Unique Contribution of the Cell Wall-Binding Endoglucanase G to the Cellulolytic Complex in *Clostridium cellulovorans*

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The cellulosomes produced by *Clostridium cellulovorans* are organized by the specific interactions between the cohesins in the scaffolding proteins and the dockerins of the catalytic components. Using a cohesin biomarker, we identified a cellulosomal enzyme which belongs to the glycosyl hydrolase family 5 and has a domain of unknown function 291 (DUF291) with functions similar to those of the surface layer homology domain in *C. cellulovorans*. The purified endoglucanase G (EngG) had the highest synergistic degree with exoglucanase (ExgS) in the hydrolysis of crystalline cellulose (EngG/ExgS ratio = 3:1; 1.71-fold). To measure the binding affinity of the dockerins in EngG for the cohesins of the main scaffolding protein, a competitive enzyme-linked interaction assay was performed. Competitors, such as ExgS, reduced the percentage of EngG that were bound to the cohesins to less than 20%; the results demonstrated that the cohesins prefer to bind to the common cellulosomal enzymes rather than to EngG. Additionally, in surface plasmon resonance analysis, the dockerin in EngG had a relatively weak affinity (30- to 123-fold) for cohesins compared with the other cellulosomal enzymes. In the cell wall affinity assay, EngG anchored to the cell surfaces of *C. cellulovorans* using its DUF291 domain. Immunofluorescence microscopy confirmed the cell surface display of the EngG complex. These results indicated that in *C. cellulovorans*, EngG assemble into both the cellulolytic complex and the cell wall complex to aid in the hydrolysis of cellulose substrates.

In plants, cell wall polysaccharides composed primarily of the polymers cellulose and hemicellulose are a major reservoir of carbon and energy. However, the chemical and physical complexity of plant cell walls is major obstacle to recycling and utilization of the photosynthetically fixed carbon, and thus considerable research has been performed to overcome the inefficient process (1). Some microorganisms which are capable of efficient saccharification by producing enzymes that degrade the cellulose and hemicelluloses have been reported and characterized. Research studies have focused on several anaerobic microorganisms that produce large extracellular enzyme complexes called cellulosomes (2).

The cellulosome has been most widely studied in anaerobic clostridia, namely, *Clostridium thermocellum* (3), *Clostridium cellulovorans* (4), and *Clostridium cellulolyticum* (5). Cellulosomes are formed by highly specific interactions between the repeated cohesin modules of the scaffolding proteins and the dockerin modules of the cellulosomal catalytic subunits (6). Cohesins are 150-residue modules that are usually present as tandem and multiple copies in scaffoldings (1). All cellulosomal catalytic subunits contain a twice-repeated sequence (called dockerin) which is not found in noncellulosomal enzymes (7). The recognition between cohesins and dockerins is mediated mainly by hydrophobic interactions. These tight protein-protein interactions allow the integration of the cellulosomal enzymes into the complex (8). In *C. cellulovorans*, the bacterial surface layer homology (SLH) domains in the scaffolding proteins mediate binding between the cellulosome and the cell surface (4).

In *C. cellulovorans*, several cellulosomal enzyme genes have been cloned and sequenced. All enzymes have dockerins at their C termini, except for mannanase with its dockerin at its N terminus, including its duplicated 22-amino-acid sequence (9). Thus far, several cellulosomal cellulases from *C. cellulovorans* have been studied and are classified into three glycosyl hydrolase families (family 5, family 9, and family 48). Endoglucanase E (EngE) and

endoglucanase B (EngB) belong to glycosyl hydrolase family 5, and exoglucanase S (ExgS) belongs to glycosyl hydrolase family 48. The previous results suggested that *C. cellulovorans* degrades cellulose by the cooperative activities of at least three different glycosyl hydrolase families of cellulases: families 5, 9, and 48 (10).

In a previous study that was conducted to detect novel cellulosomal subunits, a fluorescent cohesin module was used as a biomarker for its interaction with the dockerin-containing proteins, which were then identified by their partial amino acid sequences using electrospray ionization-tandem mass spectrometry (ESI-MS/MS) (11, 12). We detected a cellulosomal protein, referred to as endoglucanase G, that contained the catalytic domains of glycosyl hydrolase family 5 (48% sequence similarity to endoglucanase A from *Caldocellum saccharolyticum*) and domain of unknown function 291 (DUF291), which may be a putative cell surface binding domain. Intensity analysis of the fluorescent proteins indicated that the fluorescent signal of EngG was weaker at the cohesin module than that of the other cellulosomal enzymes. This result suggested that the relatively low binding affinity of EngG to the cohesin is caused by the unique characteristic of EngG.

In this study, to characterize the role of EngG in the cellulolytic complex, we analyzed the enzymatic properties of EngG and its synergistic effects with the exoglucanase in the formation of the cellulolytic complex. We also investigated the binding properties

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of cohesin-dockerin interactions and confirmed the ability of the putative cell surface binding domain to interact with the cell surface using surface plasmon resonance (SPR) and the competitive enzyme-linked interaction assay (cELIA). Taken together, our results demonstrate the importance and contribution of EngG to the assembly of the cellulosome and to the hydrolysis of cellulose in *C. cellulovorans*.

MATERIALS AND METHODS

DNA manipulations. *C. cellulovorans* strain ATCC 35296, which was used for isolation of genomic DNA, was grown under strict anaerobic conditions at 37°C in 1-liter round-bottom flasks in a medium containing 1% (wt/vol) cellobiose (Sigma-Aldrich) (13). *Escherichia coli* DH5 α bacteria were used as host cells for DNA manipulation, and *E. coli* strain BL21(DE3) (Real Biotech Corporation) was used as a host for the pET22b(+) (Novagen) derivative plasmid pET22b-EngG. A partial peptide sequence (SQIDYALGSTGR) matched the residues of a putative protein of glycosyl hydrolase family 5. The mature EngG DNA sequence (NCBI reference sequence, YP_003844073.1) was amplified in a reaction mixture containing 50 mM Tris-HCl, 50 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), and 0.5 mM phenylmethylsulfonyl fluoride (PMSF) (pH 7.9) from the genomic DNA of *C. cellulovorans* using forward primer 5'-TACGGAATTCATGAAAAATTAGGTTCTATCTTAGCTAG-3' (the EcoRI site is underlined) and reverse primer 5'-AGCTCTCGAGGAACTAGTTATTG-3' (the XhoI site is underlined). The PCR product was digested with EcoRI and XhoI and was ligated into the corresponding restriction sites in the pET22b(+) vector to generate pET22b-EngG. To enable the secretion of EngG, we used the commercial secretion pET22b(+) vector, which contains a *pelB* signal sequence and a polyhistidine (His₆) tag at its C terminus under the control of the T7 promoter (14).

Preparation of the cellulosomal enzyme. *E. coli* BL21(DE3) cells that harbored the pET22b-EngG plasmid were grown at 37°C in Luria-Bertani broth containing ampicillin (50 μ g/ml) to an optical density at 600 nm (OD₆₀₀) of 0.9 (15). The culture was then cooled to 30°C, and isopropylthio- β -D-galactoside (IPTG) was added to the culture extract at a final concentration of 1 mM. After an additional overnight growth period, the cells were collected via centrifugation (4,500 $\times$ g for 10 min at 4°C) and resuspended in 10 ml of lysis buffer (50 mM Na₂HPO₄, 400 mM NaCl, and 10 mM imidazole, pH 8.0). The cells were collected via centrifugation (4,500 $\times$ g for 30 min at 4°C) and lysed using ultrasonication. The supernatant from the crude cell extract was applied to an Ni-nitrilotriacetic acid (NTA) column, which bound to the His tag of the recombinant protein, and the column was washed with wash buffer (50 mM NaH₂PO₄, 400 mM NaCl, and 25 mM imidazole, pH 8.0). The bound recombinant EngG was eluted using an elution buffer (50 mM Na₂HPO₄, 400 mM NaCl, and 250 mM imidazole, pH 8.0). The purified protein was collected and dialyzed against phosphate-buffered saline (PBS) (pH 7.3). The dialyzed protein was concentrated via ultrafiltration (Millipore; molecular mass cutoff, 10 kDa) (14). The concentration of the purified protein was measured using the Bradford assay from the Bio-Rad protein assay kit, and bovine serum albumin (BSA) was used to generate the standard curve (16).

SDS-PAGE and zymogram analyses. SDS-PAGE analysis was performed with a 12% SDS-polyacrylamide gel using the Laemmli gel system (17). Proteins were visualized by Coomassie blue staining. The zymograms for the carboxymethyl cellulase were performed using 0.2% (wt/vol) carboxymethyl cellulose (CMC) that was incorporated into the polyacrylamide gel. After SDS-PAGE, the gel was washed for 2 h at room temperature with two buffer changes, which contained 0.1 M succinate (pH 6.3), 1 mM dithiothreitol, and 10 mM CaCl₂, before staining in 0.1% (wt/vol) Congo red solution for 30 min. Yellow halos emerged against the red background of the gel after it was destained with 1 M NaCl (18).

Enzyme assays. The hydrolytic activity of the recombinant protein was measured after 2 h of incubation at 40°C in 50 mM sodium acetate buffer (pH 6.0) with 1.0% (wt/vol) polysaccharides. The polysaccharides tested were β -glucan, CMC, phosphoric acid-swollen cellulose (PASC),

and Avicel. The reactions were terminated by boiling for 5 min and adding 1% 3,5-dinitrosalicylic acid (DNS) reagent. After centrifugation, the supernatants were assayed for the release of the reducing sugars. The concentrations of the reducing sugars released from the substrates were determined using the 3,5-dinitrosalicylic acid reagent as described by Miller et al. (19). One unit of hydrolytic activity was defined as the amount of enzyme that released 1 μ mol of glucose equivalents per minute from the substrate (14).

Assembly and purification of the recombinant cellulosomes. The assembly of the recombinant cellulosomes was performed by the method of Murashima et al. (20). Mini-CbpA (mCbpA) originated from CbpA from *C. cellulovorans*; the mCbpA contained one cellulose binding domain, one hydrophilic domain, and two cohesin domains. Since one cohesin domain binds one cellulosomal enzyme molecule, one mCbpA molecule could bind at most two cellulosomal enzyme molecules. ExgS, which is major exoglucanase in *C. cellulovorans*, degraded the nicked cellulose chain in an endo-processive manner (10). Recombinant EngG and ExgS (10.0 nmol) (12), as well as the purified mini-CbpA (5.0 nmol) (21), were mixed in 100 μ l of binding buffer (50 mM Tris-HCl buffer [pH 7.4] and 15 mM CaCl₂) and kept for 1 h at 4°C. Size exclusion chromatography was performed to purify the assembled cellulosomes on an AKTA fast protein liquid chromatography (FPLC) system using a HiLoad 16/60 Superdex 30 column (GE Healthcare). The recombinant cellulosomes (EngG-mCbpA complex and ExgS-mCbpA complex) were injected into the column using a 10-ml sample loop with an injection volume of 10 ml. Protein was eluted isocratically with 50 mM Tris-HCl (pH 7.4) at a flow rate of 0.5 ml/min. The reliability of the analysis was confirmed by at least two independent measurements under the same conditions. Detection was achieved by monitoring the UV absorbance at 280 nm (22). The purified assembly of the recombinant cellulosomes was confirmed by native PAGE analysis using a 12% polyacrylamide gel without SDS (Bio-Rad).

Synergistic effect of the cohesin-dockerin interaction on crystalline cellulose. The enzyme activities were assayed by measuring the liberated reducing sugars in 100 μ l of the reaction mixtures (0.5% substrate in 50 mM sodium acetate buffer [pH 7.0]) at 37°C for 16 h using the method of Miller et al. (19). PASC and filter paper were used as cellulosomal substrates. The enzyme concentrations in the reaction mixtures were 2 nmol/ml for the cohesin-dockerin interaction (14).

cELIA. For the competitive enzyme-linked interaction assay (cELIA), the purified cohesins, including cohesin 6 (Coh6), were prepared as in our previous study (12). Microtiter plates were coated overnight with *C. cellulovorans* cohesins (200 μ l/well, 270 nM Coh6). The plates were blocked for 2.5 h with the blocking solution (as described above) and washed 3 times with Tris-NC buffer (50 mM Tris, 100 mM NaCl, 2 mM CaCl₂, and 0.02% sodium azide, pH 7.5). The cohesin-dockerin interaction was initiated by the addition of 100 μ l of the desired dockerin competitor (up to a maximum of 1.3 μ M ExgS dockerin), followed immediately by the addition of dockerin solution (100 μ l of EngG, ExgS, and EngE to a final concentration of 47 nM). Dilutions of the dockerin competitors were performed in Tris-NC buffer containing BSA to maintain a constant protein concentration. After incubation for 2.5 h, the wells were washed 5 times, and the amount of dockerin bound to the cohesin coating was detected by measuring the cellulase activity, as described above. The results are presented as a relative percentage of binding, which was derived from the mean optical density values of three repetitions for each competitor concentration (relative percentage of binding = 100 $\times$ optical density with the competitor concentration/optical density without the competitor). Data were analyzed using a 4-parameter fit from the Grafit 5 software (23).

SPR analysis. The interactions between the cohesins (Coh2, Coh6, and Coh9) and the dockerins of EngG proteins in *C. cellulovorans* were analyzed by surface plasmon resonance (SPR) using a BiaCore 2000 instrument (GE Healthcare) (6). In brief, the cohesin protein was immobilized on a dextran matrix with free carboxylic groups (CM5 chip) using conventional carbodiimide coupling chemistry and subsequent deactiva-

FIG 1 SDS-PAGE and zymogram analyses of EngG. Purified EngG is shown in lane 1. The zymogram analysis of EngG in lane 2 was performed with 0.2% (wt/vol) CMC that was incorporated into the polyacrylamide. Lane M, protein standards.

tion of excess active esters with ethanolamine (EDC/NHS coupling kit; GE Healthcare). The ligands were diluted in running buffer (50 mM Tris-maleate, 10 mM CaCl_2 , and 0.005% Surfactant P20, pH 6.5) and allowed to interact with the sensor surface during a 240-s injection. In all cases, at least three different concentrations (0.57 to 100 nM) of the ligand were injected at a flow rate of 30 ml per min. Both the association rate constant (k_{on}) and the dissociation rate constant (k_{off}) were calculated using the BIAEVALUATION 3.2 software. The dissociation constant (K_D) was defined as $k_{\text{off}}/k_{\text{on}}$ (6).

Preparation of NPCS and HF-EPCS. Native peptidoglycan-containing sacculi (NPCS) and hydrofluoric acid-extracted cell wall polymer (HF-EPCS) were prepared from *C. cellulovorans*. Total cell envelopes for the preparation of NPCS were collected by centrifugation. The cells were initially incubated in 50 mM Tris-HCl buffer (pH 7.4) containing 0.5% (vol/vol) Triton X-100 and 5 M guanidine-HCl for 1 h at 20°C, followed by centrifugation at $40,000 \times g$, 8 washes with 50 mM Tris-HCl buffer (pH 7.2), and four additional washes with 50 mM phosphate buffer (pH 7.4). The isolated pellets were resuspended and incubated in 50 mM phosphate buffer (pH 7.4) containing 1% SDS at 100°C for 1 h, washed 6 times with distilled water, and resuspended in 50 mM phosphate buffer (pH 7.4). For the preparation of HF-EPCS, NPCS was incubated with 48% hydrofluoric acid (HF) at 4°C for 170 h. The pellets were washed once with 48% HF and 5 times with distilled water before resuspension in 50 mM phosphate buffer (pH 7.4) (24).

Interactions between the cellulases and cell wall fractions of *C. cellulovorans*. NPCS and HF-EPCS (25 μl each) were incubated with 25 μl of cellulases (EngG or ExgS) in 50 μl of 20 mM sodium phosphate buffer (pH 7.4) at 4°C for 1 h with occasional shaking. Following the incubation, insoluble fractions were pelleted by centrifugation ($4,500 \times g$ for 15 min at 4°C), and both supernatant and pellet fractions were analyzed by SDS-PAGE (25).

Immunofluorescence microscopy. The recombinant EngG was expressed and purified using the *E. coli* BL21(DE3) cells that harbored the pET22b-EngG plasmid. The recombinant ExgS (12), which does not contain an SLH or putative SLH domain, was used as a negative control, and the recombinant mCbpA was used as a positive control. *C. cellulovorans* cells were grown under strict anaerobic conditions at 37°C in a medium containing 1% (wt/vol) cellobiose. The cells were incubated with recombinant EngG protein for 4 h in PBS. Twenty microliters of suspended cells was then aspirated onto a polylysine-coated microscope slide and dried. The slides were then blocked by adding a 2% solution of BSA protein dissolved in PBS. After incubation for 15 min, the slides were washed with PBS. EngG display was probed using an anti-His₆ immunoglobulin G antibody (1.25 $\mu\text{g}/\mu\text{l}$; Santa Cruz). After incubation for 1 h, the slides were washed with PBS and incubated for 1 h with goat anti-rabbit immunoglobulin G conjugated with AF488 dye (0.2 $\text{ng}/\mu\text{l}$; Santa Cruz). After

FIG 2 Native PAGE analyses of the purified mini-CbpA and the purified cellosomal enzymes (EngG and ExgS) complex. (A) Native PAGE analysis of the mixture of mini-CbpA and EngG. Purified mini-CbpA and EngG are shown in lane 1 and lane 2, respectively. The mixture of EngG and mini-CbpA is shown in lane 3. (B) Native PAGE analysis of the mixture of mini-CbpA and ExgS. Purified mini-CbpA and ExgS are shown in lane 1 and lane 2, respectively. The mixture of mini-CbpA and ExgS is shown in lane 3.

the slides were washed again with PBS, a 10- μl solution containing 70% glycerol and 5 $\mu\text{g}/\text{ml}$ 4',6-diamidino-2-phenylindole (DAPI) was added prior to imaging. Photographs were taken using an immunofluorescence microscope (Carl Zeiss) (26).

RESULTS

Purification and substrate-specific activity of EngG. *E. coli* BL21(DE3) cells harboring pET22b-EngG were collected for protein expression analysis. The expressed EngG proteins were purified with Ni-NTA. The results of the SDS-PAGE analysis showed that EngG has a molecular mass of 76 kDa (Fig. 1, lane 1), which corresponds to its predicted molecular mass calculated based on its nucleotide sequence. By zymogram analysis, we also measured the enzyme activity of EngG (Fig. 1, lane 2). To ascertain the specific activity of EngG with various substrates, four types of substrates were tested: β -glucan, CMC, PASC, and Avicel. The hydrolytic activity of EngG toward β -glucan was approximately 17.78 ± 0.27 U/ μmol . EngG activity toward CMC was 5.31 ± 0.07 U/ μmol . With PASC and Avicel as its substrates, the specific activities of EngG were 0.12 ± 0.01 and 0.14 ± 0.01 U/ μmol , respectively. However, EngG had no hydrolytic activity toward xylan from birch wood (data not shown).

Assembly of the recombinant cellulosomes. The purified EngG was assembled with mCbpA to improve EngG cellulose-hydrolytic activity by its stronger proximity to the substrates, because the scaffolding protein mCbpA may provide the structural basis for the highly specific activity of the cellulosome (2). We performed size exclusion chromatography to remove unbound cellosomal subunits from cellulosome complexes. The association of mCbpA with the cellosomal enzymes, such as EngG and ExgS, was confirmed by native PAGE analysis (Fig. 2). This observation suggested that the cellosomal enzymes EngG and ExgS had successfully bound to mCbpA to make recombinant cellulosomes.

TABLE 1 Properties of cellulosomal enzyme EngG

Substrate	Activity (U/ μ mol of enzymatic subunit) ^a :		Synergy degree
	With mini-CbpA	Without mini-CbpA	
CMC	5.69 $\pm$ 0.07	5.31 $\pm$ 0.07	1.1
Avicel	0.23 $\pm$ 0.01	0.14 $\pm$ 0.01	1.6
PASC	0.18 $\pm$ 0.02	0.12 $\pm$ 0.03	1.4
Filter paper	0.59 $\pm$ 0.03	0.39 $\pm$ 0.01	1.4

^a Values are means and standard deviations of triplicate measurements.

somes *in vitro*. The cellulase activities of EngG with or without mCbpA were measured (Table 1). The hydrolytic activities of EngG with mCbpA toward crystalline cellulose (Avicel, PASC, and filter paper) significantly increased (1.6-, 1.5-, and 1.4-fold, respectively) compared to those with no mCbpA added. However, compared to that in the absence of mCbpA, the activity of EngG against insoluble cellulose was not significantly changed by binding with mini-CbpA (1.1-fold). As a result of binding to mCbpA, the hydrolytic activities of EngG against insoluble cellulose increased compared with those without mCbpA.

Synergistic effects on crystalline cellulose of the recombinant cellulosomes of EngG and ExgS. The enzymatic activities on crystalline cellulose of recombinant EngG and ExgS cellulosomes with various compositions were measured to test their endo-exo synergy (Fig. 3). Among the different conditions tested, the mixture of recombinant EngG and ExgS cellulosomes that showed the highest specific activity for hydrolyzing PASC (0.72 U/ μ mol) and highest degree of synergy (2.18-fold) was that with a 1:3 molar ratio of EngG to ExgS (Fig. 3A). A 1:3 molar ratio of the recombinant EngG to ExgS cellulosomes also showed the highest hydrolytic activity (1.25 U/ μ mol) toward filter paper; this combination

FIG 3 Specific activities and synergy degrees of various recombinant cellulosome mixtures against PASC (A) and against filter paper (B). The horizontal axes indicate the molar ratios of EngG and ExgS in the various recombinant cellulosome mixtures. The vertical axes indicate the specific activities and synergy degrees against PASC and filter paper. The error bars represent the standard deviation of triplicate measurements.

FIG 4 Determination of the relative binding affinities between the cohesins and the cellulosomal enzymes by competitive enzyme-linked interaction assays (cELIAs). Microtiter plates were coated with cohesin 6. The immobilized cohesins interacted with EngG (circles) in the presence of competitors ExgS (squares) and EngE (triangles). The error bars represent the standard deviation of triplicate measurements.

also showed the highest degree of synergy (1.71-fold) (Fig. 3B). These results indicated that EngG, when combined with ExgS in the proper ratio, is able to contribute synergistically to the degradation of crystalline cellulose.

Quantification of the binding affinity of EngG dockerin with competitive cellulosomal enzymes by cELIA. To quantitatively assess the competitive binding affinities of the cellulosomal enzymes (EngG, EngE, and ExgS), a competitive form of the enzyme-linked immunosorbent assay (ELISA) (competitive enzyme-linked interaction assay [cELIA]) was used to determine the high binding constants in a comparative manner. Microtiter plates were coated with Coh6 because Coh6 shares high sequence identity with the other cohesins, which ranges from 40 to 98%. The coated microtiter plates were then mixed with three cellulosomal enzymes (EngG, EngE, and ExgS). The dockerin of ExgS was used as a competitor of the cellulosomal enzymes. When the concentrations of the cellulosomal enzymes were equivalent, we confirmed that more than 80% of EngG was inhibited in binding with the Coh6 coating, which is proportional to the dissociation constant (K_D) of the competitor (Fig. 4). On the other hand, nearly 50% of EngE and ExgS bound to Coh6, based on the 1:1 ratio of the cellulosomal enzyme to its competitor. The assay results indicated that the dockerin of EngG has relatively weaker binding affinity for the cohesin modules than the other cellulosomal enzymes (EngE and ExgS).

Quantitative analysis of the cohesin-dockerin interactions by SPR. To quantitatively measure the real-time interactions of the cellulosomal enzymes and the cohesins, surface plasmon resonance (SPR) analysis was performed. Coh2, Coh6, and Coh9 modules were covalently immobilized to the dextran carboxymethyl groups on the surface of a CM5 sensor chip using the amine coupling method. We used the *C. cellulovorans* CbpA, protein that consists of nine cohesin modules (Coh). Among the cohesin modules, Coh3, Coh4, Coh5, Coh6, Coh7, and Coh8 are highly homologous to each other. Specifically, the Coh4, Coh5, and Coh6 modules displayed greater than 95% sequence identity. Conversely, the Coh1, Coh2, and Coh9 modules have relatively less homology with the other cohesin modules. Therefore, we chose Coh2, Coh6, and Coh9 to measure the cohesin-dockerin interactions and to compare the binding affinities of different cohesins for the dockerin-containing proteins (12). The running

TABLE 2 SPR analysis of binding between cohesin modules and EngG

Cellulosomal enzyme	Cohesin	k_{on} ($\text{s}^{-1} \text{M}^{-1}$) ^a	k_{off} (s^{-1}) ^a	K_D (M) ^b
EngG	Coh2	$(4.62 \pm 0.25) \times 10^4$	$(4.78 \pm 0.43) \times 10^{-2}$	6.56×10^{-7}
	Coh6	$(2.6 \pm 0.37) \times 10^4$	$(3.30 \pm 0.29) \times 10^{-3}$	1.24×10^{-7}
	Coh9	$(5.63 \pm 0.54) \times 10^4$	$(5.03 \pm 0.42) \times 10^{-2}$	8.93×10^{-7}
EngE	Coh2	$(6.37 \pm 0.64) \times 10^4$	$(4.85 \pm 0.38) \times 10^{-3}$	7.62×10^{-8}
	Coh6	$(9.56 \pm 0.84) \times 10^5$	$(4.78 \pm 0.62) \times 10^{-3}$	6.94×10^{-9}
	Coh9	$(7.51 \pm 0.41) \times 10^4$	$(4.29 \pm 0.51) \times 10^{-2}$	5.71×10^{-7}
ExgS	Coh2	$(7.81 \pm 0.18) \times 10^5$	$(4.16 \pm 0.29) \times 10^{-3}$	5.33×10^{-9}
	Coh6	$(6.72 \pm 0.25) \times 10^5$	$(2.77 \pm 0.31) \times 10^{-3}$	4.12×10^{-9}
	Coh9	$(6.43 \pm 0.40) \times 10^5$	$(3.20 \pm 0.36) \times 10^{-3}$	4.98×10^{-9}

^a Values are means and standard deviations.
^b K_D values were calculated as $k_{\text{off}}/k_{\text{on}}$.

buffer contained 10 mM CaCl₂ because calcium ions are required for the binding of cellulosomal enzymes to the cohesin modules (27). In this study, EngG showed little difference in its binding to the various cohesin modules (Table 2). Interestingly, the dockerin of EngG has a relatively weaker affinity (~100-fold) for the cohesin modules than those of the other cellulosomal enzymes (EngE and ExgS). For example, the K_D values of EngE and ExgS with Coh6 are 6.94×10^{-9} M and 4.12×10^{-9} M, respectively, but the K_D value of EngG with Coh6 is 1.24×10^{-7} M. These results suggested that the dockerin in EngG bound weakly to the main scaffolding proteins compared with those of the other cellulosomal enzymes (EngE and ExgS).

Anchoring of EngG to the cell wall fractions of *C. cellulovorans*. Sequence analysis revealed that a domain with unknown function (DUF291) in EngG was similar to the SLH domain (70% similarity). Moreover, the putative three-dimensional (3D) structure of DUF291 analyzed by the PHYRE2 Protein Fold Recognition Server matched with cell adhesion domains from *Bacteroides thetaiotaomicron* and *Dictyostelium discoideum*. Therefore, we investigated whether DUF291 in EngG has the ability to bind to the cell wall fractions. The cell wall fractions were prepared as NPCS and as hydrofluoric HF-EPCS, which have their secondary cell wall polymers (SCWPs) removed from the NPCS (25). The protein bands on the SDS-polyacrylamide gel indicated that the cell wall fractions from *C. cellulovorans* interacted with EngG (Fig. 5, lane 1), whereas ExgS, as a negative control, did not bind to the cell surface (28) (Fig. 5, lane 5). In addition, EngG was unable to interact with the HF-EPCS because the missing SCWPs consist primarily of peptidoglycans (Fig. 5, lane 3). This observation dem-

onstrated that EngG has the ability to attach to the cell surface by interacting with SCWPs through a binding site in its DUF291 domain.

Identification of cell surface display by immunofluorescence analysis. EngG on the *C. cellulovorans* cell surface was visualized with immunofluorescence microscopy to confirm the binding activity of EngG (Fig. 6). The *C. cellulovorans* cells were grown to an OD₆₀₀ of 1.2, and protein display on the cell surface was observed using immunofluorescence microscopy (26). The anti-His antibody against the C-terminal His tag in proteins EngG, ExgS, and mCbpA was used. After incubation of cell with His-tagged proteins, bright fluorescence signals were observed on the surface of the cells displaying EngG and mCbpA. However, no signals were observed with the negative control (ExgS). These results indicated that not only mCbpA but also EngG is

FIG 5 Binding of EngG and ExgS to NPCS and HF-EPCS. Lanes 1 and 2, EngG bound to NPCS; lanes 3 and 4, EngG bound to HF-EPCS. EngG was incubated with NPCS and HF-EPCS from *C. cellulovorans*. After centrifugation, the protein mixtures in the precipitates (lanes 1 and 3) and the supernatant fractions (lanes 2 and 4) were analyzed by SDS-PAGE. The control samples (ExgS with NPCS) are shown in lane 5 (precipitate) and lane 6 (supernatant).

FIG 6 Immunofluorescence micrographs of *C. cellulovorans* cells displaying His₆-tagged EngG, ExgS (negative control), and mCbpA (positive control). The recombinant protein-displaying cells were probed with an anti-His6 antibody.

able to display on *C. cellulovorans* cells and that the DUF291 in EngG has same function with the SLH domain in CbpA to display the cell surface specifically.

DISCUSSION

The cohesin-dockerin interaction plays a key role in the assembly of the cellulosome in anaerobic bacteria (29). In a previous study, a fluorescence-labeled Coh6, which shares a high degree of sequence identity with the other cohesins, was used as a biomarker to detect the primary binding step of the cellulosomal subunits (12). The cellulosomal enzymes were detected by their cohesin-dockerin interactions with the cohesin biomarker. The analysis of fluorescence intensity indicated that EngG bound less efficiently to the cohesin module than the other cellulosomal enzymes (11). These results suggested that, in *C. cellulovorans*, EngG plays a distinct role in the cellulosome and in the cellulolytic process. This study describes the functional analysis of EngG in *C. cellulovorans*, including its role in the cellulosomal system.

In *C. cellulovorans*, EngE, which belongs to glycosyl hydrolase family 5, is generally thought to be one of major cellulosomal enzymes (21). In a previous study, synergistic effects of the recombinant ExgS and EngE cellulosomes on their hydrolytic activities against crystalline cellulose were detected. The hydrolytic activities against crystalline cellulose were measured with cellulase mixtures of various compositions. The mixtures of the recombinant ExgS and EngE cellulosomes showed a maximum synergy degree of 1.79 (1:3 molar ratio of ExgS to EngE) (10). In this study, the cellulosomal mixture of recombinant EngG and ExgS exhibited the highest synergy degree (2.18) at a 1:3 molar ratio of EngG to ExgS. The results demonstrated that the synergy degree of EngG and ExgS in hydrolyzing crystalline cellulose was higher than the synergy degree of EngE and ExgS. The results also indicated that, in *C. cellulovorans*, EngG plays an important role in the cellulosome system to degrade crystalline cellulose.

In the previous study, a fluorescence intensity analysis and an enzyme-linked interaction assay were performed, and the results showed selectivity of the cohesin-dockerin interactions between certain cellulosomal enzymes and cohesins in *C. cellulovorans*. We performed SPR analysis to detect individual protein-protein interactions. The results showed that the various cellulosomal enzymes exhibited different binding affinities, depending on the cohesin modules of the main scaffolding proteins in *C. cellulovorans*. Specifically, the results revealed that EngE exhibits significant differences in its binding affinities to various cohesin modules (12). Interestingly, EngE, which possesses SLH domains, was suggested to be an anchor for the cell surface attachment and assembly to the scaffolding protein CbpA.

Sequence analysis revealed that EngG also possesses a putative SLH domain called DUF291, suggesting that EngG has the ability to anchor to the cell surface of *C. cellulovorans*. DUF291 in EngG exhibited extensive sequence similarity with the SLH domain in CbpA. On the other hand, only slight homology at the amino acid level was observed, when DUF291 in EngG was compared with the SLH domains of EngE. Therefore, the results of sequence alignment studies suggested that DUF291 in EngG may have a function similar to that of the SLH domains of CbpA. As shown in Fig. 5, EngG is bound to the SWCP in *C. cellulovorans* (25). In previous study, it was observed that not only native *C. cellulovorans* cellulosomes but also EngE had the ability to bind to the SWCP. However, the binding affinities of the SLH domain in CbpA to the cell

surface are relatively weaker than those of the SLH domain in EngE (30). These reports indicated that EngG is a dual-function protein that has the abilities to anchor to the cell surface weakly and to associate with the scaffolding protein. Moreover, the previous reports suggested that EngE functioned mainly as an anchoring protein for CbpA through its cohesin-dockerin interaction and through its SLH domains, and these results can be applicable to elucidate the function of EngG in the *C. cellulovorans* cellulosome (31). Synthetically, there are two possible explanations about the role of EngG: (i) EngG binds both to CbpA through the cohesin-dockerin interaction and to the cell surface via its SLH domain, assisting the attachment of CbpA to the cell surface, and (ii) due to the relatively low binding affinity for the cohesin modules, EngG binds either CbpA or the cell surface, depending on the extracellular conditions.

The mechanism for the different binding affinities of EngG and the other cellulosomal enzymes is unclear. Nevertheless, it is possible that the different binding affinities originated from the dockerin sequence of EngG. The sequence duplication of the dockerin module is reflected in its near-perfect 2-fold structural symmetry (8, 32). The bidirectional binding of dockerin is termed the “dual-binding mode.” Previous results showed that the cohesin recognition occurs predominantly through an α -helix (α 3) in the second segment of the dockerin in *C. thermocellum* (32). EngG has a unique sequence in its second segment compared with the other cellulosomal proteins; therefore, it is possible that the second, but not the first, segment of dockerin has influence on the binding affinity of the cohesin-dockerin interactions in the assembly of EngG and the scaffolding proteins. Further studies on the contributions of the first and second segments to the different binding affinities of the cellulosomal enzymes for the cohesin modules in *C. cellulovorans* should be pursued.

Conclusion. The protein-protein interactions required for the assembly of cellulolytic complexes in anaerobic microbe for the hydrolysis of cellulose are relatively poorly understood. We successfully detected EngG using a fluorescent cohesin as a biomarker, which was previously used in the 2D PAGE analysis and profiling of dockerins of cellulosomal subunits that associated with the cohesin. EngG showed synergistic effects with exoglucanase as a cellulolytic complex in *C. cellulovorans*. Furthermore, EngG exhibited weak binding affinities for the cohesin modules of the main scaffolding proteins (30- to 123-fold) because EngG has the unique ability to anchor to the cell surface of *C. cellulovorans* using its novel SLH domain (DUF291). This study demonstrates the assembly of the cellulosomal subunit EngG with the scaffolding protein for lignocellulosic biomass hydrolysis and the unique contribution of EngG due to its functional domain.

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