Characterization of the Cellulolytic Complex (Cellulosome) Produced by *Clostridium cellulolyticum*

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The cellulolytic complex was isolated from *Clostridium cellulolyticum* grown on cellulose. Upon gel filtration, the complex was found to consist mainly of 600-kDa units, along with a 16-MDa aggregate. Its ability to degrade various substrates and its capacity to bind to the crystalline cellulose were measured. The results of sodium dodecyl sulfate-polyacrylamide gel electrophoresis, N-terminal sequencing, and blotting analysis showed that all of the known cellulases of this organism are present in this complex. Three major components were observed: the first component, a noncatalytic, large (160-kDa) protein, was identified based on its ability to bind to the dockerin-containing cellulases as scaffolding protein CipC. The other two components, which had molecular masses of 94 and 80.6 kDa, were identified as CelE and CelF, respectively. The identified cellulases and some other components of the cellulosome were able to bind to a miniCipC1 construct. In addition to providing an extensive description of the system, the results of the present study confirm that the dockerin-cohesin domain interaction plays an essential role in the constitution of the cellulosome.

Clostridium cellulolyticum, a mesophilic cellulolytic bacterium (32), has been extensively studied to determine its growth parameters when cultivated on glucose, cellobiose, and cellulose (19, 20). Only partial purification was achieved in an attempt to isolate some of the components of its cellulolytic system (12, 27). Studies using molecular biology techniques have yielded more information about this system (11, 31), and five cellulase genes have been entirely sequenced and one has been partially sequenced (1, 7, 13, 34, 39). Four cloned enzymes have been characterized (6, 15, 16, 35, 40), and the three-dimensional structure of one of them (CelA) has been determined (10). The gene coding for the scaffolding protein (or CipC, which stands for cellulosome integrating protein C) has been identified and partially sequenced (29, 34) and found to be part of a large cluster composed of at least six genes, two of which (*celC* and *celG*) form an operon (1). In addition, evidence that xylan is utilized by *C. cellulolyticum* was recently published and a β -xylosidase produced by this organism was characterized (37). To degrade cellulose, *C. cellulolyticum*, like *C. cellulovorans* (9), *C. papyrosolvans* (17, 33), and *C. thermocellum* (2, 3, 5, 14, 23, 24), secretes a cellulolytic complex composed of a wide range of cellulases. These enzymes form a complex called the cellulosome (24). The cellulosome of *C. thermocellum* is composed of many enzymes arranged around a scaffolding protein (CipB in strain YS and CipA in strain ATCC 27405) (2, 5). This protein does not exhibit any catalytic activity, but its cellulose-binding domain (CBD) enables it to adhere to cellulose (28). The cellulosomal enzymes mostly have a C-terminal 7-kDa domain (dockerin), which is involved in adhesion to CipA. It recognizes a specific hydrophobic domain (cohesin) on CipA (21, 26, 36, 41) and CipB (43). Evidence of a similar cellulosomal organization in *C. cellulolyticum* has been obtained (12, 27). The role of scaffolding protein

CipC was found to be identical to that of *C. thermocellum* based on the study of a fragment of CipC produced in *Escherichia coli*, miniCipC1, containing, in order, the CBD, a hydrophilic domain specific to mesophilic cellulolytic clostridia (9, 34), and one cohesin domain (29). Among the six known genes coding for cellulases in *C. cellulolyticum*, four (*celA*, *celC*, *celF*, and *celG*) have been cloned in our laboratory and overexpressed in *E. coli*.

In the present study, we attempted to establish an experimental link between the molecular data and the native cellulolytic system produced by *C. cellulolyticum*. We purified the cellulosome and studied its catalytic and binding properties towards various cellulosic substrates. By using antibodies raised against the cellulases isolated in our laboratory (CelA, CelC, CelF, and the CBD of CelG), we studied the presence and relative abundance of these four proteins in the cellulosome. By using B-miniCipC1 (biotinylated miniCipC1), we estimated the number of proteins harboring a dockerin domain. N-terminal microsequencing of the main subunits separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out to complete the description of the proteins constituting the cellulosome.

MATERIALS AND METHODS

Bacterial strain and media. *C. cellulolyticum* H10 (ATCC 35319) was routinely transferred onto a medium containing MN300 cellulose (7.5 g/liter; Serva) as the carbon source as described previously (20).

Cellulosome purification. Cultures were grown in two 500-ml flasks (Schott). After 5 days of growth, cells and residual cellulose were collected by centrifugation. The pellet was washed five times in 50 ml of 25 mM Tris-HCl (pH 8.0), filtered through a 3- μ m-pore-size glass filter (glass microfiber filter GF/D; Whatman) to remove cells, and washed on the filter, first with 30 pellet volumes of 25 mM Tris-HCl (pH 8.0), and second with 12.5 mM Tris-HCl (pH 8.0). The cellulosome was eluted with 150 ml of water on the filter. The eluted fraction was concentrated on Millipore PTGC (10-kDa cutoff) and centrifuged to remove all of the insoluble material, and the supernatant was filtered on a 0.22- μ m-pore-size filter (SLGV 013 OS; Millipore). Gel filtration experiments were carried out with CL-4B Sepharose gel (Pharmacia) in a column (40 by 0.72 in.) with 50 mM Tris-HCl (pH 8.0)–100 mM NaCl as the buffer.

Substrates. Medium-viscosity carboxymethyl cellulose (CMC), oat spelt xylan, and laminarin (*Laminaria digitata*) were purchased from Sigma, barley glucan was

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from Megazyme (Sydney, Australia), and Avicel PH101 was from Fluka. Swollen Avicel was obtained by the method developed by Walseth (42).

SDS-PAGE and zymograms. SDS-PAGE was performed by the procedure developed by Laemmli (22), with precast 4 to 20% polyacrylamide (Novex) gels. The gels were run on a refrigerated (10°C) Hoefer slab gel apparatus. The samples used for the SDS-PAGE analysis were boiled in sample buffer and stored at -20°C.

CMC, barley glucan, and laminarin zymograms were performed by using a 0.25% concentration of each substrate incorporated into polyacrylamide. The samples used for the zymograms were incubated at 37°C in sample buffer for 1 h and immediately run on SDS-PAGE at 4°C. The proteins were fixed by soaking the gel in 50 mM Na₂PO₄-12.5 mM citric acid (pH 6.3)-25% isopropanol for 1 h, and the residual SDS was removed by soaking the gel in 50 mM Na₂PO₄-12.5 mM citric acid (pH 6.3) for a further hour. The gel was then incubated at 37°C for 2 h, and the activity was revealed with a 1% Congo red solution by standard procedures (38). With xylan and swollen Avicel, which are insoluble, the imprinting technique (4) was used and the substrate concentration was 0.5%.

Enzyme assay. The protein concentrations were determined by using either the method of Lowry et al. (25) or that of Bradford (8) with bovine serum albumin (BSA) as the standard. CMCase and xylanase activities were determined as described previously (15, 19), by using the Park and Johnson ferricyanide method (30) with a 1% CMC or xylan-25 mM phosphate buffer (pH 7.0) solution. One international unit of activity corresponds to 1 μ mol of D-glucose equivalent released per min.

B-miniCipC1. Labeling was performed in accordance with the manufacturer's instructions (supplied with the Boehringer biotin labeling kit) with a pure fraction of miniCipC1 purified as described previously (29).

Antibody preparation. Polyclonal antibodies against the putative CBD of CelG (CBD-CelG) were raised in rabbits by subcutaneously injecting the pure protein (0.4 mg) mixed with 1 ml of Freund's complete adjuvant (Sigma). Booster injections were performed after 10 and 15 days, by using the same quantity of protein in incomplete adjuvant. The serum was collected, kept for 2 h at room temperature, and centrifuged at 4,000 $\times$ g. The supernatant was stored at 4°C with 0.3% Na₂S₂O₃.

Western blotting. Western blotting was carried out by standard procedures on nitrocellulose BA 83 (Schleicher & Shuell) when polyclonal antibodies were used. The dilution used with the primary antibodies was 1/1,000. Anti-rabbit immunoglobulin G conjugated with horseradish peroxidase (Amersham) was used as the secondary antibody, in line with the manufacturer's instructions. To prevent any contamination or residual signals from occurring between the various hybridization experiments, each blot was incubated with only one antibody. In some cases, prior to being incubated with the primary antibody, the blots were preincubated with 10 μ g of CelA2 (with dockerin domain) or CelA3 (without dockerin domain) purified as previously described (15) for 1 h in 50 mM Tris-HCl (pH 7.5)-100 mM NaCl-2% milk.

Glycoprotein detection. To detect glycosylated proteins, we used a protocol for labeling protein in solution in accordance with the manufacturer's instructions (supplied with the glycoprotein detection system from Amersham), and the transfer was carried out with polyvinylidene difluoride membranes (Boehringer).

Cellulose-binding experiments. Binding experiments were performed with microcrystalline cellulose (Merck) in 50 mM Tris-HCl (pH 8.0) buffer giving a final volume of 1 ml. The cellulosome was incubated with the cellulose for 1 h at 4°C under slow shaking. After centrifugation (4,000 $\times$ g, 5 min), the supernatant was collected, filtered through a 0.22- μ m-pore-size filter (SLGV 013 BS; Millipore) and then tested to determine its protein concentration (free fraction). The bound fraction was calculated by subtracting the concentration of the free fraction from the initial cellulosome concentration. Experiments involving miniCipC1 were carried out in the same way.

N-terminal sequencing. The N-terminal amino acid sequence was obtained with an Applied Biosystems 470A sequence analyzer with the proteins blotted onto PVDF.

RESULTS

Purification of the cellulosome. After 5 days of growth on 7.5 g of MN300 per liter, the culture was harvested by centrifugation. Ninety percent of the CMCase activity was located in the pellet (cells and cellulose). After extensive buffer washing, cells and nonspecifically adsorbed proteins were eliminated. The cellulosomal proteins were then released by extensive washing with distilled water and concentrated.

The purification procedure led to the recovery of 50 mg of cellulosome per liter of culture with a specific CMCase activity of 2.6 IU/mg in the H₂O fraction. This fraction also contained around 70% of the total CMCase units bound to cellulose. Table 1 gives the activity levels of the purified cellulosome measured on various substrates.

TABLE 1. Cellulosome preparation activities on various substrates

Substrate ^a	Sp act (IU/mg)
Medium viscosity CMC.....	2.6
Xylan	1.7
Washed xylan.....	0.9
Swollen Avicel	0.5
Avicel.....	0.2

^a 1% (wt/vol) in 25 mM Tris-HCl, pH 8.0.

SDS-PAGE analysis. When subjected to SDS-PAGE, the cellulosome was found to consist of at least 14 proteins (Fig. 1) with molecular masses ranging from 160 to 35 kDa (Table 2). Among these proteins, the 160-, 94-, and 80.6-kDa fractions were the most abundant.

The cellulosome fraction prepared in this way showed some lack of stability when stored at 4°C, resulting in the loss of more than 50% of the total CMCase and Avicelase activities after 3 to 5 days, and exhibited great SDS-PAGE profile changes, especially at the level of the high-M_r bands. All of the experiments described here therefore involved freshly prepared (i.e., less than 3-day-old) cellulosome. When the cellulosome fraction was kept bound to the cellulose, it showed greater stability, however, in terms of its activity and protein composition.

Gel filtration analysis. The apparent size and homogeneity of the cellulosome preparation were analyzed by gel filtration on CL-4B Sepharose (Fig. 2). Two fractions were isolated. The first (I) corresponded to an apparent molecular mass of 16 MDa, and the second corresponded to an apparent molecular mass of 600 kDa. The 16-MDa value estimated for peak I is not an absolute value, but in several experiments this fraction was eluted in a significantly larger volume than the void volume. The value given was estimated by extrapolating it from the calibration curve obtained with the standards. Since both fractions were similar in composition based on SDS-PAGE analysis, it seems likely that the first one resulted from the assembly of several (20 to 25) 600-kDa cellulosome units.

FIG. 1. Electrophoretic characterization of components of the cellulosome from *C. cellulolyticum*. The purified cellulosome was analyzed by SDS-PAGE under the conditions described in Materials and Methods. Lanes: 1 and 8, molecular mass markers (high- and low-molecular mass calibration kits from Sigma and Pharmacia, respectively); 2, Coomassie blue-stained subunits of the cellulosome (14 bands were clearly visible and named S1 to S14; see table 2 for the respective sizes); 3, interaction of cellulosome subunits with β -miniCipC1; 4 to 7, Western blots showing patterns obtained with CelA, CelC, CelF, and CBD-CelG antisera, respectively. In lane 7, the first band, corresponding to 77.9 kDa, is barely visible.

TABLE 2. Summary of descriptions and identities of components of the *C. cellulolyticum* cellulosome and the various methods used

Apparent mol mass (kDa)	SDS-PAGE colored fraction	CMCase activity	CipC-binding ability	Antibody used for identification	Glycosylated proteins	N-terminal sequence	Known component	Theoretical mol mass (kDa)
160	S1				+	CipC	CipC	
114.4	S2							
94	S3		+		+	CelE	CelE	94
89.6	S4	+	+	CBD-CelG		QSTTPP		
80.6	S5		+	CelF	+	CelF	CelF	78
77.9	S6		+	CBD-CelG			CelG	76
72.6	S7		+					
67.7	S8		+		+		CelD	69
61	S9							
58.9	S10	+						
54.2			+					
53	S11	+	+	CelA			CelA	52
49	S12		+					
44.5		+	+	CelC			CelC	47
43	S13		+					
35	S14	+						
29.5		+	+					

Cellulose-binding properties of the cellulosome. The ability of the purified cellulosome fraction to bind to cellulose was studied (Fig. 3). It turned out that despite the use of freshly prepared fractions, only a fraction (65 to 70%) of the proteins kept their binding properties. This finding indicates that cellulosome release from cellulose during the purification procedure was probably accompanied by some changes (such as partial fragmentation or partial denaturation), resulting in the lack of stability mentioned above.

The apparent binding parameters were determined and compared with the relevant properties of the miniCipC1 construct containing the CBD of CipC (Fig. 4a and b). It was observed in both cases that a single category of adsorption sites was apparently involved. The binding parameters were different, however. The calculated dissociation constants indicate that the cellulosome fraction has a higher affinity (10-fold) for Avicel, with an apparent K_d of 1.4×10^{-8} M, than miniCipC1,

for which a value of 1.4×10^{-7} M was obtained, whereas Avicel bound to 35-fold more molecules of miniCipC1 (0.4 μ mol/g) than of cellulosome (11.4 nmol/g). These values, obtained by using the CBD of *C. thermocellum* scaffolding protein CipA, were very close to those obtained with miniCipC1; binding occurred at a maximum number of sites with 0.54 μ mol/g of cellulose, and a K_d value of 4.0×10^{-7} M was recorded (28). Per unit of cellulosome assembly, it is not possible to determine whether only the CBD of Cip or, in addition, the CBDs known to be present in hydrolases interacted with cellulose. Comparisons between the apparent binding parameters (the cellulosome was more tightly bound but at fewer sites) suggested that multiple interactions (involving several CBDs) occurred between each cellulosome unit and cellulose.

The effects of the presence of CMC on binding of the cellu-

FIG. 2. Gel filtration chromatography of the water-eluted cellulosome fraction on Sepharose CL-4B. Protein load, 10 mg; flow rate, 0.17 ml/min; buffer, 50 mM Tris-HCl (pH 8.0)–100 mM NaCl; protein recovery, 1 mg in peak I and 8 mg in peak II. Arrows indicate the retention times of some standards. a, blue dextran (indicating void volume); b, thyroglobulin (669 kDa); c, ferritin (440 kDa).

FIG. 3. Adsorption of the cellulosome to Avicel. The capacity of a constant amount (31.5 μ g per tube) of the purified cellulosome to bind to various amounts of crystalline cellulose (0.1 to 10 mg per tube) was determined as described in Materials and Methods. The results are expressed as percentages of bound protein as a function of various amounts of Avicel.

(Avicel/bound cellulosome) (g/mmol) $\times 10^4$

(1/free cellulosome) (mM^{-1}) $\times 10^4$

(Avicel/bound miniCipC1) (g/mmol)

(1/free miniCipC1) (mM^{-1})

FIG. 4. (a) Double-reciprocal plot of cellulosome adsorption to Avicel. Various amounts of purified protein were added to 5-mg samples of Avicel. The other conditions are described in Materials and Methods. The following apparent binding results were obtained: maximum number of sites, 11.4 nmol/g; K_d , 1.4×10^{-8} M. (b) Double-reciprocal plot of miniCipC1 adsorption to Avicel. The experimental conditions were the same as those used for panel a. The following apparent binding results were obtained: maximum number of sites, 0.4 $\mu\text{mol/g}$; K_d , 1.4×10^{-7} M.

losome or miniCipC1 (Fig. 5a and b) to Avicel were investigated. The fact that inhibition of adsorption was observed in both cases, indicates that the CBD from CipC (which is present in both miniCipC1 and the cellulosome) probably has an affinity for CMC. The solubility of CMC made it impossible to determine the parameters of the possible binding of miniCipC1 and cellulosomes to this substrate. However, the binding parameters, determined in the presence of CMC, of the cellulosome ($K_{d\text{CMC } 0.5}$, 2.1×10^{-8} M and $K_{d\text{CMC } 0.25}$, 2.47×10^{-8} M;

maximum number of sites with 0.5 mg of CMC, 4.1 nmol/g and maximum number of sites with 0.25 mg of CMC, 8.2 nmol/g) and of miniCipC1 ($K_{d\text{CMC } 0.5}$, 2.54×10^{-7} M; maximum number of sites with 0.5 mg of CMC, 0.23 $\mu\text{mol/g}$) indicate that the main effect consists of a decrease in the apparent number of sites.

Zymograms. Zymograms (data not shown) were performed on CMC with various quantities of the pure cellulosome. The results showed the existence of five (or five groups of) proteins harboring CMCase activity. There were three major bands with apparent molecular masses of 89.6, 53, and 44.5 kDa and two

(Bound cellulosome / Avicel) (mmole/g) $\times 10^5$

(Bound miniCipC1 / Avicel) (mmole/g) $\times 10^3$

FIG. 5. (a) Binding of the cellulosome to Avicel in the absence (●) and presence of 0.25 (■) or 0.5 (▲) mg of CMC. Various amounts of purified protein were added to 5-mg samples of Avicel. The other conditions are described in Materials and Methods. The results are expressed as described in the legend to Fig. 3. (b) Binding of miniCipC1 to Avicel in the absence (●) and presence (■) of 0.5 mg of CMC. The experimental conditions were the same as those used for panel a.

minor bands corresponding to 35 and 29.5 kDa. Similar experiments were carried out with swollen Avicel, xylan, and laminarin. No activity was ever detected in these cases, even with a longer incubation time (36 h). With barley glucan, a single band corresponding to a 50-kDa protein was detected.

Western blotting experiments with B-miniCipC1. Pure celulosome fractions were run on SDS-PAGE. The gel was blotted onto a membrane by standard procedures. For the interaction with B-miniCipC1, we used a 5 to 20% precast gel gradient to detect a wide range of proteins (between 10 and 200 kDa) with no loss of resolution. All of the blots were incubated under the same conditions (for 1 h at room temperature) with the same quantity of B-miniCipC1 (7 μ g). The specificity of B-miniCipC1 has been tested against *E. coli* extract, BSA, CelA2, and CelA3 but interacted with CelA2. It has been demonstrated that this interaction is highly specific. In this experiment, 13 bands (94, 89.6, 80.6, 77.9, 72.6, 67.7, 58.9, 54.2, 53, 49, 44.5, 43, and 29.5 kDa) yielded a positive signal. The 160-kDa band assumed to correspond to CipC was never recognized by B-miniCipC1. The experiment was repeated by incubating the blot with 15 mM CaCl_2 or 5 mM EDTA. The hybridization patterns were similar in both cases. It turned out that CaCl_2 is probably not necessary for binding of the cohesin domains to the dockerin domains. On this point, our results differ from those obtained by Yaron et al. (43) with *C. thermocellum*, where the cohesin-dockerin domain interaction was found to be calcium dependent.

Antibody experiments. The blots of celulosome subunits were incubated with various antibodies obtained previously by using purified recombinant proteins (15, 16, 35). The 80.6-, 53-, and 44.5-kDa proteins were recognized specifically by CelF, CelA, and CelC antibodies, respectively. The 89.3- and 77.9-kDa proteins were recognized by CBD-CelG antibodies (Fig. 1). The measured relative molecular masses of CelF, CelA, and CelC were in agreement with the theoretical molecular masses (Table 2). In the CBD-CelG experiment, the antibodies yielded a barely detectable signal corresponding to a 77.9-kDa protein. This is in agreement with the expected molecular mass of CelG. They also cross-reacted with an 89.3-kDa protein. The third experiment consisted of incubating the blot with CelA2 prior to using antibodies raised against CelA. Two bands were revealed on the film: the first corresponded to CelA (49 kDa), and the second corresponded to a large protein with a molecular mass of 160 kDa (data not shown). When CelA3 was used, only the 49-kDa band was revealed (data not shown). The 160-kDa form therefore appears to be scaffolding protein CipC. It is worth noting that no degradation of the scaffolding protein occurred with our purification procedure.

Glycosylation experiments. The total proteins of the celulosome were oxidized with metaperiodate to detect the glycosylated ones. Four proteins seemed to be glycosylated in the celulosome: CipC, CelE, CelF, and the 67.7-kDa protein (data not shown). When less drastic oxidation was applied to detect only the sialic groups in the proteins, no signal was obtained.

N-terminal sequencing. N-terminal sequencing was carried out on the bands corresponding to 80.6, 89.6, 94, and 160 kDa. The 80.6-kDa protein band gave the N-terminal sequence (ASSPAN) of CelF (34), which confirmed the results of the Western blotting experiments. As expected, the 160-kDa N terminus (AGTGVV) matches the N-terminal sequence deduced from the study of the cloned *cipC* gene (29). The N-terminal sequence (LVGAGD) of the 94-kDa protein corresponds to that of the CelE protein, which is encoded by a gene just downstream of *celG* in the cluster (1). The N-terminal sequence (QSTTPP) of the 89.6-kDa subunit did not correspond to any known cellulase.

DISCUSSION

The cellulolytic complex secreted by *C. cellulolyticum* consists of stable assemblies of proteins constituting celulosome units with a molecular mass of 600 kDa. After its purification, it is still able to bind to crystalline cellulose, and its binding properties are qualitatively similar to those of miniCipC1 (the present study) and of those of the CBD from *C. thermocellum* CipA (28). Madarro et al. (27) have established that the Avicel-adsorbed celulosome fraction of *C. cellulolyticum* comprises three major components (160 to 170, 95, and 72 kDa). Gehin et al. (18) found the molecular masses of the three major components to be 135, 90, and 68 kDa. Here we obtained results similar to those of Madarro et al. (27), since 160-, 94-, and 80.6-kDa subunits appeared to be the most abundant fractions in our celulosome preparation. The 135-kDa protein described by Gehin et al. (18) probably corresponds to a degraded form of CipC due to the lack of stability of the celulosome after purification mentioned above. Our zymogram analysis showed that the main active band corresponded to 89.6 kDa instead of the 95 kDa observed by Madarro et al. (27). Another difference with respect to their findings was the size of the celulosome units: Madarro et al. (27) indicated that the size was more than 20 MDa, and we observed that the celulosome units belonged to fractions with two apparent sizes, a 16-MDa fraction (10 to 15%) and a mainly 600-kDa fraction (85 to 90%), the latter having the same molecular mass as that reported by Pohlshröder et al. (33) for the celulosome units of *C. papyrosolvans*. These differences may be due to some variations between the bacterial strains studied and/or to differences between the growth conditions and the methods of celulosome preparation used. In addition, our data demonstrate that CelA, CelC, CelG, CelF, and CelE (previously identified as cellulases by recombinant DNA techniques) are components of these celulosome units and that the scaffolding protein corresponds to the 160-kDa form visible on SDS-PAGE. A new cellulase with a domain cross-reacting with CBD-CelG antibodies is, moreover, involved in the celulosome. All of these findings confirm the homology between this system and that of *C. thermocellum* (2, 3, 23, 24), as well as those described in *C. cellulovorans* (9) and *C. papyrosolvans* (17, 33).

The main difference between the celulosome of *C. cellulolyticum* and that of *C. thermocellum* is the lack of calcium enhancement of the activities and the binding properties of the former (23, 24). *C. cellulolyticum* is a mesophilic strain, and it is generally recognized that Ca^{2+} reinforces the thermostability of proteins. In addition, previous studies on CelA and CelC have shown that neither calcium nor EDTA has any effect on the catalytic activities of these proteins (15, 16). The present study therefore confirms that the cellulolytic system of *C. cellulolyticum* does not require any calcium.

The results of the N-terminal sequencing experiments provided evidence for the existence of a new 89.6-kDa cellulase, and attempts to clone its gene will soon be made. The number of celulosomal subunits of *C. cellulolyticum* was assessed on the basis of B-miniCipC1 experiments and found to be at least 13 proteins in addition to CipC. These results cannot be attributable to any partial degradation of proteins: the experiments involving antibodies showed that CelA, CelC, CelF, and CelG were not degraded by H_2O elution and SDS boiling. Moreover, we reported previously that the main event in *C. cellulolyticum* cellulase proteolysis was excision of the dockerin domain (15, 16, 35). Cellulases lacking this domain cannot interact with CipC and cannot give a signal in the B-miniCipC1 experiment. In this case, CipC, in which the number of cohesin

domains was estimated to be nine based on sequence homologies with CbpA from *C. cellulovorans* (9), is not able to bind to all of the subunits simultaneously. It can therefore be assumed that the cellulosomes do not all have the same composition, as was previously thought to be the case in *C. papyrosolvens* (33). Two findings reinforce this hypothesis. (i) At the longer exposure times used with B-miniCipC1, some discrete bands were observed which might correspond to proteins produced in small quantities that are able to bind to CipC. These proteins may be more abundantly produced under other culture conditions or on another growth substrate. (ii) The intensity of the bands obtained in the B-miniCipC1 experiments and the patterns of Coomassie blue coloring were, in some cases, completely different. For example, the 29.5-kDa protein, which gives a strong signal with B-miniCipC1 hybridization, was completely absent from the Coomassie blue staining profile. This suggests that the cohesin-dockerin domain interactions do not occur with the same affinity in all cases. The present paper gives an instantaneous picture of the apparent composition of the cellulosome under our experimental conditions. The relative proportions of the various components might be different under other culture conditions (with different substrates, starvation times, etc.) and/or at various stages of culture (exponential or linear growth). This regulation of the production of the various cellulosomal components is very interesting and will be dealt with further in studies in our laboratory.

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