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Comparison of Family 9 Cellulases from Mesophilic and Thermophilic Bacteria[▽]

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Cellulases containing a family 9 catalytic domain and a family 3c cellulose binding module (CBM3c) are important components of bacterial cellulolytic systems. We measured the temperature dependence of the activities of three homologs: *Clostridium cellulolyticum* Cel9G, *Thermobifida fusca* Cel9A, and *C. thermocellum* Cel9I. To directly compare their catalytic activities, we constructed six new versions of the enzymes in which the three GH9-CBM3c domains were fused to a dockerin both with and without a *T. fusca* fibronectin type 3 homology module (Fn3). We studied the activities of these enzymes on crystalline cellulose alone and in complex with a miniscaffoldin containing a cohesin and a CBM3a. The presence of Fn3 had no measurable effect on thermostability or cellulase activity. The GH9-CBM3c domains of Cel9A and Cel9I, however, were more active than the wild type when fused to a dockerin complexed to scaffoldin. The three cellulases in complex have similar activities on crystalline cellulose up to 60°C, but *C. thermocellum* Cel9I, the most thermostable of the three, remains highly active up to 80°C, where its activity is 1.9 times higher than at 60°C. We also compared the temperature-dependent activities of different versions of Cel9I (wild type or in complex with a miniscaffoldin) and found that the thermostable CBM is necessary for activity on crystalline cellulose at high temperatures. These results illustrate the significant benefits of working with thermostable enzymes at high temperatures, as well as the importance of retaining the stability of all modules involved in cellulose degradation.

Microorganisms employ diverse strategies for converting cellulose into simple sugars (18, 28). Aerobic microorganisms secrete large amounts of free cellulases that act in synergy to solubilize cellulose. These enzymes usually include a carbohydrate binding module (CBM) that promotes binding to the solid substrate (3, 18, 28). Anaerobic microorganisms, on the other hand, produce high-molecular-weight complexes called cellulosomes in which multiple enzymes are gathered on a scaffoldin protein (1). A typical scaffoldin contains a CBM and several anchoring domains called cohesins, which tightly bind cellulases via complementary dockerin domains in the cellulases.

As part of our overall effort to generate sets of highly active, highly stable cellulases by structure-guided recombination of natural homologs (14), we have investigated the stabilities and activities of three homologous glycoside hydrolase (GH) family 9 cellulases: Cel9G from the mesophile *Clostridium cellulolyticum* (10) and Cel9A (15, 17) and Cel9I (11) from the thermophiles *Thermobifida fusca* and *Clostridium thermocellum*. These enzymes are endoglucanases with a processive mode of action. We selected these enzymes for their high activity on crystalline substrates. Indeed, *T. fusca* Cel9A has the highest activity of any individual *T. fusca* enzyme on crystalline cellulose (17). It has been reported that *C. thermocellum* Cel9I alone is capable of solubilizing filter paper (11). Similarly, Cel9G in complex with a truncated scaffoldin, a small protein composed of a cohesin domain and a CBM3a which promotes attachment of the dockerin-containing cellulases to cellulose, releases more soluble sugars than other cellulases from *C. cellulolyticum* (22). *C. cellulolyticum* Cel9G has an optimal pH of 7.0, whereas that of *C. thermocellum* Cel9I is 5.5 (10, 11). *T. fusca* Cel9A is reported to retain more than 70% of its activity from pH 4.7 to pH 10.1 (15). All experiments in the present study were performed at pH 6.0.

These three cellulases contain a GH9 catalytic domain of 49 to 55 kDa joined by a short linker to a CBM3c of 16 to 17 kDa. Unlike typical CBMs that bind crystalline cellulose, CBM3c does not; it assists in catalysis by disrupting the cellulose hydrogen bond network and directing the freed chain toward the enzyme's active site (4, 20, 24). The crystal structures of *C. cellulolyticum* Cel9G (20) and *T. fusca* Cel9A (24) show a GH9 domain that consists of an $(\alpha/\alpha)_6$ barrel catalytic domain joined with limited flexibility to the antiparallel β-sandwich fold CBM3c. Cel9G's GH9-CBM3c is additionally fused to a dockerin domain, which allows it to form cellulosome complexes. In contrast, Cel9A and Cel9I are not complexed; the GH9-CBM3c is instead fused to typical high-affinity CBMs (CBM2 and CBM3b, respectively) that allow them to bind the solid substrate (Fig. 1). *T. fusca* Cel9A also contains a fibronectin type 3 homology module (Fn3). Zhou et al. have shown that a deletion of this domain leads to decreased activity of the protein on cellulose (29). Fn3 domains are found in a wide variety of cellulases. Kataeva et al. have shown that the Fn3 of *Clostridium thermocellum* CbhA increases the hydrolysis of filter paper by modifying the surface of the cellulose fibers (16). We were interested in knowing whether addition of the Fn3 module of *T. fusca* Cel9A would enhance the activity of other cellulases.

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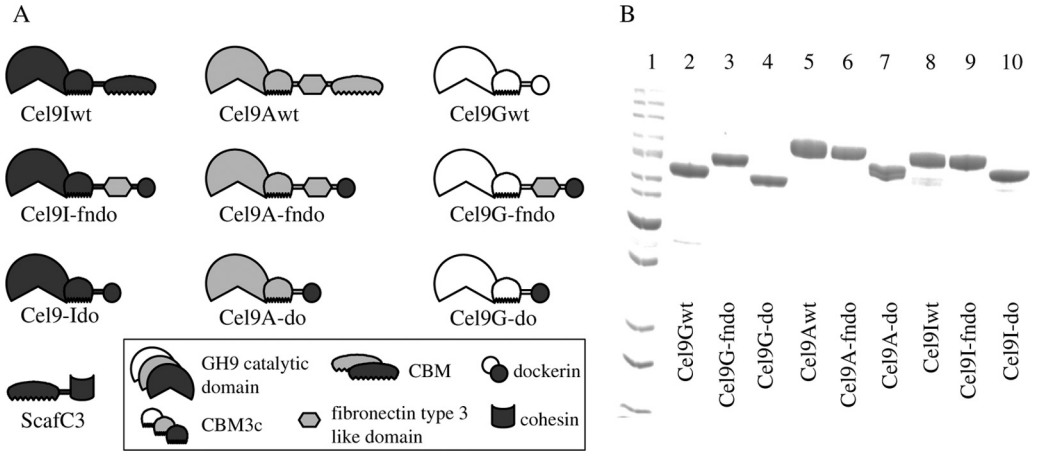

FIG. 1. Recombinant cellulases used in this study. (A) Schematic representation of the Cel9 cellulase constructs. White (*C. cellulolyticum*), gray (*T. fusca*), and black (*C. thermocellum*) denote the sources of the respective modules (see boxed key to symbols). (B) SDS-PAGE of the purified enzymes. Lane 1, molecular size markers (260, 160, 110, 80, 60, 50, 40, 30, and 15 kDa); lane 2, Cel9Gwt (77 kDa); lane 3, Cel9G-fndo (90 kDa); lane 4, Cel9G-do (78 kDa); lane 5, Cel9Awt (92 kDa); lane 6, Cel9A-fndo (89 kDa); lane 7, Cel9A-do (77 kDa); lane 8, Cel9Iwt (96 kDa); lane 9, Cel9I-fndo (95 kDa); lane 10, Cel9I-do (83 kDa).

To compare these three enzymes all in the same context, we constructed six new enzymes by fusing the GH9-CBM3c domains of Cel9G, Cel9A, and Cel9I to a *C. thermocellum* dockerin domain both with and without the *T. fusca* Fn3 module (Fig. 1). We determined the thermostabilities of the wild-type Cel9 enzymes and the new fusion enzymes and compared their activities on both soluble and insoluble substrates. We also determined the temperature-dependent activity profiles of these enzymes on crystalline cellulose and looked at the effects of combining the dockerin-appended enzymes with a miniscaffoldin. Finally, we compared the activities on crystalline cellulose of our most stable Cel9 enzymes to that of *Hypocrea jecorina* cellobiohydrolase II (CBH II, also known as Cel6A), an efficient CBH of industrial interest.

Studies that have compared cellulolytic systems from thermophilic and mesophilic organisms (19, 25, 27) show that thermophilic systems tend to outperform their mesophilic counterparts at elevated temperatures, but few comparisons of individual cellulases from these two biotopes have been made (2, 26). Thermostable enzymes are of interest due to the fact that enzyme activity usually increases with temperature. In addition, thermostable enzymes generally exhibit longer lifetimes at all temperatures. We thus compared thermophilic and mesophilic bacterial Cel9 cellulases to determine to what extent the thermophilic enzymes outperform their counterparts that function at lower temperatures.

MATERIALS AND METHODS

Construction of plasmids encoding the wild-type proteins. *C. cellulolyticum* Cel9Gwt, *T. fusca* Cel9Awt, *C. thermocellum* Cel9Iwt, *C. cellulolyticum* ATCC 35319 (kindly provided by James C. Liao), and *C. thermocellum* ATCC 27405 (from the global resource center, ATCC, Manassas, VA) were used as sources of genomic DNA. The DNA encoding *C. cellulolyticum* Cel9G was amplified by PCR from genomic DNA using the forward primer GNdeIF (5′-TTTT**CATATG**GCAGGAA CATATAACTATGGAGAAGC-3′) (boldface indicates a restriction site) and the reverse primer GXhoIR (5′-AA**CTCGAG**GCCTTGAGGTAATTGGGTGATTG-3′). An NdeI site in the wild-type gene was removed by direct mutagenesis using primers GsupNdeIF (5′-GATTTCT**C**T**T**ATGATGGATTACCAACCACC-3′) (underlining indicates a mutation) and GsupNdeIR (5′-GGTGGTTGGTAATCCAT **CATA**A**G**AGAAATC-3′). The DNA encoding *C. thermocellum* Cel9I was amplified by PCR from genomic DNA using primers INdeIF (5′-GATATA**CATATG**CTAT GGGGGCGAAAGACTTCAAG-3′) and IXhoIR (5′-AAA**CTCGAG**AGGTTCT TTGCCGTAAACAAGC-3′). Two BsaXI sites in the wild-type gene were removed by direct mutagenesis using primers IsupBsaF1 (5′-GGTATGATGCGGGTGATC ACG-3′) and IsupBsaR1 (5′-CGTGATCACCCGCATCATACC-3′) for the first BsaXI and primers IsupBsaF2 (5′-GAATTACATATACACCGAAGGGACTTG C-3′) and IsupBsaR2 (5′-GCAAGTCCCTTCGGTGTATATGTAATTC-3′) for the second BsaXI site. DNA encoding *T. fusca* Cel9A with NdeI and XhoI sites at the 5′ and 3′ ends, respectively, was synthesized by DNA2.0 (Menlo Park, CA) with *Escherichia coli* codon bias. The fragments were cloned into NdeI-XhoI-linearized pET22b (Novagen, Madison, WI), generating plasmids pET-cel9Gwt, pET-cel9Iwt, and pET-cel9Awt.

Construction of plasmids encoding the cellulases fused to a dockerin domain. The DNA encoding the dockerin domain of *C. thermocellum* cellulase Cel48S was amplified from *C. thermocellum* genomic DNA using primers doBamF (5′-AAA**GGATCC**ATGACATATAAAGTACCTGGTACTCC-3′) and doXhoIR (5′-AAA**CTCGAG**GTTCTTGTACGGCAATGTATC-3′). The fragment was cloned into BamHI-XhoI-linearized pET22b, generating pET-do. The DNA encoding the catalytic domain and the CBM3c of Cel9Gwt, Cel9Iwt, and Cel9Awt was amplified from plasmids pET-cel9Gwt, pET-cel9Iwt, and pET-cel9Awt using primers GNdeIF, INdeIF (see above), and ENdeIF (5′-TT**CATA TG**GAACCGGCGTTTAAC-3′) and GBamR (5′-AAACTCGAG**GGATCC**AC CTGCGGGTTCGTTACC-3′), IBamR (5′-AAACTCGAG**GGATCC**TGAGCC CGGTTCCCTTCC-3′), and EBamR (5′-AAACTCGAG**GGATCC**TTCTTCCG GTGCGGTGC-3′). The three fragments were cloned into NdeI-BamHI-linearized pET-do to generate pET-cel9G-do, pET-cel9I-do, and pET-cel9A-do.

Construction of plasmids encoding the cellulases fused to a fibronectin type 3 homology module and a dockerin module. The DNA encoding the fibronectin type 3 homology module of *T. fusca* Cel9Awt was amplified from pET-cel9Awt using primers FnF1 (5′-TTT**GGATCC**GAGCCGGGTGGTGGTG-3′) and FnDoR2 (5′-*GGAGTACCAGGTACTTTATATGTCAT*GTTCTCCGCCAAGG TGG-3′; the sequence matching the 3′ end of the DNA encoding the fibronectin type 3 module is underlined, and the sequence matching the 5′ end of the DNA encoding the *C. thermocellum* Cel48S dockerin module is in italics). The DNA encoding the *C. thermocellum* Cel48S dockerin module was amplified from pET-do using primers FnDoF3 (5′-CCACCTTGGCGGAGAAC*ATGACATAT AAAGTACCTGGTACTCC*-3′; the sequence matching the 3′ end of the DNA encoding the fibronectin type 3 module is underlined, and the sequence matching the 5′ end of the DNA encoding the *C. thermocellum* Cel48S dockerin module is in italics) and doXhoIR (see above). The two resulting overlapping fragments were mixed, and a combined fragment was synthesized using primers FnF1 and doXhoIR. The fragment was cloned into BamHI-XhoI-linearized pET22b, generating pET-fndo. The DNA encoding the GH9 catalytic domain and the CBM3c of Cel9G, Cel9I, and Cel9A was digested from pET-cel9G-do, pET-cel9I-do, and pET-cel9A-do with NdeI and BamHI and cloned into NdeI-BamHI-linearized

pET-fndo, generating plasmids pET-cel9G-fndo, pET-cel9I-fndo, and pET-cel9A-fndo.

Construction of plasmids encoding the miniscaffoldin. The DNA encoding the CBM and the third cohesin of the *C. thermocellum* scaffoldin protein CipA was amplified from *C. thermocellum* genomic DNA using primers ScafC3F (5′-GATA TA**CATATG**GTATCAGGCAATTTGAAGGTTGAATTCTAC-3′) and ScafC3R (5′-GATG**CTCGAG**ATCTCCAACATTTACTCCACCGTC-3′). The PCR fragments were digested with NdeI and XhoI and cloned into NdeI-XhoI-linearized pET22b, generating pET-scafC3.

Production and purification of recombinant proteins. Plasmids were introduced into strain BL21(DE3). The bacteria were grown in Terrific Broth medium supplemented with glycerol (4 g/liter) and 200 mg/liter ampicillin to an A_{600} of 1.8. The culture was cooled to 18°C, and isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 100 μM. After 16 h, cells were harvested by centrifugation (5,000 × *g*, 10 min, 4°C), resuspended in 30 mM Tris-HCl (pH 8.0)–1 mM $CaCl_2$–0.1 mg/ml DNase I, and lysed by sonication. Cell debris was harvested by centrifugation (5,000 × *g*, 20 min, 4°C), and C-terminally His-tagged proteins, contained in the soluble fraction, were purified on a HisTrap HP column (GE Healthcare, Uppsala, Sweden). After elution, proteins were concentrated using a Vivaspin spin column with a 10,000 molecular weight cutoff (Sartorius AG, Goettingen, Germany), resuspended in 10 mM Tris-HCl (pH 8.0)–1 mM $CaCl_2$, and stored at −80°C. The purity of the recombinant proteins was tested by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% acrylamide gels (Fig. 1B). The protein concentration was estimated by measuring absorbance at 280 nm in 6 M guanidine hydrochloride–25 mM sodium phosphate, pH 6.5, according to the estimated values calculated using the ProtParam tool (www.expasy.org/tools/protparam.html).

Hypocrea jeronica CBH II was kindly produced, purified, and provided by Indira Wu (14). The gene encoding C-terminally His_6-tagged *H. jeronica* CBH II was designed with the *Saccharomyces cerevisiae* codon bias, synthesized by DNA2.0 (Menlo Park, CA), and cloned into yeast expression vector YEp352/PGK91-1-αss. The fungal cellulase was produced and secreted by *S. cerevisiae* strain YDR483W BY4742 (14). His_6-tagged CBH II was purified from the supernatant on a HisTrap HP column (GE Healthcare, Uppsala, Sweden). After elution, proteins were concentrated using a Vivaspin spin column with a 10,000 molecular weight cutoff (Sartorius AG, Goettingen, Germany), resuspended in 50 mM sodium acetate (pH 5.0)–1 mM phenylmethylsulfonyl fluoride–0.02% NaN_3, and stored at −20°C.

Enzyme activity. Cellulase activity on carboxymethyl cellulose (CMC, sodium salt, low viscosity; Sigma, St. Louis, MO) and Avicel (Avicel PH101; Fluka, Buchs, Switzerland) was determined by the Park and Johnson ferricyanide method using D-glucose as the standard (23).

We defined T_{50} as the temperature at which an enzyme loses 50% of its activity after 30 min of incubation. The enzymes at a concentration of 10 μM in 10 mM Tris-HCl (pH 8.0)–1 mM $CaCl_2$ were incubated at 40, 43, 46, 49, 52, 55, 58, and 61°C for the different versions of Cel9G; 58, 61, 64, 67, 70, 73, 76, at 79°C for the different versions of Cel9A, and at 64, 67, 70, 73, 76, 79, 82, and 85°C for the different versions of Cel9I. Thermal inactivation was carried out in a PCR block. The residual activity was determined with 10 nM enzyme on 4 g/liter CMC–50 mM succinate (pH 6.0)–1 mM $CaCl_2$ for 20 min at 40°C.

Specific activity was calculated after 20 min of incubation of 10 nM enzyme on 4 g/liter CMC–50 mM succinate (pH 6.0)–1 mM $CaCl_2$ at 40°C. One unit (IU) of activity corresponds to 1 μmol of D-glucose equivalent liberated per min.

T_{50} values of Cel9Iwt and Cel9I-do on Avicel were estimated by inactivating 10 μM enzyme in 10 mM Tris-HCl (pH 8.0)–1 mM $CaCl_2$ at 64, 67, 70, 73, 76, 79, 82, and 85°C for 30 min. The residual activity was measured on 3 g/liter Avicel–50 mM succinate (pH 6.0)–1 mM $CaCl_2$ for 1 h at 60°C, with Cel9Iwt at 100 nM and Cel9I-do at 200 nM.

The temperature-activity profile was determined by measuring the quantity of released soluble sugar after 20 min of incubation of 100 nM enzyme alone or in complex on 10 g/liter Avicel–50 mM succinate (pH 6.0)–1 mM $CaCl_2$ at 30, 40, 50, 60, 70, 80, and 90°C. Complexes were obtained by mixing 200 μl of dockerin-containing enzymes at 20 μM in 10 mM Tris-HCl (pH 8.0)–1 mM $CaCl_2$ with 200 μl of miniscaffoldin ScafC3 at 20 μM in 10 mM Tris-HCl (pH 8.0)–1 mM $CaCl_2$. Complexes were stored at 10 μM at −80°C.

The effect of CBMs on activity was determined by incubating Cel9I-do alone or in complex with ScafC3 (Cel9I-do/ScafC3). The final concentration of Cel9I-do and of the complex during the test was 100 nM. The quantity of released soluble sugar was examined after incubation at 60°C for 1 h on 1, 3, or 10 g/liter Avicel in 50 mM succinate (pH 6.0) and 1 mM $CaCl_2$.

The activity of dockerin-containing enzymes (Cel9A-do, Cel9A-fndo, Cel9I-do, and Cel9I-fndo) in complex with the miniscaffoldin ScafC3 (Cel9A-do/ScafC3, Cel9A-fndo/ScafC3, Cel9I-do/ScafC3, and Cel9I-fndo/ScafC3) was compared to that of the wild-type enzymes Cel9Awt and Cel9Iwt. The activity of the complexes and the wild-type enzymes was determined by incubating 100 nM Cel9Awt, Cel9Iwt, Cel9A-do/ScafC3, Cel9A-fndo/ScafC3, Cel9I-do/ScafC3, and Cel9I-fndo/ScafC3 for 1 h on 10 g/liter Avicel–50 mM succinate (pH 6.0)–1 mM $CaCl_2$ at 50°C.

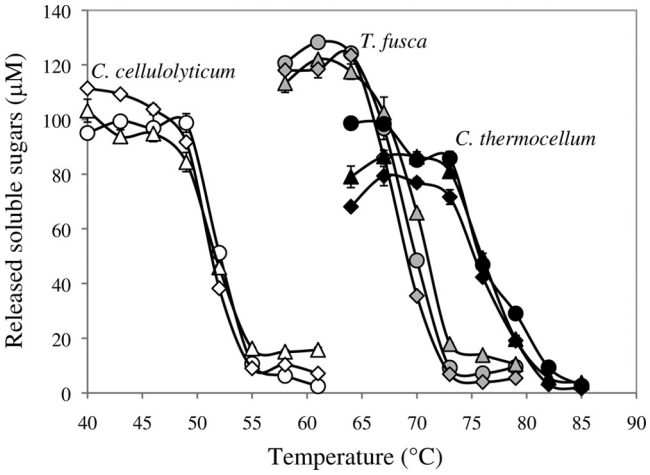

FIG. 2. Residual activities of the recombinant cellulases on CMC after incubation for 30 min at different temperatures. Curves are labeled as follows (colors refer to organisms, and shapes represent different constructs): ○, wild-type enzymes; ◇, enzymes fused to a dockerin; △, enzymes fused to an Fn3 module and a dockerin. The bars indicate the standard deviations of two independent experiments. Enzymes were incubated at different temperature for 30 min. Residual activities were assayed after 20 min on 4 g/liter CMC at 40°C with a final protein concentration of 10 nM.

RESULTS

Cellulase thermostability. The relative thermostabilities of the wild-type and newly constructed Cel9 fusion enzymes (summarized in Fig. 1) were examined by comparing their T_{50} values, which were defined as the temperatures at which the enzymes lose 50% of their activity on CMC after 30 min of incubation (Fig. 2). All three Cel9Gs (wild-type Cel9G, Cel9G with the dockerin Cel9G-do, and Cel9G-fndo containing the dockerin and the Fn3 domain) have a T_{50} value of 52°C. The thermophilic Cel9 enzymes are more stable: *T. fusca* Cel9A and its Cel9A-do and Cel9A-fndo derivatives all have a T_{50} value of 69°C, while the *C. thermocellum* Cel9I, Cel9I-do, and Cel9I-fndo T_{50} value is even higher, 76°C. The presence of the Fn3 domain or a dockerin or deletion of the high-affinity CBM (CBM2 in Cel9A and CBM3b in Cel9I) has little or no impact on the T_{50}. These results indicate that the binding or Fn3 modules appended to the wild-type cellulases do not influence the thermostability of the catalytic GH9-CBM3c domain in the presence of a soluble substrate.

Activities on cellulosic substrates. After a 20-min incubation on 4 g/liter CMC at 40°C, Cel9Awt, Cel9A-do, and Cel9A-fndo release 120 μM soluble sugars, Cel9Gwt, Cel9G-do, and Cel9G-fndo release 100 μM soluble sugars, and Cel9Iwt, Cel9I-do, and Cel9I-fndo release 80 μM soluble sugars. The specific activity on CMC of Cel9Awt, Cel9A-do, and Cel9A-fndo is 600 IU/μmol [μmol of reducing sugar/(μmol of enzyme × min)], that of Cel9Gwt, Cel9G-do, and Cel9G-fndo is 500 IU/μmol, and that of Cel9Iwt, Cel9I-do, and Cel9I-fndo is

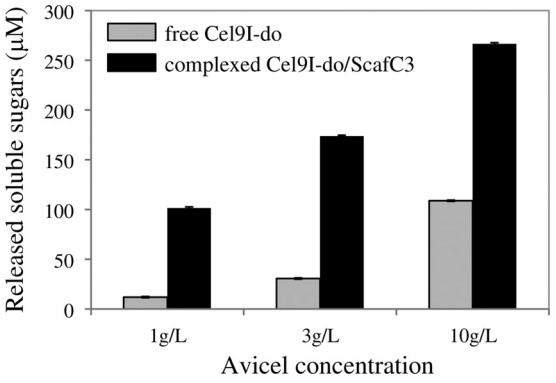

FIG. 3. Contribution of the CBM to activity on different concentrations of crystalline cellulose. The avicelase activities of the free enzyme Cel9I-do and the Cel9I-do/ScafC3 complex were determined on different concentrations of Avicel. The data are the amount of released soluble sugars after 1 h at 60°C. The final protein or complex concentration was 100 nM. The standard deviation of two independent experiments is indicated at the top of each bar. The miniscaffoldin alone, which contains a CBM, does not release soluble sugars. The data demonstrate the impact of the high-affinity CBM on the activity of Cel9Ido, an effect that depends on the Avicel concentration.

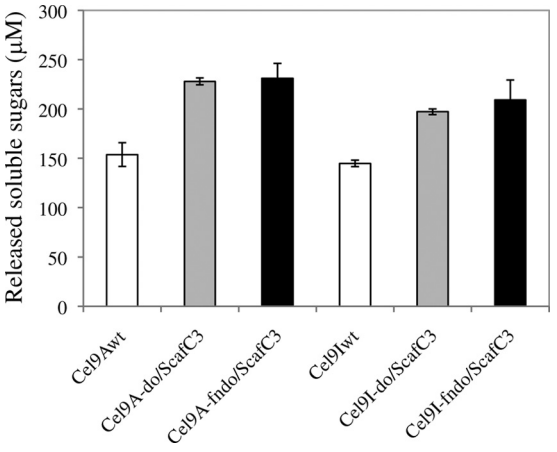

FIG. 4. Activities of wild-type enzymes Cel9Iwt and Cel9Awt and their dockerin-appended proteins in complex with ScafC3 on crystalline cellulose (Avicel). The white bars represent the activities of wild-type enzymes Cel9Iwt and Cel9Awt. The gray bars represent the activities of Cel9A-do and Cel9I-do in complex with ScafC3. The black bars represent the activities of Cel9A-fndo and Cel9I-fndo in complex with ScafC3. Bars indicate the standard deviations of two independent experiments. The enzymes or the proteins contained in the complexes are indicated at the bottom of the graph. The final concentration of enzymes or complexes was 100 nM, and the Avicel concentration was 10 g/liter. Released soluble sugars were assayed after 1 h at 50°C.

400 IU/μmol. Activities on soluble CMC therefore followed the order Cel9A > Cel9G > Cel9I, and deletion of the Fn3, dockerin, and CBM domains had no effect on activity under the conditions tested.

High-affinity CBMs are known to strongly influence cellulase activity on crystalline substrates (1). To compare the activities of the different enzymes on crystalline cellulose in the presence of a high-affinity CBM, we used the miniscaffoldin protein ScafC3, containing CBM3a and the third cohesin of the *C. thermocellum* scaffoldin CipA (Fig. 1). The interaction between the third cohesin of *C. thermocellum* CipA and the dockerin of *C. thermocellum* cellulase Cel48S is well characterized and has a K_A greater than 10^{11} M^{-1} (8). Mixing a scaffoldin-containing cohesin with a dockerin-containing enzyme at an equimolar ratio in the presence of $CaCl_2$ leads to the formation of a complex (8, 12). All of our engineered enzymes contain this *C. thermocellum* Cel48S dockerin, which allows us to investigate the effects of attaching a strong CBM.

We first compared the activity of free Cel9I-do to that of Cel9I-do complexed with ScafC3 on various concentrations of Avicel (Fig. 3). Cel9I-do/ScafC3 is 2.4, 5, and 8 times as active as Cel9I-do alone on 10, 3, and 1 g/liter Avicel, respectively. In general, the high-affinity CBM enhances cellulose hydrolysis and this effect is most pronounced at lower concentrations of crystalline substrate.

We also compared a directly fused high-affinity CBM to one formed by complexation with scaffoldin. In recent studies, free enzymes from aerobic microorganisms were altered by the addition of a dockerin to create new cellulosomal or "complexed" enzymes (5, 6, 21, 22). When in a complexed mode, enzymes could exhibit either increased or reduced activities on crystalline cellulose. Here we analyzed the effect of converting *C. thermocellum* Cel9I and *T. fusca* Cel9A into complexed enzymes. As shown in Fig. 4, the activities of the complexes (Cel9A-do/ScafC3, Cel9A-fndo/ScafC3, Cel9I-do/ScafC3, and Cel9I-fndo/ScafC3) are significantly different from those of the wild-type enzymes (Cel9Awt and Cel9Iwt). The Cel9A-do/ScafC3 and Cel9A-fndo/ScafC3 complexes are 1.5 times as active as Cel9Awt, while the Cel9I-do/ScafC3 and Cel9I-fndo/ScafC3 complexes are 1.3 times as active as Cel9Iwt. Thus, converting these noncomplexed GH9-CBM3c cellulases to a complexed mode enhances their activity on crystalline cellulose. The Fn3 module, however, does not affect activity: during a 1-h incubation on Avicel, we found no significant difference between the complexes that had the Fn3 module and those that did not.

The temperature-activity profiles of the enzymes fused to a dockerin in complex with the miniscaffoldin ScafC3 (Cel9G-do/ScafC3, Cel9A-do/ScafC3, and Cel9I-do/ScafC3) were determined on cellulose between 30 and 90°C (Fig. 5). Cel9A-do/ScafC3 is 1.2 to 1.5 times as active as the Cel9G-do and Cel9I-do complexes with ScafC3, which have similar activities between 30 and 60°C. The optimal temperatures (T_{opt}s) of Cel9G-do/ScafC3, Cel9A-do/ScafC3, and Cel9I-do/ScafC3 are 60, 70, and 80°C, respectively. These results are consistent with the relative thermostabilities of the Cel9 enzymes (Fig. 2). Furthermore, these results show that high temperatures lead to improvements in cellulose hydrolysis. Indeed, Cel9I-do/ScafC3 is nine times more active at 80°C than at 30°C. This enzyme's Q_{10} (the relative increase in activity for each 10°C increment in temperature) is consistently 1.5, up to 70°C.

Activity and stability of the different Cel9I cellulase constructs. The T_{opt} for the activity of complexed Cel9I-do/scafC3 is 80°C. To determine whether activity at elevated temperature is limited by any single component of the complex—i.e., the GH9-CBM3c, dockerin, cohesin, or ScafC3 domain—the activity of Cel9I-do alone was determined at various temperatures (Fig. 6). Like the Cel9I-do/ScafC3 complex, Cel9I-do alone has a T_{opt} of 80°C, which suggests that the loss of activity

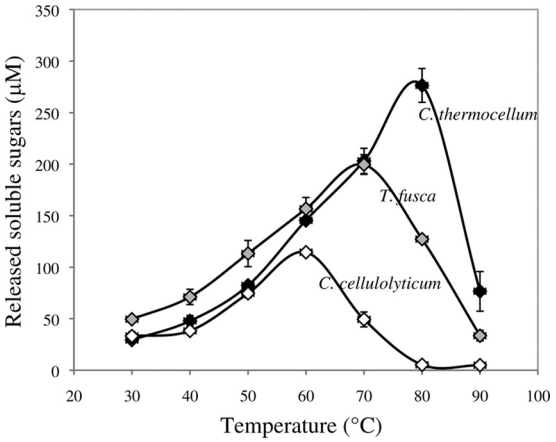

FIG. 5. Temperature dependence of the activities of three homologous Cel9 cellulases in complex with a miniscaffoldin on Avicel. Curves are labeled as follows: white for *C. cellulolyticum* Cel9G-do/ScafC3, gray for *T. fusca* Cel9A-do/ScafC3, and black for *C. thermocellum* Cel9I-do/ScafC3. Bars indicate the standard deviations of two independent experiments. Released soluble sugars were assayed after 20 min with 100 nM complex and on 10 g/liter Avicel.

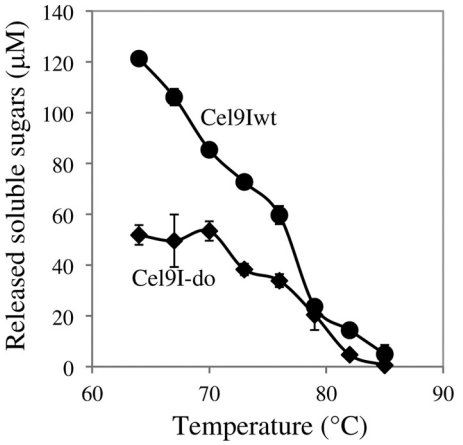

FIG. 7. Residual activities of Cel9I-do and Cel9Iwt on Avicel after incubation for 30 min at different temperatures. Cel9Iwt and Cel9I-do were incubated for 30 min at different temperatures. The residual activities of the enzymes after thermal inactivation were determined on Avicel at 60°C. Released soluble sugars were assayed after 1 h. A lower concentration (3 g/liter) of Avicel was used to amplify the CBM effect. The final concentrations of Cel9Iwt and Cel9I-do were 100 nM and 200 nM, respectively. The bars indicate the standard deviations of two independent experiments.

at high temperatures occurs as a result of denaturation of the GH9-CBM3c catalytic module rather than inactivation of other modules in the complex.

A previous study reported that Cel9Iwt is optimally active at 70°C (11). We determined the activity of Cel9Iwt between 30 and 90°C and also observed a T_{opt} of 70°C on crystalline cellulose (Fig. 6). The difference between Cel9Iwt (T_{opt} = 70°C) and Cel9I-do alone or in complex with ScafC3 (T_{opt} = 80°C) could be explained if the CBM3b of the wild-type cellulase is less stable than the CBM3a of the scaffoldin protein. Our results showed that the presence of CBM3b fused to GH9-CBM3c does not affect the stability of the latter, as determined by the T_{50} on CMC (Fig. 2).

To investigate whether the stability of CBM3b of Cel9Iwt indeed limits activity at high temperature, Cel9Iwt and Cel9I-do were incubated at a series of temperatures and their residual activities were measured on Avicel. As shown in Fig. 7, the activity of Cel9I-do shows no change after 30 min of inactivation at 73°C, while that of Cel9Iwt is already lower after 30 min of inactivation at 67°C. This suggests that CBM3b of Cel9Iwt is less thermostable than the GH9-CBM3c module.

Comparison of Cel9I-do/ScafC3 and *Hypocrea jecorina* CBH II on Avicel at different temperatures. Previous reports have shown that Cel9I is one of the most efficient cellulases produced by *C. thermocellum*. We compared the activity of Cel9I-do/ScafC3 to that of an efficient fungal cellulase of industrial interest, *H. jecorina* CBH II (14). At 30, 40, and 50°C, *H. jecorina* CBH II is 1.7, 1.6, and 1.4 times as active as the Cel9I-do/ScafC3 complex, on a molar basis (Fig. 8). As ex-

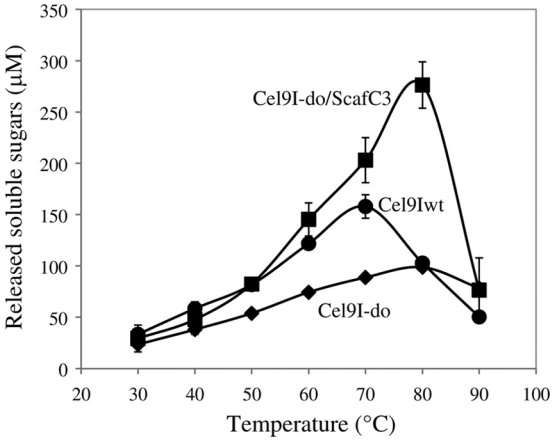

FIG. 6. Temperature dependence of the activities of Cel9Iwt and Cel9I-do alone or in complex with a miniscaffoldin on Avicel. Circles represent the wild-type enzyme Cel9Iwt; diamonds represent Cel9I-do free; squares represent Cel9I-do in complex with ScafC3. Bars indicate the standard deviations of two independent experiments. The final enzyme and complex concentration was 100 nM. Released soluble sugars were assayed after 20 min on 10 g/liter Avicel.

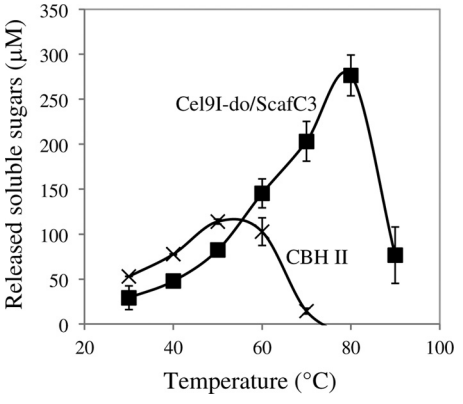

FIG. 8. Activities of Cel9Ido in complex with a miniscaffoldin and of *H. jecorina* CBH II on Avicel at different temperatures. The activity of Cel9I-do in complex with ScafC3 (■) was compared with the activity of *H. jecorina* CBH II (×). Bars indicate the standard deviations of two independent experiments. Released soluble sugars were assayed after 20 min on 10 g/liter Avicel with 100 nM protein or complex.

pected, however, the highly thermostable Cel9I-do/ScafC3 complex outperforms the fungal enzyme at elevated temperatures. The T_{opt} of CBH II is 50°C; it loses stability at higher temperatures and has a half-life of only 2 min at 63°C (13, 14). At their respective T_{opt}s, the *C. thermocellum* Cel9I-do/ScafC3 complex is 2.5 times as active as *H. jecorina* CBH II.

DISCUSSION

Numerous cellulases from different biotopes have been sequenced and characterized. However, comparing them based on literature data is highly problematic when activities have been measured under different conditions. Meaningful comparisons are also difficult because these enzymes are multimodular and often vary in module composition. In this study, we have measured the thermostabilities and activities of three homologous GH9-CBM3c cellulases using a consistent set of experimental conditions and by creating derivatives of these cellulases having the same domain compositions.

In a previous study, Voutilainen et al. compared homologous family 7 CBHs with different thermostabilities (26). They observed that these enzymes had different avicelase activities at low temperature and found little or no increase in activity going from 45°C to 70°C. Here, in contrast, we found that the activities of the three homologous bacterial family 9 enzymes containing a dockerin domain in complex with a miniscaffoldin are all similar at low temperature and increase significantly with temperature until reaching a T_{opt} above which the enzymes lose function. The T_{opt}s of Cel9G-do/ScafC3, Cel9A-do/ScafC3, and Cel9I-do/ScafC3 are 60°C, 70°C, and 80°C. At their respective T_{opt}s, Cel9I-do/ScafC3 is more active than Cel9G-do/ScafC3 by a factor of 2.5. These enzyme complexes all contain the same do/ScafC3 module; thus, differences in activity result from differences in the GH9-CBM3c domain. These enzymes all have roughly the same activity at low temperature, and the 2.5-fold increase in activity between Cel9G-do/ScafC3 and Cel9I-do/ScafC3 reflects the benefit of performing catalysis at a higher temperature. This observation at the level of individual enzymes illustrates a simple reason why entire thermophilic cellulolytic systems can be more efficient at their T_{opt} than mesophilic ones (18, 27).

To date, no comparison of the entire cellulolytic systems of *C. cellulolyticum* and *C. thermocellum* has been carried out. However, data from the literature suggest that the complete *C. thermocellum* cellulolytic system also outperforms the *C. cellulolyticum* one at the respective T_{opt}s (18). Nonetheless, mesophilic cellulolytic bacteria are perfectly able to grow on cellulose. The facts that cellulolytic systems are less efficient at low temperatures and that cellulose hydrolysis limits the rate of microbial cellulose utilization might explain why the specific growth rate of the mesophile *C. cellulolyticum* is lower than that of the thermophile *C. thermocellum* (the specific growth rates in continuous culture on crystalline cellulose are 0.083 h^{-1} at 34°C and 0.17 h^{-1} at 60°C, respectively [18]).

We also compared the activities of the complexed bacterial cellulase Cel9I-do/ScafC3 and the fungal enzyme *H. jecorina* CBH II. On a molar basis, CBH II is more active at lower temperatures (≤60°C). When they are compared at their respective T_{opt}s, Cel9I-do/ScafC3 is the more active. However, the observed molecular mass of CBH II is only 55 kDa (47 kDa for the nonglycosylated polypeptide chain [14]) versus 121 kDa for Cel9I-do/ScafC3 (38 kDa for the miniscaffoldin and 83 kDa for Cel9I-do), and thus, CBH II is considerably more active than the bacterial enzyme per unit of mass. The high specific activities of fungal cellulases and their high production levels in fungal hosts make them particularly interesting for industrial applications. The observation that bacterial cellulase activity can increase significantly at elevated temperatures motivates our efforts to generate highly active thermostable fungal cellulases suitable for higher-temperature applications (13, 14).

In this study, we also investigated the impact of the different modules appended to the wild-type enzymes. Fn3 modules, for example, are common among bacterial extracellular GHs. In most cases, the GC content of these modules corresponds to that of the host genome, suggesting they have been in place long enough to acquire the character of the host organism and to play a biological role (16). Zhou et al. showed that deletion of the Fn3 module between GH9-CBM3c and CBM2 in *T. fusca* Cel9A decreased activity on CMC and on the crystalline substrate bacterial microcrystalline cellulose (29). Here we created two mutant forms of *T. fusca* Cel9A. In the first, we deleted only CBM2, and in the second, we deleted CBM2 and the Fn3 domain. With these mutant proteins, we did not observe any impact of the Fn3 domain on the hydrolysis of CMC. Nor did we see differences in activity on Avicel when these two mutant proteins were in a complex with the miniscaffoldin ScafC3. Zhou et al. determined activity after incubating the enzymes for 16 h, whereas we incubated the enzymes for 20 min on CMC or for 1 h on Avicel. Thus, we cannot exclude the possibility that the Fn3 module can have an effect after a longer time. The Fn3 module of *C. thermocellum* CBH CbhA appears to erode the surface of filter paper fibers (16). We cannot exclude the possibility that CBM3a contained in the miniscaffoldin ScafC3 plays a similar role of eroding cellulose fibers and masks the impact of the Fn3 module.

Dockerin is an important module of anaerobic GHs, where it is critical for the assembly of the cellulosome, a complex in which multiple enzymes anchor via their dockerin modules to a high-affinity CBM-containing scaffoldin protein (12). Despite secreting a relatively small quantity of enzymes, anaerobic microorganisms efficiently utilize cellulose by producing a mixture of "complexed" and "noncomplexed" enzymes. On the other hand, aerobic microorganisms secrete only free enzymes in large quantities. The ability of cellulosomes to gather enzymes in proximity to one another enhances their synergistic effect (7, 9) and could provide an advantage to anaerobic microorganisms that secrete less enzyme. Here we have compared homologous enzymes from aerobic (*T. fusca*) and anaerobic (*C. cellulolyticum* and *C. thermocellum*) bacteria under the same set of conditions. The individual catalytic components of anaerobic and aerobic cellulolytic systems display similar activities on crystalline cellulose, up to a temperature of 60°C. This suggests that the efficiency of anaerobic complexes is not a result of higher inherent catalytic activity but is more likely a result of enhanced activity due to enzyme proximity in the cellulosome.

Solubilization of cellulose involves several cellulases acting in synergy. To be efficient at high temperature, all of the enzymes of the cellulolytic system have to be thermostable, and having a system of cellulases with comparable thermostabilities

is more important than having a few individually thermostable components (27). Similarly, cellulases are usually multimodular enzymes, and function at high temperature requires that all of the modules be folded and functional. Wild-type *C. thermocellum* Cel9I has a T_{opt} on crystalline cellulose lower than that of the engineered version in complex with a scaffoldin, which we attribute to the CBM3b of the wild-type enzyme being less stable than the CBM3a of the scaffoldin protein. The importance of the CBM to cellulose hydrolysis becomes more pronounced at low substrate concentrations. Using a stable scaffoldin and stable CBM in complex with a cellulase can circumvent problems with loss of CBM function at high temperature. Our results also show the remarkable stability of the dockerin and cohesin modules, which maintain function at 80°C.

Altogether, these experiments highlight the benefits of working at high temperatures with thermostable cellulases on crystalline substrates and the importance of the stability of all of the modules in the enzyme complex.

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