Applied and Environmental Microbiology, Apr. 2010, p. 2582–2588 Vol. 76, No. 8
0099-2240/10/$12.00 doi:10.1128/AEM.02735-09


# Increased Crystalline Cellulose Activity via Combinations of Amino Acid Changes in the Family 9 Catalytic Domain and Family 3c Cellulose Binding Module of *Thermobifida fusca* Cel9A[▽]

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Received 10 November 2009/Accepted 11 February 2010

**Amino acid modifications of the *Thermobifida fusca* Cel9A-68 catalytic domain or carbohydrate binding module 3c (CBM3c) were combined to create enzymes with changed amino acids in both domains. Bacterial crystalline cellulose (BC) and swollen cellulose (SWC) assays of the expressed and purified enzymes showed that three combinations resulted in 150% and 200% increased activity, respectively, and also increased synergistic activity with other cellulases. Several other combinations resulted in drastically lowered activity, giving insight into the need for a balance between the binding in the catalytic cleft on either side of the cleavage site, as well as coordination between binding affinity for the catalytic domain and CBM3c. The same combinations of amino acid variants in the whole enzyme, Cel9A-90, did not increase BC or SWC activity but did have higher filter paper (FP) activity at 12% digestion.**

Cellulases catalyze the breakdown of cellulose into simple sugars that can be fermented to ethanol. The large amount of natural cellulose available is an exciting potential source of fuels and chemicals. However, the detailed molecular mechanisms of crystalline cellulose degradation by glycoside hydrolases are still not well understood and their low efficiency is a major barrier to cellulosic ethanol production.

*Thermobifida fusca* is a filamentous soil bacterium that grows at 50°C in defined medium and can utilize cellulose as its sole carbon source. It is a major degrader of plant cell walls in heated organic materials such as compost piles and rotting hay and produces a set of enzymes that includes six different cellulases, three xylanases, a xyloglucanase, and two CBM33 binding proteins (12). Among them are three endocellulases, Cel9B, Cel6A, and Cel5A (7, 8), two exocellulases, Cel48A and Cel6B (6, 19), and a processive endocellulase, Cel9A (5, 7).

*T. fusca* Cel9A-90 (Uniprot P26221 and YP_290232) is a multidomain enzyme consisting of a family 9 catalytic domain (CD) rigidly attached by a short linker to a family 3c cellulose binding module (CBM3c), followed by a fibronectin III-like domain and a family 2 CBM (CBM2). Cel9A-68 consists of the family 9 CD and CBM3c. The crystal structure of this species (Fig. 1) was determined by X-ray crystallography at 1.9 Å resolution (Protein Data Bank [PDB] code 4tf4) (15). Previous work has shown that E424 is the catalytic acid and D58 is the catalytic base (11, 20). H125 and Y206 were shown to play an important role in activity by forming a hydrogen bonding network with D58, an important supporting residue, D55, and Glc(−1)O1. Several enzymes with amino acid changes in subsites Glc(−1) to Glc(−4) had less than 20% activity on bacterial cellulose (BC) and markedly reduced processivity. It was proposed that these modifications disturb the coordination between product release and the subsequent binding of a cellulose chain into subsites Glc(−1) to Glc(−4) (11). Another variant enzyme with a deletion of a group of amino acids forming a block at the end of the catalytic cleft, Cel9A-68 Δ(T245-L251)R252K (DEL), showed slightly improved filter paper (FP) activity and binding to BC (20).

The CBM3c domain is critical for hydrolysis and processivity. Cel9A-51, an enzyme with the family 9 CD and the linker but without CBM3c, had low activity on carboxymethyl cellulose (CMC), BC, and swollen cellulose (SWC) and showed no processivity (4). The role of CBM3c was investigated by mutagenesis, and one modified enzyme, R557A/E559A, had impaired activity on all of these substrates but normal binding and processivity (11). Variants with changes at five other CBM3c residues were found to slightly lower the activity of the modified enzymes, while Cel9A-68 enzymes containing either F476A, D513A, or I514H were found to have slightly increased binding and processivity (11) (see Table 1). In the present work, CBM3c has been investigated more extensively to identify residues involved in substrate binding and processivity, understand the role of CBM3c more clearly, and study the coordination between the CD and CBM3c. An additional goal was to combine amino acid variants showing increased crystalline cellulose activity to see if this further increased activity. Finally, we have investigated whether the changes that improved the activity of Cel9A-68 also enhanced the activity of intact Cel9A-90.

## MATERIALS AND METHODS

**Strains and plasmids.** *Escherichia coli* DH5α (Stratagene) was used as the host strain for plasmid extraction, *E. coli* BL21(DE3) (Stratagene) was the host strain for Cel9A-68 protein expression, and *Streptomyces lividans* TKM31 (9, 19) was the host for Cel9A-90 protein expression. The gene for Cel9A-68 (613 amino acids) with the *T. fusca* Cel6A signal sequence (MRMSPRPLRALLGAAAAA LVSAAALAFPSQAA) in the pET-26b+ vector (Novagen) was used as the

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▽ Published ahead of print on 19 February 2010.

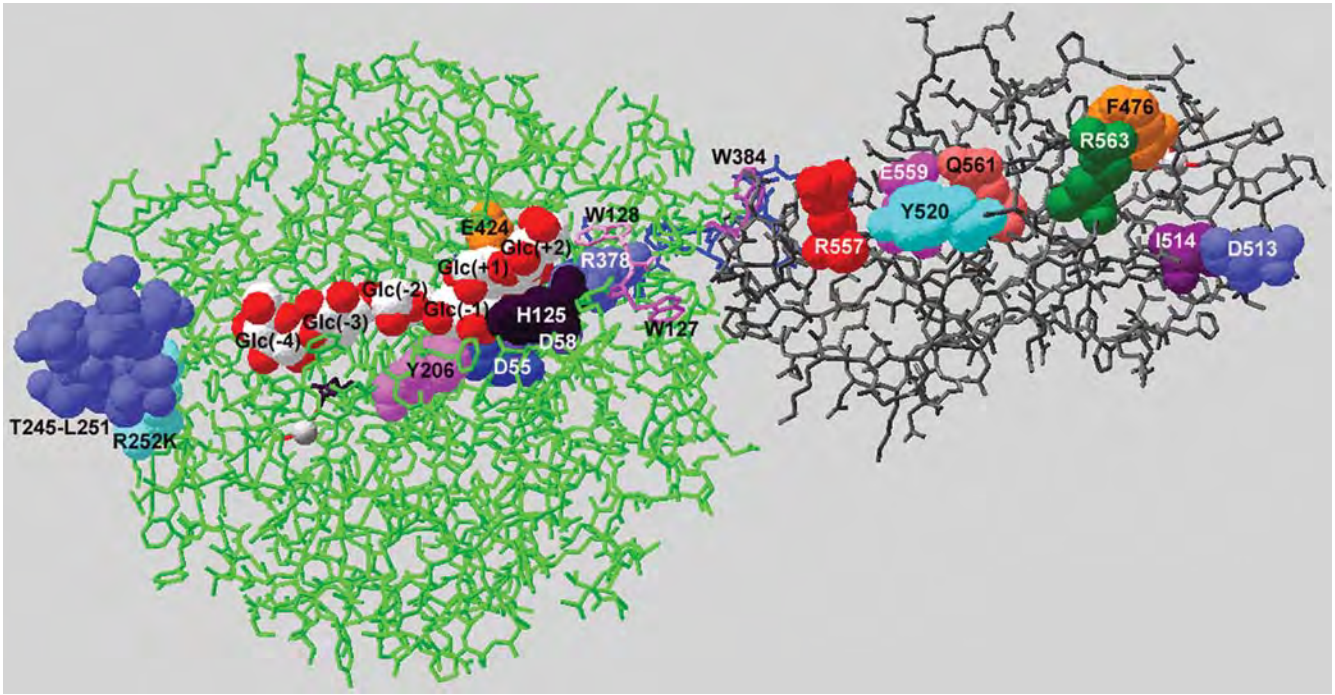


FIG. 1. Crystal structure of Cel9A-68 (PDB code 4tf4) showing the locations of the variant residues, catalytic acid E424, catalytic base D58, hydrogen bonding network residues D55, H125, and Y206, and six glucose residues, Glc(−4) to Glc(+2). Part of the linker is visible in dark blue.

template for mutagenesis (Fig. 2A). The plasmid coding for Cel9A-68 Δ(T245-L251)R252K, removing the block at one end of the catalytic cleft, was constructed as previously described (20).

**Mutagenesis and DNA manipulations.** Standard nucleic acid techniques were used as described by Sambrook and Russell (16). All point mutations were generated using the QuikChange site-directed mutagenesis method (Stratagene) as described previously (11).

Cel9A-68 mutant plasmids and the Cel9A-68 Δ(T245-L251)R252K plasmid were digested by restriction enzymes BsrGI and FseI (Fig. 2A), and the gel-purified fragments were combined using T4 DNA ligase to construct double-mutant genes.

In order to construct Cel9A-90 cross CD and CBM3c mutant plasmids, the Cel9A FNIII domain and the CBM2 gene were amplified by PCR from Cel9A-90 DNA in the pET-26b+ vector using forward primer 5′-GCGGCCCCGGATG GGACCCCTCCAACGACTGGT-3′ and reverse primer 5′-GTGGTGGTGGT GCTCGACTAGTGCCGCCCGCGGGGCGGACG-3′. The PCR product was digested by SanDI-SpeI and inserted into the desired SanDI-SpeI-digested Cel9A-68 double-mutant plasmids (Fig. 2A). The DNA sequences of all mutated Cel9A genes were determined by the Cornell Biotechnology Resource Center using a Perkin-Elmer/Applied Biosystems automated sequencer to confirm the presence of the desired mutations and that no other mutations were present. The Cel9A-90 proteins could not be expressed in *E. coli*, and the Cel9A-90 mutant genes were cloned into *S. lividans-E. coli* shuttle plasmid pSZ46 (4) as follows. The HindIII-SpeI fragment of pSZ46 codes for the Cel9A upstream DNA (including the promoter), the Cel9A signal peptide, and the Cel9A-68 structural protein. There is a unique BsrGI restriction site located prior to any desired mutations (Fig. 2A). A three-piece ligation was run using the HindIII-BsrGI fragment from pSZ46, the BsrGI-SpeI fragment from Cel9A-90 Δ(T245-L251)R252K in the pET plasmid, and pBluescript digested with HindIII and SpeI to produce strain D1642. An SspI site was placed just after the HindIII site using linkers to produce strain D1643. Plasmid DNA from each of the other desired Cel9A-90 mutant genes in pET26 was digested with BsrGI and SpeI (1.85 kb) and ligated to the (3.87-kb piece) plasmid DNA from D1643 also digested with BsrGI and SpeI. Plasmid DNA from these transformants was sequenced to be sure that only the desired mutations were present. Each of the above clones was cut with HindIII, SpeI, and AlwnI, and the unique 2.8-kb piece containing the SspI site and coding for the Cel9A promoter, the Cel9A signal sequence, and each Cel9A-90 variant protein was ligated to *S. lividans-E. coli* shuttle vector pSES1 DNA prepared from strain D851 (pSZ46) also digested with HindIII-SpeI (Fig. 2B).

Plasmid DNA from each construction was transformed into nonmethylating host strain D1194 (ET12567/pUZ8002 $Str^r$ $Tet^r$ $Cml^r$ $Kan^r$), and plasmid DNA was prepared. In order to increase plasmid stability in *S. lividans*, each plasmid was cut with SspI to remove most of the pUC vector present in the shuttle vector (Fig. 2B). This DNA was prepared for *Streptomyces* transformation by heat treatment at 65°C for 20 min to inactivate SspI, overnight ligation, ethanol precipitation, and resuspension in 10.3% sucrose at 1 μg/μl. Transformation into protoplasts of *S. lividans* low-protease strain TKM31 was performed according to standard protocols (3). All solutions and media were maintained at pH 7.1 to 7.5. Single transformant colonies were transferred into 200 μl of tryptic soy broth (TSB), dispersed by trituration with stirring, and spread successively on three plates to generate single colonies. This is necessary because *S. lividans* grows in puffballs consisting of linked tangled threads of bacteria. Single colonies of transformants were transferred onto duplicate LB (20 g/liter)–nutrient broth (8 g/liter)–0.02 M *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES; pH 7.2)–thiostrepton (tsr; 25 μg/ml) agar plates (LB-NUT plates) and grown for 2 or 3 days. The colonies on one plate were tested with a CMC overlay activity assay. This selection procedure was repeated until 24 out of 24 colonies were uniform and had good CMC activity. Each strain was grown in TSB–0.02 M TES (pH 7.2) buffer–tsr (10 μg/ml) liquid medium, tested again for uniformity, and finally preserved by freezing in 16% glycerol at −70°C.

**Enzyme production and purification.** Cel9A-68 variant enzymes were produced in *E. coli* BL-21(DE3) as described previously (11). Cel9A-90 variant enzymes were purified from the *S. lividans* TKM31 strains as follows. A 300-ml overnight culture was grown in TSB–0.02 M TES buffer (pH 7.2)–10 μg/ml tsr at 30°C with shaking. This culture was used to inoculate 10 liters of TSB plus tsr in a New Brunswick ML114 fermenter. The fermentation conditions were pH 7.2 using 5 M ammonium hydroxide and 5 M HCl, 40% dissolved oxygen, 30°C, agitation at 250 rpm, and a 12-liter/min air flow. After 36 to 40 h of growth, the culture supernatant was recovered by centrifugation at 4,050 × g for 30 min. Phenylmethylsulfonyl fluoride was added to 0.1 mM to inhibit proteases, and ammonium sulfate was added to 1 M. The supernatant was further centrifuged at 7,025 × g and filtered through a 0.2-μm NOM filter cartridge AU09Z13NG020 Beta pure polypropylene/polyester filter (CUNO) at <2 lb/in$^2$. The clarified supernatant was loaded onto a 100-ml (5-cm-diameter) phenyl Sepharose (Sigma) hydrophobic affinity column overnight. The column was

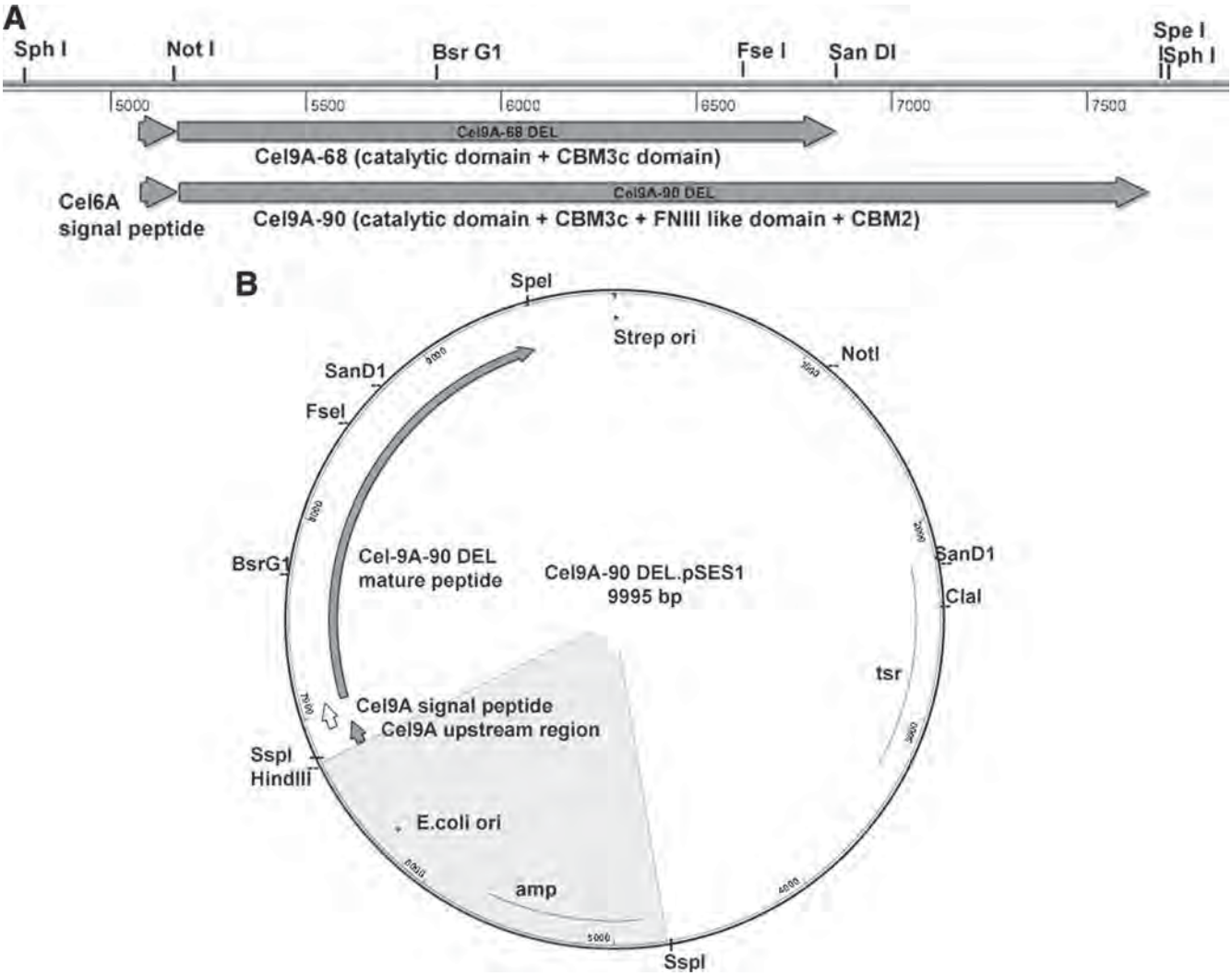


FIG. 2. Cel9A plasmid constructions. (A) Cel9A DEL insert as cloned into pET26b+. (B) Configuration of Cel9A DEL as cloned into *E. coli-S. lividans* shuttle plasmid pSES1. The shaded area denotes the SspI-SspI fragment deleted before transformation into *S. lividans*.

washed with 200 ml of 1 M ammonium sulfate, followed by a 300-ml (total volume) gradient of 0.5 M ammonium sulfate–0.01 M NaCl–20 mM $KH_2PO_4$/$K_2HPO_4$ (Kpi; pH 6) to 20 mM Kpi (pH 6) to remove some contaminating proteins and pigments. Cel9A proteins were eluted with 5 mM Kpi (pH 6). Fractions containing the highest Cel9A-to-total protein ratios as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis were combined. This material was diluted with 5 mM Bis-Tris (pH 5.6) buffer at 1:2 and loaded onto a 100-ml (5-cm-diameter) Q Sepharose anion-exchange column. Protein was eluted with a 1,500-ml (total volume) gradient of 5 mM Bis-Tris (pH 5.6)–0.05 M NaCl–10% glycerol to 5 mM Bis-Tris (pH 5.6)–0.6 M NaCl–10% glycerol. The purest fractions were combined and concentrated with buffer exchange to 5 mM ammonium acetate (pH 6.8)–10% glycerol in an Amicon stirred cell concentrator. A typical yield was 150 mg of purified protein from a 10-liter fermentation.

**Activity assays.** The activities of wild-type and variant Cel9A enzymes were determined on CMC (10 mg/ml), BC (2.5 mg/ml), phosphoric acid-SWC (2.5 mg/ml), and Whatman no. 1 FP (8 mg/ml). All assays were run in a volume of 0.4 ml in triplicate at 50°C for about 16 h in 0.05 M Na acetate–0.015 M $CaCl_2$ (pH 5.5) using from 0.5 to 600 pmol of protein, as needed. Reducing sugars were measured using the dinitrosalicylic acid (DNS) reagent (2) and a standard curve of Sigma 635-100 glucose standard solution. To calculate activity, the optical density at 600 nm was converted to micromoles of cellobiose by comparing a cellobiose standard curve and a glucose standard curve. A major advantage of the DNS reagent is that it fits the assay range well and the differing amounts of enzyme in the reaction do not produce a signal. The disadvantage of the DNS reagent, used with a cellobiose standard, is that glucose will be about 30% underestimated and cellotriose will be slightly overestimated. Variant enzyme activities were determined concurrently with wild-type enzyme activity. The nanomoles of protein used ($X$) were plotted versus the micromoles of cellobiose produced ($Y$) and fitted to the equation $Y = m_1X/(m_2 + X)$ (where $m_1$ and $m_2$ are constants generated) using the program KaleidaGraph from Synergy Software, and this curve was used to determine the amounts of enzyme required to digest each substrate to the various target values. If this curve fit was poor, a smooth curve fit was used. This allows the use of multiple data points to calculate the specific activity at a target percent digestion of the insoluble and variable substrates. For those enzymes that could not reach the target digestion, activity was calculated from the micromoles of cellobiose produced by 0.6 nmol (1.5 μM) enzyme. The average coefficients of variation among triplicates were 5.7, 6.4, 6.0, and 5.5% for the CMC, SWC, BC, and FP activity assays, respectively.

Processivity was determined from the distribution of reducing ends between the FP (insoluble) and the supernatant (soluble) by following a published procedure (5). Thin-layer assays of sugar products and thermostability tests were performed as previously described (20).

## RESULTS

**Activity of pertinent CBM3c and CD single-variant enzymes.** Cel9A CBM3c lacks the sequence of aromatic residues

TABLE 1. Activities of Cel9A-68 CBM3c variant enzymes and CD variant enzymes used to create the double variants

| Enzyme | Activity (% of wild type) on: | | | | % Processivity | % BC binding | Reference |
|---|---|---|---|---|---|---|---|
| | CMC | SWC | BC | FP[a] | | | |
| Wild type | 100 | 100 | 100 | 100 | 100 | 15 | This work |
| R378K | 98 | 91 | 103 | 93 | 139 | 20 | 11 |
| DEL[b] | 98 | 101 | 101 | 128 | 96 | | 20 |
| F476A | 97 | 105 | 79 | 100 | 145 | 21 | 11 |
| D513A | 100 | 115 | 121 | 107 | 119 | 20 | 11 |
| I514H | 104 | 91 | 112 | 104 | 110 | 23 | 11 |
| Y520A | 108 | 78 | 33[a] | 79 | 87 | 14 | 11 |
| R557A | 103 | 98 | 60[a] | 93 | 90 | | This work |
| E559A | 86 | 90 | 30[a] | 70 | 94 | | This work |
| R557A+E559A | 90 | 75 | 15[a] | 75 | 106 | 15 | 11 |
| Q561A | 103 | 56 | 51[a] | 78 | 74 | | This work |
| R563A | 97 | 70 | 52[a] | 93 | 129 | 20 | 11 |

[a] The target percent digestion could not be reached; activity was calculated using 1.5 μM enzyme.
[b] DEL refers to deletion of T245 to L251 and R252K.

contributing to strong substrate binding which exists in most CBM family 3a members. However, it has the flat face made up of strands 1, 2, 4, and 7 and appears to directly interact with cellulose (15). The width of the CBM is about 25Å, that of a cellulose chain plus a hydroxyl group is about 6Å, and that of a hydrogen bond is about 2Å (13, 17). Therefore, the flat face of CBM3c is potentially wide enough to hold three crystalline cellulose chains. In order to identify the residues interacting with cellulose chains, 15 residues on the flat face (β1, β2, β4, and β7 strands), chosen based on sequence alignment (11), a model (1), and consideration of the structure, were screened by alanine-scanning mutagenesis (10). The CBM3c modifications that affected activity are summarized in Table 1. Replacement of R557, E559, Y520, Q561, and R563 with A produced lower BC activity (Fig. 1), showing that they are important for crystalline hydrolysis. However, all of these substitutions except Q561A produced almost wild-type processivity. These amino acids are "in line" with the catalytic cleft, and this CBM probably interacts with only one chain of crystalline cellulose, as predicted by molecular docking and molecular dynamic simulation (14).

A catalytic cleft R378K variant enzyme, which normally hydrogen bonds to Glc(+1)O2, had increased processivity (Table 1). Deletion of a group of residues which appeared to block the catalytic cleft after the Glc(−4) residue [Δ(T245-L251)R252K (DEL)], resulted in increased Cel9A-68 FP activity. The CBM3c D513A and I514H variant enzymes had slightly improved BC activity and processivity, and the F476A variant had markedly improved processivity.

**Activity of CD and CBM3c double-variant enzymes.** In an attempt to construct an enzyme with improved activity on crystalline cellulose, two double-variant, R378K+D513A and R378K+I514H, enzymes were studied to see if the changes had additive effects. Unexpectedly, both of these enzymes had decreased activity on all of the substrates tested (Table 2) and processivity was impaired dramatically. A DEL+R378K enzyme was constructed to test the balance of binding on the two

TABLE 2. Specific activities of wild-type and double-variant enzymes at different target digestion levels

| Enzyme | Activity (μmol cellobiose/min-μmol enzyme) on: | | | | | | Processivity (soluble/insoluble reducing ends) |
|---|---|---|---|---|---|---|---|
| | CMC, 7.5% digestion | SWC, 15% digestion | BC, 15% digestion | FP, 16 h[b] | FP, 42 h, 10% digestion | FP, 42 h, 12% digestion | |
| Cel9A-68 | | | | | | | |
| Wild type | 36 | 3.1 | 1.9 | 0.27 | | | 3.1 |
| R378K+D51A | 28 | 0.67[b] | 0.36[b] | 0.14 | | | 1.8 |
| R378K+I514H | 28 | 0.66[b] | 0.37[b] | 0.12 | | | 1.5 |
| R378K+DEL | 88 | 1.2 | 0.43[b] | 0.21 | | | 2.2 |
| DEL+F476A | 140 | 6.7 | 3.3 | 0.34 | | | 3.7 |
| DEL+D513A | 110 | 6.3 | 3.0 | 0.36 | | | 3.4 |
| DEL+I514H | 87 | 7.0 | 2.5 | 0.33 | | | 4.2 |
| Cel9A-90 | | | | | | | |
| Wild type | 62 | 36 | 9.7 | | 0.96 | 0.52 | 13 |
| DEL[a] | 87 | 18 | 5.5 | | 1.02 | 0.76 | 10 |
| DEL+F476A | 77 | 24 | 6.2 | | 1.02 | 0.76 | 11 |
| DEL+D513A | 100 | 27 | 5.5 | | 0.94 | 0.68 | 10 |
| DEL+I514H | 69 | 15 | 6.0 | | 1.06 | 0.84 | 11 |

[a] DEL refers to deletion of T245 to L251 and R252K.
[b] The target percent digestion could not be reached; activity was calculated using 1.5 μM enzyme.

sides of the cleavage site in the CD, and this enzyme also had reduced processivity and BC activity.

Three variant Cel9A-68 enzymes, DEL+D513A, DEL+I514H, and DEL+F476A, were constructed to further study coordination between the CD and CBM3c, and each of these enzymes had markedly improved activities on all of the substrates, as well as improved processivity (Table 2). Their BC activity is clearly higher, well above 20% digestion (Fig. 3A).

In nature, crystalline cellulose degradation usually is carried out by a mixture of exo- and endoenzymes with synergistic activities (18). Cel9A is a processive endocellulase, and it can synergize with both exocellulases and endocellulases. Therefore, it was of interest to see if the improved enzymes had improved synergism in crystalline cellulose degradation. Wild-type Cel9A-68 and the DEL+D513A, DEL+I514H, and DEL+F476A variants were tested with two endocellulases, Cel6A and Cel5A, and two exocellulases, Cel6B and Cel48A, on FP as the substrate (Table 3). The results showed that the variant enzymes could increase crystalline cellulose activity as mixtures with both endocellulases and exocellulases. The activity of the mixture of the DEL+F476A enzyme plus Cel48A was 330% of that of the wild-type mixture.

Cel9A-90 cannot be expressed efficiently in *E. coli*; therefore, the same combinations of variants of full-length Cel9A were expressed and purified from *S. lividans*. Table 2 shows that their CMC activities were somewhat increased but the SWC and BC activities were lower than the wild-type activity, although in some cases, they were higher than that of the DEL enzyme (Fig. 3B). FP digestion by Cel9A-90 is much more efficient than that of Cel9A-68, which cannot even reach 5% digestion. The Cel9A-90 double variants had about wild-type FP activity at 10% digestion, and at 12% digestion, they had higher activity than the wild-type enzyme (Table 2 and Fig. 4). The enzymes were tested for thermostability and found to be the same as the wild-type enzyme (data not shown). The cellopentaose cleavage products of the wild-type and variant enzymes were estimated by thin-layer assays to be the same (data not shown). Synergism assays showed that the wild-type Cel9A-90 enzyme combined with either Cel5A or Cel48A had higher activity than any of the variant enzyme combinations (Table 3).

## DISCUSSION

**Understanding the mechanism of crystalline cellulose digestion by Cel9A.** These experimental results for the variant enzymes show that coordination is needed among cleavage of the cellulose molecule, product release, and movement of the cellulose chain into the catalytic cleft. Processivity is largely controlled by binding in the catalytic cleft, as amino acid changes in CBM3c do not affect processivity very much while a number of amino acid changes in the catalytic cleft drastically lowered processivity (11). Three new CBM3c variants reported in this work, R557A, E559A, and Q561A, lowered BC activity but did not affect processivity very much, supporting this conclusion.

Combining two CD amino acid variants that increased activity, R378K plus DEL, which are on opposite sides of the cleavage site, resulted in drastically lowered activity. This demonstrates the importance of a balance between release of the product and binding of the cellulose molecule into position for

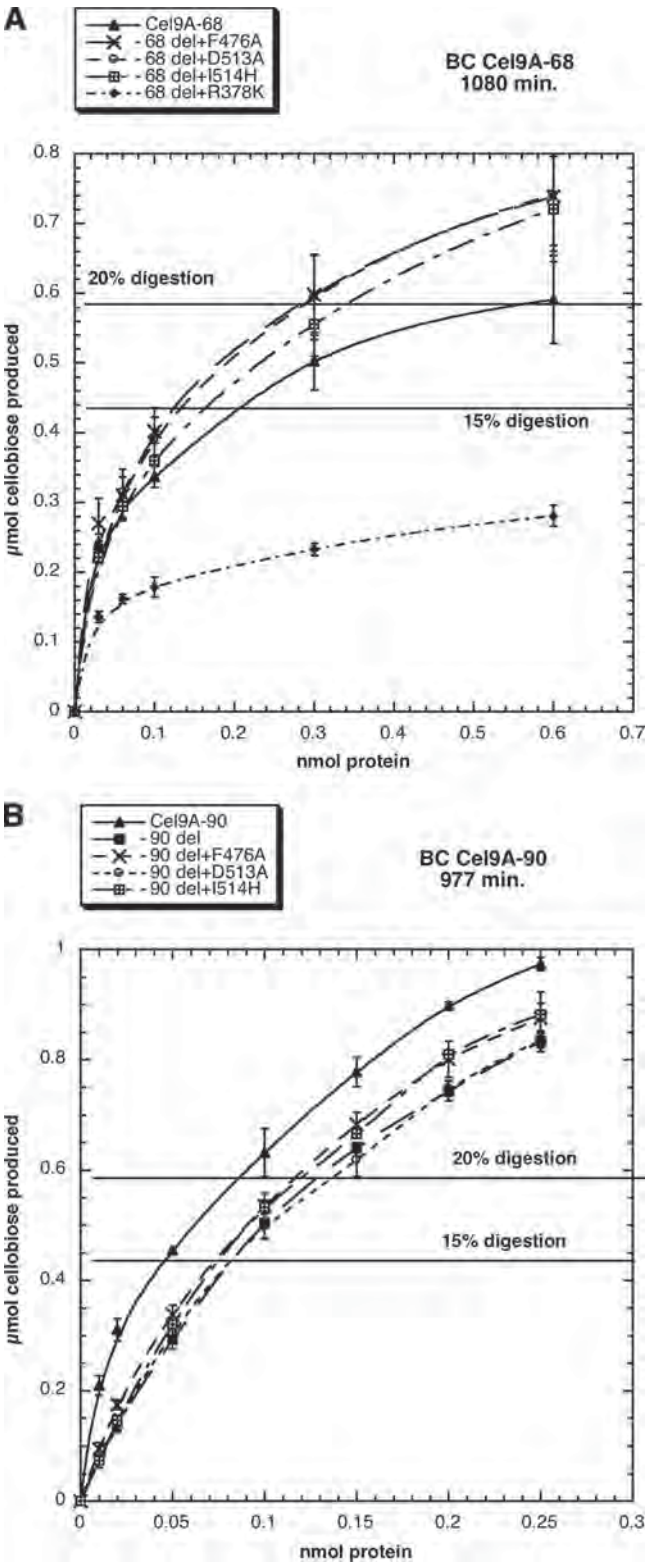


FIG. 3. BC activities of Cel9A-68 (A) and Cel9A-90 (B) variant enzymes compared to those of the wild-type enzymes.

TABLE 3. Synergistic activity between Cel9A enzymes and endocellulases Cel5A and Cel6A and exocellulases Cel6B and Cel48A

| Enzyme | FP activity (μmol cellobiose/min-μmol enzyme) | | | | | |
|---|---|---|---|---|---|---|
| | 9:1[b] Cel9A-68 + Cel5A, 3%[c] | 14:1[b] Cel9A-68 + Cel6A, 5%[c] | 1:1[b] Cel9A-68 + Cel6B, 5%[c] | 2:3[b] Cel9A-68 + Cel48A, 3%[c] | 3:2[b] Cel9A-90 + Cel5A, 12%[c] | 2:3[b] Cel9A-90 + Cel48A, 12%[c] |
| Wild type | 1.1 | 0.35 | 0.36 | 0.26 | 2.45 | 1.81 |
| DEL[a] | | | | | 1.57 | 1.14 |
| DEL+F476A | 1.32 | 0.63 | 0.72 | 0.87 | 2.24 | 1.14 |
| DEL+D513A | 1.35 | 0.57 | 0.71 | 0.75 | 1.99 | 0.86 |
| DEL+I514H | 1.15 | 0.46 | 0.48 | 0.57 | 1.80 | 1.04 |

[a] DEL refers to deletion of T245 to L251 and R252K.
[b] Mole ratio.
[c] Digestion level.

further cleavage. The poor activity of the R378K+D513A and R378K+I514H double-variant enzymes indicates that coordination between the CD and CBM substrate affinities needs to be precisely controlled.

Combinations of CBM3c variant enzymes with improved binding and processivity (F476A, D513A, I514H) and the CD (DEL) variant enzyme resulted in markedly improved CMC, SWC, and BC activities. However, in intact Cel9A-90 enzymes, these combinations were no longer advantageous. This suggests that better binding of the CD and CBM3c (Table 1) is good if those domains are used without a strong binding CBM2 domain, but with the strong binding domain, additional binding to the first two domains is a disadvantage. Perhaps the difference in CBM3c binding disturbs a cooperative interaction between CBM2 and the rest of the enzyme.

**Developing enzymes with improved activity.** Studies of amino acid point variations are very helpful in understanding how an enzyme carries out catalysis. However, it is unlikely that a single change will result in an enzyme with increased activity. Rather, a complicated array of changes is likely to be required and directed evolution is one possible method to create better enzymes. The CMC overlay method is useful as a first screen for functional enzymes, as most of the products produced by directed evolution will be defective. However, higher CMC activity is not a predictor of higher crystalline cellulose activity and an easy screening method for crystalline cellulose activity is needed. Using single or double variants in the initial step of directed evolution could reduce the size of the library needed to find improved enzymes and provide a better chance of success.

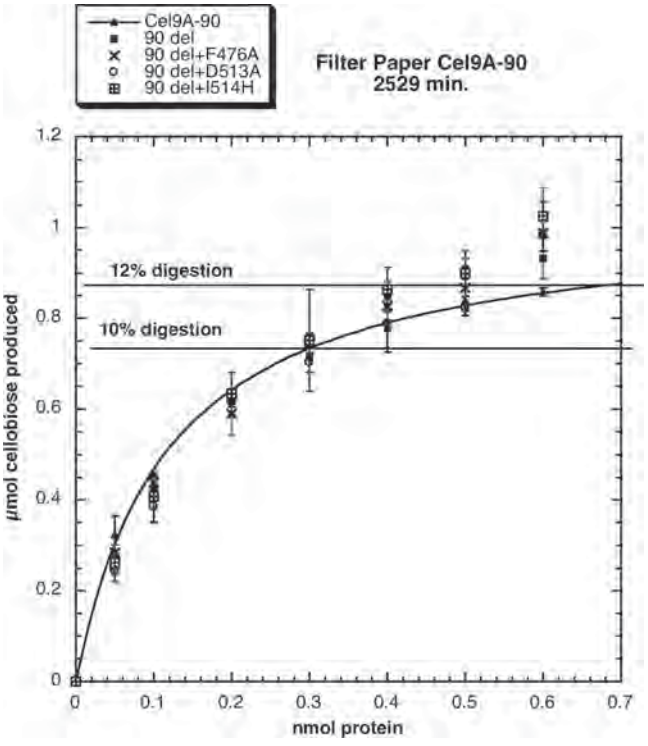


FIG. 4. FP activities of variant and wild type Cel9A-90 enzymes.

**ACKNOWLEDGMENT**

This work was supported by the DOE Office of Biological and Environmental Research Genomes to Life Program through the BioEnergy Science Center.

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