

Lawrence Berkeley National Laboratory

Biological Systems & Engineering

Title

Targeted Discovery of Glycoside Hydrolases from a Switchgrass-Adapted Compost Community

Permalink

<https://escholarship.org/uc/item/0f34r5px>

Journal

PLoS one, 5, 1, 1-9, January 2010

Author

Reddy, Amitha

Publication Date

2010-01-21

Targeted Discovery of Glycoside Hydrolases from a Switchgrass-Adapted Compost Community

Martin Allgaier^{1,3}, Amitha Reddy^{1,2}, Joshua I. Park^{1,6}, Natalia Ivanova³, Patrik D'haeseleer^{1,4}, Steve Lowry³, Rajat Sapra^{1,6}, Terry C. Hazen^{1,5}, Blake A. Simmons^{1,6}, Jean S. VanderGheynst^{1,2}, Philip Hugenholtz^{1,3}*

¹ Deconstruction Division, Joint BioEnergy Institute, Emeryville, California, United States of America, ² Department of Biological and Agricultural Engineering, University of California Davis, Davis, California, United States of America, ³ Department of Energy (DOE) Joint Genome Institute, Walnut Creek, California, United States of America, ⁴ Microbial Systems Biology Group, Lawrence Livermore National Laboratory, Livermore, California, United States of America, ⁵ Earth Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, California, United States of America, ⁶ Biomass Science and Conversion Technology Department, Sandia National Laboratories, Livermore, California, United States of America

Abstract

Development of cellulosic biofuels from non-food crops is currently an area of intense research interest. Tailoring depolymerizing enzymes to particular feedstocks and pretreatment conditions is one promising avenue of research in this area. Here we added a green-waste compost inoculum to switchgrass (*Panicum virgatum*) and simulated thermophilic composting in a bioreactor to select for a switchgrass-adapted community and to facilitate targeted discovery of glycoside hydrolases. Small-subunit (SSU) rRNA-based community profiles revealed that the microbial community changed dramatically between the initial and switchgrass-adapted compost (SAC) with some bacterial populations being enriched over 20-fold. We obtained 225 Mbp of 454-titanium pyrosequence data from the SAC community and conservatively identified 800 genes encoding glycoside hydrolase domains that were biased toward depolymerizing grass cell wall components. Of these, 10% were putative cellulases mostly belonging to families GH5 and GH9. We synthesized two SAC GH9 genes with codon optimization for heterologous expression in *Escherichia coli* and observed activity for one on carboxymethyl cellulose. The active GH9 enzyme has a temperature optimum of 50°C and pH range of 5.5 to 8 consistent with the composting conditions applied. We demonstrate that microbial communities adapt to switchgrass decomposition using simulated composting condition and that full-length genes can be identified from complex metagenomic sequence data, synthesized and expressed resulting in active enzyme.

Citation: Allgaier M, Reddy A, Park JI, Ivanova N, D'haeseleer P, et al. (2010) Targeted Discovery of Glycoside Hydrolases from a Switchgrass-Adapted Compost Community. PLoS ONE 5(1): e8812. doi:10.1371/journal.pone.0008812

Editor: Francisco Rodriguez-Valera, Universidad Miguel Hernandez, Spain

Received November 6, 2009; Accepted December 24, 2009; Published January 21, 2010

This is an open-access article distributed under the terms of the Creative Commons Public Domain declaration which stipulates that, once placed in the public domain, this work may be freely reproduced, distributed, transmitted, modified, built upon, or otherwise used by anyone for any lawful purpose.

Funding: This work was part of the DOE Joint BioEnergy Institute (<http://www.jbei.org>) supported by the U. S. Department of Energy, Office of Science, Office of Biological and Environmental Research, through contract DE-AC02-05CH11231 between Lawrence Berkeley National Laboratory and the U. S. Department of Energy. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: phughholtz@lbl.gov

Introduction

Enzymatic hydrolysis is one of the most expensive steps in biofuel production from lignocellulosic biomass primarily due to the need for high enzyme loading caused by low catalytic efficiencies [1,2]. Microorganisms including bacteria and fungi are well-known plant biomass decomposers in nature making them attractive targets for enzyme discovery. Since a variety of biomass sources are envisioned for future biofuel production (e.g. switchgrass, miscanthus, poplar), a broad spectrum of lignocellulolytic enzymes (cellulases, hemicellulases, ligninases) is required to meet future demands. These enzymes are highly modular and usually classified by their domain structure [3]. Glycoside hydrolases (GHs) are a prominent group of enzymes that hydrolyze the glycosidic bond between carbohydrate molecules. The GH families 5, 7 and 9 are the most diverse of the 115 currently recognized GH families, and are of great interest for industrial applications due to their plant cell wall depolymerizing activities [4]. Despite extensive efforts to engineer existing glycoside hydrolases to improve activity and stability, there is still a great need

to expand the current enzyme repertoire as well as improve our understanding of how these enzymes function in complex environments [5].

In the present study, we incubated compost-inoculated switchgrass under high-solids and thermophilic conditions to facilitate the enrichment of switchgrass-adapted organisms and associated lignocellulolytic enzymes using a sequencing-based metagenomic approach. Composting is a very dynamic high-solids decomposition process in which microorganisms break down organic matter into carbon dioxide, water, and stable humus-like materials throughout mesophilic and thermophilic phases [6]. Therefore, compost microbial communities can tolerate large changes in temperature, redox conditions, and water activity, recovering quickly from major environmental perturbations. This adaptation to extremes in operating conditions suggests the potential for discovering robust lignocellulolytic enzymes that will also tolerate harsh pretreatment approaches under industry-relevant production standards (e.g. dilute acid, ionic liquid, ammonia fiber expansion).

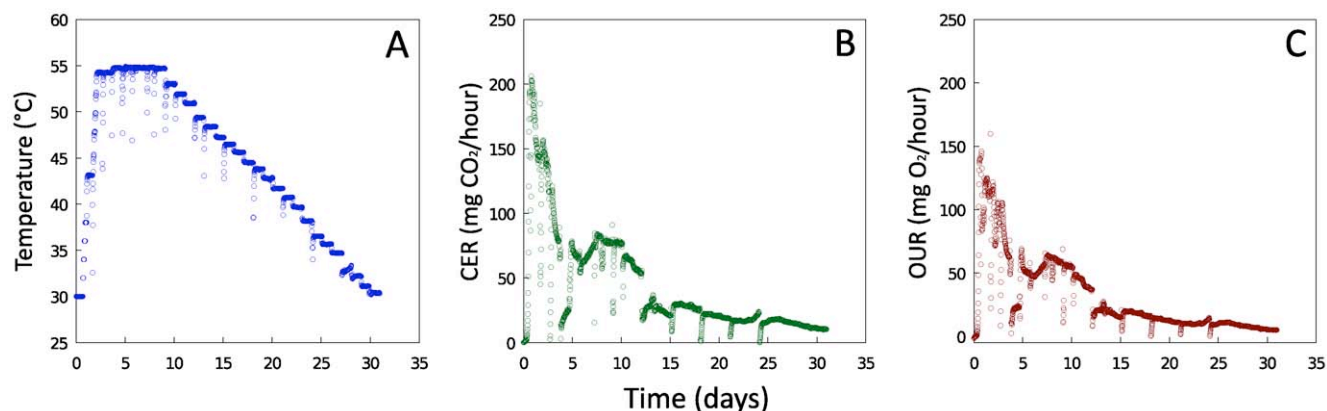


Figure 1. Temperature and respiration profiles during switchgrass incubation. A, temperature; B, carbon dioxide evolution rates (CER); C, oxygen uptake rates (OUR). Brief drops in temperature, CER and OUR levels every three days indicate mixing and water addition.
doi:10.1371/journal.pone.0008812.g001

Results

The bioreactors were established using a switchgrass feedstock inoculated with green-waste compost at a ratio of 9:1. During a 31-day incubation period, temperature was controlled to simulate a typical composting process: the temperature was maintained at 30uC for 14 hours to allow compost microorganisms to establish, then the temperature was increased from 30uC to 54uC over the course of two days to simulate the self-heating phase, maintained at 54uC for 7 days to simulate the thermophilic phase and slowly decreased back to 30uC over the course of the remaining 21 days to simulate the cooling and maturation phase (Figure 1A). Carbon dioxide evolution (CER) and oxygen uptake rate (OUR) were calculated from continuous measurements of carbon dioxide and oxygen. Both CER and OUR peaked after one day of composting corresponding to initial consumption of sugars [7] and again after eight days during the thermophilic phase (Figure 1B & 1C). This second peak in respiration corresponds to the increased activity of thermophiles [8]. Respiration rates decreased by the end of the incubation, but microorganisms were still active indicating that substrate degradation continued. Periodic mixing and water addition resulted in drops in respiration, followed by rapid recovery (Figure 1B & 1C).

The initial and final samples were analyzed for substrate composition. Total solids decreased 34% during the 31-day incubation. Furthermore, total lignin decreased 17% and sugars

associated with hemicellulose and cellulose decreased 28% on average, with the exception of mannose, which was present in low levels in the initial sample and was not detected in the final sample (Table 1).

Microbial Community Composition and Dynamics

Microbial community structure was determined for the initial (day 0) and final (day 31) bioreactor sample and for a sample of the compost inoculum using small-subunit (SSU) rRNA gene amplicon pyrosequencing. The day 0 sample had a similar microbial community structure to the inoculum (Bray-Curtis dissimilarity 0.501), with the exception that the day 0 community was dominated by switchgrass sequences (69.7% nuclear and 8.2% chloroplast). This suggests that the indigenous microbiota on the switchgrass contributed negligibly to the microbial biomass in the day 0 system. By day 31, the microbial community profile had no discernable correlation to the day 0 profile (Bray-Curtis dissimilarity 0.854) suggesting adaptation of the compost community to the switchgrass feedstock. Also, switchgrass sequences were drastically reduced in the day 31 sample (0.2% nuclear and chloroplast) suggesting that at least degradation of the switchgrass DNA had occurred. Figure 2 shows the rank abundance of the day 0 sample phylotypes overlaid with the day 31 phylotypes. Numerous phylotypes (labeled in Figure 2) increased over the 31-day incubation with up to 23-fold enrichments in relative abundance, including taxa that were below the detection threshold

Table 1. Substrate analysis of initial and final sample.

	Initial (g/kg switchgrass + compost)	Final (g/kg switchgrass + compost) ^a	Loss after incubation (%)
Acid-soluble lignin	64	23	64
Acid-insoluble lignin 250 237 5			
Total lignin	314	260	17
Glucose 260 191 27			
Xylose	170	118	31
Galactose 18 12 33			
Arabinose	23	16	30
Mannose	0.4	0	100

^aFinal composition is presented assuming there was no depletion of ash during incubation. Initial and final ash contents were 11.5% and 17.5% of the total dry solids, respectively.

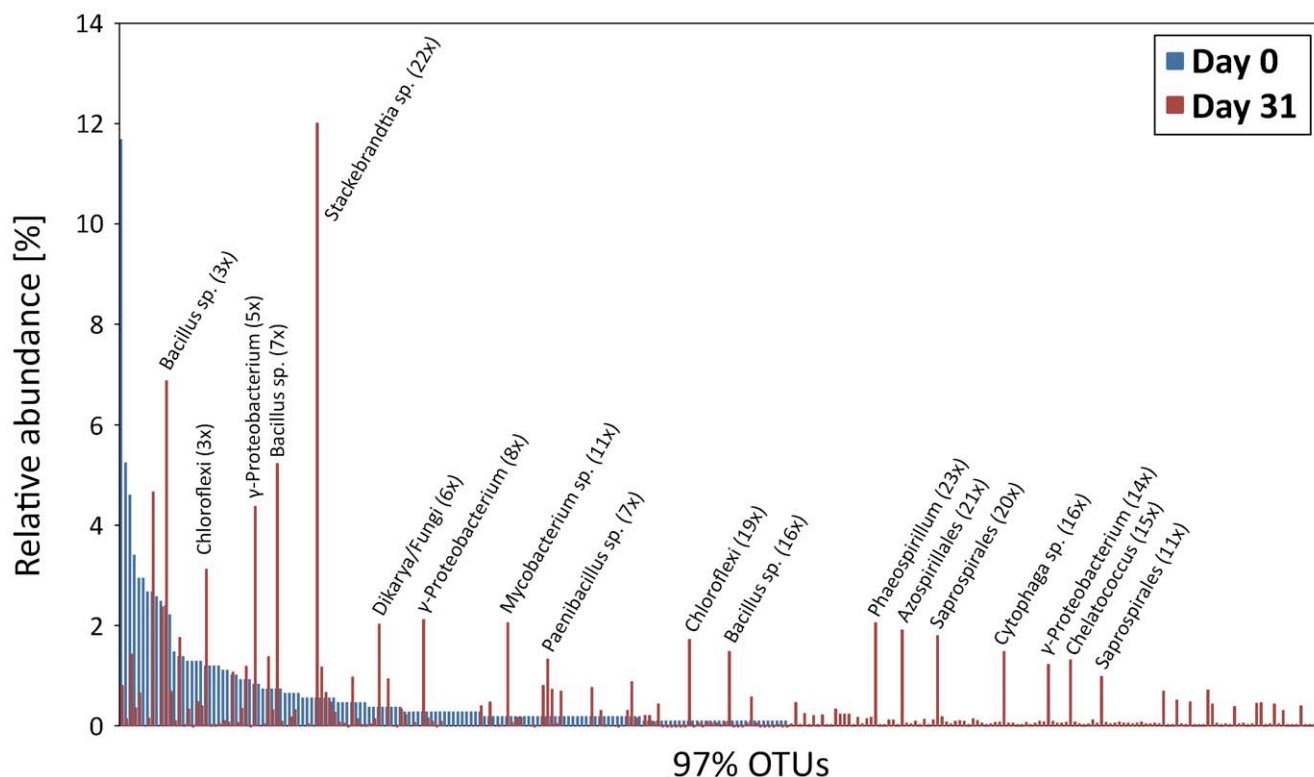


Figure 2. Rank abundance profiles of SSU rRNA phylotypes identified in the initial (day 0) and final (day 31) samples of the compost bioreactor. Overlapping bars indicate the same taxa. doi:10.1371/journal.pone.0008812.g002

(0.09% populations) in the initial sample (Figure 2). The dominant phylotype in the day 31 sample is closely related to the actinobacterial genus, *Stackebrandtia*, a member of which, *S. nassauensis* (acc. no. NZ_ABUS000000000) contains genes encoding cellulases and hemicellulases (Table 2).

Metagenome Analysis

To investigate the diversity of genes encoding glycoside hydrolases in the switchgrass-adapted compost (SAC) community, we shotgun sequenced DNA extracted from the day 31 sample using 454-titanium technology. Metagenome sequencing resulted in 548,733 reads with an average read length of 4326108 bp totaling 225 Mbp of sequence data. A considerable proportion of the reads could be assembled into contigs 1 kb (a total of 8,268 contigs) with the largest contig of 49,537 bp. This contig is circular (and therefore complete) and encodes 84 putative genes, of which 27% have a highest match to, and shared gene order with, the genome of a novel circular virus, Iodobacteriophage (NCBI acc. no. NC_011142), including capsid, baseplate and tail fiber proteins (Figure S1). Interestingly, this virus also encodes a putative family 43 glycoside hydrolase with 43% similarity to an arabinosidase from the fungus *Armillariella mellea*. To compare the global functional content of the SAC community metagenome to other lignocellulosic habitats and to non-cellulosic habitats, we performed a correspondence analysis using SEED [9] annotation. The SAC metagenome did not cluster with other lignocellulosic systems (Figure S2), and indeed the SAC community was most closely related to non-lignocellulosic systems including a hypersaline mat, whalefall and a phosphorus-removing bioreactor community. This suggests that genes involved in or associated with lignocellulose metabolism do not contribute

enough functional signal in this type of global analysis to cluster lignocellulosic systems together.

Lignocellulosic enzymes were identified by pfam HMMs and grouped according to major functional role (Table 2). Like other communities adapted to lignocellulose degradation, the SAC community had .05% of its genes involved in cellulose and hemicellulose deconstruction. Of these genes, 10.6% were putative cellulases, mainly belonging to glycoside hydrolase families GH5 and GH9. This relative abundance of cellulases in the SAC community was .5-fold higher than in cow rumen but only half of that in a termite hindgut community. Similar to the cow rumen, a high proportion of carbohydrate-active enzymes found in the SAC community are involved in hemicellulose degradation, particularly in side chain processing (debranching and cell wall elongation enzymes), that may reflect the common substrate type – grass – degraded in these ecosystems. Consistent with this inference is the enrichment of GH families likely involved in depolymerization of the major grass hemicellulose, glucuronarabinoxylan [10], in the SAC community. This includes putative α-arabinofuranosidases (GH51 and 62) that cleave off arabinose side chains, α-glucuronidases (GH67) that remove glucuronic acid side chains and xylanases (GH10 and 11) that would break down the xylan backbone of glucuronarabinoxylan (Table 2). Additionally, putative α-rhamnosidases (GH78) were enriched in the SAC and rumen communities relative to the drywood-eating termite hindgut microbiome, although the main rhamnose-containing heteropolysaccharide, pectin, is more prevalent in dicot cell walls than grass cell walls [10]. Oligosaccharide processing enzymes reflect to some degree the inferred polymer breakdown. For example, putative β-xylosidases or β-arabinosidases (GH43) that are involved in breakdown of glucuronarabinoxylan oligosaccharides are enriched in the SAC community (Table 2).

Table 2. Inventory of putative glycoside hydrolases (GHs) identified in the SAC microbiome.

CAZy family	known activity	pfam domain	SAC	Cow rumen*	Termite hindgut ^{†181}	<i>Stackebrandtia nassauensis</i> **
Cellulases						
GH5 cellulase PF00150 3.2 1.0 16.3						
GH6	endoglucanase	PF01341	2.1	0	0	5.7
GH7 endoglucanase PF00840 0.1 0 0						
GH9	endoglucanase	PF00759	4.3	0.9	3.9	0
GH44 endoglucanase NA 0.4 0 1.0						
GH45	endoglucanase	PF02015	0	0	0.6	0
GH48 endo-processive cellulases PF02011 0.5 0 0 0						
Total			10.6	1.9	21.8	11.4
Endohemicellulases						
GH8	endo-xylanases	PF02011	0.5	0.6	2.7	0
GH10 endo-1,4-b-xylanase PF00331 8.9 1.0 12.1 0						
GH11	xylanase	PF00457	1.4	0.1	2.5	0
GH12 endoglucanase & xyloglucan hydrolysis PF01670 0.6 0 0 0						
GH26	β -mannanase & xylanase	PF02156	1.5	0.7	2.8	2.9
GH28 galacturonases PF00295 0.9 0.7 1.7 0						
GH53	endo-1,4- β -galactanase	PF07745	0.2	2.5	2.7	0
Total						
			14	5.6	24.5	2.9
Cell wall elongation						
GH16 xyloglucanases & xyloglycosyltransferases PF00722 2.0 0 0.3 11.4						
GH17	1,3- β -glucosidases	PF00332	0.1	0	0	0
GH74 endoglucanases & xyloglucanases NA 1.6 0 1.0 0						
GH81	1,3- β -glucanase	PF03639	0.3	0	0	0
Total						
			4	0.3	11.4	
Debranching enzymes						
GH51 a-L-arabinofuranosidase NA 7.8 9.9 3.5 1						
GH54	α -L-arabinofuranosidase	PF09206	0	0.2	0	0
GH62 a-L-arabinofuranosidase PF03664 1.7 0 0 0						
GH67	α -glucuronidase	PF07477, PF07488	3.6	0	2.3	0
GH78 a-L-rhamnosidase PF05592 8.1 5.1 0.9 2.9						
Total			21.2	15.2	6.7	2.9
Oligosaccharide-degrading enzymes						
GH1	β -glucosidase and many other β -linked dimers	PF00232	9.2	1.5	3.1	5.7
GH2 b-galactosidases and other b-linked dimers PF02836, PF00703, PF02837 8.6 28.6 8.8 2.9						
GH3	mainly β -glucosidases	PF00933	12.2	27.0	12.8	17.1
GH29 a-L-fucosidase PF01120 2.1 4.2 0 0						
GH35	β -galactosidase	PF01301	0.6	1.8	0.7	0
GH38 a-mannosidase PF01074, PF07748 2.6 2.6 5.6 5.7						
GH39	β -xylosidase	PF01229	1.0	0.3	1.3	0
GH42 b-galactosidase PF02449, PF08533, PF08532 2.5 1.7 4.8						
GH43	arabinases & xylosidases	PF04616	11.3	9.4	8.3	17.1
GH52 b-xylosidase PF03512 0 0 0.4						
Total			50.1	77.1	45.8	71.4
Total GHs 801 651 653 35						
%GHs in total ORFs			0.72	0.78	0.78	0.54

GHs are grouped according to major functional role and compared to other lignocellulosic systems. Both partial and full-length sequences are included. The indicated values are percentages weighted according to species abundance distribution, meaning that the contribution of dominant populations is larger than rare populations. *indicated numbers are average values of the four cow rumen metagenome data sets published in [19]. ***Stackebrandtia nassauensis* LLR-40K-21, DSM 44728 (GenBank acc. no. NZ_ABUS000000000). NA: no pfam domains available for these GH families. doi:10.1371/journal.pone.0008812.t002

Complete genes are desirable for enzyme characterization but difficult to obtain from highly fragmented metagenomic datasets [11], such as the SAC metagenome. After frame-shift corrections (an artifact of 454-titanium data), we identified 25 candidate enzymes with a significant match to characterized cellulases or hemicellulases in the CAZy database [3], Table S1). These include two divergent GH9 representatives (36% amino acid similarity to each other) that are most closely related to members of the Alphaproteobacteria (gene JMC20181_1; 68% similarity) and Actinobacteria (gene JMC00312_1; 84% similarity) (Figure S3).

Cellulase Characterization

The two full-length GH9 catalytic domains were synthesized (GenScript, Piscataway, NJ) and codon-optimized for expression in *E. coli*. Protein expression was detected for both enzymes with much lower amounts of JMC00312_1 being produced. Soluble extracts of both enzymes were used to test for enzymatic activity on carboxymethyl-cellulose (CMC), 4-nitrophenyl-b-D-cellobioside (pNPC) and 4-nitrophenyl-b-D-glucopyranoside (pNPG). Activity on CMC was only detected for JMC00312_1 despite its low expression level and neither enzyme was active on pNPC or pNPG (Table S2) suggesting that JMC00312_1 is an endoglucanase lacking cellobiosidase or b-glucosidase activity. Furthermore, we suspect that JMC00312_1 is truncated at the C-terminal because its closest homolog (76% similarity) is a much longer actinobacterial endo-/exocellulase (ZP_04475820.1) comprising a conserved domain arrangement; GH9-CBM2-fn3-CBM3. These additional domains would likely enhance endoglucanase activity of the enzyme or may confer endo-/exoglucanase activity to the compost-derived enzyme. Soluble extracts of *E. coli* containing over-expressed enzyme were used to determine temperature and pH profiles of the JMC00312_1 cellulase. The enzyme had an activity optimum of 50uC and pH 7, but retained .50% of its optimum activity over a range of temperatures (30 to 55uC) and pH (5.5 to 8) (Figure 3).

Discussion

The current dependency on fossil fuels for transportation has put remarkable focus on sources of alternative renewable liquid transportation fuels in recent years [12,13,14]. Much of the current research in this area is focusing on so-called second-generation biofuels made from cellulosic biomass of non-food crops. Switchgrass is one of the leading feedstock candidates (others are miscanthus and sorghum) for biofuel production [15,16,17]. The goal of this study was to establish a switchgrass-

adapted compost (SAC) community using simulated composting conditions in order to select for enzymes capable of degrading switchgrass lignocellulose. We chose to use random shotgun sequencing (metagenomics) for enzyme discovery, an approach that has been successfully used to mine other lignocellulosic ecosystems for plant cell wall depolymerizing enzymes (e.g. termite hindgut [18], cow rumen [19]).

Over the course of a 31-day composting experiment, measurements of reduction in solids, sugar content (Table 1) and O₂ uptake and CO₂ evolution rates (Figure 1) indicated active degradation of the switchgrass biomass. In a study examining the decomposition of *Miscanthus* straw, 10–20% degradation of both cellulose and hemicellulose was observed during the first three months of composting [20]. During composting of ryegrass straw, lignin loss measured using the Klason method was 15% over a 30day period [21]. Comparable decomposition levels for lignin (17% loss in total lignin) and cellulose and hemicellulose (28%) in this study (Table 1) indicate that the bioreactor management approach sufficiently simulated an environment that might be encountered in a straw-based composting process. Switchgrass can be effectively degraded by microorganisms as shown in a previous study investigating deconstruction of the leaf blade, leaf sheath and stem of this species by cow rumen communities [22]. Results from microcosm studies in which stems and leaves from switchgrass (Sunburst) were separated, incorporated into soil and incubated at 25uC for 498 days also demonstrated that leaves and stems will decompose in a high-solids soil environment [23]. Microbial community composition changed dramatically between the initial and final bioreactor sample (Figure 2) suggesting selection of specific populations to degrade the switchgrass biomass. Composting is a highly dynamic process selecting for different species during the various composting stages [6,24,25]. Mesophilic bacteria and fungi dominate the initial composting microbial communities utilizing the soluble and easily metabolized carbohydrates from the fresh organic substrates [7]. During the subsequent thermophilic phase, Actinobacteria feeding on recalcitrant plant cell wall components dominate communities [26,27]. Consistent with these generalized compost observations, we noted an increase in Actinobacteria from 16% to 23% between the initial and switchgrass-adapted communities. Moreover, the dominant population in the SAC community, enriched 22-fold from the initial sample (Figure 2), was an actinobacterium related to the genus *Stackebrandtia*. From only two time points, we cannot tell when the *Stackebrandtia*-like population became enriched, i.e. during the thermophilic or cooling and maturation phases. Adaptation of the compost microbial community to the switchgrass biomass is reflected in the number of glycoside hydrolases identified in the metagenomic dataset which account for .05% of all genes called (Table 2). This is characteristic of ecosystems that have evolved to degrade lignocellulosic substrates [18,19]. Unlike dicots, the major hemicellulose in grass cell walls is glucuronarabinoxylan composed of a b-1,4-linked xylose backbone with single arabinose and glucuronic acid side chains [10]. We identified a high proportion of genes encoding enzymes that are likely to degrade this type of hemicellulose including debranching GH families specific to the arabinose and glucuronic acid side chains (GH51, 62, 67). These same families are present in much lower proportions in the drywood-feeding termite hindgut (Table 2). Conversely, the termite hindgut microbiome has a higher proportion of cellulases than the SAC and rumen communities possibly reflecting the typically higher cellulose content in dicots than grasses [10]. Of the putative cellulases identified in the SAC community, the highest proportion belongs to glycoside hydrolase family 9.

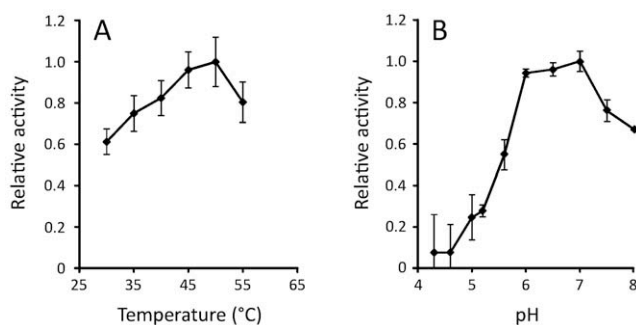


Figure 3. Temperature and pH profiles of the GH9 cellulase JMC00312_1. Soluble extracts from *E. coli* were used to determine temperature (A) and pH (B) profiles of the heterologous expressed enzyme.
doi:10.1371/journal.pone.0008812.g003

Samples for DNA extraction were stored at 280uC. Before extraction, samples were homogenized repeatedly using a TissueLyser (Qiagen, Inc., Valencia, CA) for 30 seconds at 27.7 Hz until uniform particle size was achieved. Prior to each homogenization jars holding the samples were frozen in liquid nitrogen. Approximately 0.5 g of homogenized material was loaded into bead-beating tubes (Lysing Matrix E; MP Biomedicals Life Sciences Division, Solon, OH) and extracted by adding CTAB buffer (equal volumes 10% hexadecyltrimethylammonium bromide in 1M NaCl and 0.5 M phosphate buffer, pH 8 in 1 M NaCl), 0.1 M ammonium aluminum sulfate, and phenol:chloroform:isoamylalcohol (25:24:1) followed by bead-beating for 30 seconds at 5.5 m/s [35]. This extraction was repeated two times and the aqueous phases of both steps were

further purified using chloroform:isoamylalcohol (24:1) followed by precipitation of the nucleic acids in 30% PEG 6000 (30% wt/vol Poly(ethylene glycol) 6,000 in 1.6 M NaCl). DNA pellets were washed in 70% ethanol and resuspended in nuclease-free Tris-EDTA buffer.

Community Profiling

Small subunit (SSU) rRNA gene sequences were amplified using the primer pair 926f/1392r as described in Kunin et al. [36]. The reverse primer included a 5 bp barcode for multiplexing of samples during sequencing. Emulsion PCR and sequencing of the PCR amplicons was performed following manufacturer's instructions for the Roche 454 GS FLX Titanium technology, with the exception that the final dilution was $1e^{28}$. Sequencing tags were analyzed using the software tool PyroTagger (<http://pyrotagger.jgi-psf.org/>) using a 220 bp sequence length threshold.

Metagenome Sequencing, Assembly and Analysis

Genomic DNA extracted from the day 31 sample was used for sequencing library construction following the DOE Joint Genome Institute standard operating procedure for shotgun sequencing using the Roche 454 GS FLX Titanium technology. Obtained sequencing reads were quality trimmed and assembled using the Newbler assembler software (version 2) by 454 Life Sciences. For assembly, minimum acceptable overlap match (mi) was set to 0.95. Quality filtered sequence reads and assembled contigs \$100 bp totaling 110 Mbp were used for further analysis. For global functional analysis, the metagenomic data set was loaded into MG-RAST [37] and compared to other annotated metagenomes that are publicly available in the metagenome analysis platform. Correspondence analysis was performed using the R software package ade4 [38].

Glycoside hydrolases of selected functional classes (e.g. cellulases, endohemicellulases, debranching enzymes) were identified using pfam HMMs (Pfam version 23.0 and HMMER v2.3). For the 3 GH families 44, 51 and 74 that are not represented in pfam, HMMs were generated (two for each, since they are 2-domain proteins) and treated similar to the pfam HMMs. For GH families covered by multiple pfams (e.g. GH2 or GH42) only the best scoring hit was taken into account in case there were multiple hits to the same contig. Contig read depth was factored as following: based on the Newbler output, the number of reads in each was determined and multiplied by the median read length of 400 bp and divided by the contig length. This weighting corrects approximately for differences in species abundance distribution (i.e. dominant populations producing higher depth contigs will be weighted in the analysis).

To extract potential full-length glycoside hydrolases from the metagenome data, we ran BLASTX on all contigs \$1 kb against the CAZy [3] and FOLy [39] databases ($E, 1e^{-210}$), and filtered out hits matching the target enzyme over at least 90% of its length, and for which the target enzyme has a known enzymatic function (EC number listed in CAZy or FOLy). Frameshifts (most likely introduced by homopolymers during sequencing) were delineated by BLASTX of the targeted contigs against the non-redundant NCBI nucleotide database and corrected by deleting or duplicating single bases so as to maximize the BLAST scores. After manual frameshift correction, genes were called using fgenesb (<http://www.softberry.com>). For phylogenetic analysis, peptide sequences of the two full-length GH9 enzymes were aligned to reference sequences using ClustalX [40] and imported into the ARB software package [41] for phylogenetic reconstructions using the PROML function of the integrated Phylip package.

Cellulase Protein Expression and Activity Screening

The nucleotide sequences of two putative cellulases (contigs JMC00312_1, JMC20181_1) were codon-optimized for protein expression in *E. coli* (GenScript, Piscataway, NJ). The PCR primers were designed to amplify genes without the putative signal peptide sequences (SignalP 3.0 server, <http://www.cbs.dtu.dk/services/SignalP/>). The amplicons were cloned into the pET DEST42 vector via the Gateway cloning method (Invitrogen, Carlsbad, CA). The plasmids containing the cellulase genes were transformed into the BL21 (DE3) Star strain (Invitrogen, Carlsbad, CA) of *E. coli* for protein expression. Small-scale protein expression was done in 5 mL culture volume for each cellulase gene by auto-induction at 30uC (Overnight Express AutoinductionTM System, Novagen, Gibbstown, NJ). After protein expression, the cells were harvested by centrifugation at 6,000g for 10 min. The cell lysates were prepared using the BugBuster reagent. The volume of the BugBuster used for each cell pellet was normalized to an OD600 nm reading of the culture (50 mL OD600 nm). The overnight cultures without protein induction reagent were also prepared for uninduced controls. The cell lysates were centrifuged at 10,000g for 30 min to separate soluble proteins from insoluble materials. The supernatants (soluble proteins) were collected for SDS-PAGE and enzyme activity screens. To test enzyme activity on carboxymethyl-cellulose (CMC), 2 mL of the supernatant was spotted on an agar plate containing 0.1% CMC. The plate was incubated at 37uC for 2 hours. The enzyme activity was detected by Congo red assay [42]. To determine whether the cellulases have cellobiosidase or b-glucosidase activity, 4-nitrophenyl-bD-cellobioside (pNPC) or 4-nitrophenyl-b-D-glucopyranoside (pNPG) were used as substrates [43]. Soluble protein extract from *E. coli* containing expressed JMC00312_1 was used to determine temperature and pH activity profiles. All reactions were performed in 50 mL volumes. To measure the pH optimum, a standard pH solutions containing 100 mM sodium acetate, 50 mM MES, and 50 mM HEPES between pH 4 and 8 were used as buffer for the enzyme reaction at 50uC. At the end of a 30 min incubation, 120 mL of DNS reagent [44] was added to the reaction mixture and incubated at 95uC for 5 min to label the reducing ends of hydrolyzed CMC. The absorbance at 540 nm was measured to determine the relative activity across the tested temperature and pH ranges.

Sequence Data Submission

The raw sequencing reads and the assembled metagenome dataset have been deposited at GenBank and the NCBI Short Read Archive under Genome Project ID 41493 and accession number SRA010300.1, respectively. The SSU rRNA amplicon pyrosequencing reads are deposited under the accession numbers GU178033 -GU178768.

Supporting Information

Table S1 Putative full-length cellulase and hemicellulase enzyme sequences extracted from the SAC metagenome data set. The two GH9 cellulases in bold were tested for activity on CMC, pNPC and pNPG. (a) Best BLASTX hit against any sequence in CAZy with a validated EC number indicating a lignocellulolytic enzyme. Number of frameshift corrections required, based on alignments with homologs in NR. The prevalence of frameshifts complicates assembly, gene calling, and annotation of genes in low-coverage 454-titanium metagenomic data. For example, we noticed that none of the manually corrected frameshifts for the full-length catalytic domains were caught by the MG-RAST annotation, resulting in truncated genes. (c) Length (in aa) of

potential truncation at the N terminal (N) or C terminal (C), due to the end of the contig, based on closest homolog in NR. (d) Contig JMC02101 was originally selected because of a CAZy hit against a GH30 b-xylosidase, but also contains a GH5 endoglucanase gene. *Reference species recently sequenced by the US DOE Joint Genome Institute as part of the Genome Encyclopedia of Bacteria and Archaea. Found at: doi:10.1371/journal.pone.0008812.s001 (0.09 MB DOC)

Table S2 Domain structure, protein expression and activity profiles of the two full-length genes belonging to family GH9. U: uninduced negative control; I: IPTG induced sample. Found at: doi:10.1371/journal.pone.0008812.s002 (0.03 MB DOC)

Figure S1 Chromosome of a circular phage recovered from the SAC community metagenome (contig JMC02169) related to Iodobacteriophage. Genes were predicted using fgenesV (www.softberry.com). Found at: doi:10.1371/journal.pone.0008812.s003 (1.12 MB EPS)

Figure S2 Correspondence analysis of the compost bioreactor microbiome to other lignocellulosic (pentagons) and non-lignocellulosic (circles) microbiomes. The total variance extracted by the coordinate axes was 46.8% (31.1%+15.7%). Metagenome IDs given in the legend correspond to references metagenomes in MG-

References

- Margeot A, Hahn-Hagerdal B, Edlund M, Slade R, Monot F (2009) New improvements for lignocellulosic ethanol. *Curr Opin Biotechnol* 20: 372–380.
- Wen F, Nair NU, Zhao H (2009) Protein engineering in designing tailored enzymes and microorganisms for biofuels production. *Current Opinion in Biotechnology* 20: 412–419.
- Cantarel BL, Coutinho PM, Rancurel C, Bernard T, Lombard V, et al. (2009) The Carbohydrate-Active EnZymes database (CAZy): an expert resource for Glycogenomics. *Nucleic Acids Res* 37: D233–238.
- Himmel ME, Ding SY, Johnson DK, Adney WS, Nimlos MR, et al. (2007) Biomass recalcitrance: engineering plants and enzymes for biofuels production. *Science* 315: 804–807.
- Heinzelman P, Snow CD, Wu I, Nguyen C, Villalobos A, et al. (2009) A family of thermostable fungal cellulases created by structure-guided recombination. *Proc Natl Acad Sci U S A* 106: 5610–5615.
- Ryckeboer J, Mergaert J, Vaes K, Klammer S, De Clercq D, et al. (2003) A survey of bacteria and fungi occurring during composting and self-heating processes. *Annals of Microbiology* 53: 349–410.
- Marshall MN, Reddy AP, VanderGheynst JS (2004) Microbial Ecology of Compost. In: Lens P, Hamelers B, Hoitink H, Bidlingmaier W, eds (2004) Resource recovery and reuse in organic solid waste management. London: IWA Publishing. pp 193–224.
- Nakasaki K, Shoda M, Kubota H (1985) Effect of Temperature on Composting of Sewage-Sludge. *Applied and Environmental Microbiology* 50: 1526–1530.
- Overbeek R, Begley T, Butler RM, Choudhuri JV, Chuang HY, et al. (2005) The subsystems approach to genome annotation and its use in the project to annotate 1000 genomes. *Nucleic Acids Res* 33: 5691–5702.
- Vogel J (2008) Unique aspects of the grass cell wall. *Curr Opin Plant Biol* 11: 301–307.
- Li LL, McCorkle SR, Monchy S, Taghavi S, van der Lelie D (2009) Bioprospecting metagenomes: glycosyl hydrolases for converting biomass. *Biotechnol Biofuels* 2: 10.
- Gomez LD, Steele-King CG, McQueen-Mason SJ (2008) Sustainable liquid biofuels from biomass: the writing's on the walls. *New Phytol* 178: 473–485.
- Tilman D, Socolow R, Foley JA, Hill J, Larson E, et al. (2009) Energy. Beneficial biofuels—the food, energy, and environment trilemma. *Science* 325: 270–271.
- Gura T (2009) Driving biofuels from field to fuel tank. *Cell* 138: 9–12.
- Bouton JH (2007) Molecular breeding of switchgrass for use as a biofuel crop. *Curr Opin Genet Dev* 17: 553–558.
- Rubin EM (2008) Genomics of cellulosic biofuels. *Nature* 454: 841–845.
- Simmons BA, Loque D, Blanch HW (2008) Next-generation biomass feedstocks for biofuel production. *Genome Biol* 9: 242.
- Warnecke F, Luginbuhl P, Ivanova N, Ghassemian M, Richardson TH, et al. (2007) Metagenomic and functional analysis of hindgut microbiota of a wood-feeding higher termite. *Nature* 450: 560–565.
- Brule JM, Antonopoulos DA, Miller ME, Wilson MK, Yannarell AC, et al. (2009) Gene-centric metagenomics of the fiber-adherent bovine rumen microbiome reveals forage specific glycoside hydrolases. *Proc Natl Acad Sci U S A* 106: 1948–1953.
- Eiland F, Klamer M, Lind AM, Leth M, Baath E (2001) Influence of initial C/N ratio on chemical and microbial composition during long term composting of straw. *Microbial Ecology* 41: 272–280.
- Horwath WR, Elliott LF (1996) Ryegrass straw component decomposition during mesophilic and thermophilic incubations. *Biology and Fertility of Soils* 22: 243–252.
- Twidwell EK, Johnson KD, Patterson JA, Cherney JH, Bracker CE (1990) DEGRADATION OF SWITCHGRASS ANATOMICAL TISSUE BY RUMEN MICROORGANISMS. *Crop Science* 30: 1321–1328.
- Johnson JMF, Barbour NW, Weyers SL (2007) Chemical composition of crop biomass impacts its decomposition. *Soil Science Society of America Journal* 71: 155–162.
- Takaku H, Kodaira S, Kimoto A, Nashimoto M, Takagi M (2006) Microbial communities in the garbage composting with rice hull as an amendment revealed by culture-dependent and -independent approaches. *J Biosci Bioeng* 101: 42–50.
- Yu H, Zeng G, Huang H, Xi X, Wang R, et al. (2007) Microbial community succession and lignocellulose degradation during agricultural waste composting. *Biodegradation* 18: 793–802.
- Ryckeboer J, Mergaert J, Coosemans J, Deprins K, Swings J (2003) Microbiological aspects of biowaste during composting in a monitored compost bin. *J Appl Microbiol* 94: 127–137.
- Kirby R (2006) Actinomycetes and lignin degradation. *Adv Appl Microbiol* 58: 125–168.
- Watanabe H, Tokuda G (2009) Cellulolytic Systems in Insects. *Annu Rev Entomol*.
- Gill SR, Pop M, Deboy RT, Eckburg PB, Turnbaugh PJ, et al. (2006) Metagenomic analysis of the human distal gut microbiome. *Science* 312: 1355–1359.
- Xie H, King A, Kilpelainen I, Granstrom M, Argyropoulos DS (2007) Thorough chemical modification of wood-based lignocellulosic materials in ionic liquids. *Biomacromolecules* 8: 3740–3748.
- Reddy AP, Jenkins BM, VanderGheynst JS (2009) The Critical Moisture Range for Rapid Microbial Decomposition of Rice Straw during Storage. *Transactions of the ASABE* 52: 673–676.
- Templeton D, Ehrman T (1995) Determination of Acid-Insoluble Lignin in Biomass. NREL CAT Task Laboratory Analytical Procedure #003.
- Ehrman T (1996) Determination of Acid-Soluble Lignin in Biomass. NREL CAT Task Laboratory Analytical Procedure #004.
- Ruiz R, Ehrman T (1996) Determination of Carbohydrate in Biomass by High Performance Liquid Chromatography. NREL CAT Task Laboratory Analytical Procedure #002.
- Brodie EL, Desantis TZ, Joyner DC, Baek SM, Larsen JT, et al. (2006) Application of a high-density oligonucleotide microarray approach to study

- bacterial population dynamics during uranium reduction and reoxidation. *Appl Environ Microbiol* 72: 6288–6298.
36. Kunin V, Engelbrektson A, Ochman H, Hugenholtz P (2009) Wrinkles in the rare biosphere: pyrosequencing errors lead to artificial inflation of diversity estimates. *Environ Microbiol*.
 37. Meyer F, Paarmann D, D'Souza M, Olson R, Glass EM, et al. (2008) The metagenomics RAST server -a public resource for the automatic phylogenetic and functional analysis of metagenomes. *BMC Bioinformatics* 9: 386.
 38. Dray S, Dufour AB (2007) The ade4 package: Implementing the duality diagram for ecologists. *Journal of Statistical Software* 22: 1–20.
 39. Levasseur A, Piumi F, Coutinho PM, Rancurel C, Asther M, et al. (2008) FOLY: an integrated database for the classification and functional annotation of fungal oxidoreductases potentially involved in the degradation of lignin and related aromatic compounds. *Fungal Genet Biol* 45: 638–645.
 40. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, et al. (2007) Clustal W and Clustal X version 2.0. *Bioinformatics* 23: 2947–2948.
 41. Ludwig W, Strunk O, Westram R, Richter L, Meier H, et al. (2004) ARB: a software environment for sequence data. *Nucleic Acids Res* 32: 1363–1371.
 42. Teather RM, Wood PJ (1982) Use of Congo red-polysaccharide interactions in enumeration and characterization of cellulolytic bacteria from the bovine rumen. *Appl Environ Microbiol* 43: 777–780.
 43. Deshpande MV, Eriksson KE, Pettersson LG (1984) An assay for selective determination of exo-1,4,-beta-glucanases in a mixture of cellulolytic enzymes. *Anal Biochem* 138: 481–487.
 44. Miller GL (1959) Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. *Analytical Chemistry* 31: 426–428.

This document was prepared as an account of work sponsored by the United States Government. While this document is believed to contain correct information, neither the United States Government nor any agency thereof, nor The Regents of the University of California, nor any of their employees, makes any warranty, express or implied, or assumes any legal responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by its trade name, trademark, manufacturer, or otherwise, does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof, or The Regents of the University of California. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof or The Regents of the University of California.

Contract Number:

DE-AC02-05CH11231