

Substrate Perturbation Alters the Glycoside Hydrolase Activities and Community Composition of Switchgrass-Adapted Bacterial Consortia

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ABSTRACT: Bacteria modulate glycoside hydrolase expression in response to the changes in the composition of lignocellulosic biomass. The response of switchgrass-adapted thermophilic bacterial consortia to perturbation with a variety of biomass substrates was characterized to determine if bacterial consortia also responded to changes in biomass composition. Incubation of the switchgrass-adapted consortia with these alternative substrates produced shifts in glycoside hydrolase activities and bacterial community composition. Substantially increased endoglucanase activity was observed upon incubation with microcrystalline cellulose and trifluoroacetic acid-pretreated switchgrass. In contrast, culturing the microbial consortia with ionic liquid-pretreated switchgrass increased xylanase activity dramatically. Microbial community analyses of these cultures indicated that the increased endoglucanase activity correlated with an increase in bacteria related to *Rhodothermus marinus*. Inclusion of simple organic substrates in the culture medium abrogated glycoside hydrolase activity and enriched for bacteria related to *Thermus thermophilus*. These results demonstrate that the composition of biomass substrates influences the glycoside hydrolase activities and community composition of biomass-deconstructing bacterial consortia.

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KEYWORDS: cellulase; xylanase; SSU amplicon sequencing; compost; perturbation

Introduction

Mixtures of glycoside hydrolase (GH) enzymes, often referred to as cellulase or hemicellulase cocktails, are essential for the biochemical conversion of lignocellulosic biomass to biofuels (Gao et al., 2010a,b; Lynd et al., 2002; Meyer et al., 2009). Thermophilic enzymatic cocktails for biomass deconstruction are an attractive alternative to commercial enzyme cocktails based on mesophilic fungal enzyme mixtures (Rosgaard et al., 2006). These thermophilic cocktails may be more amenable to harsh industrial conditions, including: high temperature, extremes of pH, and residual pretreatment chemicals (Datta et al., 2010). Enzymes recovered from both thermophilic fungal and bacterial sources have been explored in the hydrolysis of purified biomass substrates and complex biomass (Bhat and Maheshwari, 1987; Folan and Coughlan, 1978; Liang et al., 2010, 2009; Margaritis and Merchant, 1983; Rastogi et al., 2010; Wongwilaiwalin et al., 2010). Generally, fungi secrete higher levels of enzymes than bacteria; however, bacterial enzymes tolerate a broader range of reaction conditions.

Compost-derived thermophilic bacterial consortia enriched with switchgrass as the sole carbon source were shown to be low-diversity bacterial communities that

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produced high levels of glycoside hydrolase activities (Gladden et al., 2011). The glycoside hydrolases produced by these consortia were active at higher temperature and in the presence of higher concentrations of pretreatment chemicals compared to commercial fungal enzymatic cocktails. Measurement of individual glycoside hydrolase activities indicated that these consortia had higher hemicellulase activities compared to cellulose activities. Since cellulose is typically the most abundant polysaccharide in biomass and glucose is the primary substrate for fermentation to biofuels, increasing the titer of cellulases produced by these thermophilic consortia would produce more useful enzymatic cocktails that efficiently liberate glucose from pretreated biomass (Blanch et al., 2008; Simmons et al., 2008).

Studies of bacterial biomass deconstruction have shown that bacterial expression of glycoside hydrolases is often dependent on the chemical composition of the biomass substrate. *Thermobifida fusca* cultures were grown on corn fiber, xylan, and Solka Floc and shown to have different levels of glycoside hydrolase activities for each substrate (Irwin et al., 2003). Quantitative proteomic measurements on cellulosomes isolated from cultures of *Clostridium thermocellum* demonstrated that the glycoside hydrolase content in cellulosomes was altered when the *C. thermocellum* was grown on acid-pretreated switchgrass compared to crystalline and amorphous cellulose (Raman et al., 2009). These precedents suggest that the biomass composition may influence the expression of glycoside hydrolases and may also change the composition of the consortia. Therefore, the previously characterized thermophilic switchgrass-adapted consortia were perturbed by cultivation on several different types of biomass-derived substrates with different xylan:glucan ratios to determine how the thermophilic consortia respond to changes in the biomass composition.

Materials and Methods

Cultivation Conditions

Switchgrass-adapted thermophilic bacterial consortia derived from compost (collected at Jepson Prairie Organics in Vacaville, CA) were maintained on 1% switchgrass in M9 minimal media (50 mL total volume) for ~6 months by culturing in a rotary shaker at 60°C/200 rpm. The cultures were maintained by inoculation at 4% (v/v) into fresh media every 2 weeks (Gladden et al., 2011). For the perturbation experiments, inocula were dispensed at 10% (v/v) into multiple cultures with alternative substrates as described in Table I. Microcrystalline cellulose and oat spelts xylan were used as received. Intact switchgrass was extracted with ethanol and water as previously described (Gladden et al., 2011) and pretreated switchgrass was generated as described below. Chemical analysis of switchgrass samples was performed as previously reported (Li et al., 2010). M9 media was formulated as previously described (DeAngelis

Table I. Substrate-perturbed thermophilic bacterial consortia

Sample name	Biomass substrate
SG-M9 ^a	Switchgrass ^b
SG-R2A ^a	Switchgrass ^b
ILSG-M9	[C2mim][OAc]-pretreated switchgrass ^c
TFASG-M9	Trifluoroacetic acid-pretreated switchgrass ^d
McCel-M9	Microcrystalline cellulose
McCel-R2A	Microcrystalline cellulose
Xy-M9	Oat spelts xylan ^e

^aM9 and R2A media formulations are described in Materials and Methods section.

^bThe switchgrass was composed of 31% glucan and 17% xylan.

^c[C2mim][OAc]-pretreated switchgrass was composed of 41% glucan and 12% xylan.

^dTrifluoroacetic acid-pretreated switchgrass was composed of 68% glucan and 2% xylan.

^eThe soluble fraction of oat spelts xylan was used for cultivation.

et al., 2010). R2A media contained (g/L): Proteose peptone (0.5), casamino acids (0.5), yeast extract (0.5), dextrose (0.5), soluble starch (0.5), K₂HPO₄ (0.3), MgSO₄ × 7H₂O (0.05), sodium pyruvate (0.3). All inoculated cultures were incubated on a rotary shaker for 2 weeks at 60°C/200 rpm.

Pretreatment of Switchgrass

Switchgrass was obtained as a gift from Dr. Ken Vogel at USDA-ARS-Lincoln (University of Nebraska, Lincoln) and was pretreated using the ionic liquid 1-ethyl-3-methylimidazolium acetate ([C₂mim][OAc]) (BASF, Florham Park, NJ), in a 10:1 mass ratio at 120°C for 3 h (Gladden et al., 2011), and was reconstituted and recovered by multiple washes in water, leaving <0.1% [C₂mim][OAc] as measured by capillary electrophoresis mass spectrometry (data not shown). The wet biomass was then dried by lyophilization.

Trifluoroacetic acid (TFA)-pretreated switchgrass was prepared as previously described (Dong et al., 2009) with the following amendments: (i) pretreated switchgrass was precipitated by the addition of 0.5 volumes isopropanol/9 volumes of water and collected by centrifugation at room temperature; (ii) precipitated biomass was washed 200 mL of 95% ethanol (iii) and dried at 60°C overnight.

Residual cellulose and xylan concentrations were measured by hydrolysis with concentrated trifluoroacetic acid and measurement of the monosaccharides by a Dionex DX600 HPAEC (High Performance Anion Exchange Chromatography, Dionex, Sunnyvale, CA) as previously described (Li et al., 2010).

Glycoside Hydrolase Assays

Endoglucanase and endoxylanase activity were measured by microscale DNS assay (Singer et al., 2011). Each reaction was composed of 1–40 μL of culture supernatant (brought up to a total volume of 40 μL with water) mixed with 40 μL of 2%

carboxymethyl cellulose (CMC) for endoglucanase activity or soluble birchwood xylan for endoxylanase activity in 100 mM sodium acetate pH 5.0. Reactions were incubated at 70°C for 30 min, 80 μL of DNS reagent were added, the samples were incubated for 5 min at 95°C, and the absorbance was detected at 540 nm. Zymograms were performed as previously described (Gladden et al., 2011).

Bacterial Community Analysis

DNA extraction and amplicon pyrosequencing was performed as previously described (Gladden et al., 2011). Amplicon pyrosequencing data were analyzed using Pyrotagger software (Engelbrekton et al., 2010). Data were analyzed using the MOTHUR software package (Schloss et al., 2009), normalized to the smallest pyrosequencing library (n = 10,060). The Pyrotagger cluster classification output file was modified to a shared file, which was used as the input for MOTHUR. Bacterial richness, which is a measure of the number of different species, was estimated using Chao1 and Abundance Coverage Estimator (ACE) at the operational taxonomic unit (OTU) of 0.03 which correlates to a sequence similarity of 97% (hereafter referred to as OTU₉₇). Bacterial diversity, which is a combined measure of the number of different species along with the relative abundance of those species, was estimated using the Shannon and Simpson indices (transformed using the equation, $-\ln D$) at OTU₉₇.

Chemicals

All chemicals were reagent grade and were received from Sigma (St. Louis, MO) unless otherwise noted.

Results

Glycoside Hydrolase Activity of Perturbed Cultures

A switchgrass-adapted bacterial community was perturbed by cultivation on several different biomass substrates (Table I). After 2-week incubations on these alternative substrates, the culture supernatants were recovered and glycoside hydrolase activities were determined (Table II).

Table II. Glycoside hydrolase activities of perturbed culture supernatants

Sample	Endoglucanase (U/mL) ^a	Endoxylanase (U/mL) ^a
SG-M9	0.07 ± 0.002	4.37 ± 0.34
SG-R2A	0.02 ± 0.001	0.17 ± 0.03
ILSG-M9	0.11 ± 0.006	40.9 ± 4.08
TFASG-M9	0.40 ± 0.035	5.62 ± 0.80
McCel-M9	0.89 ± 0.026	1.12 ± 0.09
McCel-R2A	ND	ND
XY-M9	0.05 ± 0.001	1.28 ± 0.05

ND, not detected.

^aThe activity assays were done at pH 5.0 and 70°C for 30 min.

Endoglucanase and xylanase activities were used as representative cellulase and hemicellulase activities, respectively. Cellobiohydrolase, β-glucosidase, and alpha-L-arabinofuranosidase activities, as measured by release of *p*-nitrophenolate from *p*-nitrophenyl-β-D-cellobioside, *p*-nitrophenyl-*p*-D-glucopyranoside and 4-nitrophenyl-α-L-arabinofuranoside, were generally low (<0.05 μmol/mim/mL) and did not show substrate-dependent patterns (data not shown).

The switchgrass control culture (SG-M9) had comparable, but slightly lower, endoglucanase and xylanase activities compared to previous measurements on the switchgrass-adapted consortia (Gladden et al., 2011). The replacement of the minimal media with R2A media, a medium with simple organic substrates, reduced the production of both endoglucanase and xylanase activities. This cessation of glycoside hydrolases activity was also observed in the culture amended with microcrystalline cellulose (MCC-R2A).

Perturbation with substrates containing a high proportion of crystalline cellulose, such as trifluoroacetic acid-pretreated switchgrass (TFA-M9) and microcrystalline cellulose (McCel-M9), increased endoglucanase activity up to ~13× relative to the switchgrass control. In contrast, the culture with [C₂mim][OAc]-pretreated switchgrass (ILSG-M9), which has a lower glucan:xylan ratio and a cellulose structure that is largely amorphous (Li et al., 2010; Singh et al., 2009), had increased xylanase activity but showed no increase in endoglucanase activity. Cultivation of the consortia on soluble oat spelts xylan (Xy-M9) resulted in lowered recovery of both endoglucanase and endoxylanase activity.

Zymography of the Perturbed Cultures

Zymography was performed on the supernatants to compare endoglucanase and xylanase profiles (Fig. 1).

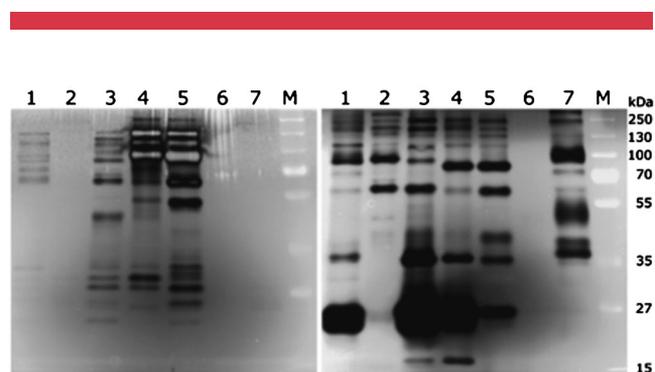


Figure 1. Zymography of glycoside hydrolase activities produced by substrate perturbation of the switchgrass-adapted consortia (1) SG-M9, (2) SG-R2A, (3) ILSG-M9, (4) TFASG-M9, (5) McCel-M9, (6) McCel-R2A, (7) Xy-M9, (M) protein ladder. Each lane was loaded with the same supernatant volume (10 μL) to visualize relative differences in activity. The left panel depicts an SDS-PAGE gel embedded with CMC and the right panel contains xylan, indicating endoglucanase and endoxylanase activity, respectively. A negative image of the gel was scanned; the dark regions correspond to endoglucanase or endoxylanase activity. The zymograms were incubated at pH 5.0 and 60°C for 2 h.

Zymography on the switchgrass-adapted consortia had previously demonstrated that multiple endoglucanases and xylanases were present in the culture supernatants (Gladden et al., 2011). The SG-M9 culture supernatant had an endoglucanase profile similar to the previously reported zymogram for the adapted consortia, with the majority of the active bands in the molecular weight range of 70–200 kDa; comparable profiles were observed for the three culture supernatants with significant endoglucanase activity (ILSG-M9, TFASG-M9, McCel-M9). The supernatants with the largest increases in observed enzymatic activity, TFASG-M9 (~6× compared to the switchgrass supernatant) and McCel-M9 (~13×) showed intensification of multiple active bands >100 kDa, suggesting that cultivation on cellulose-rich substrates enriches for multi-domain glycoside hydrolases with endoglucanase activity. The SG-M9 supernatant displayed active xylanase bands comparable to the previously reported switchgrass-adapted culture. A prominent band at ~25 kDa was dramatically enhanced in the culture supernatants with increased xylanase activity, especially the ILSG-M9 supernatant (~9× increase in activity). In the SG-R2A and Xy-M9 supernatants, which possessed low but measurable xylanase activity, this band was absent. In addition, the xylanase profile of the Xy-M9 supernatant was notably different than the other culture supernatants, with prominent bands at ~40 and ~55 kDa that were unique to this sample.

Community Composition Profile of Perturbed Cultures

SSU rRNA amplicon pyrosequencing was employed to assess the effect of substrate perturbation on the bacterial diversity, richness, and community composition of the perturbed cultures (Fig. 2, Tables III and IV). Switchgrass-amended cultures (SG-M9, SG-R2A) had the highest

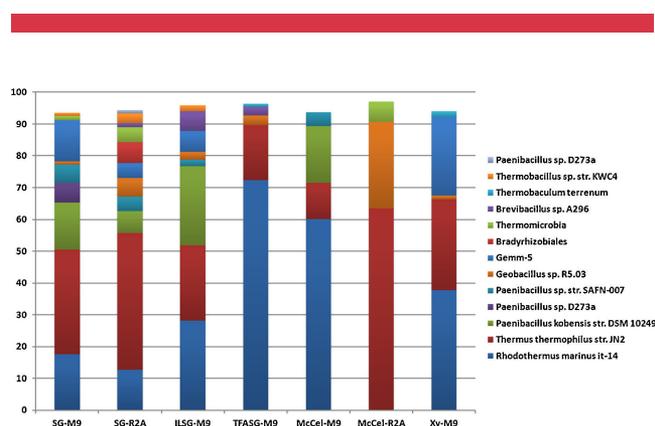


Figure 2. Plot of the relative abundance (%) of SSU rRNA amplicon clusters from the feedstock-perturbed consortia and the original switchgrass-adapted consortia (SG-M9 culture). Each cluster is labeled by the closest matching taxon to the SSU sequence. A cutoff of 1% relative abundance was chosen to highlight the most abundant organisms present in the communities. Values and GenBank accession numbers associated with the clusters are described in Table III.

estimates of richness (avg. Chao = 178, avg. ACE = 322) and diversity (avg. Simpson = 1.6, avg. Shannon = 2.1) (Table III). Cultures amended with substrates with a high proportion of crystalline cellulose had the lowest estimates of richness and diversity: TFA-pretreated switchgrass (TFA-M9) (Chao1 = 120, ACE = 172, Shannon = 1.0, Simpson = 0.6) and microcrystalline cellulose (McCel-M9, McCel-R2A) (avg. Chao1 = 100, ACE = 163, Shannon = 1.2, Simpson = 0.8) (Table III).

The community composition across the perturbed cultures primarily varied across five bacterial taxa (Table IV). The composition of the SG-M9 culture was similar to the ancestral switchgrass-adapted community containing abundant amplicons related to thermophilic *Bacilli* (*Paenibacillus*, *Thermobacillus*, *Brevibacillus*, *Geobacillus*), *Rhodothermus marinus* and *Thermus thermophilus* (Gladden et al., 2011). Replacing the M9 media with R2A in the SG-R2A switchgrass culture shifted the community to have a higher proportion of amplicons related to *T. thermophilus*. The predominance of *T. thermophilus*-related amplicons was also observed in the microcrystalline cellulose culture with R2A media (McCel-R2A). In contrast, the composition of the minimal media culture (McCel-M9) was primarily populated with amplicons related to *R. marinus*. *R. marinus*-related amplicons were also enhanced in the TFA-pretreated switchgrass (TFA-M9) culture. Although the [C2mim][OAc]-pretreated switchgrass (ILSG-M9) culture had high levels of xylanase activity compared to the SG-M9 culture, the same bacterial taxa were present in both consortia. Cultivation of the consortia on soluble oat spelts xylan (Xy-M9) increased amplicons for an uncultivated subdivision of the *Gemmatimonadetes* phylum (*Gemm-5*) relative to the cultures with switchgrass as the substrate.

Discussion

This study illustrates that a bacterial consortia adapted to grow on one biomass feedstock responds to perturbation with different biomass substrates by shifting its community composition and enzymatic activity profiles. Biomass enriched in crystalline cellulose (TFA-pretreated switchgrass, microcrystalline cellulose) generated consortia with increased endoglucanase activities, while biomass containing insoluble xylan ([C₂mim][OAc]-pretreated switchgrass) had increased xylanase activity. Despite the observed growth on microcrystalline cellulose and acid-pretreated switchgrass, exocellulase activities (cellobiohydrolase and β-glucosidase) were low, so it is not clear how the bacteria hydrolyze these cellulose substrates. Comparison of richness and diversity estimates indicate that the shift toward increased endoglucanase activity corresponded with a reduction in community complexity. Replacing minimal media with media containing simple organic molecules shifted the community and greatly lowered glycoside hydrolase activity in the culture supernatants.

Table III. Bacterial community composition of perturbed cultures^a

Closest taxon	SG-M9	SG-R2A	ILSG-M9	TFASG-M9	McCel-M9	McCel-R2A	Xy-M9
<i>Rhodothermus marinus</i> it-14 (EU214602.1)	17.6	12.7	28.2	72.4	60.1	0.03	37.7
<i>Thermus thermophilus</i> str. JN2 (AY554280.1)	32.8	43.1	23.7	17.2	11.3	63.4	28.6
<i>Paenibacillus kobensis</i> str. DSM 10249 (AB073363.1)	14.9	6.87	24.9	0.21	17.9		
<i>Paenibacillus</i> sp. D273a (FJ430033.1)	6.15	0.24	0.36	0.10	0.31	0.02	
<i>Paenibacillus</i> sp. str. SAFN-007 (AY167820.1)	5.67	4.56	1.95	0.37	4.28	0.25	0.38
<i>Geobacillus</i> sp. R5.03 (EF105452.1)	1.08	5.82	2.46	3.06	0.03	27.3	1.17
<i>Gemm-5</i> (AY493977.1) ^b	12.9	4.66	6.58	0.01		0.01	24.7
<i>Bradyrhizobiales</i> (EU491413.1)	0.82	6.65	0.11	0.03	0.02		0.16
<i>Thermomicrobia</i> (DQ490006.1)	1.37	4.66	0.01	0.11		6.38	0.33
<i>Brevibacillus</i> sp. A296 (FJ429992.1)	0.41	1.31	6.19	2.72	0.50		0.51
<i>Thermobaculum terrenum</i> (AF391972.1)	0.87	0.18	0.07	1.01	0.02	0.33	1.77
<i>Thermobacillus</i> sp. str. KWC4 (AB254031.3)	1.00	3.02	1.91	0.54	0.57	0.19	0.51

^aValues in the table are the % relative abundance of each taxon within each bacterial community. Each cluster is labeled by the closest matching taxon to the SSU sequence based on comparison to the Greengenes database (<http://greengenes.lbl.gov>). A cutoff of 1% relative abundance (in at least one of the seven consortia) was chosen to highlight the most abundant organisms present in the communities.

^b*Gemm-5* represents *Gemmatimonadetes* subdivision 5.

Comparing the enzymatic activity data and zymogram profiles with analysis of the microbial community compositions uncovered several trends that suggest roles for individual bacterial taxa in biomass deconstruction. Perturbing the adapted consortia with crystalline cellulose-rich substrates enriched for bacteria related to *R. marinus*. Isolates of *R. marinus* have previously been demonstrated to secrete endoglucanases when grown in culture with carboxymethylcellulose, a derivatized form of cellulose that is soluble (Hreggvidsson et al., 1996). However, there are no reports of isolated *R. marinus* growing on microcrystalline cellulose or other recalcitrant forms of cellulose (Bjornsdottir et al., 2006). Perturbing the microbial consortia with [C₂mim][OAc]-pretreated switchgrass did not significantly increase the endoglucanase activity and only demonstrates moderate enrichment for *R. marinus*, suggesting that the *R. marinus* population in this study may be particularly responsive to cellulose with a high level of crystallinity found in both TFA-pretreated switchgrass and microcrystalline cellulose (Cheng et al., 2011). An important goal of these perturbation studies was to identify culturing conditions that induce the consortia to increase cellulase production for potential use in thermophilic cellulase cocktails. Therefore, the supernatants recovered from the culture with microcrystalline cellulose

are currently being tested for their ability to saccharify [C₂mim][OAc]-pretreated switchgrass at 60–80°C, both alone and in combination with recombinant enzymes obtained from thermophilic bacteria.

The culture perturbed with [C₂mim][OAc]-pretreated switchgrass had an increased level of xylanase activity that did not correlate with the increase in the proportion of a specific taxon (OTU) as identified by pyrosequencing. The increase in xylanase activity was correlated with an increase in the intensity of a protein band at ~25 kDa. This active band was recovered in almost all the cultures incubated with minimal media except the culture perturbed with soluble oat spelts xylan. Low molecular weight xylanases (20–30 kDa) have been identified in numerous *Bacillus* isolates, including *Thermobacillus xylaniticus*, which is closely related to the thermophilic *Bacilli* populations present in the switchgrass-adapted consortia (Paes and O'Donohue, 2006). Therefore, the *Bacilli* present in these consortia may be responsible for the increased xylanase activity. Interestingly, approximately the same amount of xylanase activity was recovered from the perturbation with acid-pretreated switchgrass as from intact switchgrass despite the very low levels of xylan remaining after acid-pretreatment (17% before pretreatment vs. 2% after pretreatment), suggesting that other factors besides the presence of xylan in the substrate may affect the recovery of xylanase activity.

The proportional increase in the amplicons for *T. thermophilus* when M9 media was replaced with R2A culture medium demonstrates that the *T. thermophilus* population is responsive to the presence of simple organic substrates in the R2A medium, including soluble starch and dextrose. These results suggest that the *T. thermophilus* populations may not have an important role in primary biomass deconstruction, which is consistent with previous observations that the absence of *T. thermophilus* from some of the switchgrass adapted-consortia did not correlate with a decrease in glycoside hydrolase activity (Gladden et al., 2011). However, *T. thermophilus* may metabolize intermediates in polysaccharide hydrolysis that may be inhibitory

Table IV. Summary of species richness and diversity estimates^a

Sample name	Richness estimates		Diversity estimates	
	Chao1	ACE	Shannon	Simpson
SG-M9	176	221	2.1	1.7
SG-R2A	180	245	2.1	1.5
ILSG-M9	141	150	1.9	1.6
TFASG-M9	120	172	1.0	0.6
McCel-M9	111	172	1.4	0.9
McCel-R2A	88	154	1.0	0.7
Xy-M9	123	172	1.6	1.3

^aAll data were normalized to 10,060 amplicons.

to biomass deconstruction, allowing them to persist in these cultures.

In conclusion, perturbing the switchgrass-adapted thermophilic bacterial consortia with other biomass substrates has provided evidence for the functions of individual populations in the consortia in polysaccharide hydrolysis and metabolism. These results imply that cultivating thermophilic consortia in parallel on a variety of biomass substrates will enrich for distinct bacterial communities. These communities will possess complementary glycoside hydrolase activities that will be useful in defining protein constituents of an efficient thermophilic bacterial biomass-deconstructing enzymatic cocktail.

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