Diversity of Bacteria and Glycosyl Hydrolase Family 48 Genes in Cellulolytic Consortia Enriched from Thermophilic Biocompost

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The enrichment from nature of novel microbial communities with high cellulolytic activity is useful in the identification of novel organisms and novel functions that enhance the fundamental understanding of microbial cellulose degradation. In this work we identify predominant organisms in three cellulolytic enrichment cultures with thermophilic compost as an inoculum. Community structure based on 16S rRNA gene clone libraries featured extensive representation of clostridia from cluster III, with minor representation of clostridial clusters I and XIV and a novel Lutispora species cluster. Our studies reveal different levels of 16S rRNA gene diversity, ranging from 3 to 18 operational taxonomic units (OTUs), as well as variability in community membership across the three enrichment cultures. By comparison, glycosyl hydrolase family 48 (GHF48) diversity analyses revealed a narrower breadth of novel clostridial genes associated with cultured and uncultured cellulose degraders. The novel GHF48 genes identified in this study were related to the novel clostridia Clostridium straminisolvens and Clostridium clariflavum, with one cluster sharing as little as 73% sequence similarity with the closest known relative. In all, 14 new GHF48 gene sequences were added to the known diversity of 35 genes from cultured species.

The exploration and understanding of cellulose fermentation capabilities in nature could inform and enable industrial processes converting cellulosic biomass to fuels and other products. Enrichment of microbial communities that can utilize cellulose is useful in this context for the identification of novel organisms, novel metabolisms, and novel functions. Of particular interest are communities that can utilize cellulose at high temperatures and under anaerobic conditions, featuring high rates of solubilization under conditions where the energy and the reducing power of substrates are conserved in potentially useful fermentation products.

Some evidence indicates that cocultures may be able to utilize cellulose more fully and produce higher concentrations of ethanol than pure cultures of model cellulolytic organisms such as Clostridium thermocellum and Clostridium straminisolvens (16, 20, 34). An initial step toward understanding the functional roles of community members in cooperative cellulose degradation is answering the question of what organisms are present in cellulolytic consortia obtained from nature. Currently, diversity estimation methods applied to cellulolytic communities range from traditional methods targeting the 16S rRNA gene (4, 12) to complex metagenomic analyses targeting the breadth of functional genes present in genomes of mixed cultures and the environment (3).

From a functional gene standpoint, cellulose systems are complex assemblages of multifunctional glycosyl hydrolases. Even particularly relevant families, such as family 5 and family 9, tend to include hydrolases with multiple substrate specificities, deep evolutionary roots, and extensive sequence diversity within the same organism (19). However, family 48 glycosyl hydrolases include a select group of cellulosomal and unbound cellulases thought to play an essential role in cellulose solubilization by model cellulolytic clostridia (5, 7, 15), actinobacteria (6, 13), and anaerobic fungi (31). One key feature of this family of glycosyl hydrolases (mostly exoglucanases) is their ability to enhance cellulose solubilization in synergistic interactions with family 9 glycosyl hydrolases (2, 13). But unlike the latter, and with the notable exception of CelS and CelY in Clostridium thermocellum, family 48 hydrolases are present mostly in single copies in the genomes of cellulolytic microbes, making family 48 hydrolyase genes a desirable target for primer design and molecular characterization.

In this paper we describe the enrichment of microbial communities from a thermophilic compost pile and provide an assessment of diversity in stable cellulolytic enrichments by addressing total bacterial diversity using the 16S rRNA gene as well as introducing a novel method to assess functional diversity in cellulolytic consortia by targeting glycosyl hydrolase family 48 (GHF48) genes.

MATERIALS AND METHODS

Sampling site. Compost samples were collected at the Middlebury College composting facility in May 2008. Samples were collected with a T-shaped steel coring tube (diameter, 2 cm) from specific locations in the compost pile where temperatures were determined to be the highest, 40 to 50 cm below the surface of the compost pile. Temperatures at each of these hot spots ranged from 52 to 72°C, and samples were designated CO-4, CO-5, and CO-6.

Enrichment protocol and media. Three samples of approximately 15 g of compost were used as inocula in bottles with 100 ml of mineral medium containing 1 g of Avicel (PH-105; FMC Corp., Philadelphia, PA) as the carbon source. Bottles were flushed with nitrogen gas on site immediately after sampling and inoculation, thus ensuring strict anaerobic conditions from primary sampling. The primary enrichment (PE) medium consisted of the following, in grams
per liter: KH₂PO₄, 2.08; K₂HPO₄, 2.22; MgCl₂·6H₂O, 0.1; NH₄Cl, 0.4; CaCl₂·2H₂O, 0.05. Primary enrichment cultures were incu- bated at 55°C upon arrival at the laboratory and were transferred to maintenance (M) medium after 4 to 6 days of incubation. M medium consisted of 3 g of Avicel/liter, 1.04 g of KH₂PO₄/liter, 1.11 g of K₂HPO₄/liter, 2.5 g of NaHCO₃/liter, 0.2 g of MgCl₂·6H₂O/liter, 0.4 g of NH₄Cl/liter, 0.05 g of CaCl₂·2H₂O/liter, 0.05 g of FeCl₃·6H₂O/liter, 1 ml of SL-10 trace elements/liter (1), 0.5 g of L-cysteine HCl/liter, and 4 ml of 0.025% resazurin/liter. Vitamins were added in the following concentrations (in milligrams per liter): pyridoxine dihydrochloride, 0.8; p-aminobenzoic acid (PABA), 0.4; o-biotorin, 0.2; vitamin B₁₂, 0.2; thiamine-HCl, 0.05; folic acid, 0.2; pantothentic acid calcium salt, 0.5; nicotinic acid, 0.5; pyridoxine-HCl, 0.1; thioctic acid, 0.5; riboflavin, 0.05. Phosphates and other minerals were prepared and autoclaved separately to avoid precipitation and chemical interactions during autoclaving. Vitamins were sterilized by filtration. A separate stock solution (100%) of L-cysteine HCl, FeCl₃·6H₂O, MgCl₂·6H₂O, NH₄Cl, and CaCl₂·2H₂O was flushed with N₂ immediately after dissolving and was autoclaved. Serum bottles containing sterile medium were placed in an anaerobic glove box, cooled down, mixed with a reducing agent solution, closed with sterile rubber stoppers, and capped with aluminum seals.

**Batch cultivation in fermentors.** After 10 consecutive transfers, anaerobic cultivation of each enrichment culture was carried out at 55°C in 1.6-liter Biostat Aplus fermentors (Sartorius Stedim, Gottingen, Germany), with a working volume of 1 liter and without a pH control. Fermentors were equipped with Norprene tubing (Cole Palmer Instrument Company, Vernon Hills, IL) to minimize oxygen diffusion. Cultures were grown in the same maintenance (M) medium, with 3 g liter⁻¹ Avicel (PH-105; FMC Corp., Philadelphia, PA) and with reducing agents and vitamins added separately after the autoclaving of fermentor vessels. Cultures were grown in the cultures at 100 rpm. Unless indicated otherwise, all cultures were fed with 85% nitrogen gas. Each fermentation experiment was performed in duplicate and with 2% (vol/vol) inoculum.

**Analytical methods.** Samples were taken every 3 h from each fermentation experiment for determination of the residual dry weight of cellulose, pH, pellet nitrogen, and fermentation products. Residual cellulose levels were determined by dry weight measurements, using triplicate 3-ml samples after filtration (0.2-μm Millipore filter). Washing, and drying for 24 h at 60°C. The concentrations of fermentation products and soluble sugars (acetate, ethanol, formate, lactate, succinate, cellulose, and glucose) were analyzed by high-performance liquid chromatography using an Aminex HPX-87H column (Bio-Rad, Hercules, CA). Pellet nitrogen was measured in centrifuged pellet samples by using a TOC-V combustion analyzer coupled with a TNM-1 total-nitrogen module (Shimadzu Corporation, Columbia, MD) and measuring the results to a 1-g liter⁻¹ nitrogen standard.

**DNA extraction and PCR amplification of the 16S rRNA gene.** After 10 consecutive transfers using M medium with Avicel as the growth substrate, DNA was extracted from enrichment cultures using the ZR soil microbe DNA kit (Zymo Research Corp., Orange, CA) with minor modifications. Triplicate 1.5-ml samples from each enrichment were subjected to DNA extraction. Ballistic explosion times in a Mini-Beadbeater (Biospec Products, Bartlesville, OK) were extended to 5 min, and bead-beating speed was lowered to 2,500 rpm to maximize the DNA yield. The DNA obtained from each set of triplicate extractions was pooled for amplification. The extracted DNA was used in triplicate PCR amplification of the 16S rRNA gene, using universal oligonucleotide primers 8F and 1492R, designed to anneal to conserved regions of bacterial 16S rRNA (9, 10, 13). Four sets of primers were tested, and PCR amplification was optimized using a PCR gradient with genomic DNA of C. thermocellum DSM 16021 and C. cellulolyticum DSM 1313.

**RESULTS**

**Dynamic of cellulose utilization and fermentation.** After 8 to 10 consecutive transfers, anaerobic compost-derived enrichments were made up in 3% CO₂ and 97% N₂ at 55°C (5). In batch fermentor experiments using 3 g liter⁻¹ Avicel, between 88% and 97% of the cellulose was utilized in 60-h fermentations (Fig. 1A, C, and E). Cellulose utilization slowed down in all fermentations after 40 h of incubation and consisted mostly of acetate, ethanol, and formate, with no residual sugars detected. Acetate and ethanol were the dominant products in the CO-5 and CO-6 fermentations, yielding between 0.4 and 0.5 g liter⁻¹. However, CO-4 enrichment had the lowest diversity, with a total of 18 OTUs. Rarefaction analysis showed that 99% of the taxa was accounted for in CO-4, 99.7% in CO-5, and 99.5% in CO-6. Fermentation products accumulated between 20 and 40 h of incubation and consisted mostly of acetate, ethanol, and formate, with no residual sugars detected. Acetate and ethanol were the dominant products in the CO-5 and CO-6 fermentations, yielding between 0.4 and 0.5 g liter⁻¹. However, formate was equally dominant in CO-4 enrichment fermentations. Based on the products measured, we were able to account for most of the carbon in fermented carbohydrates: at least 94.1% of the CO-4 had the highest, with a total of 98.2% (Fig. 2). Rarefaction analysis of 16S rRNA gene libraries (Fig. 2) showed that while
the CO-4 and CO-5 enrichments had both attained a plateau in the number of retrievable OTUs, the CO-6 enrichment was still yielding new OTUs after 100 clones. However, additional sampling of 50 more clonal sequences from the CO-6 enrichment contributed only two new sequences as singletons (occurrence of 1 clone per OTU) and, given the repetition of previously found OTUs, reduced the slope of the curve (Fig. 2).

All of the 16S rRNA gene sequences retrieved from the CO-4, CO-5, and CO-6 enrichments belonged to the family Clostridiaceae (Fig. 3). The great majority of these sequences belonged to cluster III clostridia and were closely related to Clostridium straminisolvens, Clostridium stercorarium, and Clostridium clariflavum. However, differences in the distribution of sequences across clostridial clusters were also observed between enrichments. All OTUs from the CO-4 enrichment grouped within cluster III, as did 7 out of 8 OTUs from the CO-5 enrichment. However, not only did the CO-6 enrichment have the largest number of detected OTUs, but they were spread across clusters I, III, and XIV and a novel cluster of which the novel clostridium Lutispora thermophila (29) is the main representative.

FIG. 1. Dynamics of residual dry weight of cellulose and product formation (A, C, and E) and changes in pH and total pellet N levels (B, D, and F) for the CO-4 (A and B), CO-5 (C and D), and CO-6 (E and F) enrichment cultures during 3-g liter⁻¹ Avicel fermentations. (A, C, and E) Symbols: open squares, dry weight of cellulose; filled squares, acetate production; open circles, ethanol production; filled triangles, formate production. (B, D, and F) Symbols: open circles, changes in pH; filled squares, total pellet N. For the dry weight (of cellulose) and pellet N measurements, each data point represents the mean ± standard deviation calculated from triplicate samples.
Clostridium straminisolvens-like sequences were consistently found in all three enrichments, although in different proportions. The most abundant 16S rRNA gene sequences retrieved in the CO-5 and CO-6 enrichments accounted for 68% and 45% of each clone library, respectively, and were almost identical to Clostridium straminisolvens. On the other hand, the most abundant OTU in the CO-4 enrichment was most similar to Clostridium clariflavum (previously sp. EBR-02E-0045 [GenBank accession no. AB186359]), obtained from a thermophilic methanogenic reactor (28, 30). Under-scoring the differences between the CO-4 enrichment and the others, this particular sequence was not retrieved in the libraries constructed for the CO-5 and CO-6 enrichments.

In addition, a number of sequences grouped most closely to novel clostridia. Two OTUs from the CO-6 enrichment, representing 1% to 3% of this clone library each, were determined to be most similar to the newly isolated Lutispora thermophila, which is a noncellulolytic organism from a thermophilic methanogenic reactor (29). Likewise, a minor component of the CO-5 enrichment was most closely related to a proposed novel clostridium within cluster XIV, Clostridium islandicum (GenBank accession no. EF088328). A larger number of sequences in the CO-6 enrichment (representing 1% to 24% of this particular clone library) did not have any close cultured representatives, although they fell within cluster III and seemed most similar to C. stercorarium.

PCR amplification of glycosyl hydrolase family 48 gene sequences. Primers targeting a structural region essential to the function of family 48 glycosyl hydrolases were prepared based on translated amino acid sequences of C. thermocellum and C. cellulolyticum controls. This region includes the acidic residue responsible for protonating the glycosidic oxygen, identified as Glu87 in C. thermocellum CelS (10) and Glu55 in Clostridium cellulolyticum CelF (21); the Trp184, Asp255, and Tyr351 residues, associated with the water nucleophile involved in the catalytic mechanism of Clostridium thermocellum CelS (10); and the Asp230, Tyr299, Trp310, and Trp312 residues in the catalytic tunnel of C. cellulolyticum CelF (21).

An alignment of the PCR-amplified fragment with all the other sequences of GHF48 genes available in GenBank as of August 2009 and subsequent phylogenetic analyses revealed a grouping similar to the clustering observed in whole-gene alignments (Fig. 4). Sequences from the Fungi and the classes Clostridia, Actinobacteria, and Bacilli formed clear clusters, with the class Clostridia as the most divergent taxon. Known representatives of the Gammaproteobacteria (Hahella chejuensis) and the Chloroflexi (Herpetosiphon aurantiacus) each formed distinct, separate branches adjacent to the Actinobacteria cluster. A single exception to the class-based clustering was presented by the Cel48 sequence from Myxobacter sp. AL-1 (a deltaproteobacterium), which grouped with sequences from the Bacilli. It should also be noted that the GHF48 gene of Ruminococcus albus (cel48A) is distinctly different from those of all other glycosyl hydrolases from this family, including felloew clostridia and fellow member of the Ruminococcaceae family Bacteroides cellulosolvens.

Amplicons obtained from C. thermocellum ATCC 27405 and C. thermocellum DSM 1313 required further cloning in order to separate coamplified copies of the celY and celS genes prior to sequencing. For both strains, PCR amplification produced an even mixture (approximately 50:50) of the two genes, as observed in randomly selected clones used for amplification,
FIG. 3. Phylogenetic tree of 16S rRNA gene diversity in cellulolytic enrichments. Phylogenetic relationships were inferred by minimum evolution analysis of nucleotide sequences obtained from clone libraries from the CO-4 (open circles), CO-5 (filled squares), and CO6 (open triangles) enrichments. Bootstrap values are shown for 1,000 replicates. To indicate specific abundance, the percentage of each clone’s contribution to its library is specified in parentheses. The most abundant clones in each library are marked with asterisks.
FIG. 4. Phylogenetic tree of glycosyl hydrolase family 48 (GHF48) genes in cellulolytic enrichments. Phylogenetic relationships were inferred by minimum-evolution analysis of nucleotide sequences obtained from clone libraries from the CO-4 (filled circles), CO-5 (filled squares), and CO6 (open triangles) enrichments. Controls using type cultures sequenced in-house are labeled with filled diamonds. Bootstrap values are shown for 1,000 replicates. To indicate specific abundance, the percentage of each clone’s contribution to its library is specified in parentheses.
thus demonstrating the ability of these primers to amplify both cellulosomal (celS) and noncellulosomal (celY) copies of GHF48 genes present in the same organism.

**Diversity of family 48 glycosyl hydrolases in enrichments.**

All sequences retrieved from GHF48 gene libraries from enrichments belonged to the clostridia (Fig. 4). As a reference, we amplified the GHF48 genes in *Clostridium straminisovens* and *Clostridium clariflavum*, given their prevalence in 16S rRNA libraries. Two distinctly different sequences obtained from *C. straminisovens* were novel GHF48 gene fragment sequences, with 84% sequence similarity to *Clostridium thermocellum* celY and 89% sequence similarity to *Clostridium thermocellum* celS, respectively. Unlike *C. thermocellum*, where the celS/celY sequence retrieval ratio was close to 50:50, in *C. straminisovens* the ratio favored celY-like sequences, with a celS/celY sequence retrieval ratio close to 30:70. Similarly, dominant sequences in the CO-5 and CO-6 enrichments (representing 70% to 84% of the clone libraries) were most similar to the novel *Clostridium straminisovens* celY-like GHF48 gene reported here. The remaining GHF48 gene sequences from the CO-5 and CO-6 enrichments, accounting for 16% and 30% of each clone library, were almost identical to the celS-like gene identified in *C. straminisovens*. The dominant sequences from the CO-4 enrichment and a novel GHF48 gene sequence from *Clostridium clariflavum* grouped very closely together (>99% sequence similarity), with only 78% amino acid sequence similarity to their closest match found in *C. thermocellum* celY. Although they were retrieved in much lesser proportions, one sequence from the CO-4 enrichment was identical to *C. cellulolyticum* celF, and one was most closely related to *C. stercorarium* celY. Aside from the CO-4 GHF48 gene sequence that was identical to *C. cellulolyticum* celF, all the other GHF48 gene sequences reported here (including those of the *Clostridium straminisovens* and *Clostridium clariflavum* type cultures) represent novel cellulase genes.

**DISCUSSION**

Anaerobic enrichments from compost with cellulose as the sole carbon source facilitate the growth of consortia with various levels of community complexity and fermentation capabilities. By examining both the 16S rRNA and the glycosyl hydrolase family 48 gene composition, we have been able to characterize these communities and their dominant members at both the taxonomic and the functional level.

The breadth of 16S rRNA gene diversity in our enrichments, beyond the dominant sequences closely related to *Clostridium straminisovens* and *Clostridium clariflavum*, opens the question of what other functional roles are played by other clostridia within these communities. This is particularly true for enrichments resulting from multiple transfers such as those described here, where the presence of noncellulosomal clostridia has not been diluted after several transfers with cellulose as the sole carbon source. Of particular interest are the uncultivated members with moderately high abundances (such as 16S rRNA gene clone CO6-40), where the isolation of pure cultures from a mixed consortium would enable further understanding of the different functional roles within both simple and complex cellulosomal communities. Obtaining stable cellulosomal enrichments with various levels of diversity and their taxonomic characterization are the first steps toward this goal. Additionally, the development of a molecular marker targeting an essential functional gene for these enrichments, as is the case for glycosyl hydrolase family 48 genes, further expands the possibilities for future comparisons between communities and community members from a transcriptomic point of view. However, it should be noted that fermentation experiments revealed subtle yet important differences in product and cell biomass formation. The CO-4 enrichment, dominated by *C. clariflavum*-like sequences, produces comparatively larger amounts of formate with lower overall cell biomass production and is able to continue cellulose utilization beyond pH 7.0 to a final pH of 6.5. This is consistent with previous observations for *C. clariflavum* DSM 19732, where formate production is evident, in contrast to the findings for its closest relatives, *C. thermocellum* and *C. straminisovens* (30). On the other hand, the more diverse CO-5 (9 OTUs) and CO-6 (18 OTUs) enrichments are characterized by similar product profiles dominated by acetate and ethanol, higher cell biomass formation, and a final pH of 7.0, indicating that the *C. straminisovens* dominance, and not the total diversity or community structure, may be an important factor in the ability of these communities to utilize cellulose.

Glycosyl hydrolase family 48 genes appear to be promising targets as functional marker genes for environments in which active anaerobic cellulose degradation occurs. Our work confirms the consistency in GHF48 gene taxonomic grouping and demonstrates substantial scope for expanding known exoglucanase diversity. The phylogenetic clustering observed with the primers used in this study, as with whole GHF48 gene sequences, revealed clear taxonomic clustering for representative members of the Clostridia, Actinobacteria, Bacilli, Gammaproteobacteria, Chloroflexi, and Fungi (Fig. 4). The exceptional grouping of *Myxobacter* sp. AL-1, a deltaproteobacterium, with sequences from the Bacilli is interesting from an evolutionary point of view but is not unexpected. Similarities between *Myxobacter* sp. AL-1 and different members of the Bacilli (in particular Bacillus licheniformis), in terms of unique structural similarities in family 48 glycosyl hydrolases (25) but also in terms of sequence similarities of other types of cellulases (22), have been reported previously.

Analysis based on both 16S rRNA and GHF48 gene sequences indicated that *Clostridium straminisovens* and *Clostridium clariflavum*-like organisms were major components in the microbial communities present in the enrichments studied. This paper is the first report of *Clostridium straminisovens* and *Clostridium clariflavum* possessing glycosyl hydrolase family 48 genes, and *Clostridium straminisovens* is the first organism after *C. thermocellum* to possess two distinctly different glyco- hydrolase family 48 genes (NCBI accession no. GQ265349 and GQ487568). We also found that these genes are closely associated with the noncellulosomal celY gene and the cellulosomal celS gene in *C. thermocellum*. Given their close association with *C. thermocellum* counterparts, we speculate that the CelY-like cellulase in *C. straminisovens* may act as a non- cellulosomal exoglucanase in synergy with glycosyl hydrolase family 9 endoglucanases, as has been reported for *C. thermocellum* and *C. stercorarium* (2). Likewise, the presence of a CelY-like gene in *C. clariflavum* may indicate a similar mechanism. Further exploration of adjacent domains will be useful not only for determining the presence of cellulose-binding
domains and modes of action but also for gaining a better understanding of how conserved this family might actually be and whether horizontal gene transfer plays a role in the observed diversity in GHF48 genes from environmental strains.

A variety of novel GHF48 gene sequences have been retrieved from the cellulolytic enrichments described in this study. Aside from the dominant C. straminisolvens and C. clari-flavum sequences, two sequences associated with C. cellulolyti-
cum and C. stercorarium were also retrieved from cellulolytic enrichments and may belong to minor cellulolytic members of the community. It should, however, be noted that the mere presence of this gene does not necessarily confer functionality. For example, a GHF48 gene present in C. acetobutylicum, although it is expressed, does not confer cellulolytic activity on this organism (24).

With a variety of metagenomic studies addressing the diversity of glycosyl hydrolases in different environments (3, 9, 27), only one previous effort has targeted the diversity of cellulases beyond cultivated representatives by focusing on a specific family, describing the diversity and abundance of GHF5 in aquatic environments (8). More quantitative analyses of the relevance of GHF48 genes in cellulolytic microbial communities are needed, given the low abundance of these particular cellulases reported in metagenomic studies of complex cellu-
lyotic microbial communities (3), although it is possible that their presence in mostly single copies per genome has contributed to the lack of detection in metagenomic studies. Given their prevalence in transcriptomic and proteomic analyses of this organism (24), although it is expressed, does not confer cellulolytic activity on this organism (24).

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