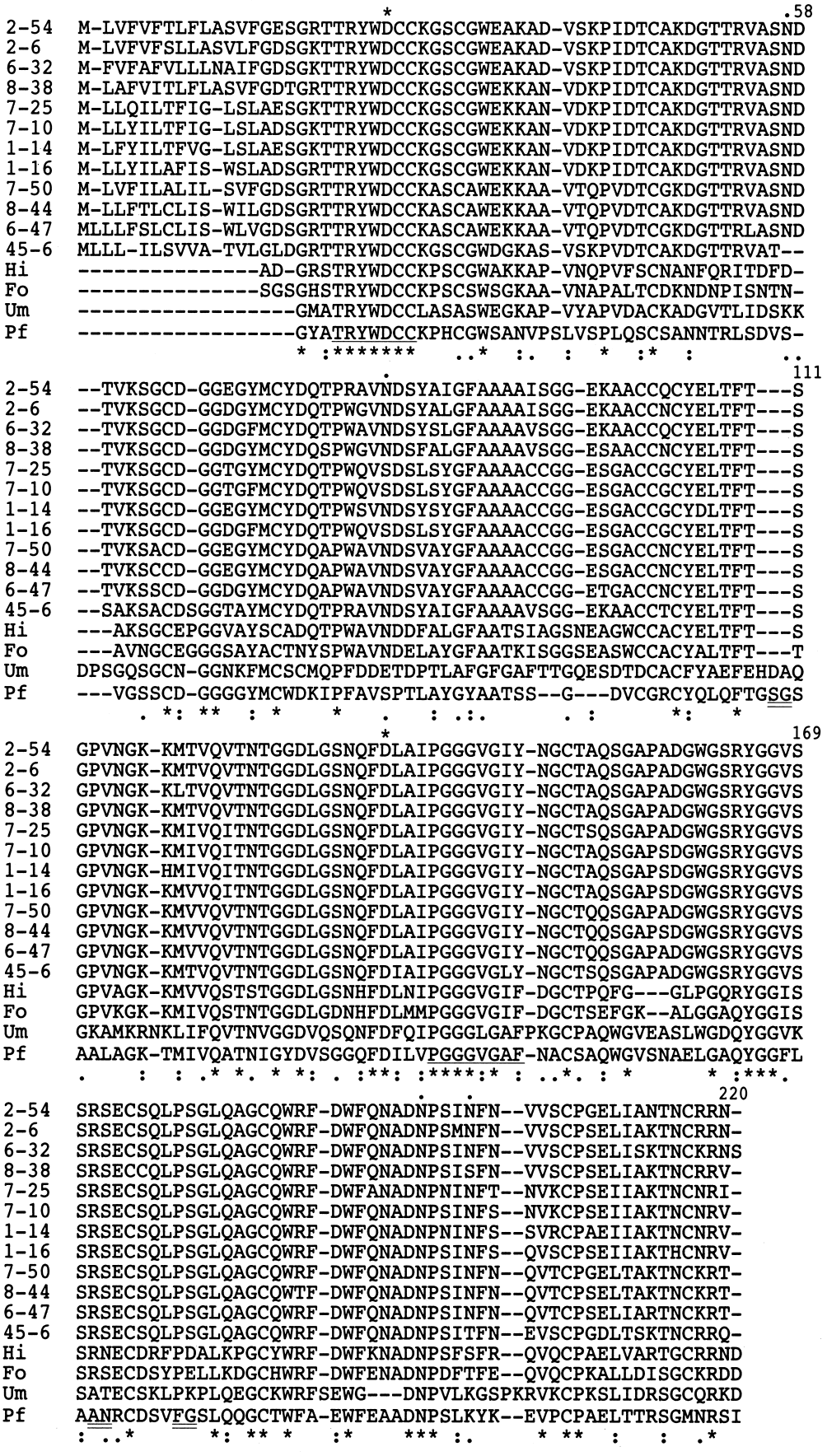

Fig. 2. An alignment of amino acid sequences of the cellulase homologues from the symbiotic protists of the termite and representative members of GHF45. Only the region of the catalytic domain is shown for the four reference sequences (the database accession numbers and corresponding amino acid positions in this alignment are indicated in the following parentheses): Hi, *Humicola insolens* (P43316; 1–203); Fo, *Fusarium oxysporum* (P45699; 19–223); Um, *Ustilago maydis* (P54424; 27–238), Pf, *Pseudomonas fluorescens* (P18126; 269–502). Gaps needed for optimal alignment are indicated by *hyphens*. In the case of *P. fluorescens*, there are three large insertions between the two *double-underlined* amino acid residues (SSYNAPGDPG, ACKQQLGY NASLSQYKSCVLN, and FGSR GLT), which are deleted from the alignment. The conserved regions used as the basis for designing the PCR primers are *underlined* in the alignment. *Asterisks* below the aligned sequences indicate amino acids identical in all the sequences. *Colons* and *dots* below the aligned sequences denote a strongly or weakly conserved substitution, respectively, at the indicated amino acid position. Two aspartate residues in the active sites of the enzymes are highlighted by *asterisks* above the aligned sequences. Putative N-glycosylation sites (N-X-S/T) in one or more sequences of the termite symbiotic protists are denoted by *dots* above the aligned sequences. *Numbers* on the right side above the aligned sequences denote the amino acid positions of the sequence of clone 2-54

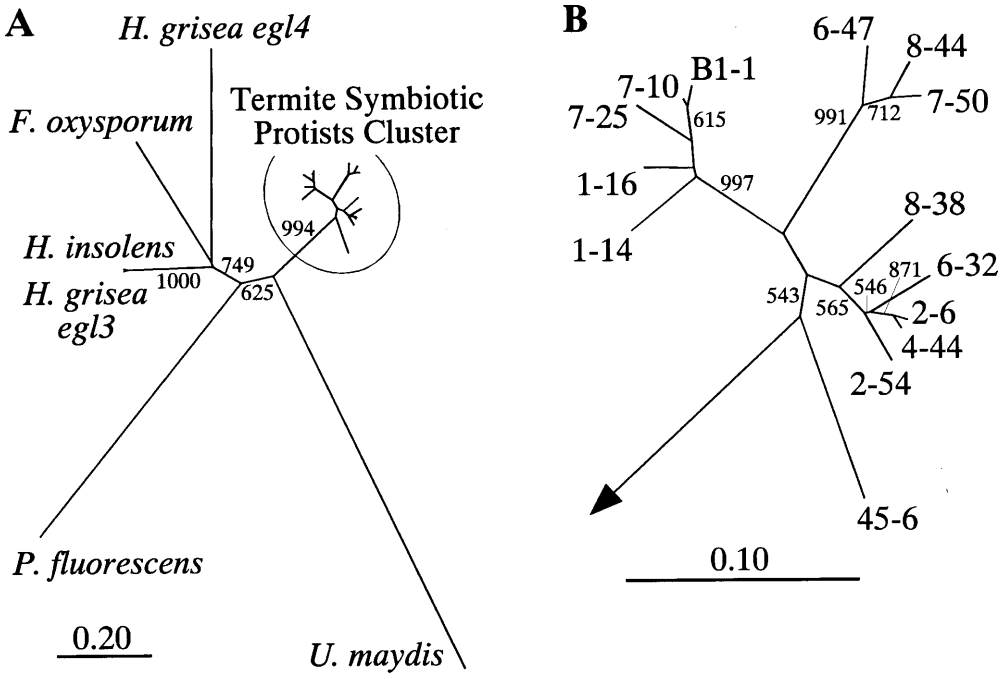

Fig. 3A,B. The phylogenetic relationship among members of GHF45. **A** The unrooted tree was inferred by the neighbor-joining method based on the amino acid sequence alignment of the catalytic domains without putative signal sequences (216 positions). The amino acid sequence of clone 8-16 is identical to that of clone 2-6. In addition to the 16 sequences included in the alignment shown in Fig. 2, two sequences from the termite symbiotic protists, clones 4-44 and B1-1, and two sequences of *Humicola grisea*, egl3 and egl4 (database accession numbers, AB003107 and AB003108, respectively), were added for the phylogenetic analysis. *Bar* shows 0.20 substitutions per site. **B** Magnification of the termite symbiotic protists cluster found in **A**. *Arrow* indicates the node connecting the other reference sequences. *Bar* shows 0.10 substitutions per site. Bootstrap values above 500 from 1000 resamplings are shown for each node in **A** and **B**

94.5% amino acid identity. The third group consisted of 2-54, 2-6, 8-16, 4-44, 6-32, and 8-38, sharing more than 88.1% amino acid identity. The sequences of two clones, 2-6 and 8-16, were completely identical, and the sequences of the clones 2-6 and 4-44 showed 97.3% identity. The clustering of the first group and that of the second group were statistically supported by the results of bootstrap analysis (100% in both cases), whereas that of the third group was not. The percent amino acid identity between members of different clusters was less than 87%. The sequence of clone 45-6 alone formed a lineage distinct from the three other groups, sharing less than 84% amino acid identity with them.

Characteristics of the amino acid sequences

In *H. insolens* EGV, a member of GHF45, two aspartate residues (Asp10 and Asp121), which have been shown to be important for catalytic activity, are positioned next to a tyrosine residue (Tyr 8) in the active site groove (Davies et al. 1993). These amino acid residues and their sequence context are conserved in all the sequences identified from the termite symbiotic protists. This observation suggests a similar mechanism of catalysis, probably as an endoglucanase because all the known members of GHF45 are exclusively endoglucanase. The amino acid regions used for the design of the PCR primers were completely conserved, suggesting that these primers would be useful to amplify any yet-unknown members of GHF45.

The polypeptides encoded by the ORFs characterized in the present study, consisting of 218–221 amino acid residues, are rather small compared to other members of GHF45, e.g., 376, 393, and 511 amino acid residues in the case of *F. oxysporum*, *U. maydis*, and *P. fluorescens*, respectively. In addition to the catalytic core, many cellulases contain a cellulose-binding domain (CBD) located either on the N-terminal side or the C-terminal side of the catalytic core. CBDs are often separated from the catalytic core by a flexible spacer sequence enriched in proline and hydroxy amino acids. The presence of a CBD apparently enhances the enzymatic activity of the catalytic core against crystalline cellulose (Tomme et al. 1995). In the sequences from the termite symbiotic protists, there was no counterpart for either CBD or the spacer. Interestingly, the termite endogenous cellulases, which belong to GHF9, also have neither CBD nor the spacer sequence (Watanabe et al. 1998; Tokuda et al. 1999).

The presence of putative signal sequences in the N-terminal parts was found to be a common feature of GHF45 cellulases. Except for the cellulase homologues of the symbiotic protists, all known members of this family are secreted extracellularly. In the case of the termite symbiotic protists, however, the cellulase activity is considered to be displayed inside the cells, associated with so-called food vacuoles, because wood components are endocytosed and

Table 1. Percent amino acid identity among members of glycosyl hydrolase family 45

		2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1.	2-54	92.7	92.7	90.9	90.9	85.4	82.6	81.7	82.2	81.7	82.2	82.2	83.1	83.3	62.0	61.5	59.1	55.1	40.6	38.5
2.	2-6		97.3	92.7	90.9	84.9	83.6	83.6	83.6	83.1	82.6	83.6	82.6	81.0	62.0	62.0	58.5	55.1	41.0	36.4
3.	4-44			92.7	91.2	83.5	82.1	81.9	84.3	83.8	82.4	84.3	83.2	80.3	61.5	61.5	58.0	54.6	41.5	35.6
4.	6-32				88.1	83.1	82.2	81.7	83.5	83.0	81.6	83.5	82.0	79.9	59.5	59.5	56.8	54.1	42.0	37.2
5.	8-38					84.9	83.6	83.1	84.1	83.6	82.2	84.5	82.7	80.5	62.7	61.8	58.5	55.4	41.7	37.0
6.	7-50						94.5	93.2	83.0	82.6	82.6	84.4	82.1	79.5	62.7	62.3	59.7	55.7	42.0	37.7
7.	8-44							94.1	84.4	83.5	83.0	86.2	82.6	77.1	61.6	61.1	58.3	55.7	41.0	37.7
8.	6-47								82.1	81.7	81.7	84.4	79.8	76.3	63.2	63.2	59.7	55.7	42.5	38.5
9.	7-10									98.6	95.9	95.9	93.6	76.3	59.3	59.8	58.5	53.4	42.5	37.9
10.	B1-1										94.5	95.4	93.6	75.3	59.8	60.3	58.5	53.4	42.9	38.3
11.	7-25											91.8	92.2	76.7	59.3	59.8	59.1	54.4	41.5	37.4
12.	1-16												90.9	76.3	60.8	60.3	58.5	53.4	41.5	37.4
13.	1-14													75.8	59.3	59.8	57.4	53.4	40.6	37.4
14.	45-6														59.6	59.1	60.0	56.2	40.5	38.4
15.	Hi															98.5	65.1	65.8	37.4	38.1
16.	Hg3																64.6	65.5	37.2	38.6
17.	Hg4																	62.3	35.9	39.3
18.	Fo																		37.4	36.3
19.	Um																			38.0
20.	Pf																			

Hi, *Humicola insolens*; Hg3, *Humicola grisea* egl3; Hg4, *Humicola grisea* egl4; Fo, *Fusarium oxysporum*; Um, *Ustilago maydis*; Pf, *Pseudomonas fluorescens*

decomposed within this organelle. In the symbiotic protist *T. agilis*, which was found to be one of the origins of the GHF45 cellulase homologues, ingestion and digestion of cellulose within the cells have been characterized (Yamaoka and Nagatani 1977). Presumably, the cellulases identified in this study are localized within the food vacuoles, although no candidate sequence corresponding to a region involved in protein sorting within the cells was found in the cellulase homologues of the symbiotic protists.

Conclusions

In this study, we have identified diverse genes encoding cellulase homologues belonging to GHF45 from the symbiotic protists of the termite *Reticulitermes speratus*. Because cultivation of the symbiotic protists is difficult, the PCR-based approach using degenerate primers specific for regions of conserved amino acid residues is thought to be an efficient way to identify genes responsible for lignocellulose decomposition in these symbiotic protists. Although the catalytic activity of these cellulases, the exact origins of the protist species involved, and the cellular localization of these cellulases remain to be clarified, the presence of diverse cellulase-homologous genes indicates that the termite symbiotic protistan community is a rich reservoir of novel cellulase sequences.

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References

Berchtold M, König H (1995) Phylogenetic position of two uncultivated trichomonads *Pentatrichomonoides scroa* Kirby and *Metadevescovina extranea* Kirby from the hindgut of the termite *Mastotermes darwiniensis* Froggatt. Syst Appl Microbiol 18:567–573

Berchtold M, Breunig A, König H (1995) Culture and phylogenetic characterization of *Trichomitus trypanoides* Duboscq & Grassé 1924, n. comb.: a trichomonad flagellate isolated from the hindgut of the termite *Reticulitermes santonensis* Feytaud. J Eukaryot Microbiol 42:388–391

Breznak JA, Brune A (1994) Role of microorganisms in the digestion of lignocellulose by termites. Annu Rev Entomol 39:453–487

Brune A, Friedrich M (2000) Microecology of the termite gut: structure and function on a microscale. Curr Opin Microbiol 3:263–269

Carninci P, Hayashizaki Y (1999) High-efficiency full-length cDNA cloning. Methods Enzymol 303:19–44

Davies GJ, Dodson GG, Hubbard RE, Tolley SP, Dauter Z, Wilson KS, Hjort C. Mikkelsen JM, Rasmussen G, Schülein M (1993) Structure and function of endoglucanase V. Nature (Lond) 365:362–364

Felsenstein J (1989) PHYLIP-phylogeny inference package (version 3.2). Cladistics 5:164–166

Gilbert HJ, Hall J, Hazlewood GP, Ferreira LMA (1990) The N-terminal region of an endoglucanase from *Pseudomonas fluorescens* subspecies *cellulosa* constitutes a cellulose-binding domain that is distinct from the catalytic centre. Mol Microbiol 4:759–767

Henrissat B, Bairoch A (1993) New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. Biochem J 293:781–788

Henrissat B, Bairoch A (1996) Updating the sequence-based classification of glycosyl hydrolases. Biochem J 316:695–696

Inoue T, Murashima K, Azuma J-I, Sugimoto A, Slaytor M (1997) Cellulose and xylan utilisation in the lower termite *Reticulitermes speratus*. J Insect Physiol 43:235–242

Moriya S, Ohkuma M, Kudo T (1998) Phylogenetic position of symbiotic protist *Dinenympha exilis* in the hindgut of the termite *Reticulitermes speratus* inferred from the protein phylogeny of elongation factor 1α. Gene (Amst) 210:221–227

Odelson DA, Breznak JA (1985a) Nutrition and growth characteristics of *Trichomitopsis termopsidis*, a cellulolytic protozoan from termites. Appl Environ Microbiol 49:614–621

Odelson DA, Breznak JA (1985b) Cellulase and other polymer-hydrolyzing activities of *Trichomitopsis termopsidis*, a symbiotic protozoan from termites. Appl Environ Microbiol 49:622–626

Ohkuma M, Ohtoko K, Grunau C, Moriya S, Kudo T (1998) Phylogenetic identification of the symbiotic hypermastigote *Trichonympha agilis* in the hindgut of the termite *Reticulitermes speratus* based on small-subunit rRNA sequence. J Eukaryot Microbiol 45:439–444

Ohkuma M, Ohtoko K, Iida T, Tokura M, Moriya S, Usami R, Horikoshi K, Kudo T (2000) Phylogenetic identification of hypermastigotes, *Pseudotrichonympha*, *Spirotrichonympha*, *Holomastigotoides*, and parabasalian symbionts in the hindgut of termites. J Eukaryot Microbiol 47:249–259

Schauwecker F, Wanner G, Kahmann R (1995) Filament-specific expression of a cellulase gene in the dimorphic fungus *Ustilago maydis*. Biol Chem Hoppe-Seyler 376:617–625

Sheppard PO, Grant FJ, Oort PJ, Sprecher CA, Foster DC, Hagen FS, Upshall A, McKnight GL, O'Hara PJ (1994) The use of conserved cellulase family-specific sequences to clone cellulase homologue cDNAs from *Fusarium oxysporum*. Gene (Amst) 150:163–167

Takashima S, Iikura H, Nakamura A, Hidaka M, Masaki H, Uozumi T (1999) Comparison of gene structures and enzymatic properties between two endoglucanases from *Humicola grisea*. J Biotechnol 67:85–97

Tokuda G, Lo N, Watanabe H, Slaytor M, Matsumoto T, Noda H (1999) Metazoan cellulase genes from termites: intron/exon structures and sites of expression. Biochim Biophys Acta 1447:146–159

Tomme P, Warren RAJ, Gilkes NR (1995) Cellulose hydrolysis by bacteria and fungi. Adv Microbiol Physiol 37:1–81

Tompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680

Watanabe H, Nakamura M, Tokuda G, Yamaoko I, Scrivener AM, Noda H (1997) Site of secretion and properties of endogenous endo-β-1, 4-glucanase components from *Reticulitermes speratus* (Kolbe), a Japanese subterranean termite. Insect Biochem Mol Biol 27:305–313

Watanabe H, Noda H, Tokuda G, Lo N (1998) A cellulase gene of termite origin. Nature (Lond) 394:330–331

Yamaoka I, Nagatani Y (1975) Cellulose digestion system in the termite, *Reticulitermes speratus* (Kolbe). I. Producing sites and physiological significance of two kinds of cellulase in the worker. Zool Mag (Tokyo) 84:23–29

Yamaoka I, Nagatani Y (1977) Cellulose digestion system in the termite *Reticulitermes speratus* (Kolbe). II. Ultrastructural changes related to the ingestion and digestion of cellulose by flagellate *Trichonympha agilis*. Zool Mag (Tokyo) 86:34–43

Yamin MA (1978) Axenic cultivation of the cellulolytic flagellate *Trichomitopsis termopsidis*.(Cleveland) from the termite *Zootermopsis*. J Protozool 25:535–538

Yamin MA (1980a) Cellulose metabolism by the flagellate *Trichonympha* from a termite is independent of endosymbiotic bacteria. Science 211:58–59

Yamin MA (1980b) Cellulose metabolism by the termite flagellate *Trichomitopsis termopsidis*. Appl Environ Microbiol 39:859–863

Yamin MA, Trager W (1979) Cellulolytic activity of an axenically-cultivated termite flagellate, *Trichomitopsis termopsidis*. J Gen Microbiol 113:417–420