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# Synergistic Effects on Crystalline Cellulose Degradation between Cellulosomal Cellulases from *Clostridium cellulovorans*

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***Clostridium cellulovorans*** **produces a multienzyme cellulose-degrading complex called the cellulosome. In this study, we determined the synergistic effects on crystalline cellulose degradation by three different recombinant cellulosomes containing either endoglucanase EngE, endoglucanase EngH, or exoglucanase ExgS bound to mini-CbpA, a part of scaffolding protein CbpA. EngE, EngH, and ExgS are classified into the glycosyl hydrolase families 5, 9, and 48, respectively. The assembly of ExgS and EngH with mini-CbpA increased the activity against insoluble cellulose 1.5- to 3-fold, although no effects on activity against soluble cellulose were observed. These results indicated that mini-CbpA could help cellulase components degrade insoluble cellulose but not soluble cellulose. The mixture of the cellulosomes containing ExgS and EngH showed higher activity and synergy degrees than the other cellulosome mixtures, indicating the synergistic effect between EngH and ExgS was the most dominant effect among the three mixtures for crystalline cellulose degradation. Reactions were also performed by adding different cellulosomes in a sequential manner. When ExgS was used for the initial reaction followed by EngE and EngH, almost no synergistic effect was observed. On the other hand, when EngE or EngH was used for the first reaction followed by ExgS, synergistic effects were observed. These results indicated that the initial reactions by EngH and/or EngE promoted cellulose degradation by ExgS.**

Cellulose is the most abundant biological polymer on the earth (34). Although the chemical composition of cellulose is very simple, consisting of only glucose residues connected by β-1,4-glycosidic bonds, no single enzyme is able to degrade crystalline cellulose. To degrade crystalline cellulose to glucose, at least three enzymes have to cooperate: endoglucanase (EC 3.2.1.4), exoglucanase (cellobiohydrolase, EC 3.2.1.91), and β-glucosidase (EC 3.2.1.21) (34, 42). Currently, some of the exoglucanases are considered to be endo-processive enzymes, which initiate their action from the end of cellulose chains and liberate cellobioses along cellulose chains (42). Henrissat et al. have classified glycosyl hydrolases, including cellulases, based on the similarity of their primary sequences (12, 14). So far, endoglucanases are classified into 13 glycosyl hydrolase families, exoglucanases are classified into 5 families, and β-glucosidases are classified into 2 families (13). Aerobic fungi are known to produce cellulase components of several different glycosyl hydrolase families (44). For example, *Trichoderma reesei* produces eight cellulase components belonging to seven different glycosyl hydrolase families (CBHI [family 7], CBHII [family 6], EGI [family 7], EGII [family 5], EGIII [family 12], EGV [family 45], BGLI [family 3] and BGLII [family 1]). These different family cellulase components are known to cooperate synergistically to degrade crystalline cellulose (3, 15).

Anaerobic bacteria, as well as anaerobic fungi, are known to produce multienzyme complexes termed cellulosomes (22, 35); in contrast, aerobic microorganisms produce their cellulase components as single enzymes (44). The cellulosome contains a variety of cellulolytic subunits attached to the nonenzymatic component, which is called the scaffolding protein (1). All cellulosomal enzymatic subunits contain a twice-duplicated sequence called the dockerin domain. These dockerin domains bind to the hydrophobic domains of scaffolding proteins termed cohesins (43, 47). The cellulosome may give anaerobic microorganisms some advantage to degrade cellulose efficiently in nature, since the specific activity of cellulosomes from *Clostridium thermocellum* is known to be higher than that of cellulase mixtures from the aerobic fungus *T. reesei* (2, 17). It is still difficult to isolate active cellulosomal components from cellulosomes (6, 46). This technical limitation has prevented a complete understanding of the mechanism of efficient cellulose degradation by cellulosomes.

*Clostridium cellulovorans* is an anaerobic mesophile (38) that produces cellulosomes (36). So far, eight cellulosomal cellulase genes (*engB*, *engE*, *engH*, *engK*, *engL*, *engM*, *engY*, and *exgS*) (41) and the gene of the scaffolding protein CbpA (37) from *C. cellulovorans* have been cloned and sequenced. These eight cellulase genes were classified into three glycosyl hydrolase families (family 5, *engB* and *engE*; family 9, *engH*, *engK*, *engL*, *engM*, and *engY*; family 48, *exgS*). Recently, we determined the subunit composition of cellulosomes partially purified from the culture supernatant of *C. cellulovorans* and confirmed the presence of EngE (family 5), EngK (family 9), and ExgS (family 48) (27). These results suggested that *C. cellulovorans* degraded cellulose by the cooperative reaction of at least three different glycosyl hydrolase family cellulases: families 5, 9, and 48.

The determination of synergistic effects on cellulolytic activity between cellulosomal cellulases should help not only to understand the cellulolytic mechanism of the cellulosome but also in the design of artificial efficient cellulase systems. To determine the mechanisms of cellulose degradation by cellulosomes, artificial cellulosomes have been assembled with recombinant cellulosomal subunits from *Clostridium* species (5,

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9, 33). In this study, we assembled three different cellulosomes in vitro with three recombinant cellulosomal cellulases (EngE, EngH, and ExgS) and mini-CbpA, a miniature part of scaffolding protein CbpA containing CBD-SLH1-Coh1-Coh2. The synergistic reactions against crystalline cellulose between these cellulosomes were determined. Also, contribution of mini-CbpA to crystalline cellulose degradation was elucidated. The results showed that these three cellulosomes degraded crystalline cellulose synergistically and that assembly with mini-CbpA increased their activity against crystalline cellulose. The possible mechanisms of synergistic reactions are discussed.

## MATERIALS AND METHODS

**Bacterial strains and media.** *Escherichia coli* BL21(DE3) (Novagen) was used as an expression host for mini-CbpA, ExgS, and EngE production with pET-22b-mini-CbpA (26), pET-22b-ExgS, and pENGE (19). *E. coli* TOP10 (Invitrogen) was used as an expression host for EngH production with pBAD/Thio-EngH. Recombinant strains were cultivated in Luria-Bertani medium supplemented with ampicillin (50 μg/ml).

**Plasmid construction for mini-CbpA, ExgS, EngE, and EngH.** Mini-CbpA, EngE, and ExgS were expressed with the pET-22b vector (Novagen). Not all of these proteins possessed their own signal peptides, and they were designed to fuse the PelA signal peptide at their N-terminal ends and the His tag at their C-terminal ends from pET-22b. The *mini-cbpA* gene and the *engE* gene were amplified by PCR and inserted into the *Nco*I and *Xho*I sites of the pET-22b vector to generate pET-22b-mini-CbpA and pENGE as described previously (19, 26). For construction of the ExgS production vector, the *exgS* gene was amplified by PCR with the genomic DNA from *C. cellulovorans* as a template with the primers exgS F (CAAGTTTCCATGGCACCAGTAGTGCCAAATAATGAG) and exgS B (GGGGGCTCGAGAGCAAGAAGTGCTTTCTTTAATAAGC). The primers exgS F and exgS B contained the *Nco*I and *Xho*I sites (underlined), respectively. The amplified PCR fragment was digested with *Nco*I and *Xho*I and inserted into pET-22b digested with the same pair of restriction enzymes to generate pET-22b-ExgS. EngH was expressed by using the pBAD/Thio vector (Invitrogen). The expressed EngH did not possess its own signal peptide and was designed to fuse the thioredoxin at its N-terminal end and a V6 epitope and His tag at its C-terminal end from the pBAD/Thio vector. For construction of the EngH production vector, the *engH* gene was amplified by PCR with the genomic DNA from *C. cellulovorans* as a template with the primers engH F (TTATCA GGAATCTTGGGTGCAACTTC) and engH B (CTGTGATAAAAGTAGTTT CTTTAAAAGAG). The fragment obtained was cloned directly into the pBAD/Thio vector by TA cloning to generate pBAD/Thio-EngH.

**Expression of recombinant proteins.** For production of recombinant mini-CbpA, ExgS, and EngE, *E. coli* BL21(DE3) harboring pET-22b-mini-CbpA, pET-22b-ExgS, or pENGE was grown and recombinant proteins were induced by adding isopropyl-β-D-thiogalactoside as an inducer. The *E. coli* cells were grown in 1 liter of medium at 37°C to an optimal density at 600 nm of 0.9. After the culture broth was cooled on ice for 30 min, isopropyl-β-D-thiogalactoside was added to a final concentration of 0.4 mM for mini-CbpA production or 0.04 mM for ExgS or EngE production. Then, the culture was grown at 18°C for 16 h. For production of the recombinant EngH, *E. coli* TOP 10 harboring pBAD/Thio-EngH was grown and recombinant proteins were induced by adding L-arabinose as an inducer. The *E. coli* cells were grown in 1 liter of medium at 37°C to an optimal density at 600 nm of 0.9. After the culture broth was cooled on ice for 30 min, L-arabinose was added to a final concentration of 0.1%. Then, the culture was grown at 18°C for 16 h.

**Purification of recombinant proteins.** The recombinant mini-CbpA, ExgS, EngE, and EngH were purified in the same manner as follows. After the *E. coli* cells grown as described above were collected by centrifugation, the cells were resuspended in 30 ml of the lysis buffer (50 mM $NaH_2PO_4$, 300 mM NaCl, 10 mM imidazole, 1 mg of lysozyme/ml, pH 8.0). The solution was incubated on ice for 30 min, then the soluble proteins were extracted by supersonication. The extracted solution was applied to 4 ml of nickel-nitrilotriacetic acid agarose resin (QIAGEN), and the proteins bound to the resin were purified and pooled according to the product manual. The pooled solution was desalted and concentrated into 1 ml of 20 mM Tris-HCl buffer (pH 8.0) by use of the Ultrafree 10-kDa membrane (Millipore). The concentrated solution was applied to an anion-exchange column Mono Q HR 5/5 (Amersham Pharmacia Biotech AB) preequilibrated with 20 mM Tris-HCl buffer (pH 8.0). After washing the column with 5 ml of the same buffer, the proteins were eluted with a linear-gradient from 20 mM Tris-HCl buffer (pH 8.0) to 1 M NaCl in 20 mM Tris-HCl buffer (pH 8.0). The fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the fractions containing recombinant proteins were collected and dialyzed against 50 mM sodium acetic acid buffer (pH 6.0).

**Protein determination.** Protein was measured by using the method of Bradford (4) with a protein assay kit from Bio-Rad with bovine serum albumin as a standard. The molar amounts of each recombinant protein were calculated by use of the theoretical molecular weights of each protein.

**Assembly of recombinant cellulosomes.** The purified mini-CbpA (5.0 nmol) and the recombinant cellulosomal cellulases (ExgS, EngE, or EngH, 10 nmol) were mixed in 100 μl of the binding buffer (25 mM sodium-acetic buffer [pH 6.0], 15 mM $CaCl_2$), and kept for 1 h at 4°C. The assembly of mini-CbpA and cellulolytic subunits was confirmed by native PAGE analysis by use of 4 to 15% or 10% ready-made gel (Bio-Rad) as described previously (26).

**Determination of cellulase activities.** The cellulase activities were assayed in 100 μl of the reaction mixtures (0.5% substrate, 50 mM sodium acetate buffer [pH 6.0]) at 37°C by measuring the liberated reducing sugars, as D-glucose equivalents, by the Somogyi-Nelson assay method (45). The substrates were Avicel (FMC Corporation) as crystalline cellulose, acid-swollen cellulose as amorphous cellulose prepared from Avicel as described previously (16), and carboxymethylcellulose (medium viscosity; Sigma) as soluble cellulose. The cellulase concentrations in the reaction mixtures were 20 nmol/ml for activities against crystalline cellulose, 2 nmol/ml for activities against amorphous cellulose, and 20 nmol/ml (ExgS), 0.2 nmol/ml (EngE), or 2 nmol/ml (EngH) for activities against soluble cellulose. The standard reaction periods were 15 h, except for the determination of activities against soluble cellulose of EngE (15 min). Activities were expressed in units, with 1 U defined as the amount of enzyme releasing 1 μmol of reducing sugar per min.

## RESULTS

**Preparation of recombinant cellulosomal subunits.** To prepare the recombinant cellulosomes in vitro, the mini-CbpA (as a scaffolding protein) and three cellulosomal cellulases (ExgS, EngE, and EngH) were expressed by *E. coli.*

The mini-CbpA contained one cellulose binding domain, one hydrophilic domain, and two cohesin domains as designated previously (26). Since one cohesin domain binds one cellulosomal enzyme, one mini-CbpA could bind at most two cellulosomal enzymes. Moreover the mini-CbpA was designed to possess a His tag at its C terminus, which allows a ready purification by Ni-affinity chromatography. The mini-CbpA was expressed as a soluble protein, and purified almost to homogeneity by combination of Ni-affinity chromatography and anion-exchange chromatography (Fig. 1).

So far, eight cellulosomal cellulase genes, which are classified into three different glycosyl hydrolase families (families 5, 9, and 48) have been cloned (41). Among them, we selected one gene from each glycosyl hydrolase family, *engE* (40) from family 5, *engH* (41) from family 9, and *exgS* (24) from family 48. Although EngE and ExgS could be expressed as soluble proteins by the pET-22b vector, EngH was expressed as an inclusion body by this vector (data not shown). To express EngH as a soluble protein, EngH was expressed as a fused protein with thioredoxin by using the pBAD/Thio vector (Invitrogen), since fusion with thioredoxin is known to help solubilize expressed proteins (23). As a result, EngH was also expressed as a soluble protein. ExgS, EngE, and EngH each possess a C-terminal dockerin domain, which is considered to be responsible for binding with cohesin domains on CbpA (47) (Table 1). When the cellulosomal enzymes from *C. cellulovorans* were expressed by *E. coli*, their dockerin domains were often deleted by *E. coli* protease(s) (26, 39). Thus, ExgS, EngE, and EngH were designed to possess His tags at their C termini, which allowed the isolation of the cellulases that retained their C-terminal dock-

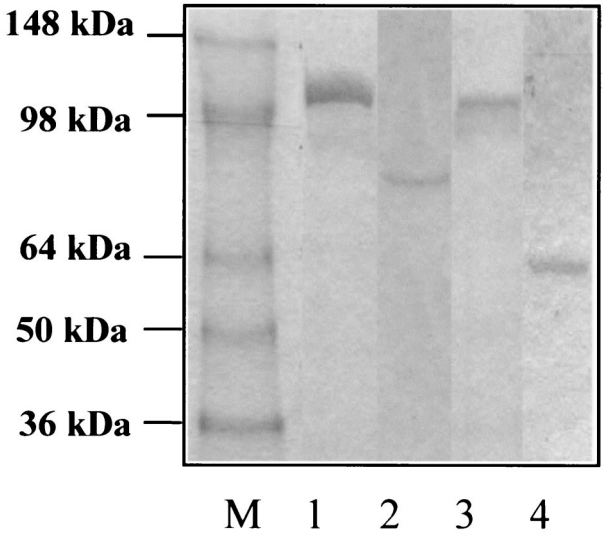


FIG. 1. Coomassie brilliant blue-stained SDS-PAGE gel of the purified cellulases and purified mini-CbpA. Lane M, molecular mass marker; lane 1, EngE; lane 2, ExgS; lane 3, EngH; lane 4, mini-CbpA.

erin domains by Ni-affinity chromatography. By combination of Ni-affinity chromatography and anion-exchange chromatography, ExgS, EngE, and EngH were purified to near homogeneity (Fig. 1). The apparent molecular weights of the purified proteins as determined by SDS-PAGE analysis were in good agreement with their theoretical molecular weights.

**Assembly of recombinant cellulosomes.** To assemble the recombinant cellulosomes between mini-CbpA and cellulosomal cellulases, the purified mini-CbpA and each of the purified cellulosomal cellulases were mixed at a molar ratio of 1 mini-CbpA to 2 cellulosomal cellulases. The molar amount of each subunit was calculated with the protein amount determined by Bradford's method (4). Since the calcium ion is known to be necessary for binding between the cohesin domain and dockerin domain (28), 15 mM of $CaCl_2$ was added to the binding mixture. The assembly of the mini-CbpA and the cellulosomal cellulases was confirmed by native PAGE (4 to 15% linear gradient gel) analysis (Fig. 2A and C). The purified mini-CbpA showed one band (Fig. 2A, lane 5, and C, lane 3). The purified ExgS (Fig. 2A, lane 1) and the purified EngE (Fig. 2A, lane 3) also showed one band, although the band of ExgS was obscure. On the other hand, the purified EngH showed four bands (Fig. 2C, lane 1). The SDS-PAGE analysis indicated that the purified EngH was almost homogeneous as described above. Thus, the four bands on native PAGE of the purified EngH suggested that the purified EngH existed in several forms, such as dimer, trimer, and tetramer. In the mixture of mini-CbpA and cellulosomal cellulases, there were no cellulase bands, and new bands appeared (Fig. 2A, lanes 2 and 4, and C, lane 2). These new bands were considered to be the bands of the recombinant cellulosomes between the mini-CbpA and cellulosomal cellulases. The mixture of mini-CbpA and EngH showed two new bands. Thus, the complex between mini-CbpA and EngH was also considered to exist in at least two forms as well as the purified EngH.

The bands of the purified ExgS and the mixture of mini-CbpA and ExgS were obscure on a 4 to 15% linear gradient gel (Fig. 2A). To further confirm assembly between mini-CbpA and purified ExgS, these samples were analyzed by native PAGE with a different gel (10% gel). As a result (Fig. 2B), both bands were shown to be clearer on the 10% gel than on the 4 to 15% linear gradient gel. In the mixture of mini-CbpA and ExgS (Fig. 2B, lane 2), a new band appeared, suggesting the purified ExgS assembled with the mini-CbpA.

Since mini-CbpA was designed to have two cohesin domains, the mixture could form two types of complexes (one or both cohesin domains of mini-CbpA could be occupied by cellulases). However, the mixtures of ExgS and EngE with mini-CbpA showed only one new band in spite of the existence of a free mini-CbpA band (Fig. 2A, lanes 2 and 4). Kataeva et al. (18) showed that the mixture of cellulosomal cellulase CelD and Cip16 containing two cohesin domains from *C. thermocellum* formed only one type of complex even if an excess amount of Cip16 was present. By sedimentation equilibrium analysis, they concluded that both cohesin domains of the complex were occupied by the CelDs. Based on the observation of Kataeva et al., the mixtures of ExgS and EngE with mini-CbpA may have formed only complexes in which both cohesin domains were occupied.

In the mixtures of mini-CbpA and cellulosomal cellulases at the molar ratio of 1:2, all mini-CbpA should exist as bound forms, since one mini-CbpA could bind two cellulosomal cellulases. However, all the mixtures of mini-CbpA and cellulosomal cellulases at a molar ratio of 1:2 showed a band at the purified mini-CbpA position (Fig. 2A, lanes 2 and 4, and C, lane 2), indicating that an excess amount of unbound mini-CbpA existed in the mixtures. Since protein determination

TABLE 1. Properties of recombinant ExgS, EngE, and EngH

| Protein (modular structure[a]) | Activity[b] (U/μmol) against: | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Crystalline cellulose | | | Amorphous cellulose | | | Soluble cellulose | | |
| | With mini-CbpA | Without mini-CbpA | Synergy by mini-CbpA[c] | With mini-CbpA | Without mini-CbpA | Synergy by mini-CbpA | With mini-CbpA | Without mini-CbpA | Synergy by mini-CbpA |
| ExgS (GH48-Doc) | 0.089 (0.003) | 0.058 (0.001) | 1.53 | 0.595 (0.181) | 0.302 (0.013) | 1.97 | 0.281 (0.001) | 0.287 (0.005) | 0.98 |
| EngE ($SLH_3$-GH5-X-Doc) | 0.054 (0.002) | 0.061 (0.001) | 0.89 | 0.780 (0.260) | 0.661 (0.013) | 1.18 | 1,243 (18.7) | 1,337 (20.9) | 0.93 |
| EngH (GH9-CBDIIIc-Doc) | 0.359 (0.003) | 0.145 (0.008) | 2.48 | 2.857 (0.240) | 0.919 (0.090) | 3.11 | 4.511 (0.122) | 4.131 (0.056) | 1.09 |

[a] GH, Doc, SLH, CBD, and X represent the catalytic domain of glycosyl hydrolase families, dockerin domain, surface layer homologous sequence, cellulose binding domain, and an unknown domain, respectively.

[b] Activity was expressed as units per nanomole of enzymatic subunit, excluding the amount of mini-CbpA added. The numbers in parentheses indicate standard deviations.

[c] The synergy by mini-CbpA was calculated by dividing the activity with mini-CbpA by the activity without mini-CbpA.

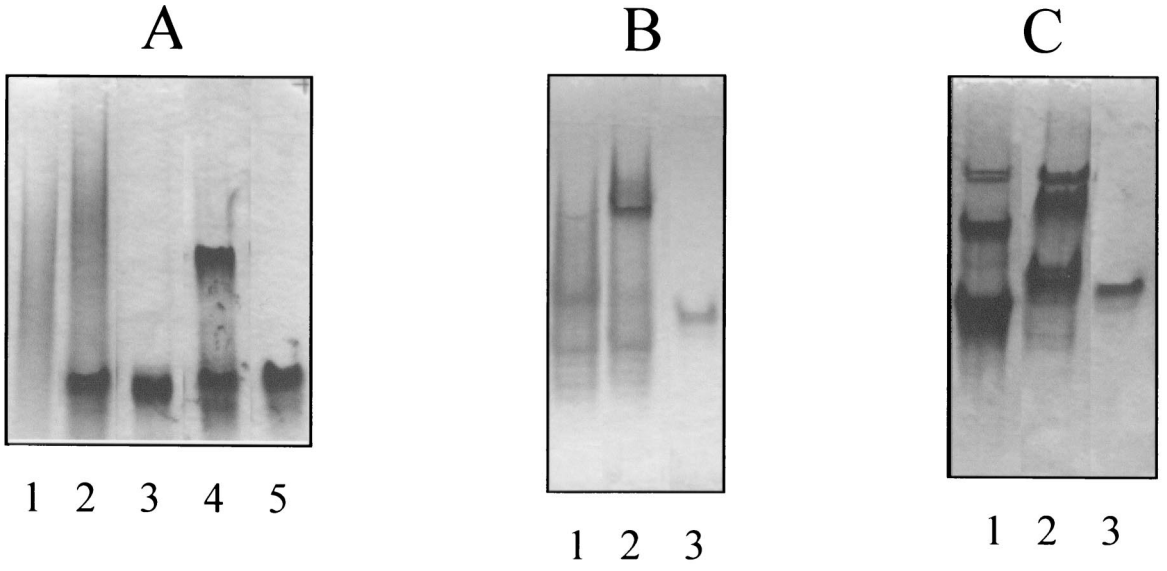


FIG. 2. Native PAGE analysis of the purified cellulosomal cellulases and the purified mini-CbpA complex. (A) Native PAGE analysis of mixtures of ExgS or EngE and mini-CbpA on a 5 to 15% gel. Lane 1, ExgS; lane 2, mixture of ExgS and mini-CbpA (molar ratio, 2:1); lane 3, EngE; lane 4, mixture of EngE and mini-CbpA (molar ratio, 2:1); lane 5, mini-CbpA. (B) Native PAGE analysis of the ExgS-mini-CbpA complex on a 10% gel. Lane 1, ExgS; lane 2, mixture of ExgS and mini-CbpA (molar ratio, 1:1); lane 3, mini-CbpA. (C) Native PAGE analysis of the EngH-mini-CbpA complex on a 5 to 15% gel. Lane 1, EngH; lane 2, mixture of EngH and mini-CbpA (molar ratio, 2:1); lane 3, mini-CbpA.

methods, such as Bradford's method, Lowry's method, and A280, usually give approximate protein amounts, it is very difficult to determine accurate molar amounts of the proteins of interest. In our experiments, the molar amount of mini-CbpA was considered to be more underestimated by Bradford's method than those of the purified cellulosomal cellulases.

Based on the results of native gel analysis, it is likely that most of the purified EngE, ExgS, and EngH successfully bound with mini-CbpA to make recombinant cellulosomes in vitro.

**Specific activities of recombinant cellulosomes of EngE, ExgS, and EngH.** The cellulase activities of the purified cellulosomal cellulases were determined with mini-CbpA (recombinant cellulosomes) or without mini-CbpA (noncomplex forms). The results are shown in Table 1. By binding with mini-CbpA, the activities of ExgS and EngH against insoluble cellulose (crystalline cellulose and amorphous cellulose) increased 1.5- to 3-fold over those without mini-CbpA. On the other hand, the activity of EngE against insoluble cellulose was not changed by binding with mini-CbpA. The activities of all ExgS, EngE, and EngH proteins against soluble cellulose were not affected by the addition of mini-CbpA.

**Synergy effects on crystalline cellulose between recombinant cellulosomes of EngE, ExgS, and EngH.** To determine the synergy effects on activities against crystalline cellulose between the recombinant cellulosome of ExgS, EngE, and EngH, activities against crystalline cellulose were determined with 2-nmol/ml cellulase mixtures with various compositions. The synergy degree (the actual activities divided by the summation of each recombinant cellulosome activity) was calculated.

First, we determined the relationships between two of three recombinant cellulosomes. Figure 3 shows the specific activities and synergy degrees of ExgS-EngE, ExgS-EngH, and EngE-EngH. Among the conditions we tested, the mixture of the recombinant cellulosomes of ExgS and EngH showed the highest specific activity (0.497 U/μmol) at a molar ratio of ExgS to EngH of 50%:50%. Also, the mixtures of the recombinant cellulosomes of ExgS and EngH showed the highest synergy degree (2.70) at a molar ratio of ExgS to EngH of 75%:25%. The mixtures of the recombinant cellulosomes of ExgS and EngE showed specific activities of only 0.122 U/μmol at the most (molar ratio of ExgS to EngE of 50%:50%), and the synergy degree reached 1.79 at the most (molar ratio of ExgS to EngE of 25%:75%). Although the mixtures of the recombinant cellulosomes of EngE and EngH showed comparatively high specific activities (2.76 to 3.46 U/μmol), these activities were lower than that of the recombinant cellulosome of EngH (0.359 U/μmol) (Table 1).

We also determined the relationship between the three recombinant cellulosomes. The specific activity and synergy degree of each mixture were mapped according to the compositions as shown in Fig. 4. The recombinant cellulosome mixtures, which contained more than 12.5% of ExgS and more than 25% of EngH, possessed specific activities of more than 0.4 U/μmol. Among the various compositions, the mixture, which contained 25% ExgS, 25% EngE, and 50% EngH, showed the highest specific activity of 0.543 U/μmol. And the activity of this cellulosomal cellulase composition without mini-CbpA was 0.276 U/μmol. Thus, the mini-CbpA is considered to increase the activity of this cellulosomal cellulase mixture almost twofold. The synergy degrees were inversely proportional to the contents of EngH, indicating that only small amounts of EngH could help ExgS and EngE to degrade crystalline cellulose. The highest synergy degree (4.92) was obtained at the recombinant cellulosome composition, which contained 37.5% ExgS, 50% EngE, and 12.5% EngH.

**Degradation of crystalline cellulose by sequential reaction**

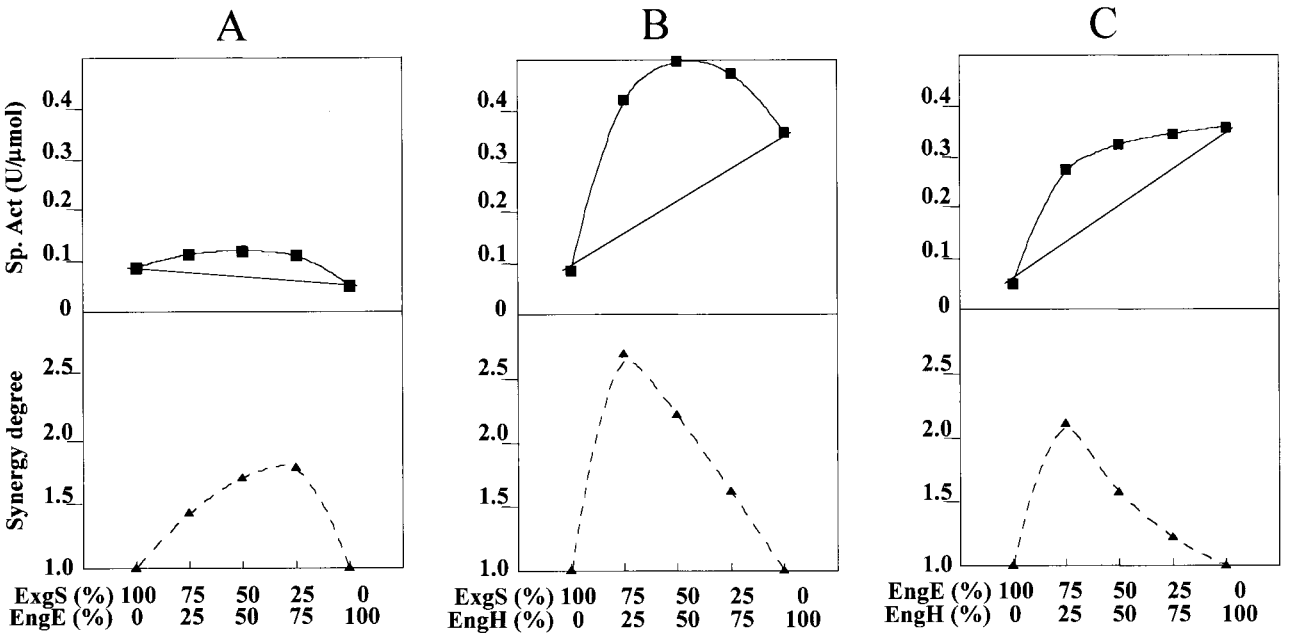


FIG. 3. Specific activities against crystalline cellulose and synergy degrees of recombinant cellulosome mixtures. The specific activities (Sp. Act) are shown in the upper boxes. The synergy degrees are shown in the lower boxes. The synergy degrees are shown as the actual activities divided by the summation of each cellulosome activity shown in Table 1. Two recombinant cellulosomes were mixed at various compositions as shown along the *x* axes. The total concentration of cellulases was fixed at 2 nmol/ml. (A) Mixture of recombinant cellulosomes of ExgS and EngE. (B) Mixture of recombinant cellulosomes of ExgS and EngH. (C) Mixture of recombinant cellulosomes of EngH and EngE.

**with recombinant cellulosomes.** To further determine the synergistic relationship between the recombinant cellulosomes of ExgS, EngE, and EngH, sequential reactions were carried out. As the first reactions, crystalline cellulose was reacted with one of three recombinant cellulosomes for 15 h. Then, the reaction mixtures were boiled for 20 min to inactivate the recombinant cellulosomes, which were used for the first reactions. By this treatment, the recombinant cellulosomes were inactivated completely (data not shown). The heat treatment was considered to have no effect on the amount of liberated reducing sugar from crystalline cellulose, since this treatment did not release any reducing sugars. After the reaction mixtures for the first reaction were boiled, the other two cellulosomes, which were not used for the first reaction, were added to the reaction mixtures and reacted for an additional 15 h. The cellulosome compositions used were 25% ExgS, 25% EngE, and 50% EngH, since this composition showed the highest specific activity as described above. The amount of liberated reducing sugars from the crystalline cellulose was determined and compared with that of simultaneous reactions, in which crystalline cellulose was reacted simultaneously with the three recombinant cellulosomes for 15 h. The synergy degrees were also determined. The results are shown in Table 2. All sequential reactions liberated less reducing sugars than the simultaneous reactions. These results indicated that sequential reactions were not sufficient to obtain the full synergy degree. The sequential reaction, in which the ExgS was used for the first reaction, showed a synergy degree of 1.28. On the other hand, the synergy degree of the sequential reaction, in which EngE or EngH was the cellulase for the first reactions, was still 1.95 or 1.73, respectively. These results indicated that almost no synergy effect on cellulose degradation was obtained by the treatment of crystalline cellulose by ExgS before the reaction with EngE and EngH; on the other hand, the treatment with EngE or EngH before ExgS treatment gave synergy effects.

## DISCUSSION

To analyze the synergistic effect between cellulosomal cellulases, three recombinant cellulosomes were assembled by use of three cellulosomal cellulases, EngE (glycosyl family 5) (40), EngH (glycosyl family 9) (41), and ExgS (glycosyl family 48) (24), and mini-CbpA (26). All of these components were expressed successfully by *E. coli*. Synergistic effects on crystalline cellulose degradation were observed between all three recombinant cellulosomes. These results suggest that EngE, EngH, and ExgS have different roles for crystalline cellulose degradation. A broad range of recombinant cellulosome compositions retained relatively high activity against crystalline cellulose, as shown in Fig. 4A. These results implied that synergy effects between three different types of cellulases could allow *C. cellulovorans* to vary the cellulosomal subunit composition while retaining high activity against crystalline cellulose. Therefore, possessing different types of cellulases in a complex might be an advantage for anaerobic microorganisms that assemble cellulosomes.

In nature, cellulosomal cellulases should degrade cellulose in plant cell walls in a simultaneous manner rather than in a sequential manner, since nine cellulosomal enzymatic subunits are present in the complex with the scaffolding protein. The simultaneous reaction with recombinant cellulosome mixtures showed higher crystalline cellulose degrading activity than the sequential reactions, as shown in Table 2. These results implied that cellulose degradation in such a simultaneous manner

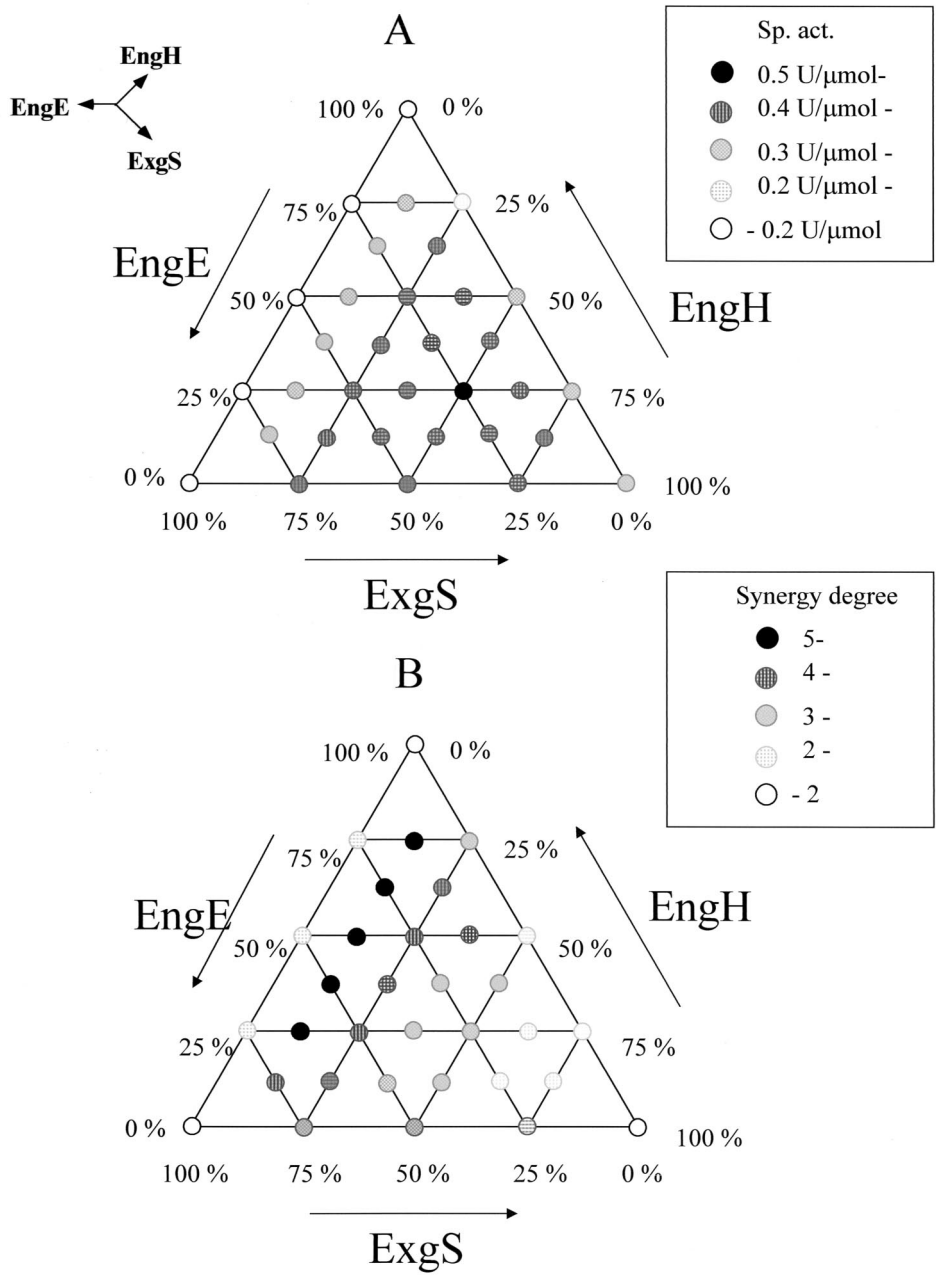


FIG. 4. Specific activities against crystalline cellulose (A) and synergy degrees (B) of recombinant cellulosome mixtures. The specific activities (Sp. act.) and the synergy degrees are indicated as the relative density of the circles, as shown on the figure. Three recombinant cellulosomes were mixed at various compositions. The content of each recombinant cellulosome is shown in the axis as a molar percentage, and the arrows indicate the directions of axis for each cellulosomal cellulase concentration. The synergy degrees are shown as the actual activities divided by the theoretical activities (sum of each cellulosome activity, as shown in Table 1).

might also be one of the advantages of assembling cellulosomes for the efficient degradation of cellulose, as suggested by Shoham et al. (35).

The mixture of ExgS (glycosyl hydrolase family 48) and EngH (glycosyl hydrolase family 9) showed much higher specific activity and synergy degrees than the other mixtures. Therefore, the synergistic effect between ExgS and EngH on the degradation of crystalline cellulose can be considered to have the most dominant effect on the degradation of crystalline cellulose by the recombinant cellulosome mixtures of EngE, EngH, and ExgS. The cellulosomal cellulases, which belong to glycosyl family 48, are considered to be typical endo-processive cellulases by both biochemical (21, 24, 32) and structural analyses (30, 31). On the other hand, the enzymes belonging to glycosyl hydrolase family 9 were proposed to be endoglucanases, although they could also degrade crystalline cellulose, as well as soluble cellulose, efficiently (10). In the sequential reactions for which data are shown in Table 2, almost no synergistic effect was obtained when ExgS was used for the first reaction followed by the EngH reaction. In contrast, when

TABLE 2. Sequential and simultaneous reactions against crystalline cellulose by recombinant cellulosomes of ExgS, EngE, and EngH

| Reaction[a] | Amt of liberated reducing sugar (μmol/ml)[b] | Synergy degree[c] |
|---|---|---|
| ExgS + EngE + EngH, 15h | 490.3 (3.9) | 2.76 |
| ExgS, 15h → Boiling → EngE + EngH, 15h | 227.6 (4.9) | 1.28 |
| EngE, 15h → Boiling → ExgS + EngH, 15h | 345.8 (10.9) | 1.95 |
| EngH, 15h → Boiling → ExgS + EngE, 15h | 306.6 (2.2) | 1.73 |

[a] The amounts of enzyme in the reaction mixtures were 0.5 nmol of ExgS/ml, 0.5 nmol of EngE/ml, and 1.0 nmol of EngH/ml. All enzymatic subunits were mixed with mini-CbpA (50% molar amount of each enzymatic subunit) before starting the reactions. The first reaction listed is the simultaneous reaction; the other three-reactions are sequential reactions.

[b] The numbers in parentheses indicate standard deviations.

[c] The synergy degrees were calculated by dividing the actual activities by the sum of the individual activities (with mini-CbpA) of ExgS (for 0.5 nmol/ml), EngE(for 0.5 nmol/ml), and EngH (for 1.0 nmol/ml), as shown in Table 1.

EngH was used for the first reaction, significant synergy effects were obtained. Based on the cellulolytic properties of glycosyl hydrolase families 9 and 48 and the results of the sequential reactions, a possible explanation for the synergistic effect between EngH and ExgS is that the EngH initially nicked the cellulose chains by endo-type reactions, then ExgS degraded the nicked cellulose chain in an endo-processive manner.

Although EngE is considered one of the main components of cellulosomes of *C. cellulovorans* (25, 27), the results of this study indicated that the contributions of EngE to crystalline cellulose degradation were limited. Recently, we found that the hydrophilic domains of EngE were responsible for binding the cellulosomes to the cell wall of *C. cellulovorans* (19). Thus, the main role of EngE on the cellulosome might be to connect cellulosomes to the cell wall. Moreover, degradation of soluble cellulose might also be a major role for EngE, since the specific activity of EngE against soluble cellulose was much higher than those of ExgS and EngH.

By assembling with mini-CbpA, the activities of EngH and ExgS against insoluble cellulose increased significantly, although activities against soluble cellulose were not changed. These results indicated that mini-CbpA could help ExgS and EngH degrade insoluble cellulose. By binding with scaffolding proteins, cellulosomal enzymes are considered to obtain binding ability for insoluble cellulose through the cellulose binding domain of the scaffolding protein (11, 20, 26). Thus, enhanced cellulose binding ability might increase the activity of ExgS and EngH against insoluble cellulose. The cellulose binding domain of *Cellulomonas fimi* is known to disrupt insoluble cellulose by itself (7, 8). Therefore, it is also possible that mini-CbpA could help disrupt insoluble cellulose. In this context, the large excess of free mini-CbpA in the reaction mixtures could also enhance the activities of ExgS and EngH against insoluble cellulose, as reported by Pages et al. (29).

Among the recombinant cellulosome mixtures determined in this study, the mixture containing 25% EngE, 50% EngH, and 25% ExgS showed the highest specific activity against crystalline cellulose. Conidering the specific activity of the partially purified cellulosome fractions from *C. cellulovorans* culture supernatant determined recently (27), the specific activity of this recombinant cellulosome mixture was still about five times lower than that of purified cellulosome fractions from culture supernatants. The whole scaffolding protein CbpA of *C. cellulovorans* possesses one cellulose binding domain, four hydrophilic domains, and nine cohesin domains (37). However, because of the difficulty in expressing the whole CbpA in *E. coli*, we used a mini-CbpA, which contained one cellulose binding domain, one hydrophilic domain, and two cohesin domains. Garcia-Campaya and Beguin reported that the activity against crystalline cellulose of endoglucanase CelD increased 10-fold by binding with the whole scaffolding protein CipA from *C. thermocellum* (11). The mini-CbpA used in this study increased the activity of the recombinant cellulosome mixture only twofold. Therefore, assembly with the whole CbpA might increase the activity of recombinant cellulosomes to levels comparable to those of native cellulosomes. Also, optimization of the alignment of the enzymatic subunits on CbpA might increase the activity of recombinant cellulosomes.

One of our ultimate goals is the preparation of designer cellulosomes, which could degrade cellulose efficiently for industrial purposes. The results obtained in this study have provided some hints for designing the enzymatic subunit composition for efficient cellulose degradation, although we need further studies to increase the cellulolytic activity of recombinant cellulosomes as discussed above.

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## REFERENCES

1. **Bayer, E. A., L. J. Shimon, Y. Shoham, and R. Lamed.** 1998. Cellulosome structure and ultrastructure. J. Struct. Biol. **124:**221–234.
2. **Boisset, C., H. Chanzy, B. Henrissat, R. Lamed, Y. Shoham, and E. A. Bayer.** 1999. Digestion of crystalline cellulose substrates by the *Clostridium thermocellum* cellulosome: structural and morphological aspects. Biochem. J. **340:** 829–835.
3. **Boisset, C., C. Petrequin, H. Chanzy, B. Henrissat, and M. Schulein.** 2001. Optimized mixtures of recombinant *Humicola insolens* cellulases for the biodegradation of crystalline cellulose. Biotechnol. Bioeng. **72:**339–345.
4. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
5. **Carrard, G., A. Koivula, H. Soderlund, and P. Beguin.** 2000. Cellulose-binding domains promote hydrolysis of different sites on crystalline cellulose. Proc. Natl. Acad. Sci. USA **97:**10342–10347.
6. **Choi, S. K., and L. G. Ljungdahl.** 1996. Dissociation of the cellulosome of *Clostridium thermocellum* in the presence of ethylenediaminetetraacetic acid occurs with the formation of truncated polypeptides. Biochemistry **35:**4897–4905.
7. **Din, N., H. G. Damude, N. R. Gilkes, R. C. Miller, Jr., R. A. Warren, and D. G. Kilburn.** 1994. C1-Cx revisited: intramolecular synergism in a cellulase. Proc. Natl. Acad. Sci. USA **91:**11383–11387.
8. **Din, N., N. R. Gilkes, B. Tekant, R. C. Miller, R. A. Warren, and D. G. Kilburn.** 1991. Non-hydrolytic disruption of cellulose fibers by the binding domain of a bacterial cellulase. Bio/Technology **9:**1096–1099.
9. **Fierobe, H. P., A. Mechaly, C. Tardif, A. Belaich, R. Lamed, Y. Shoham, J. P. Belaich, and E. A. Bayer.** 2001. Design and production of active cellulosome chimeras. Selective incorporation of dockerin-containing enzymes into defined functional complexes. J. Biol. Chem. **276:**21257–21261.
10. **Gal, L., C. Gaudin, A. Belaich, S. Pages, C. Tardif, and J. P. Belaich.** 1997. CelG from *Clostridium cellulolyticum*: a multidomain endoglucanase acting efficiently on crystalline cellulose. J. Bacteriol. **179:**6595–6601.
11. **Garcia-Campayo, V., and P. Beguin.** 1997. Synergism between the cellulosome-integrating protein CipA and endoglucanase CelD of *Clostridium thermocellum*. J. Biotechnol **57:**39–47.
12. **Henrissat, B., and A. Bairoch.** 1993. New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. Biochem. J. **293:**781–788.
13. **Henrissat, B., and A. Bairoch.** 1996. Updating the sequence-based classification of glycosyl hydrolases. Biochem. J. **316:**695–706.
14. **Henrissat, B., M. Claeyssens, P. Tomme, L. Lemesle, and J. P. Mornon.**

1989. Cellulase families revealed by hydrophobic cluster analysis. Gene **81:** 83–95.
15. **Henrissat, B., H. Driguez, C. Viet, and M. Schulein.** 1985. Synergism of cellulases from *Trichoderma reesei* in the degradation of cellulose. Bio/Technology **3:**722–726.
16. **Ichiishi, A., S. Sheweita, and R. H. Doi.** 1998. Characterization of EngF from *Clostridium cellulovorans* and identification of a novel cellulose binding domain. Appl. Environ. Microbiol. **64:**1086–1090.
17. **Johnson, E. A., M. Sakajoh, G. Halliwell, A. Madia, and A. L. Demain.** 1982. Saccharification of complex cellulosic substrates by the cellulase system from *Clostridium thermocellum*. Appl. Environ. Microbiol. **43:**1125–1132.
18. **Kataeva, I. A., G. Guglielmi, and P. Beguin.** 1997. Interaction between *Clostridium thermocellum* endoglucanase CelD and polypeptides derived from the cellulosome-integrating protein CipA: stoichiometry and cellulolytic activity of the complexes. Biochem. J. **326:**617–624.
19. **Kosugi, A., K. Murashima, and R. H. Doi.** 2002. Cell surface anchoring role of N-terminal surface layer homology domains of *Clostridium cellulovorans* EngE. J. Bacteriol. **184:**884–888.
20. **Kruus, K., A. C. Lua, A. L. Demain, and J. H. Wu.** 1995. The anchorage function of CipA (CelL), a scaffolding protein of the *Clostridium thermocellum* cellulosome. Proc. Natl. Acad. Sci. USA **92:**9254–9258.
21. **Kruus, K., W. K. Wang, J. Ching, and J. H. Wu.** 1995. Exoglucanase activities of the recombinant *Clostridium thermocellum* CelS, a major cellulosome component. J. Bacteriol. **177:**1641–1644.
22. **Lamed, R., and E. A. Bayer.** 1988. The cellulosome of *Clostridium thermocellum*. Adv. Appl. Microbiol. **33:**1–46.
23. **Lavallie, E. R., Z. Lu, E. A. Diblasio-Smith, L. A. Collins-Racie, and J. M. Mccoy.** 2000. Thioredoxin as a fusion partner for production of soluble recombinant proteins in *Escherichia coli*. Methods Enzymol. **326:**322–340.
24. **Liu, C. C., and R. H. Doi.** 1998. Properties of *exgS*, a gene for a major subunit of the *Clostridium cellulovorans* cellulosome. Gene **211:**39–47.
25. **Matano, Y., J. S. Park, M. A. Goldstein, and R. H. Doi.** 1994. Cellulose promotes extracellular assembly of *Clostridium cellulovorans* cellulosomes. J. Bacteriol. **176:**6952–6956.
26. **Murashima, K., C. L. Chen, A. Kosugi, Y. Tamaru, R. H. Doi, and S. L. Wong.** 2002. Heterologous production of *Clostridium cellulovorans engB*, using protease-deficient *Bacillus subtilis*, and preparation of active recombinant cellulosomes. J. Bacteriol. **184:**76–81.
27. **Murashima, K., A. Kosugi, and R. H. Doi.** 2002. Determination of subunit composition of *Clostridium cellulovorans* cellulosomes that degrade plant cell walls. Appl. Environ. Microbiol. **68:**1610–1615.
28. **Pages, S., A. Belaich, J. P. Belaich, E. Morag, R. Lamed, Y. Shoham, and E. A. Bayer.** 1997. Species-specificity of the cohesin-dockerin interaction between *Clostridium thermocellum* and *Clostridium cellulolyticum*: prediction of specificity determinants of the dockerin domain. Proteins **29:**517–527.
29. **Pages, S., L. Gal, A. Belaich, C. Gaudin, C. Tardif, and J. P. Belaich.** 1997. Role of scaffolding protein CipC of *Clostridium cellulolyticum* in cellulose degradation. J. Bacteriol. **179:**2810–2816.
30. **Parsiegla, G., M. Juy, C. Reverbel-Leroy, C. Tardif, J. P. Belaich, H. Driguez, and R. Haser.** 1998. The crystal structure of the processive endocellulase CelF of *Clostridium cellulolyticum* in complex with a thiooligosaccharide inhibitor at 2.0 A resolution. EMBO J. **17:**5551–5562.
31. **Parsiegla, G., C. Reverbel-Leroy, C. Tardif, J. P. Belaich, H. Driguez, and R. Haser.** 2000. Crystal structures of the cellulase Cel48F in complex with inhibitors and substrates give insights into its processive action. Biochemistry **39:**11238–11246.
32. **Reverbel-Leroy, C., S. Pages, A. Belaich, J. P. Belaich, and C. Tardif.** 1997. The processive endocellulase CelF, a major component of the *Clostridium cellulolyticum* cellulosome: purification and characterization of the recombinant form. J. Bacteriol. **179:**46–52.
33. **Riedel, K., and K. Bronnenmeier.** 1998. Intramolecular synergism in an engineered exo-endo-1,4-beta-glucanase fusion protein. Mol. Microbiol. **28:** 767–775.
34. **Schwarz, W. H.** 2001. The cellulosome and cellulose degradation by anaerobic bacteria. Appl. Microbiol. Biotechnol. **56:**634–649.
35. **Shoham, Y., R. Lamed, and E. A. Bayer.** 1999. The cellulosome concept as an efficient microbial strategy for the degradation of insoluble polysaccharides. Trends Microbiol. **7:**275–281.
36. **Shoseyov, O., and R. H. Doi.** 1990. Essential 170-kDa subunit for degradation of crystalline cellulose by *Clostridium cellulovorans* cellulase. Proc. Natl. Acad. Sci. USA **87:**2192–2195.
37. **Shoseyov, O., M. Takagi, M. A. Goldstein, and R. H. Doi.** 1992. Primary sequence analysis of *Clostridium cellulovorans* cellulose binding protein A (CbpA). Proc. Natl. Acad. Sci. USA **89:**3483–3487.
38. **Sleat, R., R. A. Mah, and R. Robinson.** 1984. Isolation and characterization of an anaerobic, cellulolytic bacterium, *Clostridium cellulovorans* sp. nov. Appl. Environ. Microbiol. **48:**88–93.
39. **Tamaru, Y., and R. H. Doi.** 2001. Pectate lyase A, an enzymatic subunit of the *Clostridium cellulovorans* cellulosome. Proc. Natl. Acad. Sci. USA **98:** 4125–4129.
40. **Tamaru, Y., and R. H. Doi.** 1999. Three surface layer homology domains at the N terminus of the *Clostridium cellulovorans* major cellulosomal subunit EngE. J. Bacteriol. **181:**3270–3276.
41. **Tamaru, Y., S. Karita, A. Ibrahim, H. Chan, and R. H. Doi.** 2000. A large gene cluster for the *Clostridium cellulovorans* cellulosome. J. Bacteriol. **182:** 5906–5910.
42. **Teeri, T. T.** 1997. Crystalline cellulose degradation: new insight into the function of cellobiohydrolases. Trends Biotechnol. **15:**160–167.
43. **Tokatlidis, K., P. Dhurjati, and P. Beguin.** 1993. Properties conferred on *Clostridium thermocellum* endoglucanase CelC by grafting the duplicated segment of endoglucanase CelD. Protein Eng. **6:**947–952.
44. **Tomme, P., R. A. Warren, and N. R. Gilkes.** 1995. Cellulose hydrolysis by bacteria and fungi. Adv. Microb. Physiol. **37:**1–81.
45. **Wood, W. A., and K. M. Bhat.** 1988. Method for measuring cellulase activity. Methods Enzymol. **160:**87–112.
46. **Wu, J. H., W. H. Orme-Johnson, and A. L. Demain.** 1988. Two components of an extracellular protein aggregate of *Clostridium thermocellum* together degrade crystalline cellulose. Biochemistry **27:**1703–1709.
47. **Yaron, S., E. Morag, E. A. Bayer, R. Lamed, and Y. Shoham.** 1995. Expression, purification and subunit-binding properties of cohesins 2 and 3 of the *Clostridium thermocellum* cellulosome. FEBS Lett. **360:**121–124.