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Cellulose hydrolysis ability of a *Clostridium thermocellum* cellulosome containing small-size scaffolding protein CipA

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ABSTRACT

Mutant *Clostridium thermocellum* YM72 that produces small-size scaffolding protein CipA (ssCipA) was isolated from wild-type YM4. Sequencing of ssCipA revealed that two domains, cohesin 6 and cohesin 7, were not present. Cellulosome prepared from YM72 exhibited a significant reduction of hydrolysis ability on crystalline celluloses such as Sigmacell type-20 and cellulose from *Halocynthia*. To investigate this influence *in vitro*, artificial cellulosomes were assembled as recombinant CipA (rCipA) and ssCipA (rssCipA) using native free-cellulosomal subunits. The cellulosome assembled using rssCipA showed a 1.8-fold decrease in the hydrolysis of crystalline cellulose compared with that of rCipA. However, no significant differences in the hydrolysis of carboxymethylcellulose and acid-swollen cellulose were observed. One protein band was missing from the complex that was assembled using rssCipA (confirmed by native-PAGE). The missing protein was identified as CelJ, which is a major cellulosomal subunit. This suggests that insufficient cooperation of CelJ into the cellulosome results in the significant reduction of hydrolysis toward crystalline cellulose. These results indicate that cohesin 6 and 7 may be responsible for the cooperation of CelJ through cohesin and dockerin interactions, and adequate cooperation of CelJ into the cellulosome is important for significant hydrolysis of crystalline cellulose.

1. Introduction

Clostridium thermocellum, an anaerobic, thermophilic, spore-forming bacterium, is a widely used cellulose-degrading bacterium that produces cellulosomes (2–3.5 MDa) (Bayer et al., 2004; Demain et al., 2005). The cellulosome of *C. thermocellum* consists of a large (197 kDa), non-catalytic, multimodular scaffolding protein CipA, which includes nine cohesins, four hydrophilic modules, and a family 3 carbohydrate-binding module (CBM3). The catalytic units are non-covalently attached to the scaffolding via high-affinity type I interactions between the dockerin domains of the catalytic units and cohesins on the scaffolding (Bayer et al., 2004). Additionally, genome sequencing efforts have identified more than 70 dockerin containing proteins in the genome of *C. thermocellum* ATCC27405 (Demain et al., 2005). The cellulosome of *C. thermocellum* contains a surprisingly large variety of enzymes possessing attractive enzymatic properties for the degradation of complex plant biomass. The scaffolding protein CipA has been described as molecular Lego, binding a number of cellulases through its type I cohesin modules to regulate the efficiency of the entire enzymatic cascade (Valbuena et al., 2009). Recently, structural and molecular research of type I interactions have extended this structural knowledge toward the molecular determinants driving cellulosome assembly (Carvalho et al., 2007; Carvalho et al., 2003). Small-angle X-ray scattering studies have also recently illustrated that the conformation between the scaffoldin cohesin modules allow for optimal positioning of the enzymatic subunits onto the substrate (Hammel et al., 2005). Despite these extensive attempts, a comprehensive understanding of the unique structural elements that contribute to the highly efficient cellulose-degrading properties of the cellulosome have been hindered by the piecemeal approach to reconstituting small complexes composed of a mini-scaffoldin combined with two or three recombinantly produced enzyme components. However, the enzymatic components required for cellulose breakdown and the role of complex formation remain unclear.

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Mutant YM72, which produces small-size scaffolding protein CipA (ssCipA), was obtained from wild-type *C. thermocellum* YM4 (WT) (Mori, 1990) by comparing the protein patterns of the cellulosome. *C. thermocellum* YM4, isolated from a coculture obtained from an enrichment culture, showed increased cellulolytic activity toward microcrystalline cellulose (Demain et al., 2005; Mori, 1990). In this study, we investigated cellulosome function more closely by using molecular and biochemical analyses of CipA and ssCipA.

2. Materials and methods

2.1. Organisms, media, and growth conditions

C. thermocellum YM4 was originally isolated from soil and was stored in our laboratory (Mori, 1990). Strain YM4 and the mutant YM72 were grown under an atmosphere of carbon dioxide in M medium as described by Mori (Mori, 1990) supplemented with 10 g/L microcrystalline cellulose powder (Sigmacell type-20; Sigma–Aldrich, St. Louis, MO, USA) or with 5 g/L cellobiose (Sigma–Aldrich) as the carbon source. All media for *C. thermocellum* were degassed in boiling water and bubbled with high purity carbon dioxide gas. *Escherichia coli* DH5α (Takara Bio, Shiga, Japan), BL21 (DE3), and the plasmid pET22b (Merck KGaA, Darmstadt, Germany) served as the cloning host, expression host, and vector, respectively. *E. coli* cells were grown at 37 °C in Luria–Bertani medium containing ampicillin (100 μg/ml).

2.2. Mutagenesis and mutant screening

The mutagenesis of *C. thermocellum* followed the procedure as described by Zverlov et al. (Zverlov et al., 2008). The culture was centrifuged at room temperature in an anaerobic chamber (Hirasawa Works Inc., Tokyo, Japan), and resuspended in 5 ml of medium containing 1% cellobiose. Each 1-ml sample was mixed with ethyl-methanesulfonate (EMS) at final concentrations of 10, 50, and 100 mM. The mixtures were incubated at 60 °C for 2 h under static conditions. The cells were washed three times with the medium, and resuspended in 0.5 ml of fresh medium. The cell growth was sensitive to incubation with 1–50 mM of EMS as indicated by changes in optical density at 660 nm after 24 h at 60 °C. The cell suspension was inoculated into roll tube medium containing 1% microcrystalline cellulose powder and 1.5% Bacto agar (Difco Laboratories, Detroit, MI, USA), and cultured at 60 °C for 7 days. Colonies that showed zones of clearing were randomly picked from the YM4 grown roll tube medium containing 1% microcrystalline cellulose powder and 1.5% Bacto agar (Difco Laboratories). Approximately 500 single colonies were inoculated into 5 ml of liquid medium supplemented with 1% microcrystalline cellulose for 4 days at 60 °C along with the parent strain YM4. To compare the patterns of cellulosomal proteins from the mutants, cellulosomes in cell-free broth were isolated from fermenting cultures of the mutants using an affinity digestion method, which is based on binding of cellulosomes to amorphous cellulose. Over 90% of the cellulase activity in the crude supernatant can be recovered by this method. Cultures of 5 ml were centrifuged and the cell-free broth was incubated with phosphoric acid-swollen cellulose (ASC; 100 μg/ml of cell-free broth) overnight at 4 °C to obtain cellulosomes bound to the cellulose. On the following day, the amorphous cellulose with bound enzymes was centrifuged and washed twice with 50 mM sodium phosphate buffer (pH 7.0). The pellets were resuspended in 0.5 ml of the same buffer. To identify whether a spontaneous mutant showed a different pattern of cellulosomal proteins from that of the parent strain YM4, an aliquot was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was performed using 5–20% gradient polyacrylamide gels (ATTO, Tokyo, Japan) in accordance with the manufacturer's instructions. Samples used for SDS-PAGE were boiled for 5 min in sample buffer containing dithiothreitol (Sigma–Aldrich). After electrophoresis, gels were stained with Coomassie brilliant blue R-250 (Bio-Rad Laboratories, Hercules, CA, USA). Molecular mass standards were from Bio-Rad Laboratories. The strain YM72 shown to produce small molecular size CipA (ssCipA) was isolated three times from single colonies. *C. thermocellum* strain identity was proven by PCR sequencing of the 16S rRNA gene.

2.3. *Measurement of* thermocellum *growth properties*

The growth of *C. thermocellum* was determined by measuring the optical density and total protein concentration. When the medium containing cellobiose was used for *C. thermocellum* cultures, the cell growth was measured by optical density at 660 nm. Cell growth in medium supplemented with cellulose was monitored based on the increase in pellet protein concentration. Briefly, cells were lysed in a NaOH/SDS solution of 0.2 N NaOH (Wako Pure Chemical) containing 0.2% (w/v) SDS (Wako Pure Chemical). Cell debris and residual solids were pelleted from the NaOH/SDS solution and removed by centrifugation (9,700 × *g* for 5 min), and the protein concentration in the supernatant was estimated using the Pierce BCA assay kit (Thermo Fisher Scientific, Waltham, MA, USA) with bovine serum albumin as the standard.

2.4. Recombinant DNA techniques and sequencing of mutated CipA

The preparation of chromosomal and plasmid DNA, and the transformation were carried out by standard procedures or in accordance with supplier protocols (Qiagen, Frederick, MD, USA). Restriction digests of DNA were carried out as recommended by the manufacturer's instructions (Takara Bio). Primer pairs containing artificial restriction enzyme recognition sites (underlined) were used to amplify fragments of CipA (5′-AGTCAGAGCTCATGAGAAAAGTCATCAGT-3′ for the sense primer with *Sac*I sites, and 5′-ACTGTCTAGACTGTGCGTCGTAATCACTTG-3′ for the antisense primer with XbaI sites) from *C. thermocellum* YM4 and YM72. CelJ (GenBank: BAA12070) was amplified from *C. thermocellum* YM4 genomic DNA with the following forward and reverse primers 5′-CCGCCTCGAGGCCGAAACAGTTGCTCCTGAAGGCTACAGG-3′ and 5′-ATTGCTCAGCCCAGTCAATAGCATCTACATAGATAGT-3′, respectively (*Xho*I and *Bpu* 1102I restriction sites are underlined). PCR was performed using PrimeSTAR® HS DNA Polymerase (Takara Bio) under standard conditions according to the manufacturer's instructions. The amplified fragments for CipA and ssCipA were estimated as ∼5.5 kbp and ∼4.4 kbp, respectively, and inserted between the *Sac*I and *Xba*I sites of pET22b to generate pET22-CipA and pET22-ssCipA. Similarly, the 4.7 kbp amplified fragment of CelJ was inserted between the *Xho*I and *Bpu* 1102I sites of pET19b to generate pET19-CelJ. These expression vectors were transformed into *E. coli* BL21. The amplified fragment for ssCipA was also inserted between the *Sac*I and *Xba*I sites of pUC19 and transformed into *E. coli* JM109. To identify the complete sequence of ssCipA, the inserted fragment was sequenced using the Deletion Kit for Kilo-Sequencing kit (Takara Bio). Approximately 100 clones containing deleted fragments of ssCipA were detected and sequenced from double stranded DNAs of the selected colonies. The nucleotide sequence of ssCipA has been deposited in the GenBank database under the following accession no. JX050175.