Role of the CipA Scaffoldin Protein in Cellulose Solubilization, as Determined by Targeted Gene Deletion and Complementation in *Clostridium thermocellum*

Daniel G. Olson, a,d Richard J. Giannone, b,d Robert L. Hettich, b,d Lee R. Lynd a,b,c,d

Dartmouth College, Hanover, New Hampshire, USA; Oak Ridge National Laboratory, Oak Ridge, Tennessee, USA; Mascoma Corporation, Lebanon, New Hampshire, USA; BioEnergy Science Center, Oak Ridge, Tennessee, USA

The CipA scaffoldin protein plays a key role in the *Clostridium thermocellum* cellulosome. Previous studies have revealed that mutants deficient in binding or solubilizing cellulose also exhibit reduced expression of CipA. To confirm that CipA is, in fact, necessary for rapid solubilization of crystalline cellulose, the gene was deleted from the chromosome using targeted gene deletion technologies. The CipA deletion mutant exhibited a 100-fold reduction in cellulose solubilization rate, although it was eventually able to solubilize 80% of the 5 g/liter cellulose initially present. The deletion mutant was complemented by a copy of *cipA* expressed from a replicating plasmid. In this strain, Avicelase activity was restored, although the rate was 2-fold lower than that in the wild type and the duration of the lag phase was increased. The *cipA* coding sequence is located at the beginning of a gene cluster containing several other genes thought to be responsible for the structural organization of the cellulosome, including *olpB*, *orf2p*, and *olpA*. Tandem mass spectrometry revealed a 10-fold reduction in the expression of *olpB*, which may explain the lower growth rate. This deletion experiment adds further evidence that CipA plays a key role in cellulose solubilization by *C. thermocellum*, and it raises interesting questions about the differential roles of the anchor scaffoldin proteins OlpB, Orf2p, and SdbA.

*Clostridium thermocellum* is an anaerobic thermophilic bacterium noted for its ability to rapidly solubilize crystalline cellulose, a process mediated by the cellulosome (1). The cellulosome is composed of tightly bound enzymatic and structural components. At the heart of the cellulosome is the scaffoldin protein, CipA (also known as S1 and S1) (2). This protein has been shown to be capable of crystalline cellulose solubilization in conjunction with cellulosomal cellulase Cel48S (3). Analysis of the DNA sequence of *cipA* has revealed a set of nine repeated elements known as type I cohesins (4). These cohesins bind to the type I dockerins found on cellulosomal enzymes (5). Subsequent analysis of the CipA protein has revealed three additional modules, the type II dockerin, the carbohydrate binding module (CBM), and the x domain.

The type II dockerin comprises a duplicated set of 22 amino acid residues located near the C terminus of CipA (4). The type II dockerin binds to type II cohesins located on the anchor scaffoldin proteins, OlpB, Orf2p, and SdbA. OlpB has seven type II cohesins, while Orf2p has two and SdbA has one. The anchor scaffoldins have a C-terminal sequence called the S-layer homology (SLH) domain that mediates binding to the cell surface (6). In CipA the CBM is located between the second and third type I cohesins and binds to crystalline cellulose with a $K_D$ (equilibrium dissociation constant) of 0.4 $\mu M$ (1). Thus, the current understanding of the adhesion of *C. thermocellum* to cellulose involves the following 3 interactions: (i) binding of glycoside hydrolyase enzymes in proximity to each other to promote enzyme–enzyme synergy; (ii) binding of enzymes to the cellulosic substrate via the CBM; and (iii) anchoring of the cellulosome to the cell surface, where CipA binds to the anchor scaffoldin (OlpB, Orf2p, or SdbA) via its type II dockerin and the anchor scaffoldins are attached to the cell by their SLH domains.

Finally, CipA has one additional module, located between the ninth type I cohesin and the type II dockerin, called the x module. Its function in *C. thermocellum* remains unknown, although it has been shown to improve the solubility of recombinantly expressed type II dockerins and seems to enhance the affinity of the type II cohesin-dockerin interaction (7).

Electron microscopy has revealed hemispherical protuberances on the outside of *C. thermocellum* cells, which are known as polycellulosomes (8). In their resting state they are about 200 nm in diameter, but they form a protracted conformation in the presence of cellulose (9). Immunolabeling has identified the presence of CipA (10) and OlpB (6) in the polycellulosomes, though the protuberances may contain other cellulosomal components as well.

There have been two previous reports of mutants of *C. thermocellum* deficient in cellulase activity. Both were isolated by screening for cells unable to adhere to cellulose. *C. thermocellum* AD2 was isolated by mixing cells with cellulose and allowing the cellulose to settle. Adherent cells were pulled out of solution upon binding to cellulose, thus enriching the supernatant for nonadherent cells. After five rounds of this sedimentation enrichment, strain AD2 was isolated by single-colony purification (11). The AD2 strain was analyzed by SDS-PAGE and found to be missing a band associated with CipA when grown on cellulbiose, although...
the band reappeared when the strain was grown on cellulose (8). Further analysis of AD2 by scanning electron microscopy revealed the complete absence of polycellulosomes when the strain was grown on cellobiose (12).

Strains SM1, SM4, SM5, and SM6, which also are deficient in cellulase activity, were isolated using a procedure similar to that used for strain AD2, though augmented by an initial chemical mutagenesis step followed by a screen on cellobiose plates with an Avicel overlay (13). This final screen was designed to identify cells that were deficient in cellulose solubilization. These mutants were analyzed by SDS-PAGE, and all were found to be missing a 210-kDa band associated with CipA. DNA sequence analysis revealed the presence of an IS\textsubscript{1447} insertion element disrupting the \textit{cipA} coding sequence in each mutant. Strain SM1 had an insertion in the first type I cohesin and appeared to be completely lacking functional type I cohesins. This strain was unable to grow on MN300 cellulose and exhibited a 15-fold reduction in enzymatic activity compared to the wild-type (WT) strain (13).

Previous work has shown that the abilities to bind and to solubilize cellulose are linked (11, 13). Thus, mutants deficient in cellulose binding are also deficient in cellulose solubilization. The functional link between these abilities is consistent with our understanding of the component modules of CipA. In this study, we further evaluated the extent to which \textit{cipA} is responsible for this dysfunctional phenotype and explored the cellulase activity of \textit{C. thermocellum} in the absence of a complexed cellulase system.

**MATERIALS AND METHODS**

**Strains and media.** All \textit{C. thermocellum} strains described here are derived from \textit{C. thermocellum} strain DSM 1313 and were grown in modified DSM 122 broth as described previously (14). Cellobiose or Avicel-PH105 microcrystalline cellulose (Sigma-Aldrich) was used as the primary carbon source at a concentration of either 5 or 10 g/liter. Cells were grown at 55°C. Strain M1354 was a generous gift from the Mascoma Corporation (Lebanon, NH) (15). This strain is derived from \textit{C. thermocellum} strain DSM 1313 and has a deletion of the \textit{hpt} gene (Clo1313_2927) to allow for use of the \textit{hpt} gene as a counterselectable marker with the antimetabolite 8-aza-hypoxanthine.

**Molecular biological methods.** Plasmids were constructed using yeast-mediated ligation (16), In-Fusion PCR cloning (TaKaRa Bio Inc.), or standard cloning techniques (17). Plasmids were maintained in \textit{Escherichia coli} TOP10 cells (Invitrogen Corporation) and prepared using the Qiagen plasmid minikit (Qiagen Inc.). Sequences of chromosomal DNA were obtained by PCR using genomic DNA from \textit{C. thermocellum} strain DSM 1313. Primers were designed using genome sequences provided by the Joint Genome Institute (http://www.jgi.doe.gov/). The \textit{repB} and \textit{cat} genes are derived from plasmid pMU102 (18). The \textit{hpt} gene is derived from plasmid pMQ87 (16). The pMB1 \textit{E. coli} origin of replication is derived from plasmid pMQ87 (16). The pMB1 \textit{E. coli} origin of replication is derived from plasmid pUG19 (Invitrogen Corp.). The p15A \textit{E. coli} origin of replication and arabino-lose-inducible promoter are derived from plasmid pBAD30 (19). The \textit{hpt} and \textit{tdk} genes (Tsac_0936 and Tsac_0324, respectively) are derived from \textit{Thermoanaerobacterium saccharolyticum} JW/SL-YS485. The glyceraldehyde 3-phosphate dehydrogenase promoter consists of the 525-bp region upstream of the \textit{C. thermocellum} glyceraldehyde 3-phosphate dehydrogenase gene (Clo1313_2095). The \textit{cbp} promoter consists of the 621-bp region upstream of the cellobiose phosphorylase gene (Clo1313_1954).

Plasmid pDGO-37 (GenBank accession number JX966413) was created by combining the p15A \textit{E. coli} origin of replication and Phad promoter with the thermophilic Gram-positive origin of replication from plasmid pMU102. Plasmid pDGO-40 (GenBank accession number JX966414) was created by inserting the \textit{cipA} coding sequence, including 819 bp upstream of the start codon (putative promoter region) and 67 bp downstream of the stop codon (putative terminator region), into plasmid pDGO-37 (Fig. 1; Table 1).

PCR was performed using either Taq or Phusion DNA polymerase.
(New England BioLabs Inc.) according to the directions provided by the manufacturer. When using whole cells as the PCR template, a 10-min heating step was included at the beginning of the thermocycling protocol to lyse the cells. When using Taq DNA polymerase, the lysing temperature was 95°C. When using Phusion DNA polymerase, the lysing temperature was 98°C. DNA sequencing was performed using standard techniques with an ABI model 3100 genetic analyzer (Applied Biosystems).

**Fermentation conditions.** Strains were grown in modified DSM 122 broth (18) at 55°C with cellobiose or Avicel microcrystalline cellulose as the primary carbon source. When fermentations were performed in a 125-ml glass bottle sealed with a butyl rubber stopper (22), the fermentation volume was 50 ml, 5 g/liter substrate (Avicel or cellobiose) was used, the headspace was purged with nitrogen, and the bottles were shaken at 200 rpm. When fermentations were performed in a computer-controlled fermentor (Sartorius GmbH), the fermentation volume was 2 liters, 10 g/liter substrate (Avicel or cellobiose) was used, the headspace was purged with a mixture of 20% CO₂ and 80% N₂, the vessel was stirred at 200 rpm, and the pH was controlled to 7.0 with 4 N potassium hydroxide. For some fermentations, an automated sampling device was used to take 6-ml samples at regular intervals (23).

**Analytical techniques.** Concentrations of cellobiose, glucose, lactate, acetate, ethanol, and formate were measured by high-performance liquid chromatography (HPLC) as previously described (24). Total carbon and total nitrogen concentrations (TOC) were measured with a Shimadzu TOC-V CPH elemental analyzer with TNN-M and ASI-V modules (Shimadzu Corp.) on 0.5- to 1.0-ml aliquots washed twice with water. The Avicel concentration was determined from these measurements by as-

### TABLE 1 Description of *C. thermocellum* strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Chromosomal genetic elements</th>
<th>Plasmid genetic elements</th>
<th>Genotype</th>
<th>Source or reference</th>
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<tbody>
<tr>
<td>DSM1313 (WT)</td>
<td>+</td>
<td>+</td>
<td>Wild type</td>
<td>DSMZ²</td>
</tr>
<tr>
<td>M1354</td>
<td>+</td>
<td>+</td>
<td>Δhpt</td>
<td>15</td>
</tr>
<tr>
<td>DS11</td>
<td>+</td>
<td></td>
<td>M1354 ΔcipA</td>
<td>20</td>
</tr>
<tr>
<td>DS16</td>
<td>+</td>
<td>+</td>
<td>M1354 Δ(cipA-cipA)</td>
<td>20</td>
</tr>
<tr>
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<td></td>
<td>+</td>
<td>DS16/pDGO-37</td>
<td>This study</td>
</tr>
<tr>
<td>DS19</td>
<td>+</td>
<td>+</td>
<td>DS16/pDGO-40</td>
<td>This study</td>
</tr>
<tr>
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<td>+</td>
<td>+</td>
<td>DS16/pDGO-40</td>
<td>This study</td>
</tr>
<tr>
<td>DS22</td>
<td>+</td>
<td>+</td>
<td>DS16/pDGO-40</td>
<td>This study</td>
</tr>
</tbody>
</table>

² DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany.

Mathematical analysis of fermentation data. To determine the rate of substrate consumption, the substrate consumption data points were fitted with the 5-parameter sigmoidal Richards equation (30) per the work of Holwerda and Lynd (unpublished data):

$$s(t) = A_0 + \frac{A_t - A_0}{1 + e^{(\frac{t-x_0}{\text{asym}}})}}$$

where $A_0$ is the lower horizontal asymptote, $A_t$ is the higher horizontal asymptote, $t$ is time, $x_0$ is the inflection point, slope is the slope at the inflection point, and asymm is the asymmetry parameter. The time $t$
cellobiose, the substrate consumption rate was determined in mM glucose substrate consumption. To allow ready comparison between Avicel and at the time determined by equation 2 to determine the maximum rate of The first derivative of equation 1 with respect to time was then evaluated deviation and were determined based on biological replicates, where $n \geq 2$. *, due to the difficulties of growing strains DS11 and DS16 on Avicel in fermentors, they were grown in sealed glass bottles instead.

when the slope of the fitted curve was greatest was determined by taking the second derivative with respect to time, setting it equal to zero, and solving for $t$, which yielded the following equation:

$$t_{\text{max slope}} = x_0 \ln(\text{asymm}) \ast \text{slope} \quad (2)$$

The first derivative of equation 1 with respect to time was then evaluated at the time determined by equation 2 to determine the maximum rate of substrate consumption. To allow ready comparison between Avicel and cellobiose, the substrate consumption rate was determined in mM glucose equivalent/hour.

RESULTS

Comparison of growth rates of various mutants. As expected, the wild-type (WT) strain and the $cipA$ deletion strains (DS11 and DS16) have similar substrate consumption rates when grown on either cellobiose (cb) or Avicel (Av) at an initial concentration of 5 or 10 g/liter. Antibiotic selection was used to maintain the plasmid in plasmid-containing strains. The presence of the $cipA$ coding sequence is indicated as either chromosomal (C), plasmid based (P), or both. Error bars represent one standard deviation and were determined based on biological replicates, where $n \geq 2$. * , due to the difficulties of growing strains DS11 and DS16 on Avicel in fermentors, they were grown in sealed glass bottles instead.

TABLE 1

<table>
<thead>
<tr>
<th>strain</th>
<th>substrate</th>
<th>amount (g/l)</th>
<th>antibiotic</th>
<th>plasmid</th>
<th>$cipA$</th>
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<tbody>
<tr>
<td>WT</td>
<td>cb</td>
<td>10</td>
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<td>WT</td>
<td>Av</td>
<td>10</td>
<td>yes</td>
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<td>yes</td>
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</table>

FIG 2 Strain consumption rates for strains of C. thermocellum grown on either cellobiose (cb) or Avicel (Av) at an initial concentration of 5 or 10 g/liter. The metabolic burdens of plasmid maintenance and thiamphenicol inactivation do not have an effect on the wild-type (WT) strain and the $cipA$ deletion strains (DS11 and DS16) have similar substrate consumption rates when grown on either cellobiose (cb) or Avicel (Av) at an initial concentration of 5 or 10 g/liter. Antibiotic selection was used to maintain the plasmid in plasmid-containing strains. The presence of the $cipA$ coding sequence is indicated as either chromosomal (C), plasmid based (P), or both. Error bars represent one standard deviation and were determined based on biological replicates, where $n \geq 2$. *, due to the difficulties of growing strains DS11 and DS16 on Avicel in fermentors, they were grown in sealed glass bottles instead.

The first derivative of equation 1 with respect to time was then evaluated at the time determined by equation 2 to determine the maximum rate of substrate consumption. To allow ready comparison between Avicel and cellobiose, the substrate consumption rate was determined in mM glucose equivalent/hour.

RESULTS

Comparison of growth rates of various mutants. As expected, the wild-type (WT) strain and the $cipA$ deletion strains (DS11 and DS16) have similar substrate consumption rates when grown on either cellobiose (Fig. 2). The metabolic burdens of plasmid maintenance and thiamphenicol inactivation do not have an effect on the Avicel consumption, as can be seen by comparing the wild type to the empty-vector control (strain DS19) (Fig. 2). The effect of $cipA$ overexpression can be seen by comparing the empty-vector control (strain DS19) with the $cipA$ overexpression strain (strain DS22) (Fig. 2, second column). Among the cohesin-containing proteins, only cel9P is significantly differentially expressed. Among the dockerin-containing proteins, only Cel9P is significantly differentially expressed. Among the other CAZy proteins, only licA is significantly differentially expressed.

The effect of $cipA$ overexpression is demonstrated by comparing the empty-vector control (strain DS19) with the $cipA$ overexpression strain (strain DS22) (Fig. 4, second column). Among the cohesin-containing proteins, none of them were significantly different at a $P$ value of 0.01. Although the increase in $cipA$ expression was not significant at the 0.01 level ($P = 0.015$), when the values for the wild type are included as well, the significance increases to 0.0002, suggesting that the effect would likely be confirmed if we were to perform more replicates. $cipA$ expression increased by 3-fold in strain DS22, although this was not significantly different from the mean $cipA$ expression in the empty-vector control (strain DS19) at the 0.01 level ($P = 0.015$). Among the dockerin-containing enzymes, none were significantly differentially expressed at the 0.01 level. Among the other CAZy proteins, LicA and Clo1313_0647 (CBM16, domain of unknown function) were significantly lower in abundance.

The effect of $cipA$ complementation can be determined by comparing the empty-vector control (strain DS19) with the complemented $cipA$ deletion strain (DS20) (Fig. 4, third column). Among the cohesin-containing proteins, OlpB showed significantly reduced expression and was 11-fold less abundant in the complemented deletion strain. Among dockerin-containing proteins, Cel9P and Clo1313_2861 (GH2-CBM6) showed increased expression. The significance of the Clo1313_2861 result is difficult to interpret because of the low number of spectra identified for this protein ($\leq 7$ for all samples). Among the other CAZy proteins, Clo1313_2460 (GH15) showed significantly increased abundance.

Since OlpB contains 7 type II cohesins, a dramatic decrease in OlpB levels could result in a decrease in type II cohesin availability. Type II cohesin availability was calculated by multiplying the...
abundance of each anchor scaffoldin (SdbA, Orf2p, and OlpB) by the number of type II cohesins it contains (Table 2).

**DISCUSSION**

In agreement with Zverlov et al., we have shown that *cipA* is essential for rapid solubilization of crystalline cellulose (13). However, contrary to what was reported previously, we observed that *cipA* deletion strains are able to solubilize Avicel microcrystalline cellulose (which is similar to the MN300 microcrystalline cellulose used by Zverlov et al.). Furthermore, since the solubilization of Avicel resulted in the production of lactate, acetate, and ethanol and this ability was maintained despite serial transfer, it appears that the strain was able to grow on Avicel. What is the explanation for residual ability of the *cipA* deletion strains (DS11 and DS16) to solubilize crystalline cellulose? One possibility is that components of the noncomplexed cellulase system (i.e., CelI and CelY), which have been shown to synergistically solubilize crystalline cellulose (31), can compensate for the expected loss of activity. Since each enzyme has its own CBM, there is no need for CipA to mediate binding with the cellulosic substrate. These enzymes were found at very low levels in all strains (<0.01% of cell protein) as determined by nSpC values (see Data Set S1 in the supplemental material), which reduces support for this explanation. Another possibility is that the cellulosomal components are bound directly to the cell surface via OlpA, which contains both a type I cohesin (for binding a cellulase enzyme containing a type I dockerin) and an S-layer homology (SLH) binding domain (for binding to the cell surface). Levels of OlpA were about 40% higher in the complemented *cipA* deletion strain (DS20) than in the empty-vector control (DS19), which supports this hypothesis.

Why does the *cipA* deletion and complementation strain (DS20) grow more slowly than the wild-type strain and have a longer lag phase? *cipA* expression does not seem to be a likely explanation, since minor variations in CipA abundance do not appear to be correlated with growth rate (Fig. 5). On the other hand, OlpB levels were unexpectedly low in this strain. Compared to the empty-vector control strain (DS19), the *cipA* deletion and complementation strain (DS20) had 30% fewer type II cohesins, since it seems to have partly compensated for the reduction in *olpB* expression with higher levels of *sdbA* and *orf2p* expression. Furthermore, the wild-type strain and the *cipA* overexpression strain (DS22) both had 15% fewer type II cohesins, and this change in type II cohesin number did not have a substantial effect on fermentation performance; thus, it seems unlikely that the change in type II cohesin number is the full explanation. Another possibility is that the anchor scaffoldins (OlpB, Orf2p, and SdbA) are not, in fact, interchangeable. For example, if Orf2p and SdbA are primarily used during cellulosome assembly (as has been suggested for ORFXp in *Clostridium cellulolyticum* [32]) and OlpB is the final destination for the assembled cellulosome, then a change in the abundance of OlpB might have a greater impact on cellulosome function than would be indicated simply by the overall change in type II cohesin number.

**FIG 3** Avicel consumption by 4 strains of *C. thermocellum* growing on 10 g/liter Avicel. In order to allow subsequent comparison with growth rates on cellobiose, the rate was reported in mM glucose equivalents per hour. Based on an assumed monomer mass of 162 g/mol and a 5% moisture content of Avicel, 58.6 mM glucose equivalents were present initially. Avicel consumption was measured by elemental analysis of the pellet fraction of fermentation broth corrected for cell carbon. Error bars represent one standard deviation (n = 3) for Avicel measurement of a representative fermentation. Solid lines represent the best fit of a 5-parameter logistic equation. Equation parameters are given in Table S1 in the supplemental material.

Why is *olpB* expression changed in the *cipA* deletion strain? Although *cipA* and *olpB* have been reported to be transcribed independently (33), they may, in fact, be cotranscribed. Even if *cipA* and *olpB* are expressed from individual promoters, the 1-kb region upstream of *cipA* may contain other regulatory elements that...
affect olpB expression. Replacing the native cipA promoter on plasmid pDGO-40 with a different promoter would allow cipA to be expressed from a replicating plasmid in a cipA deletion strain where the native cipA promoter has been left on the chromosome (e.g., strain DS11).

The cipA deletion and complementation system described here

**TABLE 2 Abundances of type II cohesins**

<table>
<thead>
<tr>
<th>Protein</th>
<th>No. of type II cohesins per molecule</th>
<th>Protein abundance (nSpC) in:</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>DS19</td>
<td>WT</td>
</tr>
<tr>
<td>SdbA</td>
<td>1</td>
<td>35.9</td>
<td>30.1</td>
</tr>
<tr>
<td>OlpB</td>
<td>7</td>
<td>44.1</td>
<td>42.0</td>
</tr>
<tr>
<td>Orf2p</td>
<td>2</td>
<td>106.3</td>
<td>70.8</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>557.4</td>
<td>466.0</td>
</tr>
</tbody>
</table>

**FIG 4** Comparison of protein abundance as determined by normalized spectral abundance factor (nSpC) from tandem mass spectrometry measurements of fermentation broth (combined cells and supernatant) at the end of Avicel fermentations. nSpC measurements were taken from biological duplicate experiments. Pairwise comparisons were made, and proteins with significant changes ($P < 0.01$) are indicated by filled symbols. Other proteins are indicated by unfilled symbols. The presence of cohesins, dockerins, and carbohydrate binding modules (CBMs) was determined by searching the Pfam database (29).

**FIG 5** Comparison of substrate consumption rate with the abundance of the CipA scaffoldin protein for duplicate fermentations with strains DS1, DS19, DS20, and DS22 grown on 10 g/liter Avicel.
will be useful for systematic understanding of the cellulolytic capabilities of *Clostridium thermocellum*. The ability to express *cipA* from a replicating plasmid will enable the rapid exploration of the roles of its subcomponents, including elucidation of the function of individual modules of *cipA*, exploration of alternative cellulosomal architectures, and characterization of its noncomplexed cellulase system.

**ACKNOWLEDGMENTS**

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