JOURNAL OF BACTERIOLOGY, Mar. 2008, p. 1976–1984 Vol. 190, No. 6
0021-9193/08/$08.00+0 doi:10.1128/JB.01667-07

Cel9D, an Atypical 1,4-β-D-Glucan Glucohydrolase from *Fibrobacter succinogenes*: Characteristics, Catalytic Residues, and Synergistic Interactions with Other Cellulases[▽]†

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Received 15 October 2007/Accepted 3 January 2008

The increasing demands of renewable energy have led to the critical emphasis on novel enzymes to enhance cellulose biodegradation for biomass conversion. To identify new cellulases in the ruminal bacterium *Fibrobacter succinogenes*, a cell extract of cellulose-grown cells was separated by ion-exchange chromatography and cellulases were located by zymogram analysis and identified by peptide mass fingerprinting. An atypical family 9 glycoside hydrolase (GH9), Cel9D, with less than 20% identity to typical GH9 cellulases, was identified. Purified recombinant Cel9D enhanced the production of reducing sugar from acid swollen cellulose (ASC) and Avicel by 1.5- to 4-fold when mixed separately with each of four other glucanases, although it had low activity on these substrates. Cel9D degraded ASC and cellodextrins with a degree of polymerization higher than 2 to glucose with no apparent endoglucanase activity, and its activity was restricted to β-1→4-linked glucose residues. It catalyzed the hydrolysis of cellulose by an inverting mode of reaction, releasing glucose from the nonreducing end. Unlike many GH9 cellulases, calcium ions were not required for its function. Cel9D had increased k_{cat}/K_m values for cello-oligosaccharides with higher degrees of polymerization. The k_{cat}/K_m value for cellohexaose was 2,300 times higher than that on cellobiose. This result indicates that Cel9D is a 1,4-β-D-glucan glucohydrolase (EC 3.2.1.74) in the GH9 family. Site-directed mutagenesis of Cel9D identified Asp166 and Glu612 as the candidate catalytic residues, while Ser168, which is not present in typical GH9 cellulases, has a crucial structural role. This enzyme has an important role in crystalline cellulose digestion by releasing glucose from accessible cello-oligosaccharides.

Plant material is now viewed as the bioenergy feedstock of the future. Cellulose, which accounts for the bulk of plant materials, is recalcitrant, which necessitates a combination of the classes of cellulases for biodegradation. These include endoglucanases (EC 3.2.1.4) that cut randomly at internal amorphous sites in the cellulose chain; exoglucanases (EC 3.2.1.91 and EC 3.2.1.74) that act processively on the reducing or nonreducing ends of cellulose chains, releasing either cellobiose or glucose as major products; and β-glucosidases (EC 3.2.1.21) that hydrolyze soluble cellodextrins and cellobiose to glucose (18).

Fibrobacter succinogenes is a highly cellulolytic bacterium that is closely related to the *Cytophaga-Flavobacterium-Bacteroides* group (8). It is commonly found in the rumen of ruminant animals and appears to be one of the most active rumen cellulose degraders (4). Recently, the genome was sequenced (21) and a number of cellulases (24) and cellulose binding proteins (12) were identified. We reported synergistic interactions among five cellulases of this organism (24). Although these enzymes had degrees of synergism (DoS) of up to 3.7 and the combination of Cel9B, Ce51A, and Cel8B gave the highest activity, the extent of hydrolysis of cellulose was low. In addition, Cel10A, known as the exo-acting, Cl-stimulated cellobiosidase, had limited synergistic interaction with other enzymes.

In the following study, a novel cellulase gene, *cel9D*, was cloned and the protein encoded was purified and characterized. An investigation of the synergistic effect of Cel9D with four other cellulases, Cel9B, Cel51A, Cel8B, and Cel45C, from *F. succinogenes* revealed that Cel9D acted synergistically with these glucanases. Our data also document the fact that this novel enzyme is the first glucan 1,4-β-D-glucohydrolase in glycoside hydrolase family 9 (GH9) with enhanced activity on long-chain cello-oligosaccharides.

MATERIALS AND METHODS

Bacterial strains and plasmid. *Fibrobacter succinogenes* S85 was used as the source of genomic DNA. It was maintained and cultured in chemically defined medium with either glucose or microcrystalline cellulose (Avicel) PH105 (FMC BioPolymer) as the carbon source as described previously (30). The *Escherichia coli* strain DH10B (Invitrogen) was used as the host for genetic manipulation. *E. coli* BL21(DE3) was the host for protein production. Cultures were grown at 37°C or 18°C in Luria-Bertani medium or Luria-Bertani medium supplied with 1 M sorbitol and 2.5 mM betaine glycine for enhanced solubility of the recombinant proteins (29). When required, media were supplemented with kanamycin at 34 μg/ml. Solid media contained 1.6% (wt/vol) agar.

Identification of Cel9D. *F. succinogenes* S85 was grown in 300 ml chemically defined medium with 0.3% (wt/vol) Avicel cellulose PH105 as the sole carbon source at 37°C for 24 h with reciprocating shaking at 150 rpm. After the removal

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▽ Published ahead of print on 18 January 2008.

TABLE 1. Oligonucleotide primers used in this study

Primer	Sequence[a]	Location[b]	Purpose
Cel9D_1	5′-GTCCTCCATATGCGTATCTATAAGCTTTC-3′	1–20	Cloning with NdeI
Cel9D_2C	5′-ATATGC CTCGAGCTTCACAACAAACTTCTT-3′	2143–2160	Cloning with XhoI
D166A_1	5′-CGGTTGGTATG**C**TGCGAGCGGCG-3′	487–509	Mutagenesis
D166A_2C	5′-CGCCGCTCGCA**G**CATACCAACCG-3′	487–509 (reverse)	Mutagenesis
S168A_1	5′-GGTTGGTATGATGCG**GC**CGGCGATGTCAGTAAG-3′	487–519	Mutagenesis
S168A_2C	5′-CTTACTGACATCGCCG**GC**CGCATCATACCAACC-3′	487–519 (reverse)	Mutagenesis
ΔS168_1	5′-GGTTGGTATGATGCG~GGCGATGTCAGTAAG-3′	487–519	Mutagenesis
ΔS168_2C	5′-CTTACTGACATCGCC~CGCATCATACCAACC-3′	487–519 (reverse)	Mutagenesis
E612A_1	5′-GCGCTGGGATG**C**ACAGTGGCTGC-3′	1824–1846	Mutagenesis
E612A_2C	5′-GCAGCCACTGT**G**CATCCCAGCGC-3′	1824–1846 (reverse)	Mutagenesis

[a] Underlining indicates restriction sites for cloning. Bold and tilde denote the substituted nucleotides for amino acid replacement and deletion, respectively.
[b] Values shown are nucleotide positions.

of residual cellulose by centrifugation at 300 rpm for 10 min, cells were harvested by centrifugation at 5,000 × *g* and then resuspended in 10 ml of 30 times concentrated culture fluid prepared as described before (20). The cells were then broken by three passes through a French pressure cell at 1,200 lb/in^2. The cell lysate was centrifuged at 12,000 × *g* for 30 min. The clarified supernatant containing 25 mg of protein was separated by a 2.5 × 10-cm column of DEAE-Sepharose CL-6B as described previously (20), except that imidazole buffer (20 mM) was used instead of potassium phosphate buffer. Approximately 40% of the activity eluted in the application buffer before NaCl gradient was applied, and these fractions were kept for further study. Four hundred microliters of each fraction eluted by the NaCl gradient buffer was mixed with 100 μl 2.5% Avicel PH105 suspension and incubated at 37°C for 20 h. Reducing sugar produced was determined by the *p*-hydroxybenzoic acid hydrazide methods (16).

Fractions eluting in the buffer gradient exhibiting Avicelase activities were loaded to a 7.5% (wt/vol) polyacrylamide gel without sodium dodecyl sulfate and subjected to electrophoresis at 100 V for 2 h. The gel was then laid on a plate containing 20 ml 0.1% (wt/vol) carboxymethyl cellulose (CMC) solidified with 0.7% agarose and incubated at 37°C for 4 h. The gel was then removed and stained with colloidal Coomassie blue, and the agar layer on the plate was stained by 0.1% Congo red for 30 min and destained by 1 M NaCl solution. The bands on the gel that corresponded to clear zones on the CMC plate were excised and subjected to matrix-assisted laser desorption ionization–time of flight mass spectrometry analysis, and Cel9D was identified by matching the mass spectra of peptides to the *F. succinogenes* protein database as described previously (14).

Cloning, expression, and purification of Cel9D and its mutants. Genomic DNA was prepared from *F. succinogenes* S85 by using the cetyltrimethylammonium bromide method as described before (1). The intact *cel9D* gene was amplified from the genomic DNA by using Cel9D_1 and Cel9D_2C primers (Table 1) and *Pwo* polymerase (Roche). The restriction endonuclease sites NdeI and XhoI in the 5′ primer and 3′ primer, respectively, allowed for cloning of the gene into the expression vector pET30a (Novagen). The coding sequence of Cel9D was fused in frame with a downstream sequence of the vector encoding six-histidine residues. The mutants of Cel9D, D166A, E612A, S168A, and ΔS168, were generated by the PCR overlap extension method (27) using the primers listed (Table 1) to replace amino acid residues as indicated and to remove the Ser168. The wild-type and mutant genes were sequenced to ensure the correct nucleotide changes and that there were no PCR-induced errors. Cel9D and its mutants were produced in *E. coli* BL21(DE3) and purified by immobilized metal affinity chromatography. Wild-type Cel9D was further purified by molecular sieve chromatography as described previously (14). Cel8B, Cel9B, and Cel51A were produced and purified as described previously (24).

Enzyme assays. Glycoside hydrolase activity was assayed with various substrates as described previously (24). The standard reaction period for Cel9D was 16 h for Avicel PH105 and 2 h for all other substrates in 0.05 M sodium phosphate buffer, pH 6.5. The reducing sugar produced was measured. The substrate used was either Avicel PH105 (0.5%, wt/vol) or phosphoric acid swollen cellulose (ASC; 0.25%, wt/vol). The DoS was calculated as follows: (observed activity of a mixture of enzymes)/(additive activities of the individual enzymes making up the mixture).

To determine the kinetics parameters for hydrolysis of cello-oligosaccharides, 0.1 μg purified Cel9D enzyme was mixed with different concentrations of cello-oligosaccharide substrates in 0.05 mM phosphate buffer, pH 6.5. Samples were taken at given intervals and appropriately diluted in 0.15 M sodium hydroxide to stop the reaction and hydrolysis products were analyzed by high-performance liquid chromatography (HPLC).

Analysis of degradation products. Cello-oligosaccharides and their hydrolysis products as well as products from synthetic substrates *p*-nitrophenol (pNP) glucopyranoside (pNPG), pNP β-D-cellobioside (pNPC), and ASC were analyzed by high-performance anion-exchange chromatography coupled with a Waters model 464-pulsed amperimetric detector system with a base-resistant reference electrode as described before (14).

^{1}H NMR spectroscopy. ^{1}H nuclear magnetic resonance (NMR) spectra were generated on a Bruker Avance-400 NMR spectrometer in 5-mm-diameter sample tubes. Samples (0.6 ml) were analyzed at 27°C in 50 mM potassium phosphate buffer, pH 6.5, containing 20 mM pNPC, or 30 mM cellotetraose as the substrate. Chemicals and substrates were dissolved in D_2O. Cel9D enzyme was transferred to buffer containing D_2O by ultrafiltration by using a PM-10 (Amicon) membrane. Spectra were collected in 32-k data points using a spectral width of 5 KHz and a relaxation delay of 2 s. The spectra were referenced against an external standard of trimethylsilyl-2,2,3,3-tetradeuteropropionic acid (sodium salt) at 27°C. One hundred micrograms of purified Cel9D was added to each reaction mixture.

Nucleotide sequence accession numbers. The nucleotide sequences of the gene *cel9D* from *F. succinogenes* S85 have been deposited in the GenBank database under accession number EU352748.

RESULTS

Identification and sequence analysis of a novel family 9 enzyme from the genome of *F. succinogenes*. When the avicelases present in the whole-cell extract from cellulose-grown *F. succinogenes* were separated on DEAE-Sepharose, a sharp single peak of activity was eluted in the buffer gradient at 0.15 to 0.25 M NaCl, followed by several minor peaks at 0.35 M and 0.45 M NaCl (see Fig. S1 in the supplemental material). The fractions (numbers 15, 16, and 17) corresponding to the major peak were subjected to native polyacrylamide gel electrophoresis (PAGE) and zymogram analysis (see Fig. S2 in the supplemental material). The three fractions yielded similar profiles, with activity zones at about 62, 75, and 80 kDa, respectively. Proteins in the corresponding bands of fraction 16 on the native PAGE were extracted and analyzed by peptide mass fingerprinting. No proteins involved in cellulose degradation were identified from the two smaller bands, but in the 80-kDa band, a cellulase with an amino acid sequence corresponding

to FSU2558, known as *cel9D*, was identified in the genome of *F. succinogenes* S85. Matrix-assisted laser desorption ionization–time of flight mass spectrometry analysis of tryptic peptides of the 80-kDa bands accounted for 22% of the Cel9D protein and the peptide sequences were dispersed throughout the protein (see Fig. S3 in the supplemental material).

The gene *cel9D* encoded a protein that had an N-terminal signal peptide and a putative cleavage site located between Ala23 and Glu24. The mature protein contained 697 amino acid residues and had a theoretical mass of 77.0 kDa and a theoretical isoelectric point of 5.27. Following the signal peptide, there was a 100-amino-acid region that had low similarity to an immunoglobulin-like module, followed by a putative family 9 catalytic domain and a 69-amino-acid basic C-terminal domain with a theoretical pI of 10.5. The protein sequence had the highest sequence similarities to several putative endoglucanases or hypothetical proteins mainly from the genera of *Vibrio*, *Photobacterium*, and *Cytophaga*, but had low similarities to nearly all endo- or exoglucanases ("classical GH9s"). Phylogenetic analysis showed that the group of GHs in which Cel9D resides is a unique branch of the GH9 family (see Fig. S4 in the supplemental material). Multiple sequence alignment (Fig. 1) showed that the putative catalytic residues of Cel9D (D166 and D170) were separated by three other amino acids, while in all other characterized cellulases, they were separated by two. The extra residue (S168) seems to be conserved in the Cel9D group (data not shown). E612 was identified as a putative catalytic base; however, the surrounding amino acid residues, which are conserved in classical GH9s, were not aligned in the Cel9D group.

Production and purification of recombinant Cel9D. *cel9D* gene was cloned, and protein Cel9D was produced in *E. coli* BL21(DE3). Most of the recombinant Cel9D enzyme was associated with the membrane fraction perhaps in inclusion bodies. However, a small amount present in the nonsedimentable fraction was purified by immobilized metal affinity chromatography, followed by molecular sieve chromatography. About 1 mg of pure protein was obtained from 1 liter of culture. The recombinant protein had a mass of 76 kDa, as determined by sodium dodecyl sulfate-PAGE (see Fig. S5 in the supplemental material), which is in good agreement with the theoretical mass of 77 kDa based on the primary sequence.

Characterization of Cel9D. As with most other GHs identified in *F. succinogenes* S85, Cel9D had an optimum pH of 6.5 and retained 80% activity between pH 6.0 and 8.0. The temperature for maximum activity was 37°C. Detailed substrate specificity assays (Table 2) on various types of polysaccharides showed that Cel9D had activity on all polymers with a β-1,4 linkage, but not on laminarin, which has a β-1,3 linkage. By measuring the release of pNP, no activity was detected on pNP-α-L-arabinofuranoside, pNP-α-D-glucoside, pNP-β-D-cellobioside, pNP-β-D-fucoside, pNP-β-D-galactoside, pNP-*N*-acetyl-β-D-glucosaminide, pNP-β-D-glucoside, pNP-β-D-glucuronoside, pNP-β-D-lactoside, or pNP-β-D-maltoside. Cel9D had 27% lower activity on medium-viscosity (average M_w, ~250,000) CMC compared to the activity on low-viscosity CMC (average M_w, ~90,000) (Table 2). This property of Cel9D was quite different from that of the Cel9B enzyme, which had high activity on both medium- and low-viscosity CMC (24). The hydrolytic activities of Cel9D on Avicel cellulose, ASC, and CMC were tested and compared to those of Cel8B, Cel9B, and Cel51A from *F. succinogenes* (Table 3). It had less activity on CMC than on other enzymes.

Although Cel9D did not release pNP from any of the aryl-glycosides tested, it did efficiently cleave pNPC to pNPG and glucose, as indicated by HPLC analysis (see Fig. S6A in the supplemental material). This result documented the fact that Cel9D acts on the nonreducing end. Cel9D did not degrade sophorose (β1-2), laminaribiose (β1-3), or gentiobiose (β1-6), as assayed by HPLC analysis.

Hydrolytic activities on either CMC or cellopentaose of purified Cel9D were not enhanced by 1 mM $CaCl_2$ or 1 mM $MgCl_2$. EDTA (10 mM) did not inhibit the activities.

Synergism of Cel9D with other cellulases from *F. succinogenes*. To identify possible synergistic interactions among different GH families of cellulases synthesized by *F. succinogenes*, ASC and Avicel cellulose hydrolysis by different combinations of cellulases was assessed. Reducing sugars released by different combinations of enzymes are presented in Fig. 2 and Table 4. For all the binary mixtures, the greatest DoS was obtained from the reaction containing 1.2 nmol of Cel9D and 1.2 nmol of Cel51 on Avicel (Fig. 2f), in which the actual activity was four times the sum of individual enzyme activities (Table 4). The highest DoS on the ASC was from the combination of Cel9D and Cel8B, with a synergism value of 3.4 (Fig. 2p). The combinations of the two family 9 enzymes, Cel9D and Cel9B, gave a moderate DoS of 1.5, which indicates that there are differences between the two cellulases within the same family. Cellulose degradation by mixtures of up to four cellulases was also investigated (Table 4). The enzyme mixtures containing Cel9D generally yielded a higher DoS as well as a higher overall level of reducing sugar production. By incubation of the enzyme mixture containing all four enzymes for 60 h, the amount of reducing sugar released corresponded to the hydrolysis of 2.8% of the initial Avicel substrate present.

Hydrolysis products of Cel9D. Glucose was the only product released from ASC by Cel9D, as detected by HPLC analysis (see Fig. S6B in the supplemental material). This result indicated that Cel9D has β-1,4-glucosidase activity. Cellohexaose hydrolysis initially yielded cellopentaose and glucose (Fig. 3). No cellotetraose was detected (at time zero) until a larger amount of cellopentaose accumulated (at 0.5 min). Similarly, no cellotriose was detected (at 0, 0.5, and 1 min) until a larger amount of cellotetraose accumulated (at 2 min) and no cellobiose was detected (at 0, 0.5, 1, 2, and 4 min) until there was a larger amount of cellotriose accumulated (at 8 min). These results clearly showed that Cel9D catalyzed an exotype of hydrolysis reaction. Cellobiose was degraded very slowly such that less than 5% of the cellobiose accumulated at 120 min was digested to glucose after 3,000 min (Fig. 3). In contrast, for the same amount of cello-oligosaccharides with three or more glucose residues, complete hydrolysis was achieved within 2 h. The detailed relationships between the kinetic parameters (K_m, k_{cat}, and k_{cat}/K_m) and the cello-oligosaccharide substrates of Cel9D are shown in Table 5. The K_m decreased with increasing chain length of substrate. The k_{cats} for cellotetraose, cellopentaose, and cellohexaose were similar, while the k_{cats} for cellotriose and cellobiose hydrolysis were substantially smaller. Catalytic efficiency factors k_{cat}/K_m increased steadily with increasing degrees of polymerization (DP). The k_{cat}/K_m value for cellobiose was 2,300-fold lower than that for cellohexaose. The obser-

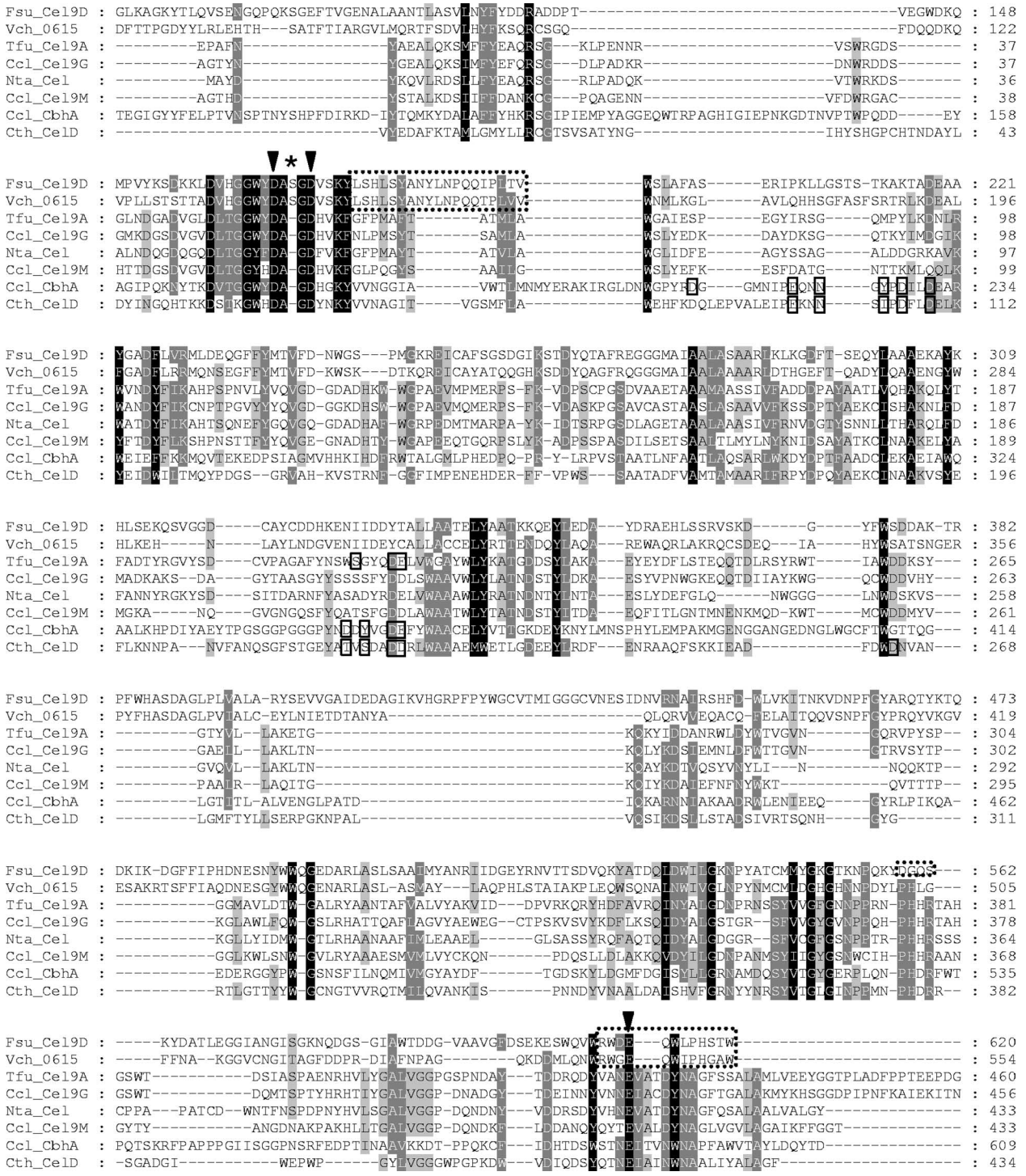

FIG. 1. Alignment of Cel9D and Vch_0615 (AAF93781) with six classical GH9s for which structures have been determined. The alignment was carried out using the ClustalX program and edited by eye. Putative catalytic sites of Cel9D are indicated by filled arrowheads. Regions of Cel9D that showed significant differences from classical GH9s are framed by dots. Amino acid residues involved in Ca^{2+} binding in Tfu_Cel9A, Cth_CbhA, and Cth_CelD were boxed with solid lines. The asterisk indicates a serine residue present in the Cel9D-like proteins. Tfu_Cel9A, *T. fusca* Cel9A, pdb:3TF4 (theme B); Ccl_Cel9G, *C. cellulolyticum* Cel9G, pdb:1G87 (theme B); Nta_Cel, termite, *Nasutitermes takasagoensis* endoglucanase, pdb:1KSD (theme A); Ccl_Cel9M, *C. cellulolyticum* Cel9M, pdb:1IA7 (theme A); Cth_CbhA, *C. thermocellum* CbhA, pdb:1RQ5 (theme D); Cth_CelD, *C. thermocellum* CelD, pdb:1CLC (theme C).

TABLE 2. Substrate specificity of Cel9D

Substrate	Activity[a] (μmol product/min/μmol protein)	% Relative activity[b]
CMC (medium viscosity)	0.275 ± 0.050[d]	100.0
ASC	0.250 ± 0.063[d]	90.4
Barley β-glucan	0.102 ± 0.031[d]	37.2
Lichenin	0.095 ± 0.040[d]	34.6
CMC (low viscosity)	0.075 ± 0.028[d]	27.1
Oat spelt xylan	0.075 ± 0.035[d]	27.1
Sigmacel 100	0.056 ± 0.022[d]	20.2
Avicel PH105	0.038 ± 0.014[d]	13.8
Mannan	0.019 ± 0.006[d]	6.9
Hydroxyethyl cellulose	0.019 ± 0.009[d]	6.9
Laminarin	<0.001	0
Acyl glycosides[b]	<0.001	0
Disaccharides[c]	0	0

[a] Glycoside hydrolase activities were determined by reducing sugar (glucose equivalents) produced from polysaccharides.

[b] Includes pNP-α-L-arabinofuranoside, pNP-α-D-glucoside, pNP-β-D-cellobioside, pNP-β-D-fucoside, pNP-β-D-galactoside, pNP-N-acetyl-β-D-glucosaminide, pNP-β-D-glucoside, pNP-β-D-glucuronoside, pNP-β-D-lactoside, pNP-β-D-maltoside at 5 mM in 0.05 M sodium phosphate buffer pH 6.5 for 2 h at 37°C. Released pNP was measured as described by Kam et al. (14).

[c] Includes sophorose (β1-2), laminaribiose (β1-3) and gentiobiose (β1-6), products detected by HPLC.

[d] Values shown are means ± standard deviations.

TABLE 3. Catalytic properties of the cellulases from *F. succinogenes* S85

Protein	Molecular mass (kDa)/pI	Family	Specific activity (μmol Glc/min/μmol protein ± SD)		
			CMC	Amorphous cellulose	Avicel
Cel9D	79.4/5.38	9	0.275 ± 0.050	0.250 ± 0.063	0.038 ± 0.003
Cel8B	81.4/5.55	8	6.38 ± 1.25	1.56 ± 0.29	0.056 ± 0.014
Cel9B	67.3/6.06	9	1680 ± 151	3.94 ± 0.18	0.069 ± 0.014
Cel45C	37.7/4.90	45	4.84 ± 1.89	0.027 ± 0.015	0.009 ± 0.003
Cel51A	119/7.81	51	1300 ± 35.9	2.93 ± 0.12	0.067 ± 0.016

vation that cello-oligosaccharides with longer DP were degraded faster than those with shorter chain lengths documented a preference for longer cello-oligosaccharides.

After we mixed Cel9D with up to 100 mM glucose and/or cellobiose and incubated it at 37°C for 24 h, cello-oligosaccharides with longer chain lengths were not detected, indicating that Cel9D does not catalyze the reverse transglycosylation reaction.

Anomeric configuration of hydrolysis products. Proton-NMR was used to investigate the anomeric configuration of oligosaccharides released during the hydrolysis of pNP-cellobioside by Cel9D (see Fig. S7 in the supplemental material). The results indicate that Cel9D catalyzes the hydrolysis of the β-(1,4) linkage with inversion of configuration.

Site-directed mutagenesis of Cel9D. To investigate whether D166 and E612 were the catalytic residues, the two amino acid residues were replaced with alanine. The mutant proteins (D166A and E612A) were produced and purified by immobilized metal ion affinity chromatography. No activity was detected on either 1% (wt/vol) CMC after 4 h of incubation or 5 mM cellopentaose after 24 h of incubation, as determined either by reducing sugar analysis or by HPLC. Thus, D166 and E612 are good candidates for catalytic residues.

To investigate whether the serine residue at position 168 had a role in catalysis, two Cel9D mutants were made. ΔS168, constructed with the Ser168 removed, had no activity on CMC or cellopentaose when assayed under the same conditions with Cel9D as the parallel positive control, although when this serine residue was replaced by an alanine residue (S168A), the mutants retained about 40% percent of activity compared to the wild type.

DISCUSSION

Cel9D is a glucan 1,4-β-glucohydrolase. The present experiments showed that Cel9D hydrolyzed cello-oligosaccharides from the nonreducing end, releasing glucose units. There is similarity between β-glucosidase (EC 3.2.1.21) and glucan 1,4-β-glucohydrolase (EC 3.2.1.74), as both cleave the nonreducing terminal glycosyl residues from cello-oligosaccharide substrates. Cel9D hydrolyzed cello-oligosaccharides with a DP greater than 2 or hydrolyzed pNPC at much higher rates than those for cellobiose or pNPG, showing that the rate of hydrolysis increases with the DP of the cello-oligosaccharides. Cel9D released glucose from the nonreducing end of the substrate, and its activity was restricted to β-1,4 linkages. Cel9D also had activity on soluble and insoluble β-glucans, including CMC, ASC, and Avicel. This capability is distinct from that of β-glucosidases (EC 3.2.1.21), which have a decreased rate of hydrolysis with increasing DP (11, 25). Thus, Cel9D is classified as a glucan 1,4-β-glucohydrolase (34).

Since HPLC showed that the enzyme removed glucose residues from the nonreducing ends of cello-oligosaccharides, the subsites for binding substrate were designated −1, +1, +2, +3, etc., where the "−1" subsite binds to the nonreducing terminal glucose and hydrolysis occurs between −1 and +1. Subsite affinities of Cel9D for glucose residues in cello-oligosaccharides were estimated (see Table S1 in the supplemental material). Highest affinity was seen at the +2 subsite, which was 15.8 kJ/mol. Due to the difference in the subsite affinities between −1 (5.7 kJ/mol) and +2 (15.8 kJ/mol), cellobiose and pNPG would prefer to stay across sites +1 and +2 or +2 and +3 rather than the "productive" sites −1 and +1, which explains the low rate of hydrolysis of cellobiose and pNPG.

Glucan 1,4-β-D-glucohydrolase is a type of exoglucanase that releases glucose from β-1→4-linked glucan. This type of enzyme has been identified in many organisms, including bacteria (15, 34), fungi (33), and plants (10, 22). Previously, all glucan 1,4-β-glucohydrolases and most β-glucosidases were classified as GH1 or GH3 (www.cazy.org/fam/GH1.html and www.cazy.org/fam/GH3.html [9]), which not only catalyzed hydrolysis with retention of anomeric configuration but also catalyzed the reverse transglycosylation reaction (17). Our data showed that Cel9D could not produce long-chain cello-oligosaccharide from glucose or cellobiose, indicating that it does not catalyze the reverse transglycosylation reaction. Thus, Cel9D is a unique inverting glucan 1,4-β-glucohydrolase.

Cel9D exhibits less than 20% identity to classical GH9s, while it shows about 35% similarity to a series of putative endoglucanases mainly from the genus *Vibrio*. Among its many relatives, Vch_BglA (Vch0615) from *Vibrio cholerae* was previously characterized and was subsequently classified as a β-glucosidase (EC 3.2.1.21) (www.cazy.org/fam/GH9.html) that releases pNP from pNPG and hydrolyzed cellobiose efficiently (23). In a manner similar to that of Cel9D, Vch_BglA degraded cello-oligosaccharides and produced glucose as the

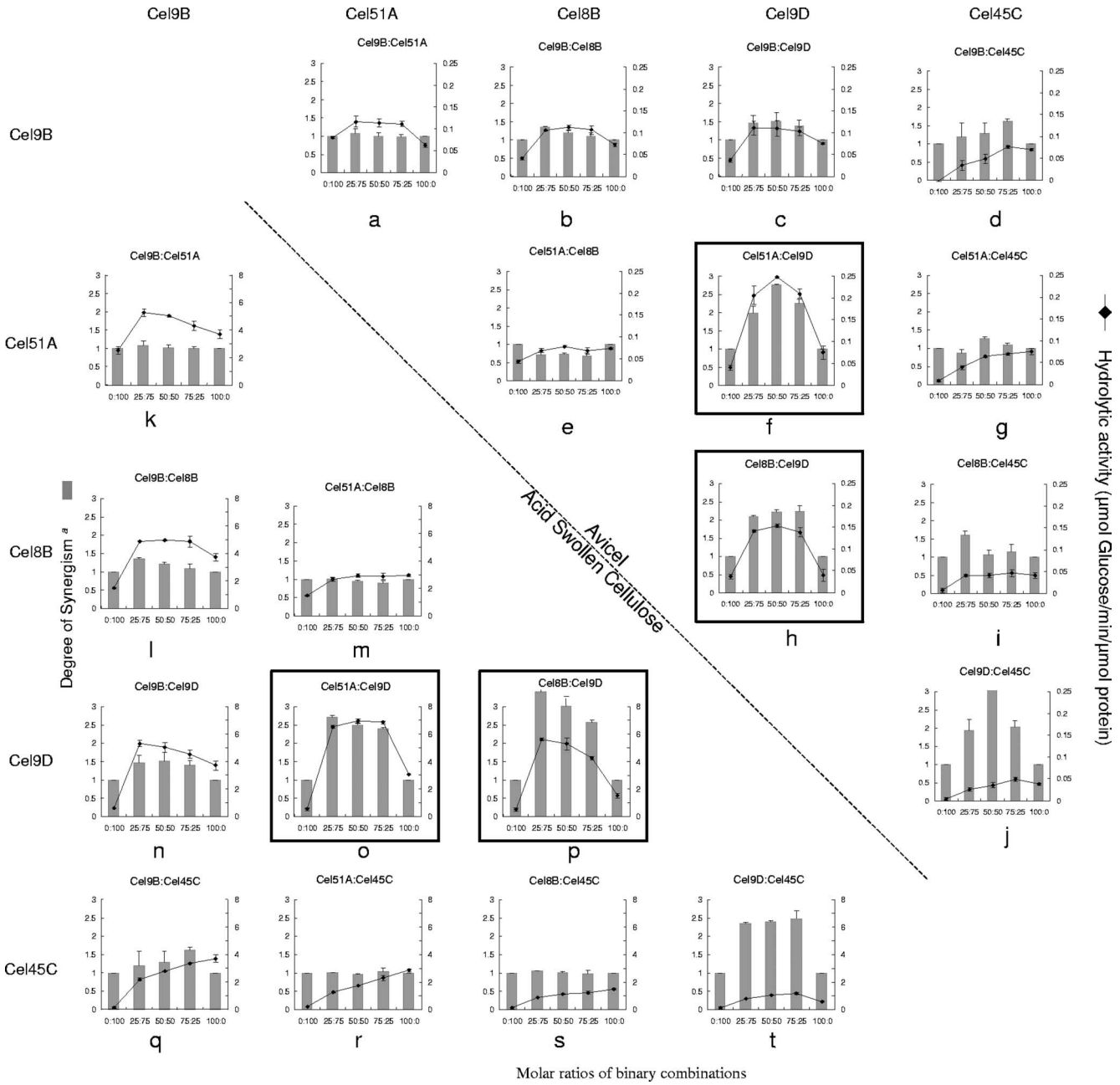

FIG. 2. Hydrolytic activity (lines and filled circles; values are indicated on right *y* axes) and DoS (gray bars; values are indicated on left *y* axes) of various binary combinations of Cel9B, Cel51A, Cel8B, Cel9D, and Cel45C at different molar ratios for the hydrolysis of either Avicel (a to j) or amorphous cellulose (k to t). The two enzymes used are indicated for each subfigure, and proportions of enzymes in each reaction are shown at the bottom of the subfigures. Note that 100 means 0.98 nmol per 100 μl of reaction for Cel9B, 1.01 nmol per 100 μl of reaction for Cel51A, 1.18 nmol per 100 μl of reaction for Cel8B, and 0.69 nmol per 100 μl of reaction for Cel9D. [a], DoS is defined as observed cellulase activity by the combination of cellulases divided by the additive activities of the individual cellulases acting alone. Error bars indicate standard deviations.

major product. Like Cel9D, Vch_BglA did not cleave β-1,2-glucosidic, β-1,3-glucosidic, and β-1,6-glucosidic linkages. Unfortunately, Park et al. (23) did not test the activity of the enzyme on cellulosic polymers. A key difference between the two enzymes is that Vch_BglA does not contain a signal peptide at the N terminus, which may indicate that it is intracellular. Cel9D has a signal peptide and presumably is exported across the cytoplasmic membrane. The difference in localization suggested a difference in their physiological functions.

Why does Cel9D have a different mode of action compared with those of other GH9s? Most members in the GH9 family are endoglucanases or exoglucanases that hydrolyze soluble or insoluble cellulosic substrate and produce cellobiose or cellotetraose as the major product (32, 36), which is further de-

TABLE 4. Synergistic effect of various combination of Cel9D with Cel9B, Cel51A, and Cel8B on the hydrolysis of Avicel cellulose

Cellulase mixture[a]	Molecular concn (nmol/ml) of enzyme	Reducing sugar produced (μg)		DoS	% Conversion
		Observed[b]	Theoretical		
Cel9D	1.2	0.35 ± 0.04		1	0.07
Cel9B	1.8	2.18 ± 0.36		1	0.44
Cel51A	1.2	1.24 ± 0.18		1	0.25
Cel8B	1.2	1.26 ± 0.37		1	0.25
Cel9D + Cel9B	1.2 + 1.8	3.00 ± 0.25	2.53	**1.19**	**0.60**
Cel9D + Cel51A	1.2 + 1.2	6.36 ± 1.24	1.59	**4.01**	**1.27**
Cel9D + Cel8B	1.2 + 1.2	3.71 ± 0.24	1.60	**2.31**	**0.74**
Cel9B + Cel51A	1.8 + 1.2	3.61 ± 0.57	3.43	1.06	0.72
Cel9B + Cel8B	1.8 + 1.2	3.27 ± 0.18	3.44	0.95	0.65
Cel51A + Cel8B	1.2 + 1.2	1.93 ± 0.57	2.50	0.77	0.39
Cel9D + Cel9B + Cel51A	1.2 + 1.8 + 1.2	6.30 ± 0.39	3.77	**1.67**	**1.26**
Cel9D + Cel9B + Cel8B	1.2 + 1.8 + 1.2	5.34 ± 0.23	3.79	**1.41**	**1.07**
Cel9D + Cel51A + Cel8B	1.2 + 1.2 + 1.2	6.62 ± 0.31	2.84	**2.33**	**1.32**
Cel51A + Cel8B + Cel9B	1.2 + 1.2 + 1.8	4.27 ± 0.13	4.68	0.91	0.85
Cel9D + Cel9B + Cel51A + Cel8B	1.2 + 1.8 + 1.2 + 1.2	7.20 ± 1.28	5.03	**1.43**	**1.44**

[a] Reaction mixtures (100 μl) were incubated at 37°C for 16 h. Bold indicates a cellulose mixture containing Cel9D.
[b] Results are shown as means ± standard deviations.

graded by β-glucosidase or cellobiase. Although Cel9D belongs to the GH9 family, the major hydrolytic product was glucose. Cel9D also has very distinct sequence features compared to those of other family 9 cellulases (classical GH9 enzymes) as indicated by sequence alignment (Fig. 1) and phylogenetic analysis (see Fig. S4 in the supplemental material). Most remarkably, the two catalytic residues (D166 and D170 in Cel9D), which are thought to deprotonate the water molecule that carries out the nucleophilic attack on the C-1 carbon of the substrate (28), were separated by three amino acid residues instead of two. The extra amino acid residue, S168, which is conserved in the Cel9D group, is not in the classical family 9 enzymes (Fig. 1). The removal of Ser168 by site-directed mutagenesis indeed completely abolished the enzyme activity, while S168A retained about 40% of the activity. These data indicated that Ser168 is a structurally, but not catalytically, important residue. Secondly, the catalytic residue, a glutamic acid (13), and surrounding amino acid residues, which are conserved in all the other classical family 9 cellulases (Glu418 in Cth_CelD and Glu424 in Tfu_Cel9A), were not easily identified based on computer-generated sequence alignment. E612 seems to be conserved within the Cel9D group and may be the catalytic residue relative to the classical ones. The substitution of E612 of Cel9D with an alanine residue caused the enzyme to lose activity on both CMC and cellopentaose; therefore, E612 probably is a catalytic residue. A third difference of Cel9D from other GH9 enzymes is at position 559 to 562, where the classical enzymes have four conserved residues, PHHR, and in Cel9D, these residues were absent (Fig. 1). The four residues are situated in the cleavage grooves of family 9 enzymes (13), and a substitution of the first histidine residue caused a 75% loss in activity of CelD from *Clostridium thermocellum* (31). The combination of these differences may result in a conversion of a classical endoglucanase or cellobiohydrolase to an exo-β-glucosidase like Cel9D, although further studies are needed to explore this possibility.

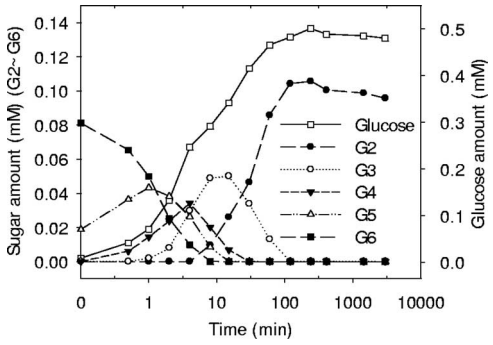

FIG. 3. Time course of hydrolysis of cellohexaose by Cel9D monitored by HPLC. Cellohexaose and their hydrolysis products were analyzed by high-performance anion-exchange chromatography coupled with a Waters model 464-pulsed amperimetric detector using a Waters 625 LC system with a base resistant reference electrode.

Many studies have reported that divalent metal ions, such as calcium and magnesium, enhanced the activity of GH9 cellulases (2, 24). However, this result was not observed for Cel9D and was corroborated by the fact that the calcium binding residues, which were identified by structural analyses of CelD (3), CbhA (28) from *C. thermocellum*, and E4 from *T. fusca* (26), were not identified in Cel9D (Fig. 1).

Despite the observation that Cel9D has many differences in the arrangement of catalytically important residues (D166, D170, and E612) and different hydrolytic products, the proton NMR results (see Fig. S7 in the supplemental material) clearly showed that Cel9D catalyzes hydrolysis of the β-1,4 linkage by

TABLE 5. Values of kinetic parameters for Cel9D during the hydrolysis of cello-oligosaccharides of DP 2 to 6[a]

Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (10^{-3} s^{-1} M^{-1})
Cellobiose	28.0 ± 6.3	0.25 ± 0.03	0.0097 ± 0.003
Cellotriose	1.52 ± 0.27	6.21 ± 0.45	4.27 ± 1.06
Cellotetraose	1.21 ± 0.11	18.6 ± 0.66	15.5 ± 1.97
Cellopentaose	0.90 ± 0.10	16.4 ± 0.65	18.5 ± 2.74
Cellohexaose	0.73 ± 0.09	16.3 ± 0.69	22.8 ± 3.76

[a] Results are shown as means ± SD.

an inverting mechanism, which is the same method as that for other GH9 members (5).

Family 9 cellulases are classified into four different themes based on their domain organization (6). Theme A enzymes have only family 9 catalytic domains. In addition to the catalytic domain, theme B enzymes have family 3 carbohydrate binding modules (CBMs) at the C termini. Theme C and D members have immunoglobulin-like domains at their N termini, and theme D enzymes have additional family 4 CBMs. Enzymes in themes B and D often have high activities toward crystalline cellulose, and enzymes in themes A and C are usually potent endoglucanases.

A phylogenic tree based on the amino acid sequences from selected family 9 cellulases was constructed (see Fig. S4 in the supplemental material). Theme A and theme B members were well separated in the phylogenic tree, indicating that in addition to the conserved domain arrangement, amino acid sequences were conserved within the themes. Theme C and D enzymes were well separated from theme A and B enzymes, but the two themes themselves could not be fully separated phylogenetically. Theme D is more like a subgroup of theme C. Theme C and D enzymes are closer to members from theme A than those from theme B. Cel9D and Vch_BglA reside in a distinct branch separated from all other family 9 themes in the tree (see Fig. S5 in the supplemental material).

During the course of evolution, a common ancestor of the members from the Cel9D branch and those in themes C and D gained the N-terminal immunoglobulin-like domain, and after that, it began to diverge. One branch contained the classical GH9s, which are mainly endoglucanases and cellobiohydrolases, including themes C and D. The other branch includes Cel9D in the present study as well as the Vch_BglA, which are exo-β-glucosidases. Interestingly, although Cel9D and Vch_BglA have similar modes of action, Cel9D has an N-terminal signal peptide, while Vch_BglA does not, indicating their different localization and physiological roles.

Mode of synergistic interaction of Cel9D. The Cel51A of *F. succinogenes*, formerly CelF (19), contains three cellulose binding modules. In the present study, the Cel51A and Cel9D were more synergistic than other pairs of proteins (Fig. 2). In the multiple enzyme mixtures, those containing both Cel51A and Cel9D gave the highest degree of cellulose degradation (Table 4). Thus, besides having the major role in cellulose binding, Cel51A may also be a central enzyme for cellulose hydrolysis. Previous studies showed that the major hydrolytic products of Cel51A were cellotetraose and cellotriose, which are the preferred substrates for Cel9D. Cel8A degraded ASC, and the major products were cellodextrins with DP ranging from 2 to 5 (24). So the synergistic effect between Cel8B and Cel9D is also expected. Cel9D has a much lower activity on cellobiose than on longer cello-oligosaccharides, which explains why Cel9D had a less synergistic effect with Cel9B, since the latter enzyme was shown to degrade cellulose mainly to cellobiose and glucose (20). Since Cel9D cannot hydrolyze cellobiose, it can be presumed that β-glucosidases (EC 3.2.1.21) would exert a synergistic effect with Cel9D. Indeed, cellobiase activity has been detected in *F. succinogenes* (7) and two GH3 β-glucosidases were identified in the genome of *F. succinogenes* (21).

A putative protein that has 37% sequence identity to Cel9D was identified from the genome of a phylogenetically related bacterium, *Cytophaga hutchinsonii* (35). Cellulase systems of *F. succinogenes* and *C. hutchinsonii* are quite similar and distinct from other cellulolytic bacteria in that both bacteria lack genes encoding proteins with similarity to members from GH6 or GH48; most cellulases in both bacteria are cell associated but lack a carbohydrate binding module, and no cellulosome structures were identified (21, 35). The Cel9D enzyme may be related to their unique but yet-to-be-elucidated cellulose degradation system.

In conclusion, Cel9D is a novel GH9 enzyme that has an important role in crystalline cellulose degradation by releasing glucose from accessible cello-oligosaccharides during cellulose degradation by other cellulases. Further structural studies of Cel9D will provide greater insight into the function of this unique GH9 enzyme.

ACKNOWLEDGMENTS

This research was supported by the Natural Science and Engineering Research Council of Canada and the Initiative for Future Agriculture and Food Systems grant no. 2000-52100-9618 from USDA-CSREES to the North American Consortium for Genomics of Fibrolytic Ruminal Bacteria. The *Fibrobacter succinogenes* genome sequencing project was conducted at The Institute for Genomic Research (now the J. Craig Venter Institute) and also supported by the USDA-CSREES funds listed above.

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