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Mutations in the Scaffoldin Gene, *cipA*, of *Clostridium thermocellum* with Impaired Cellulosome Formation and Cellulose Hydrolysis: Insertions of a New Transposable Element, IS*1447*, and Implications for Cellulase Synergism on Crystalline Cellulose[▽]

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Mutants of *Clostridium thermocellum* that had lost the ability to adhere to microcrystalline cellulose were isolated. Six of them that showed diminished ability to depolymerize crystalline cellulose were selected. Size exclusion chromatography of the proteins from the culture supernatant revealed the loss of the supramolecular enzyme complex, the cellulosome. However, denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis resulted in extracellular protein patterns comparable to those of isolated cellulosomes, except for a missing CipA band. Sequencing of the six mutant *cipA* genes revealed a new insertion (IS) element, IS*1447*, belonging to the IS*3* family. It was inserted into the *cipA* reading frame in four different locations: cohesin module 1, two different positions in the carbohydrate binding module, and cohesin module 3. The IS sequences were identical and consisted of a transposase gene and the inverted repeats IRR and IRS. The insertion resulted in an obviously nonspecific duplication of 3 base pairs within the target sequence. This lack of specificity allows transposition without the need of a defined target DNA sequence. Eighteen copies of IS*1447* were identified in the genomic sequence of *C. thermocellum* ATCC 27405. At least one of them can be activated for transposition. Compared to the wild type, the mutant culture supernatant, with a completely defective CipA protein, showed equal specific hydrolytic activity against soluble β-glucan but a 15-fold reduction in specific activity with crystalline cellulose. These results identify a genetic basis for the synergistic effect of complex formation on crystalline-cellulose degradation.

The strictly anaerobic, thermophilic bacterium *Clostridium thermocellum* exhibits one of the highest known growth rates of a microorganism on the recalcitrant substrate crystalline cellulose, a rate-limiting factor for growth. It thus produces one of the most efficient enzymatic cellulose degradation systems (17). Evidence is accumulating that this higher efficiency over other cellulolytic systems is at least in part due to the formation of a huge enzyme complex. This "cellulosome" is a distinctive feature which *C. thermocellum* shares with other highly efficient cellulose-degrading microorganisms. The complex has a diameter of ~18 nm and a mass in excess of 2×10^6 Da (14, 15, 31). About 30 dockerin-containing, cellulosome-related genes have been cloned by screening genomic libraries from *C. thermocellum* for enzymatically active colonies (5, 28, 29). In addition the scaffoldin protein CipA was identified. It contains nine type I cohesin modules to which enzymes and other protein components specifically dock by virtue of type I dockerin modules (6, 22). Type II cohesin-dockerin interactions anchor the CipA protein to cell wall-bound proteins, such as OlpB or SdbA (4, 16). The nonenzymatic component CspP is presumably involved in structure formation of the huge complex (35). However, not much is known about the structure of the complex and how it is assembled.

When the whole genomic sequence of *C. thermocellum* ATCC 27405 became accessible (GenBank accession no. CP000568), 72 cellulosomal genes, including *cipA*, were identified in the genome. The dockerin modules were used as an indicator of a cellulosomal gene (36). These dockerin-bearing hydrolytic enzymes appear to be responsible for the efficient degradation of cellulose, xylan, pectin, chitin, mannan, and other plant polysaccharides. They also contain a serine protease and its inhibitors (30), as well as a number of components with still unknown functions (for a list, see reference 36). It is unclear which of these components are required for cellulose breakdown and what role the complex formation could play. Proteomic analysis of isolated cellulosomes helped to identify the most prevalent cellulosomal components (11, 35). Nevertheless, in vitro assembly of the cellulosome and its individual components would be necessary to identify their roles in cellulolysis and fiber degradation. However, previous attempts to take apart the individual components in their native state and to reconstitute them into a functional complex have failed. Morag et al. (23) reported that soluble carboxymethyl-cellulose and amorphous cellulose were degraded by disintegrated cellulosomes, although hydrolysis of crystalline cellulose was severely diminished. Only the reconstitution of small complexes composed of a miniscaffoldin combined with two or three recombinantly produced enzyme components has been

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possible so far, showing a distinct synergistic effect on crystalline cellulose (8, 9).

The isolation and analysis of mutants impaired in the degradation of crystalline cellulose was considered to shed light on the function of complex formation. A mutant of *C. thermocellum* that did not adsorb to cellulose, called AD2, was isolated but only partially characterized in respect to its molecular mechanism (1). Its cellulose-degrading ability and cellulosome formation were impaired to some degree, but depending on the substrate, a smaller amount of CipA antigenic material and multimolecular complexes were still formed. However, they were not found to be cell associated (2). The growth of mutant AD2 on cellulose as a substrate was delayed, and although cellulose adsorption was not observed initially, it was observed after some time. The authors later interpreted this as a regulatory phenomenon (3).

Two mutants defective in cellulose degradation have been isolated from the mesophilic cellulosome-forming bacterium *Clostridium cellulolyticum* (18, 19). The two mutant strains did not degrade crystalline cellulose. They were found to be defective in the entire scaffoldin, as well as several enzymes. Cellulose-adsorbed proteins contained a truncated CipC (scaffoldin) and lacked the major cellulosomal enzyme Cel48F and other proteins encoded by downstream genes in the "*cip-cel*" cluster. The genetic organization of the *cip-cel* cluster was characterized for both strains. It showed one or two insertion (IS) elements in the seventh cohesin module-encoding sequence of CipC, which led to a strong polar effect on downstream gene transcription (19, 20).

The results with both types of *C. thermocellum* and *C. cellulolyticum* mutants do not directly shed light on the function of a complexed compared to a noncomplexed enzyme system, although the *C. thermocellum* mutant AD2 marked the beginning of the unraveling of the cellulosome paradigm. The synergistic action between selected pairs of cellulases on crystalline cellulose was revealed either by mixing the soluble components or by binding them in a polypeptide chain or through a binding scaffold (8, 24, 25, 34).

To investigate the cellulosome mechanism more closely, mutants of *C. thermocellum* defective in adsorption to and degradation of crystalline cellulose were screened. Isolation of the mutants and their molecular and biochemical analysis are described here. As expected, these mutants were found to be defective in scaffoldin production. Surprisingly, all six mutations were caused by transposition of the same IS element. One mutant had the insertion within the first cohesin module-encoding sequence and was thus completely defective in the production of scaffoldin. The analysis of the mutant phenotypes showed that the hydrolytic cellulosomal components were produced but not assembled. The hydrolysis of soluble β-glucans was not affected, whereas the degradation of crystalline cellulose was seriously disturbed.

MATERIALS AND METHODS

Strains and media. *C. thermocellum* strain DSM 1237 (corresponding to ATCC 27405, JCM 12338, and NCIB 10682) was grown at 60°C in a prereduced GS-2 medium (12) for liquid cultures or a CM3 medium (32) for agar plates (with 1.5% [wt/vol] agar) containing 1.0% (wt/vol) cellobiose or 0.5% microcrystalline cellulose (MN300; Macherey & Nagel, Dueren, Germany).

Mutagenesis and mutant screening. A single colony was inoculated at 60°C in GS-2 medium and incubated for 24 h with Whatman no. 1 filter paper as a carbon source. The culture was centrifuged at room temperature in an anaerobic hood (Coy Laboratory Products Inc., Grass Lake, MI), resuspended in the same volume of prewarmed GS-2 medium (without carbohydrate), and kept at 60°C for 2 h. Several 1-ml-samples were mixed with ethyl-methanesulfonate (EMS) (final concentration, 3 to 192 mM) and incubated at 60°C for another 2 h. The cells were washed three times with GS-2 medium by centrifugation, resuspended in 5 ml GS-2 medium supplemented with 1% (wt/vol) cellobiose, and diluted 1:100 in GS-2 medium (with cellobiose). The culture density was determined photometrically after 22 and 43 h (optical density at 600 nm). Growth was detected only at 3 to 25 mM of EMS. The same result was obtained by plating the mutagenized culture for colonies on CM3 medium supplemented with 1% (wt/vol) cellobiose. All manipulations of *Clostridium* cultures were done under anaerobic conditions.

For enrichment of cells with diminished adsorption of cellulose particles, a method similar to that described for the selection of a spontaneous nonadsorbing mutant was used (1). Cells growing in the logarithmic growth phase in liquid GS-2 medium at 60°C with 1% (wt/vol) cellobiose were gently shaken for 30 min with 10% (wt/vol) MN300 cellulose fibers to enable adsorption of the cells. After sedimentation of the cellulose with adherent cells, 2 ml of the supernatant was carefully removed with a syringe and injected into 4 ml of prewarmed fresh cellobiose GS-2 medium. The flask was incubated for 8 h with occasional shaking. This procedure was repeated nine times. The nonadsorbed cultures were diluted, plated on CM3 plates supplemented with 0.1% cellobiose, and overlaid with 5 ml of CM3 agar containing 0.5% (wt/vol) MN300. Colonies that formed a reduced clear halo or no halo around the colony in the turbid cellulose layer were streaked three times for single colonies. *C. thermocellum* strain identity was proven by sequencing a PCR amplicon of the 16S rRNA gene.

Recombinant DNA techniques. Preparation of chromosomal and plasmid DNA, endonuclease digestion, and ligation were carried out by standard procedures or according to supplier protocols. Plasmid DNA was prepared with the Qiaprep Spin Miniprep Kit (Qiagen, Hilden, Germany). Restriction digests of DNA were done as recommended by the manufacturer (MBI Fermentas, St.Leon-Rot, Germany). *Escherichia coli* cells were transformed with plasmid DNA using electroporation.

Vector pCR2.1-TOPO and the *E. coli* host strain TOP10 (Invitrogen, Karlsruhe, Germany) were used for cloning. PCR for amplification of the *cipA* gene fragments and the promoter region was carried out using the synthetic oligonucleotide primers Cip1f (5′-AAA ACT TTG CCG GAA AGT TGC), Cip2r (5′-ATA CTG ATG ACT TTT CTC ATC TAC), Cip3f (5′-AAT GGT AGA TGA GAA AAG TCA TC), Cip4r (5′-ATC ATC TGA CGG CGG TAT TG), Cip5f (5′-AAT ACC GCC GTC AGA TGA TC), Cip6r (5′-TTT CAA GGT AGG TGT CTG CG), Cip7f (5′-ATT TGA AGG TTG AAT TCT ACA ACA GC), Cip8r (5′-TTA AGT GTC AAC TTG TTC GGA G), Cip9f (5′-AAC AAG TTG ACA CTT AAG ATA GG), and Cip10r (5′-TAA TCA CTT GAT GTA GCT CCA A) with chromosomal DNA from *C. thermocellum* DSM1237 as a template and the KOD XL DNA polymerase (Novagen, Darmstadt, Germany). For PCR, the following conditions were used: 25 cycles of 30 s at 94°C, 5 s at 55°C, and 1 to 6 min at 72°C. The reaction was completed for 10 min at 74°C.

The 16S rRNA gene was amplified using the oligonucleotide primers 616V (5′-AGA GTT TGA T[CT][AC] TGG CTC) and 630R (5′-CA[GT] AAA GGA GGT GAT CC).

Plasmids were sequenced from double-stranded DNAs of selected colonies.

Preparation of supernatant proteins. Cultures were grown in GS-2 medium containing 1% cellobiose and centrifuged (1,000 × *g*; 20 min). Cleared supernatants were subjected to ultrafiltration with spin columns of 10,000-Da exclusion size (Vivaspin 500; Vivascience AG, Aubagne, France) to remove salts and small molecules from the medium. The proteins were washed with 100 mM MES (morpholineethanesulfonic acid) buffer, pH 6.0, with 10 mM $CaCl_2$ and concentrated five times. The protein concentration was determined with Coomassie brilliant blue G-250 (Bradford reagent; Bio-Rad Laboratories, Munich, Germany).

Culture supernatant proteins were separated in their native state by size exclusion chromatography using XK16/100 columns with Superose 6 prep grade (GE Healthcare, Germany). The protein concentration was detected photometrically at 280 nm. The protein composition was analyzed by denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and staining with Coomassie brilliant blue G-250.

Enzymatic assays. Enzyme samples were incubated in MES buffer (50 mM) at pH 6.8 and 60°C. Reducing sugars were quantified at least in triplicate in the linear range of the reaction by the 3.5-dinitrosalicylic acid method (33), assuming that 1 unit of enzyme liberates 1 μmol of glucose equivalent per minute. The

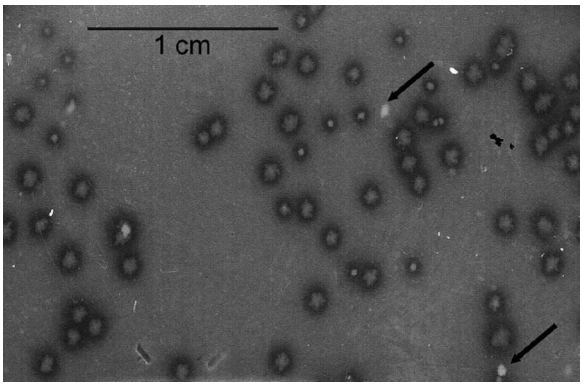

FIG. 1. Single colonies of a mutagenized culture of *C. thermocellum* enriched for nonadsorbing cells. Colonies on the turbid cellulose background produce a dark halo when degrading cellulose. The bar indicates a length of 1 cm. Colonies with different sizes and abilities in cellulose hydrolysis can be recognized. Two colonies with reduced cellulose-degrading ability (putative mutants) are indicated by arrows.

following soluble and insoluble substrates were used at 0.5% and 0.67% (wt/vol), respectively: barley β-glucan (Megazyme International, Bry, Ireland), oat spelt xylan, low-viscosity carboxymethyl cellulose (CMC) (Sigma-Aldrich, Deisenhofen, Germany), cellulose powder MN300 (Serva, Heidelberg, Germany), microcrystalline cellulose Avicel (Serva; no. 14201), and bacterial cellulose (Cellulon Microfibrous Cellulose; CP Kelco, Atlanta, GA). Phosphoric acid-swollen cellulose was prepared from Avicel CF1 (Sigma-Aldrich, Taufkirchen, Germany) in accordance with the method of Wood and Bhat (33). All enzymatic estimations were performed at least in triplicate.

Affinity blotting of truncated CipA. Proteins in SDS-PAGE slabs were renatured by incubation in 25% isopropanol, 50 mM Na-citrate for 20 min and washing with 50 mM Na-citrate for 20 min (27). After a buffer change to transfer buffer (50 mM Tris, 40 mM glycine, 1 mM SDS, and 20% methanol), the proteins were electroblotted to a polyvinylidene difluoride membrane at 1 mA/cm^2 for 2 h. The membrane was blocked overnight with 200 g/liter skim milk powder in phosphate-buffered saline buffer and washed with a washing buffer (50 mM Tris, pH 7.7, 0.05% Tween 20, 0.5% bovine serum albumin, 2 mM $CaCl_2$) for 20 min.

To detect cohesin-containing proteins on the blot, the recombinant marker protein rCel9N (*C. thermocellum* Cel9N cellulase containing a dockerin and a His tag) was purified from 400 ml of cell extract of *E. coli* (pQE-hCel9N) (35) with a 5-ml Ni-nitrilotriacetic acid superflow column (Qiagen) in accordance with the supplier's protocol. Fifty micrograms of rCel9N per ml per cm^2 of membrane surface was applied for 2 h. After the washing buffer was changed three times, the attached rCel9N protein was detected with peroxidase-conjugated anti-His antibodies (Qiagen; 1:1,000 in phosphate-buffered saline buffer). The rCel9N/antibody complex was detected with 4-nitroblue tetrazolium chloride and bromochloro-indolylphosphate (Roche Diagnostics GmbH, Mannheim, Germany) in accordance with the manufacturer's recommendations.

Nucleotide sequence accession number. The nucleotide sequences of mutated *cipA* genes containing the IS sequences were deposited under GenBank accession no. AM491039 (SM1), AM491040 (SM4), AM491041 (SM5), and AM491042 (SM6).

RESULTS

Isolation of nonadsorbing mutants. Cultures of *C. thermocellum* were mutagenized with EMS and grown further on a cellobiose-containing medium to enable the growth of cellulase-defective mutants. Cells not adsorbing to crystalline cellulose were enriched by adding excess crystalline cellulose to the cellobiose-grown culture, shaking, sedimenting cellulose and adsorbed cells, transferring the nonadsorbed cells of the supernatant to a fresh culture vial, and repeating the procedure nine times, as described previously by Bayer et al. (1). The last enrichment culture was diluted, plated on agar plates (with cellobiose), covered with a cellulose layer, and incubated for at least 5 days at 60°C (Fig. 1). Colonies with a reduced ability to form clear halos in the cellulose around the colonies were picked and purified by single-colony streaking under anaerobic conditions. Six of these "swimmer mutants" (SM1, SM4, SM5, SM6, SM10, and SM14) were selected due to their barely visible activity on Avicel and investigated further.

TABLE 1. Positions of the IS*1447* insertion in the *cipA* gene in six SM mutants

Mutant	Target[a]	Location in *cipA*[b]	Module[c]
SM1 (=SM14)	CCT	332–334 c	Coh1 (94–504)
SM4 (=SM10)	GAA	1544–1546 c	CBM3 (1111–1566)
SM5	GTT	1524–1526 c	CBM3 (1111–1566)
SM6	CTG	1727–1729 c	Coh3 (1684–2088)

[a] The target sequence is indicated as it appears in the coding strand of the transposase gene.
[b] c, complementary strand of *cipA*. The numbers indicate the bases counted from the first base of the *cipA* reading frame. These bases are duplicated on insertion.
[c] The extension of the CipA modules is given. Coh, cohesin module; CBM, carbohydrate-binding module.

The new mutant strains formed creamy-white colonies on CM3 agar plates with cellobiose as their carbon source, slightly smaller than those of the wild type. Only mutant SM6 was able to grow on MN300 cellulose as the sole carbon source without cellobiose (data not shown). To ensure that the mutants arose from the original *C. thermocellum* culture and were not contaminants, their 16S rRNA genes were amplified from genomic DNA and sequenced from both strands. The sequences were found to be identical to the wild-type *C. thermocellum* sequence.

Mutant phenotype. Four of the six *C. thermocellum* mutants, SM1, SM4, SM5, and SM6, were genetically different (Table 1). Concentrated cell culture supernatants were subjected to enzymatic tests on barley β-glucan and CMC for control and on microcrystalline cellulose MN300. The enzymatic activities on barley β-glucan and CMC were about 8.5 and 1.0 U mg protein^{-1}, respectively, in all supernatants (Table 2). In contrast, the specific activity on crystalline cellulose was reduced in the mutants SM6, SM5, SM4, and SM1 (in decreasing order of activity), exhibiting as much as 15-fold reduction compared to the wild type.

Interestingly, in all culture supernatants a protein concentration comparable to that of the wild type was found. Moreover, in denaturing SDS-PAGE, the distribution of strong and weak bands was indistinguishable between the mutants and the wild type and corresponded to the pattern of bands appearing

TABLE 2. Enzymatic activities of concentrated culture supernatants of the mutant strains and the wild type on barley β-glucan, CMC, and MN300 crystalline cellulose

Substrate	Activity (U mg protein^{-1} ± SD)				
	SM1	SM4	SM5	SM6	WT[a]
β-Glucan	7.9 ± 1.1	8.4 ± 1.6	8.6 ± 1.6	7.6 ± 1.0	9.5 ± 0.9
CMC	1.1 ± 0.1	1.0 ± 0.2	1.0 ± 0.1	1.0 ± 0.1	1.2 ± 0.1
MN300[b]	0.3 ± 0.1	0.9 ± 0.2	1.1 ± 0.1	1.7 ± 0.1	4.4 ± 0.1

[a] WT, wild type.
[b] Activities are 10^2-fold.

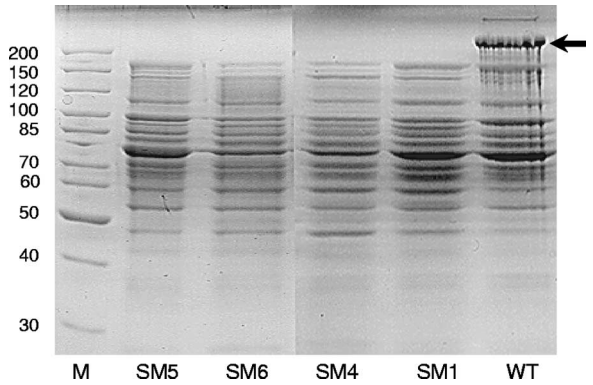

FIG. 2. SDS-PAGE of culture supernatant proteins from the mutants. Purified cellulosomes from the wild type (WT) were used. The position of CipA protein is indicated by an arrow. The left lane shows molecular mass markers (kDa).

from purified cellulosomes (Fig. 2). This indicates that in the different mutants the cellulosomal proteins are produced in similar amounts and in the distribution observed for the wild type. However, one band with a molecular mass of about 210 kDa was completely missing in the mutants (Fig. 2). This band corresponds to the CipA scaffoldin.

To analyze the presence of supramolecular complexes, the undenatured culture supernatants were subjected to size exclusion chromatography (Fig. 3). While most of the protein from the wild-type supernatant was found in high-molecular-weight complexes (the cellulosomes), the sizes of these complexes were dramatically reduced in mutant SM6. The complexes were completely missing in mutant SM1. SM5 showed an intermediate profile similar to that of SM4 (data not shown). The lack of enzymatic complexes was even more obvious in the barley β-glucan activity profiles (Fig. 3). Barley β-glucan was chosen because the vast majority of β-1,4-glucanases investigated so far exibit high activity on this substrate. The proteins from the wild type contained in the peak slightly below 2×10^6 Da exhibited β-glucanase activity. Such high-molecular-mass complexes were found to be completely absent in all mutants. The endoglucanase activity in mutants SM6 and SM4 showed the occurrence of small complexes that were not present in mutant SM1. However, such complexes were significantly smaller than the cellulosomes. Mutant SM1 appeared to produce exclusively single enzyme components that appeared in a well-defined peak around the 70-kDa calibration marker.

Truncated CipA proteins. Functionally intact cohesins in the residual, truncated CipA proteins were detected on SDS-PAGE slabs by affinity blotting with the recombinant dockerin-containing marker protein rCel9N and anti-His tag antibodies after in situ renaturation (27). Only proteins specifically interacting with rCel9N protein via cohesin-dockerin binding were detected (Fig. 4). The three mutant CipA proteins from SM6 and SM4 showed a reduced molecular size, as expected, whereas no SM1 CipA protein interacted with the rCel9N dockerin. SM5 unexpectedly showed a double band, which cannot be explained at present. The lack of binding in the SM1 extract indicates that the remaining part of the Coh1 module in this mutant was functionally defective.

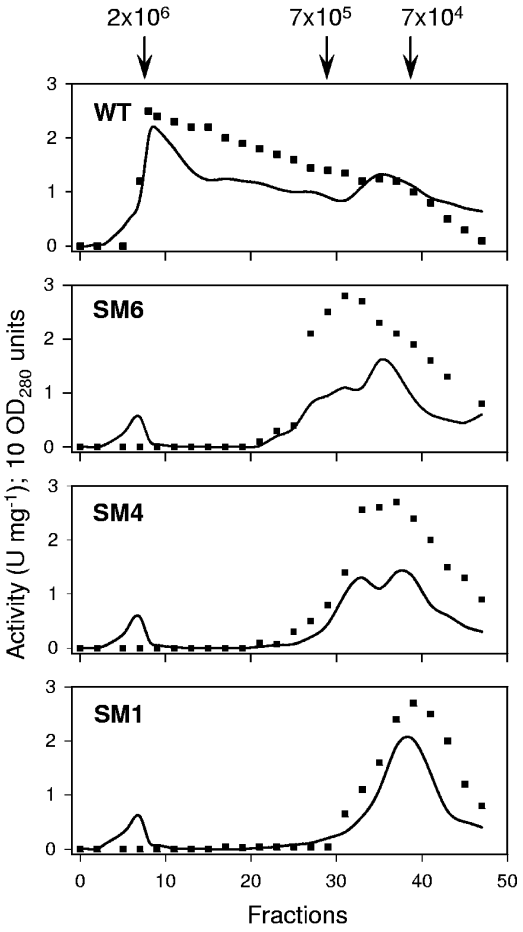

FIG. 3. Gel filtration (size exclusion chromatography) of culture supernatant proteins from the wild type (WT) and mutants. The amounts of protein (solid lines) and endoglucanase activity on barley β-glucan (dotted lines) are shown. The molecular masses of marker proteins (in kDa) are indicated above.

Molecular analysis of mutated *cipA* genes and identification of a new transposable element, IS*1447*. Due to the lack of the scaffoldin protein, CipA, it was evident that the *cipA* gene is involved in the mutant phenotype. Oligonucleotide primers were designed to investigate different regions of the *cipA* gene. DNA was amplified from the genomic DNA of the mutants. The upstream (promoter region with primer pair Cip1f-Cip2r) and the downstream (primer pair Cip9f-Cip10r) regions of the gene showed the expected fragment size, whereas the fragments amplified by primer pairs Cip3f-Cip6r (mutant SM1), Cip4r-Cip7f (mutants SM4 and SM5), and Cip5f-Cip8r (mutant SM6) produced amplification products which were about 1,500 bp larger than amplicons obtained from wild-type genomic DNA. Sequencing of the amplicons revealed the presence of a 1,447-bp IS element that was identical in all mutants. It was found to be inserted at four different locations within the *cipA* gene (Fig. 5). The insertion positions of SM1 and SM14, and SM4 and SM10, respectively, were identical (Table 1). Mutants SM14 and SM10 were thus not investigated further. Part of the IS element showed homology with transposase genes of the IS*3* family and with its inverted-repeat sequences. It was therefore called IS*1447*, referring to the length of the

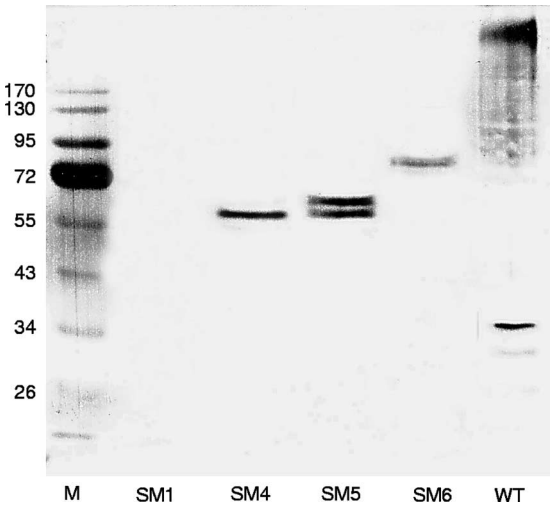

FIG. 4. Denaturing gel electrophoresis (SDS-PAGE) of concentrated culture supernatants: CipA fragments containing cohesins. Fifteen micrograms of protein was applied to the gel: SM1, 5.02 μl; SM4, 10.7 μl; SM5, 8.1 μl; SM6, 19.2 μl; and wild type (WT), 11.35 μl. Proteins containing cohesin modules were detected with His tag-Cel9N and anti-His antibodies. The numbers indicate molecular masses (kDa) of the protein mass standard (lane M).

sequence. The transposase gene was transcribed in all cases in a direction opposite to the *cipA* gene. The inverted repeats were 26 bases in length and were situated at the very ends of the IS element (Fig. 6).

The insertion at four different locations in *cipA* of one copy of IS*1447* allowed the detection of a 3-base pair target sequence that was different in each insertion event. This nonspecific target region was duplicated upon insertion.

IS*1447* copies present in the genomic sequence of *C. thermocellum* ATCC 27405. Eighteen copies of IS*1447* were identified in the genome sequence of *C. thermocellum* ATCC 27405. Earlier versions of the draft genome sequence showed that IS*1447* created severe problems with the connecting process of small contigs. Although they were corrected in later versions, incorrect contig connections could have been partially avoided with knowledge of the IS element sequence.

DISCUSSION

The detection of the loss of the scaffoldin protein (CipA) in the mutant culture supernatant allowed a straightforward genetic analysis of the mutant genes. Sequencing of the PCR-amplified putative mutated *cipA* genes revealed the introduction of a new IS element called IS*1447*. It was inserted in identical orientations in all six mutants investigated, albeit into different positions within *cipA*. This resulted in truncated scaffoldins, which were secreted but had limited or missing complexing ability. The effect of the sizes of the complexes formed in mutants SM4 to SM6 (Fig. 3) is mirrored in their increasing abilities to degrade crystalline cellulose (Table 2); larger complexes showed more activity. This is a confirmation of the cellulosome paradigm that complex formation stimulates activity of the cellulases on the crystalline substrate.

The reduction of complex size or the loss of entire complexes indicates an inability of the mutants to form normal cellulosomes. Genetic analysis showed that this was solely the result of size reduction or destruction of the scaffoldin. The proteins thus appear in mutant SM1 as dispersed, noncomplexed proteins. The SDS-PAGE pattern (Fig. 2) showed that these proteins appear to be cellulosome components, although minor components could not be recognized with confidence. Only the CipA band was certainly missing completely.

The insertion of a transposable element as the cause of all mutations in the *cipA* gene was not expected. It is not clear if the transposition events were accidental transpositions selected by the tight screening procedure or if they were induced by a stress reaction caused by the chemical mutagenesis. The appearance of six mutants out of 10^5 screened colonies appears to be far too frequent for a spontaneous event in a single gene, even if the more than 50 generations of bacterial growth during the screening procedure are taken into account. Due to the negative effect of *dam* methylation, spontaneous transposition generally occurs after DNA replication (26). Transposition events can be induced frequently, e.g., by stress reactions, which increase *recA* expression levels during SOS response. IS*10* transposition, which was shown to be induced by UV irradiation, is an example of this (7). Chemical mutagenesis could have a similar effect but was probably not necessary in the experiments described here.

Transposition activity of an IS element (IS*1447*) in *C. thermocellum* has been shown here for the first time. IS elements are known to increase the plasticity of genomes by creating genome rearrangements, insertions, deletions, or inversions and switching genes on and off (13, 21). A putative transposase gene belonging to a partial IS element differing from IS*1447* was identified upstream of the *licA* gene in *C. thermocellum*. It was discussed as a possible cause for the "module shuffling" expected for cellulosomal genes and has been proposed to be the source of their modular composition. Multiple copies of

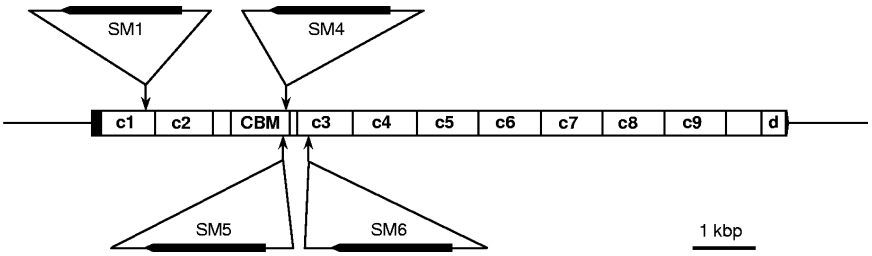

FIG. 5. Structure of the *cipA* gene and positions of IS*1447* insertions (mutant designations are indicated). 1, cohesin module 1; CBM, carbohydrate binding module; d, dockerin module.

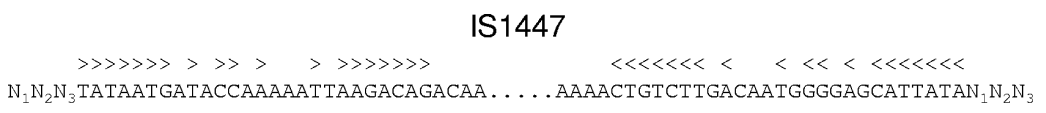

FIG. 6. Sequences of the inverted-repeat structures IRR and IRL of IS*1447*. Repeated bases are marked by arrowheads. The position of the nonspecific 3-bp repeat created on transposition is indicated by $N_1N_2N_3$.

this putative IS element were identified in the genome (10). However, no obvious connection between the locations of the cellulosomal genes and those of the IS*1447* elements was found (data not shown).

IS*1447* is a member of the IS*3* family, which is the most prevalent transposon family in bacteria. It matches the average size of IS*3* elements (around 1,300 bp). On insertion, it creates a 3-bp target repeat, as do many of its kind (Table 1). The 3-bp doubling of the target in all four mutants allowed the prediction that this is the usual insertion mechanism. This mechanism is in accordance with that described for other IS*3* family insertions. With the newly occurring transposition events, this insertion mechanism was obvious.

In all different mutants, the transposase gene of the IS*1447* copy was found in the strand opposite to *cipA*. It is not clear if insertion in the same direction as *cipA* would allow readthrough and thus expression of functional *cipA* cohesin modules with a nearly wild-type phenotype of the scaffoldin. In that case, such mutants would not have been picked up by the applied enrichment procedure.

The insertion of the IS*1447* copies within the first modules of the *cipA* gene appeared to have abolished expression of the residual gene completely. Cellulosome complexes were absent in culture supernatants of mutant SM1. Nevertheless, small complexes with increasing size were detected in the mutants SM4, SM5, and SM6, and some active cohesin modules could be produced (Fig. 3). Smaller complexes were observed to have lower activity on microcrystalline cellulose, with increasing activity detected for larger complexes. This is in agreement with the cellulosome paradigm (4) and with the finding that a ternary complex showed synergism on crystalline cellulose (8). However, the 15-fold difference in activity on crystalline cellulose found for the mutant SM1 cellulolytic enzymes compared to the wild-type cellulosomes is extraordinarily high and far beyond the synergistic effect found with so-called minicellulosomes (ternary complexes of two selected cellulases with a miniscaffoldin). This suggests involvement of more components, which should be present in a complex with optimal activity on crystalline cellulose.

The cellulosome paradigm attributes the high efficiency of the complex on crystalline cellulose to the high local concentration of synergistic components. In addition, it can be suggested that a mechanistic cooperation exists between enzyme components with identical hydrolytic activities on β-1,4-linkages but with different structural preferences on the substrate.

The difference between the cellulase activity from mutant SM1 and that from the wild type can be attributed to the complex formation via dockerin-cohesin interaction, in combination with the ability of the scaffoldin to hold the complex on the crystalline substrate via its CBM3 carbohydrate-binding module. An identical enzyme composition of simultaneously present enzyme components was found in the mutant extract. The synergism model was verified by the absence of synergism in the mutant lacking the complex. However, the high degree of synergism in the wild type was surprising. Whether this synergism is the result of the complex alone, whether the substrate binding via CipA-CBM3 is crucial, and whether certain enzymes play pivotal roles here have yet to be evaluated by further experiments.

The tight screening scheme for nonadsorbing mutants of *C. thermocellum* yielded isolates severely impaired in the hydrolysis of crystalline cellulose, but not in their abilities to hydrolyze soluble β-glucans. Consequently, the mutants were different from those isolated earlier from *C. thermocellum*, which were obviously regulation defective (3), or from *C. cellulolyticum*, which were defective in the production of a part of the cellulosomal components (19). The mutant SM1 is the first genetically defined mutant of any cellulosome-producing bacterium that produces the dockerin-containing cellulases but does not assemble them into the full complex. The mutant evidently fully retains the ability to secrete the exoenzymes in a wild-type pattern, but not complex bound. This allows the comparison of an identical native set of cellulases and other glycoside-hydrolases in complex-bound and soluble forms and the quantification of the effect of complex formation for the synergism on refractive, insoluble substrates, such as cellulose.

These "soluble" enzymes degrade soluble β-glucans as effectively as the wild type. However, the hydrolysis of microcrystalline cellulose was diminished by about 15-fold for mutant SM1 and progressively less for the other mutants. The degree of crystalline cellulose degradation was directly related to the reduction of complex size. The difference between the mutants and the wild type is likely to be even larger than indicated by the numbers in Table 2: the degradative determination of crystalline cellulose MN300 may be distorted by amorphic regions, which are degraded by endoglucanase activities. This degradation is not dependent on synergistic enzyme interaction. Moreover, the conditions used for cellulosome preparation do not necessarily allow collecting cellulosomes to be as active as they would be in vivo. Wild-type cellulosome activity could thus have been underestimated. However, the mutants form an excellent tool for future investigations of the basis for the extraordinarily high efficiency of cellulosomal cellulose degradation.

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