

Do Rumen *Bacteroidetes* Utilize an Alternative Mechanism for Cellulose Degradation?

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ABSTRACT Uncultured and therefore uncharacterized *Bacteroidetes* lineages are ubiquitous in many natural ecosystems which specialize in lignocellulose degradation. However, their metabolic contribution remains mysterious, as well-studied cultured *Bacteroidetes* have been shown to degrade only soluble polysaccharides within the human distal gut and herbivore rumen. We have interrogated a reconstructed genome from an uncultured *Bacteroidetes* phylotype that dominates a switchgrass-associated community within the cow rumen. Importantly, this characterization effort has revealed the first preliminary evidence for polysaccharide utilization locus (PUL)-catalyzed conversion of cellulose. Based on these findings, we propose a further expansion of the PUL paradigm and the saccharolytic capacity of rumen *Bacteroidetes* species to include cellulose, the most abundant terrestrial polysaccharide on Earth. Moreover, the perspective of a cellulolytic PUL lays the foundation for PULs to be considered an alternative mechanism for cellulose degradation, next to cellulosomes and free-enzyme systems.

Uncultured *Bacteroidetes* lineages dominate many lignocellulose-degrading communities. A comprehensive understanding of how plant biomass deconstruction occurs in nature has far-reaching implications related to mammalian health and nutrition, as well as development of sustainable bio-based economies. Our current understanding is severely impeded by the inability to cultivate and thus examine the majority of microbes that perform the key metabolic processes of interest. For example, the rumen of herbivores represents one of nature's most proficient plant biomass-degrading ecosystems; however, it is controlled largely by uncharacterized microbes that belong to a limited number of frequently observed bacterial phyla (1). Degradation of the most abundant plant polysaccharide (cellulose) within ruminal ecosystems has for the most part been attributed to the metabolic capabilities of species affiliated with the bacterial phyla *Firmicutes* and *Fibrobacteres*. These species produce one or more well-known cellulases that are structurally assembled on the cell surface as a cellulosome or secreted as free enzymes (2). The ruminal *Bacteroidetes* represent another numerically dominating phylum; it is not associated with cellulose degradation, but its saccharolytic reputation is based on limited case studies of noncellulolytic *Prevotella* rumen isolates (3) and renowned culturable human gut representatives, such as *Bacteroides thetaiotaomicron* and *Bacteroides ovatus* (4). The saccharolytic machineries of gastrointestinal *Bacteroidetes* species have thus far been attributed to polysaccharide utilization loci (PULs), gene clusters that encode cell envelope-associated enzyme systems that enable the bacterium to respond to, bind, and degrade specific glycans and import released oligosaccharides (5).

The numerical predominance of uncultured *Bacteroidetes* species in lignocellulose-degrading ecosystems (1, 6) and the observed abundance and diversity of PUL-encoded carbohydrate-active enzymes within *Bacteroidetes* genomes suggest that there is much to learn about the contribution of these enzymatic complexes to polysaccharide metabolism. Here we propose an alternative hypothesis regarding cellulose degradation, which was generated by the biochemical characterization of a simplistic cellulase-encoding PUL previously annotated in a high-coverage uncultured *Bacteroidetes* phylotype (here referred to as AC2a) in-

herent to the cow rumen microbiome (6, 7). The gene organization of the AC2a PUL indicates a direct targeting of cellulose, which is unique for PULs that have been described and characterized to date (Fig. 1, and see Fig. S1 in the supplemental material).

EXPERIMENTAL RATIONALE

Biochemical characterization of a putative cellulolytic PUL within the uncultured AC2a *Bacteroidetes* phylotype. The AC2a draft genome sequence (~76% complete) was one of 16 genomes previously binned using tetranucleotide signatures from a switchgrass-degrading metagenome recovered from a cow's rumen (7). The high assembly coverage of the genome (284-fold coverage; third highest) indicated that AC2a is likely a numerically abundant organism in the rumen microbiome. Our own *de novo* predictions using Support Vector Machine classifiers (8) identified AC2a as a potential cellulolytic *Bacteroidetes* species, which challenges the current idea that *Bacteroidetes* drive only noncellulosic metabolism in the rumen. AC2a's cellulolytic capabilities were predicted to be dependent on a relatively simple eight-gene PUL encoding two putative cellulases (GH5 and GH9 of the glycoside hydrolase [GH] family) and a cellobiose phosphorylase (GH94) (Fig. 1A) (6). Sequence analysis and comparisons of gene organization with the model starch utilization system (Sus) of *B. thetaiotaomicron* led to identification of a SusC-like (TonB-dependent) outer membrane transporter, SusD-like and SusE-positioned lipoproteins that putatively bind to the substrate (9, 10), an inner membrane sugar transporter, and an inner membrane sensor (4) (Fig. 1). We predicted that GH5 and GH9 could degrade cellulose to cellobiose, which would be transported to the periplasm via the SusC-positioned transporter, where the well-

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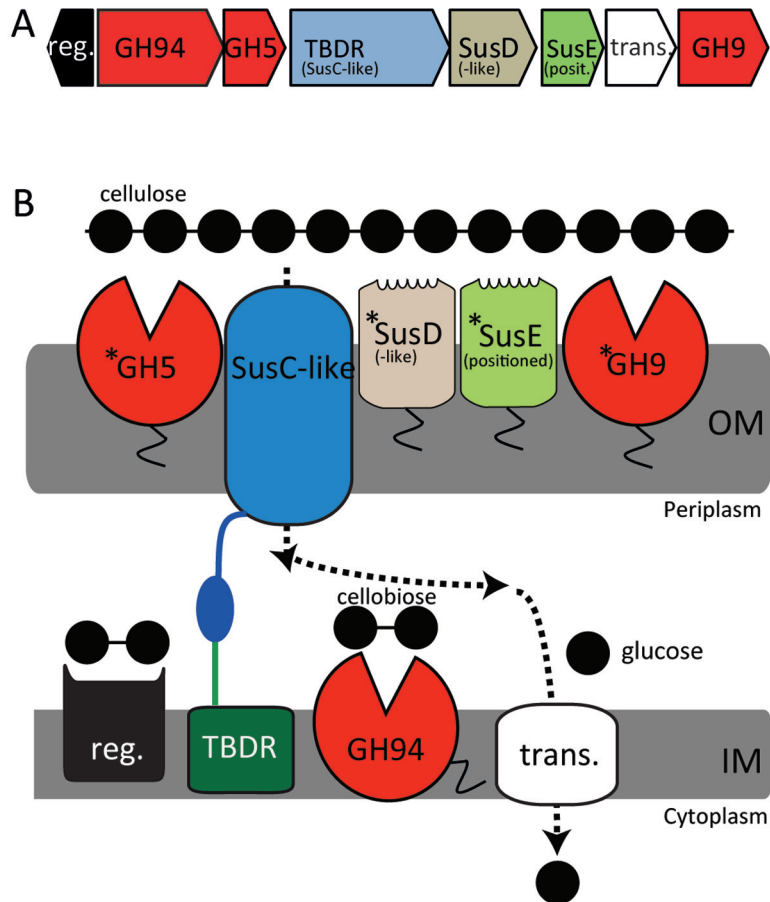


FIG 1 Putatively cellulolytic PUL recovered from the AC2a genome inherent to the cow rumen. (A) Gene organization of a cellulase (GH5 and GH9)-containing PUL identified in AC2a, which was selected for in-depth biochemical characterization. Gene identification numbers can be found in Table S2. (B) A hypothetical model, based on predicted protein locations and analogies to the model starch utilization system (Sus) of *B. thetaiotaomicron* (5), depicts a process in which glucans are bound and hydrolyzed via outer membrane lipoproteins and enzymes, whereas the generated cellobiose is transported to the periplasm, converted to glucose, and imported to the cytoplasm for cellular metabolism (see the text for more details). Proteins marked with an asterisk were subjected to biochemical characterization. Proteins marked with a “tail” are predicted to be membrane associated. The annotations of SusC (*TonB*-dependent receptor) and SusD are based on significant hits using Pfam, and these two proteins are therefore referred to as “SusC-like” and “SusD-like” in the main text. SusE is not recognized by Pfam, and its annotation is thus based on position only; this protein is referred to as “SusE positioned” (17). reg., regulator; trans., transporter; OM, outer membrane; IM, inner membrane; TBDR, *TonB*-dependent receptor.

known GH94 activity would generate monomeric sugars for transport into the cytoplasm (Fig. 1B). To test this prediction, we have biochemically characterized the GH5 and GH9 enzymes and determined the functionality of the putative SusD-like and SusE-positioned glycan-binding lipoproteins.

Initial screens with chromogenic substrates showed that the GH5 and GH9 glycoside hydrolases in the AC2a PUL are active on β -(1,4)-linked glucose units in amorphous cellulose and β -glucan (barley) (Fig. S2). The enzymes showed weak side activities on xyloglucan and xylans (Fig. S2) and were not active on β -(1,3) glucan (pachyman). These observations are consistent with the subfamily 4 classification of the GH5 enzyme (11), which typically encompasses extracellular bacterial enzymes that exhibit one or more activities categorized as endoglucanase, xyloglucan-specific endoglucanase, xylanase, and licheninase. Typically for endoglucanases, both GH5 and GH9 demonstrated higher activity on soluble cellulose and more highly accessible natural β -glucan substrates than recalcitrant crystalline cellulose (Table S1). The AC2a PUL is distinct from barley β -glucan PULs characterized from

Bacteroides cellulosilyticus (12) and *B. ovatus* (4) in that it does not contain GH3 [β -(1-3)-glucosidase] or GH16 [β -(1,3)-glucanase] (Fig. S1C). High-pH anion-exchange chromatography-pulsed amperometric detection (HPAEC-PAD) analysis demonstrated that GH5 hydrolysis of filter paper produced dimer and trimer cellooligosaccharides, whereas GH9 hydrolysis produced dimers and monomers (Fig. 2A). Interestingly, upon combination of the two enzymes, the filter paper was converted to dimers and monomers only (Fig. 2A), indicating synergism to produce cellobiose, which ultimately would be degraded by the periplasmic GH94 cellobiose phosphorylase. Further analysis of cellooligosaccharide (DP2-6) hydrolysis revealed that GH5 cannot degrade cellooligosaccharide and produces only cellobiose from cellooligosaccharide (Fig. S3). GH5 hydrolysis of cellooligosaccharide and cellooligosaccharide produced dimers and trimers. In contrast, GH9 degraded cellooligosaccharide and produced cellobiose and a small amount of glucose from DP4-6. This indicates that the two cellulases have different, complementary roles. The degradative effect of the AC2a PUL enzymes could easily be observed

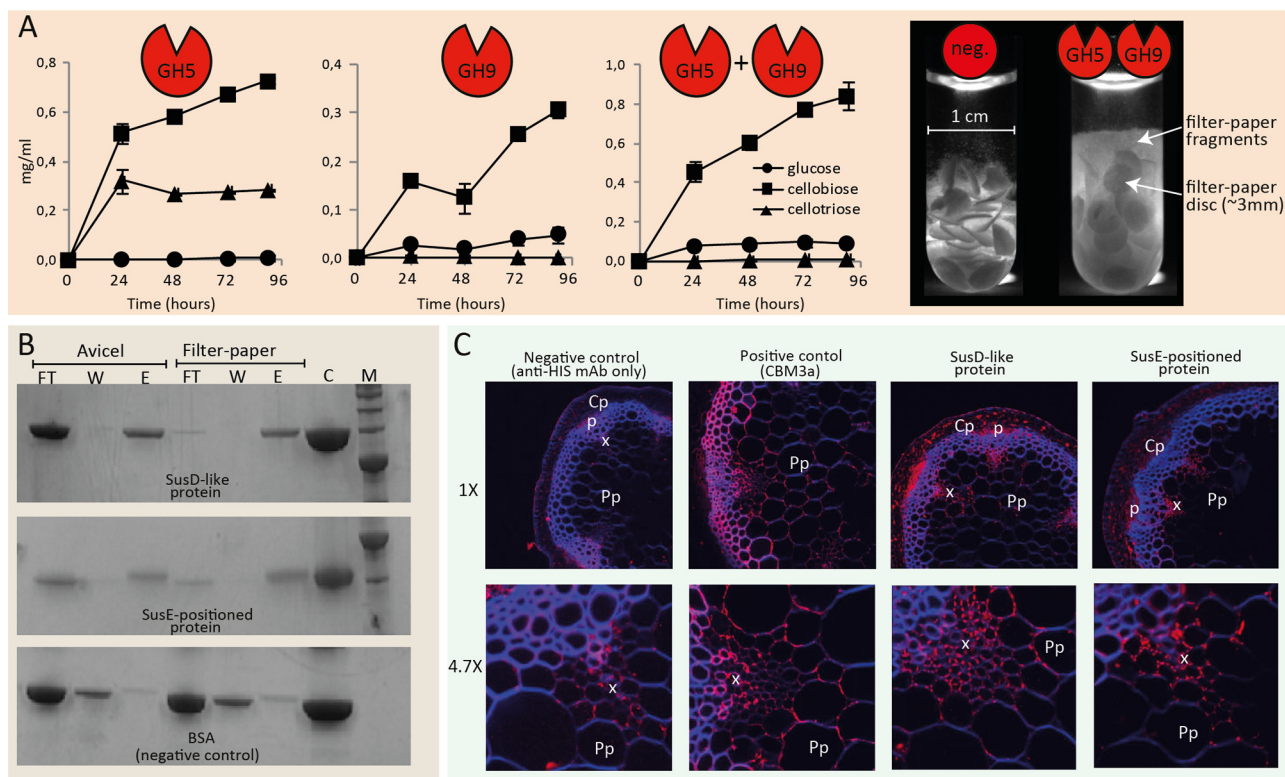


FIG 2 Biochemical characterization of cellulases and binding proteins encoded within the AC2a PUL. (A) Enzymatic activities of the GH5 and GH9 proteins determined by HPAEC-PAD analysis of products generated from filter paper (5%, wt/vol, 3 μ M total enzyme concentration, pH 6.6). Error bars represent standard deviations between results of three replicates. The image to the right visualizes partial solubilization of filter paper discs after a 6-day incubation (discs were diluted 3:1 prior to image capture). neg., negative. (B) SDS-PAGE analysis of fractions from pull-down assays using cellulosic substrates. Lanes: FT, unbound protein from supernatant fractions collected after a 1-h incubation and centrifugation; W, the wash fraction, containing protein washed off the substrate; E, eluted protein fractions where protein was released from the polysaccharides by boiling them in urea; C, control, where only the protein was loaded on the gel; M, a molecular weight standard. (C) Immunofluorescence labeling of *A. thaliana* cross sections using crystalline cellulose-binding CBM3a (positive control) as well as the SusD-like and SusE-positioned proteins from the AC2a cellulose-active PUL. Fluorescence from anti-His (in red) indicates bound protein, while autofluorescence, mainly in the interfascicular tissue, is blue. The SusD-like protein bound to cortical parenchyma (Cp)-, phloem (p)-, xylem (x)-, and pith parenchyma (Pp)-adjacent cell walls. The SusE-positioned protein showed weaker binding than the SusD-like protein and particularly targeted the intercellular junctions in the xylem tissue. The positive-control images were taken at lower gain due to high signals, while negative-control images were taken at higher gain. mAb, monoclonal antibody.

by monitoring the partial solubilization of filter paper discs (Fig. 2A).

Pull-down binding assays showed that SusD-like and SusE-positioned proteins from the AC2a PUL bind to crystalline cellulose (Fig. 2B) and also bind weakly to β -glucan (Fig. S4). To further visualize the ability of the SusD-like and SusE-positioned proteins to interact with plant cell walls, both proteins were used for indirect immunofluorescence labeling of *Arabidopsis thaliana* cross sections. The two proteins demonstrated clear binding to various sections of the plant cell walls, including xylem, phloem, and cortical parenchyma, with the SusE-positioned protein giving weaker signals than the SusD-like protein (Fig. 2C). CBM3a from *Clostridium thermocellum* (13) was included as a positive control and showed binding to cellulose-rich secondary cell walls (SCW) in the xylem and adhered to faces of adjacent pith parenchyma (PP) cells. The SusD-like protein bound to similar regions, whereas binding of the SusE-positioned protein appeared limited to the intercellular junctions of adjacent PP cells (Fig. 2C).

HYPOTHESIS

PULs represent an alternative mechanism for cellulose degradation, next to cellulosomes and free-enzyme systems. The insatiable

interest in the human gastrointestinal microbiome has provided detailed accounts of the diversity and mechanisms of PULs that are central to plant polysaccharide degradation. However, this understanding has been limited by a reliance on well-known cultivated *Bacteroidetes* species, which represent a significant minority in many saccharolytic ecosystems. By specifically targeting uncultured microbiota resident in the cow rumen with approaches that go beyond predictive annotation, we reveal a possible alternative mechanism for microbial cellulose degradation, which implies that rumen *Bacteroidetes* utilize PUL-based machinery, rather than (or in addition to) well-known mechanisms such as cellulosomes and free-enzyme systems. Broader genomic comparisons of the AC2a PUL with publicly available metagenomes and *Bacteroidetes* genomes identified sequence homology and synteny with a partial metagenomic fragment derived from the tamar wallaby foregut, a marsupial herbivore whose diet is rich in lignocellulose (14) (Fig. S1A). Partial synteny was observed with the “core” components of a well-characterized xyloglucan PUL that encodes both GH5 and GH9 representatives but which targets only xyloglucans, while lacking activity against any other hemicellulose or cellulose substrate (Fig. S1A) (15). Closer inspec-

tion of the proteins occurring in both these PULs revealed low sequence similarity (Fig. S1A) as well as different Pfam-predicted domain organizations for GH5 (AC2a lacking the BACON [*Bacteroidetes*-associated carbohydrate-binding often N-terminal] domain at the N terminus) and the SusD-like lipoprotein (AC2a with PF12771, BACOVA_02651; PF14322/PF07980). While degrading activity for soluble cellulose-analogues has been described for several endoglucanases encoded within large hemicellulosic PULs, these enzymes are devoid of activity on recalcitrant cellulose and the PULs in question bear no resemblance to the AC2a PUL (Fig. S1B) (6, 14). Interestingly the SusD-like lipoprotein from the AC2a PUL exhibited very low sequence identity to two cellulose-binding SusD-like representatives that we previously characterized from a hemicellulose-degrading PUL reconstructed from an uncultured phylotype (both exhibited less than 23% alignment coverage and 31% sequence identity) (10). This suggests that functional differences cannot necessarily be detected by the binding assays done in the present and in past studies.

Collectively, these findings expand current perceptions regarding the overall saccharolytic capacity of rumen *Bacteroidetes* affiliates, which so far have been coupled only to the degradation of noncellulosic polysaccharides. Furthermore, it adds the Earth's most abundant organic polymer to an already impressive catalogue of PUL target substrates, including starch, alginate, various hemicelluloses, and host mucin glycans (3, 4, 15–17). To conclusively determine that AC2a is indeed capable of sustaining cell metabolism and growth on recalcitrant cellulosic substrates, knockout mutagenesis studies of pure culture representatives are required. While isolating deeply branched novel affiliates of the *Bacteroidetes* has proved extremely difficult in herbivore microbiomes, the ability to mine the AC2a genome for growth requirements provides a unique opportunity to reconstruct a custom enrichment medium and isolation strategy. Similar metagenome-directed isolation approaches have ultimately proved successful for gut microbiomes in the past (18) and form the basis of our ongoing efforts.

PROCEDURES

Gene annotation of the AC2a genome. The AC2a genome was previously reconstructed from metagenome sequencing data generated from the microbiota in a cow's rumen (pH 7.0) (7). Assembled and unprocessed DNA reads previously assigned to AC2a based on tetranucleotide frequencies were retrieved from <http://portal.nersc.gov/project/jgim/CowRumenRawData/submission/> and annotated via the RAST server (19). The cellular localization of proteins was predicted using PSORTb 3.0 (20) and LipoP 1.0 (21).

Heterologous expression and purification of enzymes. Genes encoding signal peptide-free versions of AC2a GH5, GH9, SusD-like, and SusE-positioned proteins were synthesized and cloned into the pNIC-CH expression vector by ligation-independent cloning (LIC) using the primers listed in Table S2 in the supplemental material (22). Transformants were verified by sequencing. *Escherichia coli* BL21 harboring the plasmids was precultured for 8 h in Luria-Bertani broth and inoculated to 1% in an overnight culture at 18°C. Expression was induced by adding isopropyl- β -D-thiogalactopyranoside (IPTG) to a final concentration of 0.75 mM at an optical density at 600 nm (OD_{600}) of 0.5 to 1.0, followed by incubation for 24 h at 18°C. Cells were harvested by centrifugation (5,000 rpm, 10 min) and resuspended in lysis buf-

fer (100 mM Tris-HCl, pH 8.0, 500 mM NaCl, 5 mM imidazole, 0.1 mg/ml lysozyme) before a 30-min incubation on ice. Cells were disrupted by pulsed sonication, and debris was removed by centrifugation ($8,000 \times g$, 10 min), with the supernatant filtered using 0.45- and 0.22- μ m syringe filters. Proteins were loaded onto 5-ml HisTrap HP Ni Sepharose columns (GE Healthcare) and eluted with a linear gradient of 100 mM Tris-HCl, pH 8.0, 500 mM NaCl, 500 mM imidazole. The eluted fractions were concentrated, and the buffer was changed to 100 mM Tris-HCl, pH 8.0, using Sartorius Vivaspinn concentrators with a 10-kDa cutoff. Further purification steps were performed using ion-exchange chromatography (GH5, GH9, and SusD-like proteins) with a 5-ml HiTrap diethylaminoethyl (DEAE) fast-flow (FF) column (GE Healthcare) and gel filtration (HiLoad Superdex 75; GE Healthcare) in 50 mM Tris-HCl with 200 mM NaCl (SusE). Proteins were concentrated, and the buffer was changed to 10 mM Tris-HCl, pH 7.5. Protein purity was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and protein concentration was estimated by measuring the A_{280} and using the proteins' molar extinction coefficients.

Chromogenic substrates. Azurine cross-linked labeled (AZCL) substrates (Table S3) partly dissolved in isopropanol (10 mg/ml) were added to 135 μ l buffer (50 mM potassium phosphate, pH 7.5, or the positive control's preferred pH). Plates were sealed with adhesive PCR plate seals (Thermo Scientific; AB-0558) and incubated with overhead rotation (~ 20 rpm, room temperature) for 1 h. Plates were spun down (4,000 rpm, 10 min), and the absorption of the filtrate was measured against that of negative controls at 590 nm. Values reported are relative absorbance values calculated against the absorbance values of the positive controls listed in Table S3.

Enzymatic assays. The optimum pH was determined to be approximately 6.6 for both enzymes, and 20 mM Bis-Tris, pH 6.6, was used for all enzyme assays. Enzyme activities were determined for carboxymethyl-cellulose (CMC) (Sigma-Aldrich), filter paper (Whatman no. 1), Avicel (Sigma-Aldrich), and barley β -glucan (Megazyme). CMC (1%, wt/vol) and β -glucan (0.5%, wt/vol) were incubated at 40°C (900-rpm horizontal shaking) with 25 nM and 10 nM GH5, respectively, for 10 min in a total volume of 500 μ l. The reactions were stopped by adding an equal amount of 3,5-dinitrosalicylic acid (DNS) reagent, and the amounts of reducing sugars relative to a glucose standard curve were determined using the DNS assay (23). A unit of enzyme activity was defined as the amount of enzyme releasing 1 μ mol of reducing sugars per minute. For GH9, the enzyme concentration was increased to 100 nM, and the incubation time was 15 min. For filter paper and Avicel, the conditions were 1% substrate (wt/vol) and 100 nM GH5 or GH9, with an incubation time of 30 min. The reactions were stopped by boiling the mixtures (5 min) before soluble cellodextrins were quantified by HPAEC-PAD as described below. A unit of enzyme activity was defined as the amount of enzyme releasing 1 μ mol of soluble products per minute. The time course analysis of degradation of filter paper was performed using a 5% (wt/vol) concentration of the substrate and 3 μ M enzyme (GH5 plus GH9, 1.5 plus 1.5 μ M enzyme). Soluble cellodextrin products were quantified against a standard curve of cellodextrins (DP1-3) by HPAEC-PAD using a Dionex ICS-3000 system with a CarboPac PA1 column at 0.25 ml/min and 0.1 M NaOH. Oligosaccharides were eluted in a multistep linear gradient going from 0.1 M NaOH to 0.1 M NaOH-0.1 M sodium ace-

tate (NaOAc) in 10 min, to 0.1 M NaOH-0.18 M NaOAc in 8 min, to 0.1 M NaOH-0.3 M NaOAc in 1 min, and to 0.1 M NaOH-1.0 M NaOAc in 1 min, before column reconditioning by 0.1 M NaOH for 14 min. Visual assessment of the degradation of filter paper discs was performed using the same conditions as described above in glass tubes in a total volume of 1 ml, with 0.8 U/ml β -glucosidase (Megazyme) added to avoid potential cellobiose inhibition.

Binding assays. Filter paper (Whatman no. 1) milled to a 0.5-mm size, Avicel (Sigma-Aldrich), and the insoluble fraction (room temperature) of barley β -glucan (Megazyme) were washed twice in MES (morpholineethanesulfonic acid) buffer (20 mM, pH 6.0), suspended to 6% (wt/vol) in a total volume of 200 μ l along with 0.1 mg/ml protein, and incubated at 40°C with horizontal shaking (900 rpm). The substrate and bound protein were pelleted by centrifugation, and the supernatant containing unbound protein (referred to as the flowthrough) was carefully removed. The pellet was washed with 200 μ l buffer for 15 min, and the supernatant was again removed by centrifugation. To elute the proteins, the pellets were resuspended in 200 μ l 8 M urea and boiled for 10 min (filter paper and Avicel) or incubated with 200 μ l 2% SDS and incubated with shaking for 10 min (β -glucan). The flowthrough, wash, and elution fractions were analyzed by SDS-PAGE.

Binding to plant material was tested by probing transverse sections through *Arabidopsis thaliana* stems. Hand-cut sections through the stems of 4- to 5-week-old plants were labeled using a His₆ tag-based three-stage procedure essentially as previously described (24), in which binding was detected using a fluorescein isothiocyanate-conjugated tertiary antibody. Cellulose-binding CBM3a from *Clostridium thermocellum* (13) was included as a positive control.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01401-14/-/DCSupplemental>.

Figure S1, PDF file, 0.1 MB.
Figure S2, PDF file, 0.1 MB.
Figure S3, PDF file, 0.2 MB.
Figure S4, PDF file, 0.1 MB.
Table S1, PDF file, 0.1 MB.
Table S2, PDF file, 0.1 MB.
Table S3, PDF file, 0.1 MB.

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