Appl Biochem Biotechnol (2011) 163:205–214
DOI 10.1007/s12010-010-9029-6

Mining *Dictyoglomus turgidum* for Enzymatically Active Carbohydrases

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Received: 25 April 2010 / Accepted: 28 June 2010 /
Published online: 16 July 2010
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Abstract The genome of *Dictyoglomus turgidum* was sequenced and analyzed for carbohydrases. The broad range of carbohydrate substrate utilization is reflected in the high number of glycosyl hydrolases, 54, and the high percentage of CAZymes present in the genome, 3.09% of its total genes. Screening a random clone library generated from *D. turgidum* resulted in the discovery of five novel biomass-degrading enzymes with low homology to known molecules. Whole genome sequencing of the organism followed by bioinformatics-directed amplification of selected genes resulted in the recovery of seven additional novel enzyme molecules. Based on the analysis of the genome, *D. turgidum* does not appear to degrade cellulose using either conventional soluble enzymes or a cellulosomal degradation system. The types and quantities of glycosyl hydrolases and carbohydrate-binding modules present in the genome suggest that *D. turgidum* degrades cellulose via a mechanism similar to that used by *Cytophaga hutchinsonii* and *Fibrobacter succinogene*s.

Keywords *Dictyoglomus turgidum* · Biomass · Genome screening · Thermophilic cellulase · Hemicellulase · Glucosidase · Enzyme discovery

Background

The extreme thermophiles *Dictyoglomus thermophilus* and *Dictyoglomus turgidum* were isolated from hot springs of the Kamchatka peninsula in Russia [1, 2]. Both strains have very

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similar growth requirements, and both grow up to 80 °C. *Dictyoglomus* species represent a novel group of thermophilic, anaerobic chemoorganotrophic organisms with considerable biotechnological promise. These organisms are so unique that they have been given their own genus, *Dictyoglomi.* The genome of *Dictyoglomus thermophilum* has been recently sequenced and has a number of potentially commercially useful enzymes including an extremely thermophilic xylanase encoded by the *xyn*A gene [3]. *D. thermophilus* does not produce cellulase. However, the related strain, *D. turgidum*, is reported to grow on a wide range of substrates including starch, cellulose, pectin, carboxymethylcellulose, lignin, and humic acids, but not on pentose sugars such as xylose and arabinose [1]. In addition, this strain is reported to produce ethanol as a fermentation end product while growing on sugars (e.g., glucose or cellobiose). This organism was selected for library construction and screening, as well as whole genome sequencing.

Materials and Methods

Materials D. turgidum strain 6724^T bacterial cell concentrate was a kind gift of Dr. Frank T. Robb, Center of Marine Biotechnology, University of Maryland Biotechnology Institute. *D. turgidum* strain 6724^T is on deposit at Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH. 10G electro-competent *E. coli* cells, BL21(DE3) chemically competent *E. coli* cells, and pEZSeq (a lac promoter vector) were obtained from Lucigen, Middleton, WI, USA. pET28a vector and Overnight Express medium were obtained from Merck Chemicals, San Diego, CA, USA. Azurine cross-linked-labeled polysaccharides were obtained from Megazyme International (Wicklow, Ireland). 4-Methylumbelliferyl-β-D-cellobioside (MUC), 4-methylumbelliferyl-β-D-xylopyranoside (MUX), and 4-methylumbelliferyl-β-D-glucopyranoside (MUG) were obtained from Research Products International Corp. (Mt. Prospect, IL, USA). CelLytic IIB reagent, pNP-β-glucoside, pNP-β-cellobioside, 4-methylumbelliferyl-α-D-arabinofuranoside (MUA), 4-methylumbelliferyl-β-D-lactopyranoside (MUL), 5-bromo-4-chloro-3-indolyl α-D-galactopyranoside (X-α-Gal, XAG), and 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal, XG) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of analytical grade.

Growth of Organisms YT plate media (16 g/l tryptone, 10 g/l yeast extract, 5 g/l NaCl, and 16 g/l agar) were used in all molecular biology screening experiments. Terrific broth (12 g/l tryptone, 24 g/l yeast extract, 9.4 g/l K_2HPO_4, 2.2 g/l KH_2PO_4, and 4.0 g/l glycerol added after autoclaving) was used for liquid cultures.

Enzyme Assays The *endo*-glucanase specificity of enzymes was determined in 0.50 ml of 50 mM acetate buffer, pH 5.8, containing 0.2% azurine cross-linked-labeled (AZCL) insoluble substrates and 50 μl of clarified lysate. Assays were performed at 70 °C, with shaking at 1,000 rpm, for 60 min in a Thermomixer R (Eppendorf, Hamburg, Germany). Tubes were clarified by centrifugation, and absorbance values were determined using a Bio-Tek EL_x800 plate reader. The *exo*-glucanase specificity of enzymes was determined by spotting 2.0 μl of clarified lysate directly on agar plates containing 10 mM 4-methyl umbelliferyl substrate. Plates were incubated in a 70 °C incubator for 60 min; after incubation, the plates were examined using a hand-held UV lamp and compared with negative and positive controls.

Library Construction, Cloning, and Screening D. turgidum strain 6724T (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Micro-

organisms and Cell Cultures)) bacterial cell concentrate was a kind gift of Dr. Frank T. Robb, Center of Marine Biotechnology, University of Maryland Biotechnology Institute. The cell concentrate was lysed using a combination of SDS and proteinase K, and genomic DNA was purified using phenol/chloroform extraction [4]. The genomic DNA was precipitated, treated with RNase to remove residual contaminating RNA, and fragmented by hydrodynamic shearing (HydroShear apparatus, GeneMachines, San Carlos, CA, USA) to generate fragments of 2–4 kb. The fragments were purified on an agarose gel, end-repaired, and ligated into pEZSeq, a lac promoter vector (Lucigen). The recombinant plasmids were then used to transform electro-competent cells. *D. turgidum* genomic DNA was submitted to the Joint Genome Institute of the Department of Energy for whole genome sequencing; a copy of the library was used for screening experiments.

Two separate approaches were utilized for functional annotation of the carbohydrase active enzymes: (1) whole genome random shotgun expression screening [5] and (2) gene-directed expression screening. A copy of the library was screened on YT+30 mg/l kan plates containing either 0.01% MUC, MUG, X-*beta*-galactosidase (XG), or X-*alpha*-galactosidase (XAG). Positive clones were picked, grown in liquid media, lysed, and tested for activity on AZCL substrates and soluble chromogenic substrates. Enzymes from these positive clones were purified and characterized, and their genes were sequenced and subcloned. When the genome sequencing was completed, BLAST analysis [6] was used to identify the gene number.

Additional genes of interest were selected from the completed genome for amplification and expression. These genes were amplified without the secretory signal sequence leader, ligated into pET28A, and transformed into BL21(DE3) *E. coli* competent cells. Clones were cultured overnight in 96-deep well plates using Overnight Express medium (Novagen, Madison, WI, USA). Cells were pelleted by centrifugation, and the pellets were lysed using Cellytic B reagent. Protein expression was checked by SDS PAGE and enzyme activity by assay with the expected substrates.

Bioinformatics InterProScan Family analysis (http://www.ebi.ac.uk/Tools/InterProScan/) and BLASTP (Basic Local Alignment Search Tool) [7] (http://blast.ncbi.nlm.nih.gov/Blast.cgi) analysis tools were used to compare CelA with other proteins in the database. Phylogeny analysis was performed using software at http://www.phylogeny.fr/version2_cgi/index.cgi. Multiple alignments were run using ClustalW [7, 8], alignment curation was done to remove positions with gaps [9], construction of the phylogenetic tree was done using PhyML [10], and visualization was done using TreeDyn. Glycosyl hydrolase predictions were obtained from http://www.cazy.org/geno/acc_geno.html. The graph of CAZy genes was generated from the whole genome sequence using DNASTAR® GenVision software (Fig. 1).

Results

Genome Sequencing Results

Whole genome sequencing of *D. turgidum* (http://genome.ornl.gov/microbial/dtur/) resulted in a completed assembly of 1.8 Mb in one contig (GenBank: NC_011661.1). The GC content of the genome was 33.9% GC. For comparison, whole genome sequencing of *D. thermophilum* resulted in a slightly larger completed assembly of 1.9 Mb in one contig (GenBank: NC_011297). The GC content of the *D. thermophilum* genome was similar at 33.0% GC. Annotation identified 1,744 candidate protein-encoding gene models for *D. turgidum*; *D. thermophilum* contains 1,912 identified candidates.

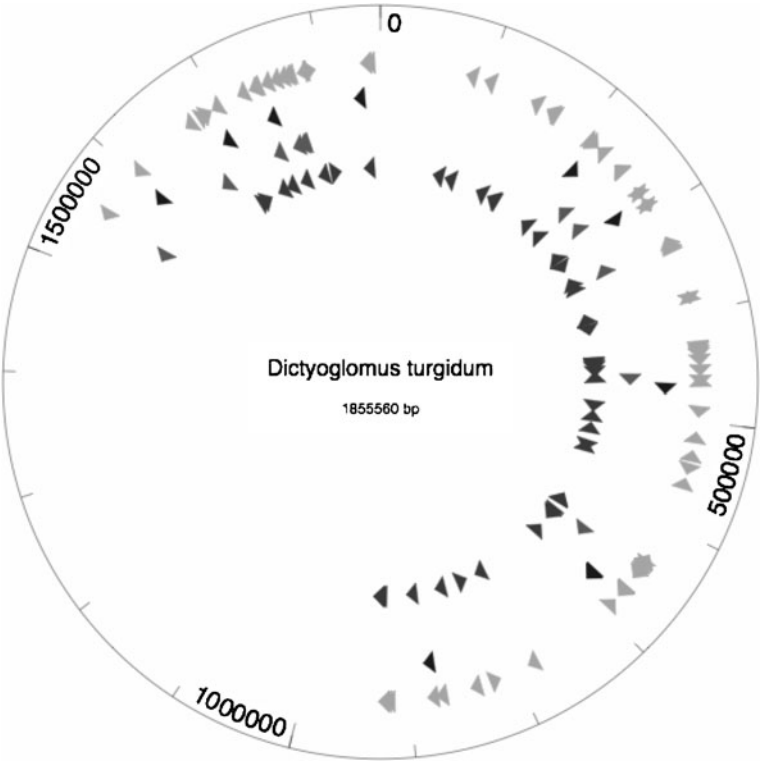

Fig. 1 CAZYme functional annotation of *Dictyoglomus turgidum*. *Arrows*, *from outmost circle to innermost circle*; (1) all annotated CAZymes, (2) cellulose-degrading enzymes, (3) xylan-degrading enzymes, (4) other carbohydrases

The CAZy database (http://www.cazy.org/Genomes.html) identifies 54 glycosyl hydrolases in *D. turgidum* genome. These 54 CAZyme genes represent 3.09% of its total genes. This percentage makes *D. turgidum* one of the top ten organisms (Table 1) when ranked by percentage of CAZymes genes in bacterial genome (Garret Suen, private communication). The genome contains only three predicted polysaccharide lyase genes and 14 predicted CBMs. None of the annotated cellulases and only one of the xylanases (Dtur_1715) is associated with CBMs.

The locations of all CAZy genes are shown in Fig. 2. The graph was generated using DNASTAR® GenVision software. CAZY genes are asymmetrically arranged in the genome, being totally absent in the 1.0- to 1.5-Mb region of the genome. A closer examination of this region of the genome shows an enrichment in DNA and protein synthesis genes and confirms the absence of CAZy genes. Cellulose-degrading and xylan-degrading genes do not appear to be clustered in separate operons. Genes for degradation of both cellulose and starch are found together in the potential operon shown in Fig. 3 and described further in Table 2.

Screening Results

Whole genome random shotgun expression screening of the Dtur library using chromogenic and fluorescent small molecule substrates resulted in the discovery of five enzymes (Table 2): a

Table 1 Top ten organisms by percentage of CAZymes in genome.

ID	CAZymes	Proteome size	Percent CAZy
Bacteroides thetaiotaomicron VPI-5482	164	4,816	3.41
Cellvibrio japonicus Ueda107	127	3,754	3.38
Fibrobacter succinogenes subsp. *succinogenes* S85	104	3,085	3.37
Saccharophagus degradans 2-40	126	4,007	3.14
Dictyoglomus turgidum DSM_6724	54	1,744	3.09
Catenulispora acidiphila DSM_44928	253	8,914	2.84
Paenibacillus JDR_2	173	6,213	2.78
Dictyoglomus thermophilum H 6 12	51	1,912	2.67
Clostridium thermocellum ATCC 27405	85	3,189	2.67
Clostridium phytofermentans ISDg	99	3,902	2.54
Clostridium cellulolyticum H10	85	3,390	2.51

Calculated percentage of genome as CAZymes for completely sequenced genomes circa March 2010 (courtesy of Garret Suen, University of Wisconsin, Madison).

cellulase, a xylanase, an *alpha*-galactosidase and two *beta*-galactosidases. Based on the electronic annotation of the genome and the substrates used for the plate screens, 22 of the 54 annotated CAZy genes could potentially have been identified by these plate screens. This is a significantly higher recovery of enzymes than with the same screens performed on a *Clostridium thermocellum* DNA library (unpublished data).

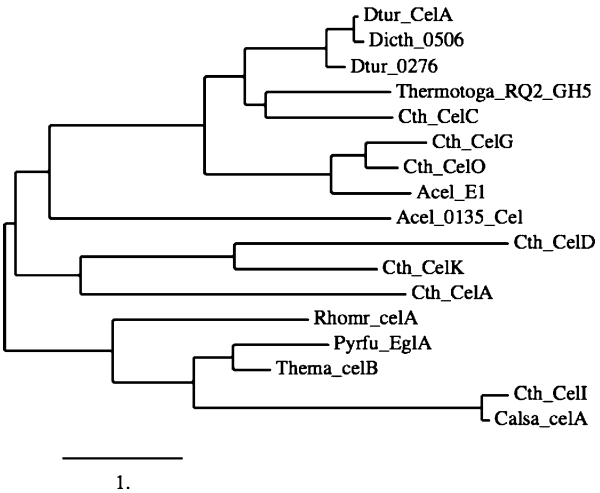

Fig. 2 Phylogenetic tree of Dtur_0670 (CelA) cellulase from *D. turgidum*. *Dtur_0276*, *D. turgidum* GeneID: 7083099; *DturCelA*, *D. turgidum* GeneID: 7083332; *Dicth_0506*, *D. thermophilum* GeneID: 6946331; *Cth_CelC*, *C. thermocellum* GeneID: 12584559; *Cth_CelG*, *C. thermocellum* GeneID: 462211; *Cth_CelO*, *C. thermocellum* GeneID: 7208816; *Cth_CelK*, *C. thermocellum* GeneID: 2978565; *Cth_CelD*, *C. thermocellum* GeneID: 40671; *Cth_CelA*, *C. thermocellum* GeneID: 144752; *Cth_CelI*, *C. thermocellum* GeneID: 7208809; *Thema_celB*, *T. maritima* GeneID: 1297062; *Thermotoga_RQ_GH5*, *Thermatoga* RQ2 Gene ID: 6092506; *Acel_E1*, *Acidothermus cellulolyticus* Gene ID: 1708075; *Acel_0135_Cel*, *Acidothermus cellulolyticus* Gene ID: 4485572; *Rhomr_celA*, *Rhodothermus marinus* Gene ID: 2304961; *Pyrfu_EglA*, *Pyrococcus furiosus* Gene ID: 5870829; *Calsa*, *Caldicellulosiruptor saccharolyticus* Gene ID: 1708078

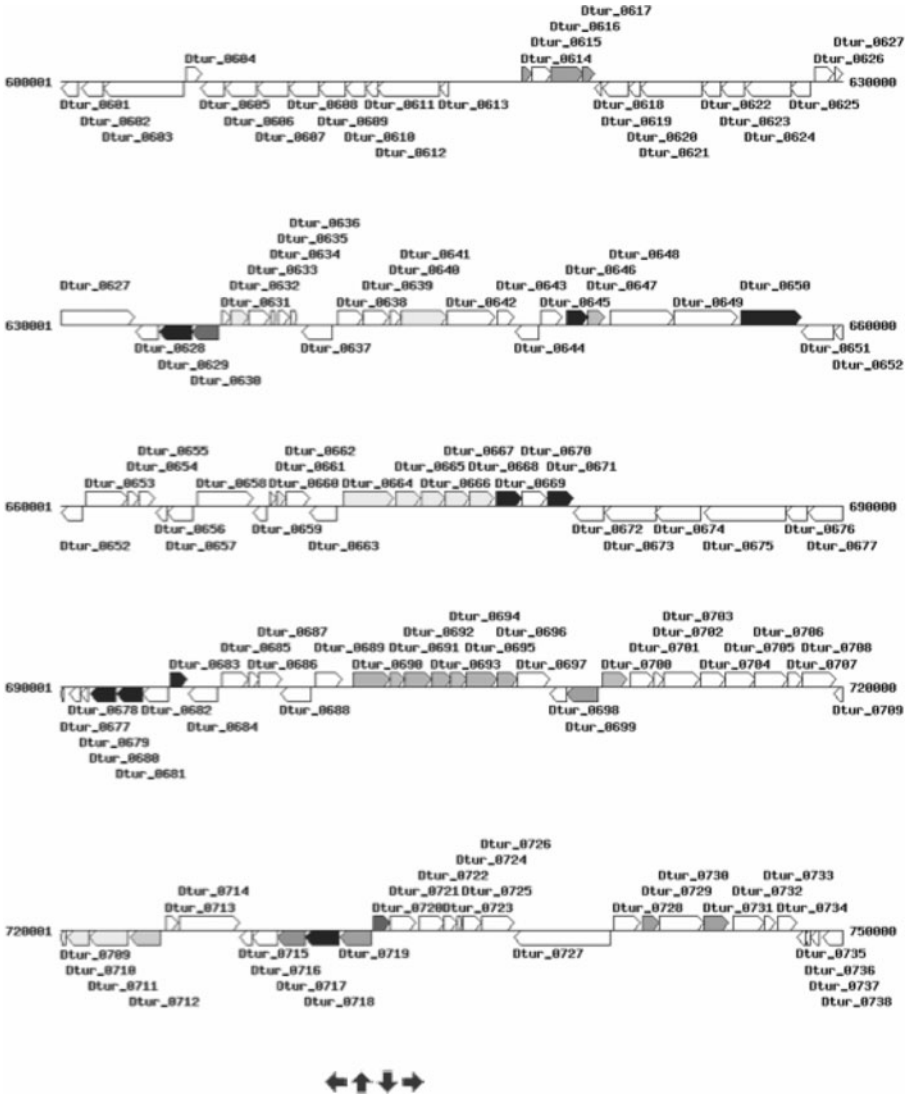

Fig. 3 Potential carbohydrate-degradation operon of *Dictyoglomus turgidum* (additional details in Table 2)

Three of the enzymes discovered in the screen (Table 2) are of particular interest because of the wide range of substrates utilized by them. The cellulase Dtur_0670 displays *endo*-glucanase activity on a wide range of *beta*-1,4-linked substrates including xyloglucan, *beta*-glucan, arabinoxylan, HE cellulose, and galactomannan. The cellulase also displays activity on a number of small, synthetic substrates, being active on MUG, MUC, and MUL. The xylanase Dtur_1647 0670 displays *endo*-glucanase activity on both *beta*-1,4-linked pentose substrates such as xylan, arabinoxylan, and linear arabinan and *beta*-1,4-linked hexose substrates such as *beta*-glucan and HE cellulose. The xylanase also displays activity on a number of small, synthetic substrates, being active on MUG, MUC, MUX, and MUL. The *beta*-galactosidase Dtur_1799 displays *exo*-glucanase activity on a wide

Table 2 Details of genes present in the potential carbohydrate-degradation operon from *Dictyoglomus turgidum.*

Gene	Assignment
0663	Transcriptional regulator, LacI family
0664	Extracellular solute-binding protein family 5
0665	ABC transporter
0666	ABC transporter
0667	ABC transporter
0668	ABC transporter
0669	Cellulase
0670	Glycoside hydrolase family 5
0671	Glycoside hydrolase family 5
0672	Hypothetical protein
0673	ABC transporter related
0674	ABC transporter related
0675	Glycoside hydrolase family 57
0676	Pullulanase
0677	Hypothetical protein-ABC transporter
0678	Protein of unknown function
0679	Transcriptional regulator, XRE family
0680	*Endo*-glucanase
0681	*Endo*-glucanase

range of substrates, being active on MUG, MUC, MUX, and MUL along with X-gal (Table 3).

The sequences of the enzymes found in the screens show little homology to enzymes outside the genus *Dictyoglomi*. For example, outside the genus *Dictoglomi*, Dtur_0670 (CelA) is most homologous to an electronically annotated family 5 glycoside hydrolase of *Thermotoga maritima* MSB8; the homology is low, with 52% amino acid identity and 68%

Table 3 Enzymes obtained by random shotgun expression screening of *Dictyoglomus turgidum genomic* DNA.

Gene detected	Predicted gene product	CAZy	Activity screen	Other enzymatic activities
Dtur_0505	*Beta*-galactosidase	GH42	XG	MUG
Dtur_0670	Glycoside hydrolase family 5	GH5	MUC	BG, HEC, AX, XYG, GM, MUG, MUL
Dtur_1647	Xylanase	GH10	MUC	AX, AR, BG, HEC, MUG, MUL, MUX
Dtur_1670	*Alpha*-galactosidase	GH36	XAG	MUC
Dtur_1799	*Beta*-galactosidase	GH1	XG	MUC, MUG, MUL, MUX

AR AZCL-arabinan, *AX* AZCL-arabinoxylan, *BG* AZCL-beta-glucan, *GM* AZCL-galactomannan, *HEC* AZCL-HE cellulose, *MUC* methylumbelliferyl-β-D-cellobioside, *MUG* 4-methylumbelliferyl-β-D-glucopyranoside, *MUL* 4-methylumbelliferyl-β-D-lactopyranoside, *MUX* 4-methylumbelliferyl-β-D-xylopyranoside, *XAG* 5-bromo-4-chloro-3-indolyl α-D-galactopyranoside, *XG* 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside, *XYG* AZCL-xyloglucan

amino acid similarity over 312 amino acids. This low homology is seen in the phyogenetic tree constructed from thermophilic cellulase genes (Fig. 1).

Gene Amplification Results

An additional 18 CAZy genes (Table 4) were selected for amplification and expression analysis. Of the 18, three potential pectinases genes (Dtur_0430, Dtur_0431, and Dtur_0435) could not be amplified from genomic DNA. Of the remaining 15 genes, seven gave clones that expressed the activity predicted by electronic annotation and gave proteins of the expected molecular weight on SDS gel electrophoresis. These seven genes expressed two cellulases, a xylanase, an *alpha*-amylase, an *alpha*-glucuronidase, a *beta*-xylosidase, and a *beta*-glucosidase. Lysates of cultures containing the remaining eight genes showed no expression of proteins of the expected molecular weight and no activity when tested against the predicted substrates. The reason(s) for the failure of genes to amplify or express is unknown, but the 67% AT-rich nature of the Dtu genome could be a contributing factor. Misfolding of the proteins or toxicity of the gene products may also contribute to the lack of apparent expression of these genes. Detailed characterization of the seven active proteins is underway.

Table 4 Gene products amplified from *Dictyoglomus turgidum* genomic DNA for expression analysis.

Gene	Predicted gene product	CAZy	Substrate screened	Expression
Dtur_0276	Cellulase	GH5	HEC	Activity
Dtur_0462	*Beta*-glucosidase	GH1	MUG	Activity
Dtur_0671	GH family 5	GH5	HEC	Activity
Dtur_0675	*Alpha*-amylase	GH57	PUL	Activity
Dtur_0852	Xylosidase/arabinfuranosidase	GH3	MUX	Activity
Dtur_1714	*Alpha*-glucuronidase	GH67		Activity
Dtur_1715	*Beta*-xylanase	CBM9	AX	Activity
		CBM22		
		GH10		
Dtur_0430	Pectinase	PL1	RH	No PCR product
Dtur_0431	Pectinase	PL1	RH	No PCR product
Dtur_0435	Pectinase	PL1	RH	No PCR product
Dtur_0451	1,3 *beta*-glucanase	CBM4	CU	No activity
		GH16		
Dtur_0669	GH family 5	GH5	HEC, AX	No activity
Dtur_0857	Arabinogalactanase	GH53	GL	No activity
Dtur_1586	GH family 5	GH5	HEC, AX	No activity
Dtur_1729	GH family 43	GH43	MUX, MUA	No activity
Dtur_1735	Arabinfuranosidase	GH51	MUA	No activity
Dtur_1739	Arabinfuranosidase	GH51	MUA	No activity
Dtur_1758	*Alpha*-mannosidase	GH38	GM, MUM	No activity

AR AZCL-arabinan, *AX* AZCL-arabinoxylan, *BG* AZCL-*beta*-glucan, *CU* AZCL-curdlan, *GM* AZCL-galactomannan, *GL* AZCL-galactan (potato), *HEC* AZCL-HE cellulose, *MUC* methylumbelliferyl-β-D-cellobioside, *MUG* 4-methylumbelliferyl-β-D-glucopyranoside, *MUL* 4-methylumbelliferyl-β-D-lactopyranoside, *MUM 4-methylumbelliferyl-β-D-mannopyranoside, MUX 4-methylumbelliferyl-β-D-xylopyranoside, PUL AZCL-pullulan, RH AZCL-rhamnogalacturonan, XAG 5-bromo-4-chloro-3-indolyl α-D-galactopyranoside, XG 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside, XYG AZCL-xyloglucan*

Conclusions

The *D. turgidum* genome at 1.8 Mb is small compared with the genome size of many other cellulolytic organisms, yet it contains one of the highest percentages (3.09%) of CAZymes. Analysis of the genome shows the presence of a number of potential carbohydrate-degradation operons, including one containing at least 19 proteins. Phylogenetic analysis of the CAZymes shows little homology to other proteins outside the genus *Dictyoglomi.* A number of the *D. turgidum* CAZymes appear to be unique to *D. turgidum* and absent from *D. thermophilum*, possibly explaining the observed inability of *D. thermophilum* to grow on cellulose.

The reported ability of the organism to grow on cellulose is difficult to reconcile with the absence of cellulases containing family 2 or family 3 CBMs. The composition (Table 5) of key CBM family members of *D. turgidum* shows no similarity to the soluble enzyme system of *Acidothermus cellulyticus*, which possesses ten CBM 2 modules and nine CBM 3 modules. The composition of *D. turgidum* CBM family members also shows no similarity to the cellulosomal system of *C. thermocellum*, which possesses no CBM 2 members, but 23 CBM 3 members. The GH and CBM composition of *D. turgidum* appears to be most similar to that of two other unusual gram-negative cellulolytic organisms, *Fibrobacter succinogenes* and *Cytophaga hutchinsonii*, two well-documented, prolific cellulose degraders. All three organisms are able to consume and grow on cellulose without any family 2 or family 3 CBMs. A number of the cellulases of *F. succinogenes* have been cloned and characterized [11]; unexpectedly, these enzymes show poor performance on

Table 5 Comparison of *Dictyoglomus turgidum* GH and CBM family members with those of other cellulolytic organisms.

Family	*Dictyoglomus turgidum*	*Clostridium thermocellum*	*Acidothermus cellulyticus*	*Fibrobacter succinogenes*	*Cytophaga hutchinsonii*
GH 1	1	2	1	0	1
GH 2	3	1	0	2	0
GH 3	5	2	3	3	6
GH 5	5	11	2	16	0
GH 6	0	0	2	0	0
GH 8	0	1	0	6	6
GH 9	0	16	2	9	7
GH 10	2	6	2	9	3
GH 11	1	1	0	4	1
GH 16	1	2	0	4	2
GH 43	1	6	0	14	2
GH 45	0	0	0	4	0
GH 51	2	1	0	2	0
CBM 2	0	0	10	0[a]	0
CBM 3	0	23	9	0	0
CBM 4	3	7	0	4	2
CBM 6	0	11	2	26	2
CBM 9	4	2	0	0	10

Data from CAZy Database, http://www.cazy.org/Genomes.html.

[a] One CBM 2 is listed. The corresponding protein sequence, GenBank AAC06197.1, does not have BLAST analysis to any protein in the *Fibrobacter* genome, suggesting a mis-identification of the protein's source.

insoluble cellulose substrates and cannot explain the rapid degradation of cellulose by this organism. One explanation for these apparently contradictory observations is that these three organisms possess cellulases and carbohydrate-binding proteins with structures sufficiently dissimilar to those reported for other organisms to not be recognized by homology-seeking software. Alternately, these three organisms may degrade cellulose by an alternate mechanism that utilizes supramolecular structures embedded in the outer membranes to depolymerize and degrade cellulose. Since these structures may involve active sites formed at interfaces of multiple proteins, the individual proteins may not be recognized as involved in cellulose binding or degradation.

Twelve novel *D. turgidum* carbohydrases were obtained by either screening of a random shotgun library or by direct amplification from genomic DNA. Expression, purification, and characterization of these enzymes reveal that they may have a significant potential in high-temperature biomass degradation processes. Preliminary results indicate that the cellulases and xylanases have broader than normal substrate specificities, making them potentially better at degrading natural biomass.

Acknowledgements This work was funded in part by DOE grant DOE DE-FG36-06GO16106, "Novel enzyme products for the conversion of defatted soybean meal to ethanol," and funded in part by the DOE Great Lakes Bioenergy Research Center (DOE Office of Science BER DE-FC02-07ER64494). The work conducted by the U.S. Department of Energy Joint Genome Institute is supported by the Office of Science of the U.S. Department of Energy under Contract No. DE-AC02-05CH11231.

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