

FIG. 1. Molecular architecture of HbpA, miniCbpA1, EngB, and EngL. SHL, SLH domain; Cat., catalytic domain; CBM, carbohydrate binding module.

Assembly of HbpA or miniCbpA1 and cellulosomal endoglucanase. The purified HbpA-His or miniCbpA1-His and the recombinant cellulosomal subunits (equal molar) were mixed in 15 μ l of binding buffer (25 mM sodium acetate buffer [pH 6.0], 10 mM CaCl₂) and kept for 1 h at 4°C. The assembly of HbpA-His6 and the cellulosomal subunit was confirmed by native polyacrylamide gel electrophoresis (PAGE) as described below.

SDS-PAGE. Sodium dodecyl sulfate (SDS)-PAGE was performed with 10% polyacrylamide by the method of Laemmli (10). Proteins were visualized by Bio-Safe Coomassie staining (Bio-Rad).

Native PAGE. Native PAGE was performed with 4 to 15% polyacrylamide (Bio-Rad).

Enzyme assays. Enzyme activity was assayed in the presence of a 0.5% (wt/vol) concentration of each substrate at 37°C in 50 mM sodium phosphate buffer (pH 7.0). Enzyme concentrations were 10 nM (HbpA-His), 5 nM (EngB-His), and 4.25 nM (EngL-His). To investigate synergistic effects, HbpA-His was added to 0, 5, 10, 20, and 50 nM for EngB-His or 0, 4.25, 8.5, 17, and 42.5 nM for EngL-His. Reaction samples (500 μ l) were collected at appropriate times and immediately mixed with 500 μ l of chilled 0.38 M sodium carbonate containing 1.8 mM cupric sulfate and 0.2 M glycine. The reducing sugars were determined by reductometry by the Dygert method (5). Incubations with acid-swollen cellulose (ASC), Avicel, and corn fiber were for 6 h, 12 h, and 24 h, respectively. All assays were repeated three times.

Interaction of HbpA-His with cell wall fragments of *C. cellulovorans*. Preparation of cell wall fragments of *C. cellulovorans* followed the method of Kosugi et al. (8). Binding experiments were carried out by incubating and cosedimenting each recombinant protein with cell wall fragments. One microgram of HbpA-His was mixed with 50 μ l of cell wall fraction, and reaction volume was increased to 200 μ l with 50 mM sodium phosphate buffer (pH 7.0). The reaction mixtures were incubated for 1 h at 37°C with shaking. The bound and free proteins were separated by centrifugation at 16,000 \times g for 20 min at room temperature. The supernatant consisted of the soluble fraction. A wash fraction was obtained after washing the cell wall with 50 mM sodium phosphate buffer (pH 7.0). The pellet, consisting of the insoluble cell wall fragments and attached proteins, was washed with the same buffer and then resuspended with 200 μ l of the same buffer. Each fraction was analyzed by SDS-PAGE and then Western blotting, as described below.

Affinity of HbpA for insoluble polysaccharides. One microgram of HbpA-His was incubated for 1 h at 37°C with shaking in 300 μ l of 50 mM sodium phosphate buffer (pH 7.0) containing Avicel, chitin (Sigma), and oat-spelled xylan (Sigma) at a concentration of 25 mg/ml as insoluble polysaccharides. Free HbpA-His was separated by centrifugation at 16,000 \times g for 5 min at room temperature. The supernatants were labeled as supernatant fractions. The pellets were suspended in 300 μ l of 50 mM sodium phosphate buffer (pH 7.0) and labeled as precipitate fractions. Each fraction was analyzed by SDS-PAGE and then Western blotting as described below.

Western blotting. Western blotting was performed by using anti-His antibody conjugated with horseradish peroxidase (HRP) (Invitrogen). After electrophoresis, the gel was soaked in transfer buffer (25 mM Tris, 192 mM glycine, 10% methanol). Proteins in the gel were transferred onto a polyvinylidene difluoride membrane (Millipore) using the semidry blotting method and then neutralized with phosphate saline buffer (PBS). Skim milk (1%) in PBS was used for blocking at room temperature for 1 h, and then anti-His antibody conjugated with HRP

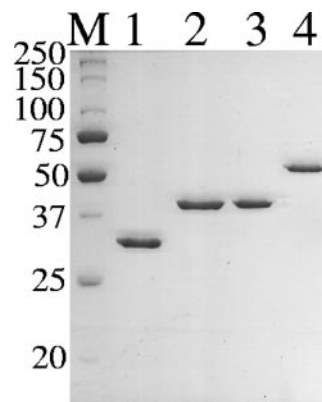


FIG. 2. Coomassie blue staining of SDS-PAGE gel of purified HbpA-His, miniCbpA1-His, EngB-His, and EngL-His. Each lane contained 1 μ g of protein. Lane M, molecular mass markers; lane 1, HbpA-His; lane 2, miniCbpA1-His; lane 3, EngB-His; lane 4, EngL-His in a 10% polyacrylamide gel. The positions of molecular mass markers (in kilodaltons) are indicated on the left.

was added (1:5,000), and the mixture was incubated at room temperature for 2 h. The membrane was washed with PBS for 5 min three times. To detect the signal, ECL-plus (Amersham) was used. The signals were exposed onto X-ray film (Fuji film) for appropriate times.

RESULTS AND DISCUSSION

Comparison of domains of HbpA with CbpA and EngE.

Since the HbpA sequence revealed domains that were similar to those of surface layer proteins, a study comparing of the SLH domains in HbpA, CbpA, and EngE and comparing the cohesin domain of HbpA with those of CbpA was done. The SLH domain in HbpA had 16.7% to 19.7% similarity to the SLH domains in CbpA and EngE. SLH domains within CbpA (about 48%) and EngE (from 50 to 70%) have higher similarity to each other than to the SLH domain in HbpA. The SLH domains in CbpA (7) and EngE (18) are able to bind to the cell surface and have been implicated in the binding of the cellulosome to the cell surface.

Since cohesins are involved in binding cellulosomal enzymes through their dockerin domains to form cohesin-dockerin interactions, we compared the cohesin sequence of HbpA to those of the nine cohesins present in CbpA. The cohesin domain in HbpA had various degrees of similarity to the cohesin domains in CbpA, from 26.9% to 32.1%. As with the SLH domain, cohesins in CbpA are very similar (about 80%) except for cohesin 9, but the cohesin of HbpA is very different from the cohesins in CbpA. From these comparisons, the origin of the SLH and cohesin domains of HbpA appears to be different from the origin of those of CbpA and EngE. These results also suggest that the properties of the HbpA domains might be different from those of the CbpA and EngE domains.

Construction of plasmids for expression of *C. cellulovorans* genes in *B. subtilis* WB800. In order to test whether the cohesin domain of HbpA was able to bind cellulosomal enzymes, we constructed expression plasmids for HbpA, miniCbpA1, and the cellulosomal enzymes EngB and EngL (see Materials and Methods) (Fig. 1). The vector pWB1000 is an *E. coli*-*B. subtilis* shuttle vector containing a P43 promoter, a *sacB* sequence encoding a signal peptide, a multiple cloning site, an ampicillin

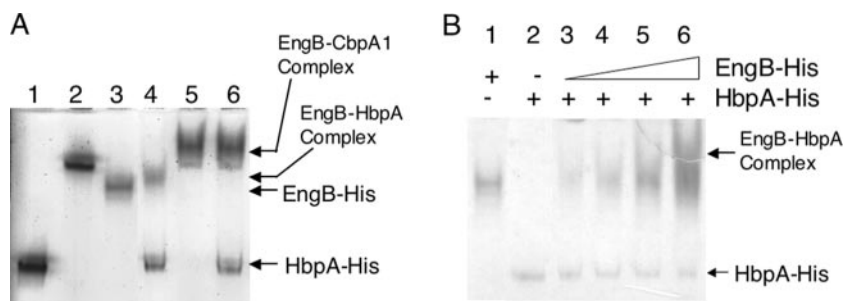


FIG. 3. Complex formation between HbpA-His and cellulosomal cellulases analyzed by native PAGE. (A) Lane 1, HbpA-His; lane 2, miniCbpA1-His; lane 3, EngB-His; lane 4, EngB-His and HbpA-His; lane 5, miniCbpA1-His and EngB-His; lane 6, miniCbpA1-His, HbpA-His, and EngB-His. Each protein was present at 25 pmol. (B) Lane 1, EngB-His (25 pmol); lane 2, HbpA-His (25 pmol); lane 3, 1:1 EngB-His–HbpA-His (25 pmol each); lane 4, 2:1 EngB-His–HbpA-His (50 pmol of EngB-His–25 pmol HbpA-His); lane 5, 4:1 EngB-His–HbpA-His (100 pmol EngB-His–25 pmol HbpA-His); lane 6, 10:1 EngB-His–HbpA-His (250 pmol EngB-His–25 pmol HbpA-His).

resistance marker from *E. coli*, and a kanamycin resistance marker from *B. subtilis*. HbpA-His was expressed with pWB1000. The His tag was added for ease of purification of the expressed protein.

The vector pDG148 is also a shuttle vector containing a SalI-SphI cloning site and an IPTG-inducible promoter. *minicbpA1*, *engB*, and *engL* were cloned into pDG148; their products had their original Shine-Dalgarno and signal peptide sequences and His₆ tags at their C termini. Expression of the *C. cellulovorans* genes had no effect on the growth rate of *B. subtilis* (data not shown). The *C. cellulovorans* genes were expressed, and their products were secreted successfully by *B. subtilis* (Fig. 2). These results indicated that the Shine-Dalgarno and signal peptide sequences from *C. cellulovorans* also worked in *B. subtilis* and that the secreted proteins were folded into active forms.

Interaction between HbpA-His and cellulosomal cellulases and their synergistic effect. To demonstrate interaction between HbpA-His and EngB-His, we mixed the proteins in binding buffer and then performed native PAGE to look for the formation of an HbpA-EngB complex. miniCbpA1-His was also used as a binding competitor of HbpA-His. In native gel, each protein formed a single band, except that the EngL-His band was very diffuse (data not shown). As shown in Fig. 3A,

lane 4, Eng B formed a complex with HbpA in vitro. In Fig. 3A, lane 5, EngB also formed a complex with miniCbpA1 as expected. However, the complex which consisted of HbpA-His and EngB disappeared when miniCbpA1-His was added to the reaction (Fig. 3A, lane 6). In Fig. 3B, mixtures of EngB-His and HbpA-His were analyzed for formation of the EngB-His–HbpA-His complex. These results indicate that the cohesin domain of HbpA has the ability to bind to the dockerin domain of EngB but that the binding is weaker than that with the cohesin in miniCbpA1-His. These properties are similar to those of OrfXp (14).

Synergistic effects between CbpA and cellulosomal cellulases and hemicellulases were observed (9, 12, 13). For the purpose of determining enzymatic activity and any synergism between HbpA and the cellulosomal enzymes, we measured the glucanase activity of the complexes against ASC, Avicel, and corn fiber. There was no apparent synergistic effect when endoglucanases and HbpA-His were present at a 1:1 molar ratio, but, at a higher ratio of HbpA-His to enzymes, the glucanase activity increased (Table 1). The synergistic activities of HbpA and EngB or EngL reached 1.58- and 1.25-fold for ASC, 2.19- and 1.74-fold for Avicel, and 1.83- and 2.40-fold for corn fiber when 10× HbpA-His was added in the reaction solution. HbpA and EngL had higher activity towards cellulose

TABLE 1. Activity of cellulases as a function of HbpA concentration^a

| Substrate | Enzyme | Free enzyme concn (μM) | 1× HbpA-His | | 2× HbpA-His | | 4× HbpA-His | | 10× HbpA-His | |
|------------|----------|------------------------|-----------------------|-----------------|-------------|------|-------------|------|--------------|------|
| | | | Amt ^b (μM) | SF ^c | Amt (μM) | SF | Amt (μM) | SF | Amt (μM) | SF |
| ASC | HbpA-His | ND ^d | | | | | | | | |
| | EngB-His | 25.7 ± 2.8 | 26.1 ± 5.7 | 1.01 | 28.8 ± 2.2 | 1.12 | 31.4 ± 1.5 | 1.22 | 40.7 ± 2.5 | 1.58 |
| | EngL-His | 56.4 ± 4.5 | 57.3 ± 4.7 | 1.02 | 59.1 ± 3.4 | 1.05 | 61.9 ± 3.1 | 1.10 | 70.3 ± 2.6 | 1.25 |
| Avicel | HbpA-His | ND | | | | | | | | |
| | EngB-His | 5.4 ± 1.1 | 5.1 ± 0.5 | 0.95 | 6.2 ± 0.5 | 1.15 | 8.2 ± 1.6 | 1.53 | 11.8 ± 0.5 | 2.19 |
| | EngL-His | 7.9 ± 1.7 | 8.4 ± 1.4 | 1.06 | 8.7 ± 0.7 | 1.10 | 9.9 ± 0.9 | 1.25 | 13.7 ± 1.3 | 1.74 |
| Corn fiber | HbpA-His | ND | | | | | | | | |
| | EngB-His | 20.1 ± 4.1 | 20.3 ± 1.1 | 1.01 | 19.0 ± 4.7 | 0.95 | 28.8 ± 3.7 | 1.43 | 36.9 ± 7.4 | 1.83 |
| | EngL-His | 16.7 ± 7.3 | 24.4 ± 7.8 | 1.46 | 23.7 ± 6.5 | 1.42 | 34.4 ± 1.7 | 2.05 | 40.2 ± 0.2 | 2.40 |

^a See Materials and Methods for assay conditions.

^b Amount of reducing sugar produced from ASC in 6 h, from Avicel in 12 h, and from corn fiber in 24 h.

^c SF, synergy factor.

^d ND, not detected.

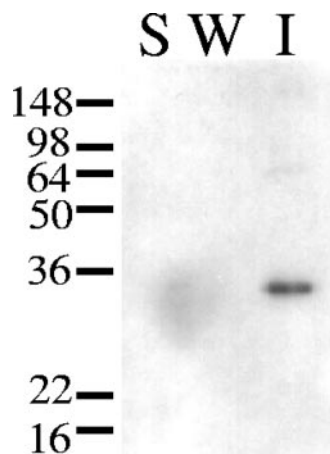


FIG. 4. Interaction between HbpA-His and the *C. cellulovorans* cell wall fraction. The materials were loaded on 10% polyacrylamide-SDS gels and blotted onto a membrane. Shown is the binding of HbpA-His to the cell wall fraction as determined by immunoblotting with anti-His antibody. Lane S, soluble fraction; lane W, wash fraction; lane I, insoluble fraction. The positions of molecular mass markers (in kilodaltons) are indicated on the left.

and corn fiber than HbpA and EngB. These results indicated not only that HbpA was capable of binding cellulosomal cellulases but also that formation of the complex resulted in higher activity of the bound enzymes. No synergy was observed when carboxymethyl cellulose was used as the substrate (data not shown).

Analysis of binding ability of HbpA-His for *C. cellulovorans* cell wall fragments. Kosugi et al. showed that SLH domains of CbpA and EngE had the ability to attach these proteins to the cell wall of *C. cellulovorans* (7, 8). HbpA has also an SLH domain, but its amino acid sequence has low similarity to those of EngE. Therefore we investigated whether HbpA has the ability to bind to the cell wall of *C. cellulovorans*. As shown in Fig. 4, HbpA did bind to the cell wall fragments of *C. cellulovorans*. In spite of the low similarity of the amino acid sequences of the SLH domains of HbpA and EngE, the results indicate that the SLH domain of HbpA plays a role similar to that of the SLH domains of EngE, i.e., to bind the protein to *C. cellulovorans* cell walls.

Binding of HbpA-His to several polysaccharides. Table 1 showed that HbpA might have the ability to bind polysaccharides. To prove that hypothesis, we examined whether HbpA-His could bind to cellulose, chitin, and xylan. As shown in Fig. 5, HbpA-His did bind to those polysaccharides. From these results, HbpA could bind not only to the cell wall of *C. cellulovorans* but also to polysaccharides such as cellulose, chitin, and xylan. Thus, HbpA can bind not only to cellulosomal enzymes but also to the cell wall and to the substrate. These properties are very similar to those attributed to CbpA (7).

In *C. cellulolyticum*, the production of ORFXp is induced by the presence of cellulose (14). The authors suggested that ORFXp had an important role in cellulose degradation or in facilitating cellulosome assembly in the vicinity of the membrane. In *C. cellulovorans*, *hbpA* is located in a large cellulosomal gene cluster and seems to be in an operon with *engL*

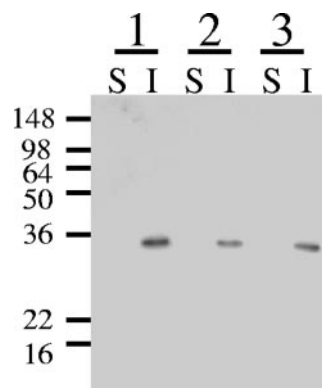


FIG. 5. Binding of HbpA-His to cellulose, chitin, and xylan. Purified HbpA-His was incubated with cellulose, chitin, and xylan. After centrifugation, proteins in supernatant (lane S) and in the precipitate (lane I) were loaded on 10% polyacrylamide-SDS gels and blotted onto a membrane. The binding of HbpA-His to the polysaccharides was determined by immunoblotting with anti-His antibody. Lanes 1, binding to cellulose; lanes 2, binding to chitin; lanes 3, binding to xylan. The positions of molecular mass markers (in kilodaltons) are indicated on the left.

(19). Han et al. showed that many cellulases were induced by cellulose (6). As in *C. cellulolyticum*, HbpA might be induced by the presence of cellulose. A paralogue also exists in a large gene cluster in *Clostridium acetobutylicum* (15). *C. acetobutylicum* cannot grow in a medium which contains cellulose as a sole carbon source. However, by analogy to *C. cellulovorans* and *C. cellulolyticum*, the OrFXp of *C. acetobutylicum* might work cooperatively with other cellulosomal subunits.

Here we demonstrated that HbpA bound to the cell wall of *C. cellulovorans* and to polysaccharides such as Avicel, chitin, and xylan. HbpA also formed a complex with EngB and EngL via a cohesin-dockerin interaction, and the HbpA-enzyme complex had higher activity. This synergistic activity is particularly interesting, since it implies that either the formation of the complex results in a conformational change of the enzyme resulting in higher activity or the HbpA-bound enzyme complex may be bound more efficiently to the substrate, resulting in higher activity.

In any case, we suggest that the role of HbpA is to anchor cellulosomal cellulases to the cell surface and to bind to polysaccharides to facilitate cellulosomal enzyme activity. Furthermore the HbpA-enzyme complexes may complement the activity of the cellulosome.

ACKNOWLEDGMENTS

We thank Kei Asai at Saitama University (Japan) for kindly providing plasmid pDG148 and Helen Chan for skillful technical assistance.

The research was supported in part by U.S. Department of Energy grant DE-FG02-04ER15553 and by the Research Institute of Innovative Technology for the Earth.

REFERENCES

- Asai, K., H. Yamaguchi, C. M. Kang, K. Yoshida, Y. Fujita, and Y. Sadaie. 2003. DNA microarray analysis of *Bacillus subtilis* sigma factors of extracytoplasmic function family. *FEMS Microbiol. Lett.* **220**:155–160.
- Bayer, E. A., J. P. Belaich, Y. Shoham, and R. Lamed. 2004. The cellulosomes: multienzyme machines for degradation of plant cell wall polysaccharides. *Annu. Rev. Microbiol.* **58**:521–554.
- 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.

4. Doi, R. H., and A. Kosugi. 2004. Cellulosomes: plant-cell-wall-degrading enzyme complexes. *Nat. Rev. Microbiol.* **2**:541–551.
5. Dygert, S., L. H. Li, D. Florida, and J. A. Thoma. 1965. Determination of reducing sugar with improved precision. *Anal. Biochem.* **13**:367–374.
6. Han, S.-O., H.-Y. Cho, H. Yukawa, M. Inui, and R. H. Doi. 2004. Regulation of expression of cellulosomes and noncellulosomal (hemi)cellulolytic enzymes in *Clostridium cellulovorans* during growth on different carbon sources. *J. Bacteriol.* **186**:4218–4227.
7. Kosugi, A., Y. Amano, K. Murashima, and R. H. Doi. 2004. Hydrophilic domains of scaffolding protein CbpA promote glycosyl hydrolase activity and localization of cellulosomes to the cell surface of *Clostridium cellulovorans*. *J. Bacteriol.* **186**:6351–6359.
8. Kosugi, A., K. Murashima, Tamaru, Y., 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.
9. Koukiekolo, R., H.-Y. Cho, A. Kosugi, M. Inui, H. Yukawa, and R. H. Doi. 2005. Degradation of corn fiber by *Clostridium cellulovorans* cellulases and hemicellulases and contribution of scaffolding protein CbpA. *Appl. Environ. Microbiol.* **71**:3504–3511.
10. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**:680–685.
11. Lamed, R., E. Setter, and E. A. Bayer. 1983. Characterization of a cellulose-binding, cellulase-containing complex in *Clostridium thermocellum*. *J. Bacteriol.* **156**:828–836.
12. Murashima, K., A. Kosugi, and R. H. Doi. 2002. Synergistic effects on crystalline cellulose degradation between cellulosomal cellulases from *Clostridium cellulovorans*. *J. Bacteriol.* **184**:5088–5095.
13. Murashima, K., A. Kosugi, and R. H. Doi. 2003. Synergistic effects of cellulosomal xylanase and cellulases from *Clostridium cellulovorans* on plant cell wall degradation. *J. Bacteriol.* **185**:1518–1524.
14. Pages, S., A. Belaich, H.-P. Fierobe, C. Tardif, C. Gaudin, and J.-P. Belaich. 1999. Sequence analysis of ORFXp, a new cohesin-containing protein in *Clostridium cellulolyticum*: comparison of various cohesin domains and subcellular localization of ORFXp. *J. Bacteriol.* **181**:1801–1810.
15. Sabathe, F., A. Belaich, and P. Soucaille. 2002. Characterization of the cellulolytic complex (cellulosome) of *Clostridium acetobutylicum*. *FEMS Microbiol. Lett.* **217**:15–22.
16. 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.
17. Spizizen, J. 1958. Transformation of biochemically deficient strains of *Bacillus subtilis* by deoxyribonucleate. *Proc. Natl. Acad. Sci. USA* **44**:1072–1078.
18. 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.
19. 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.
20. Wu, S.-C., J. C. Yeung, Y. Duan, R. Ye, S. J. Szarka, H. R. Habibi, and S.-L. Wong. 2002. Functional production and characterization of a fibrin-specific single-chain antibody fragment from *Bacillus subtilis*: effects of molecular chaperones and a wall-bound protease on antibody fragment production. *Appl. Environ. Microbiol.* **68**:3261–3269.
21. Wu, X.-C., W. Lee, L. Tran, and S.-L. Wong. 1991. Engineering a *Bacillus subtilis* expression-secretion system with a strain deficient in six extracellular proteases. *J. Bacteriol.* **173**:4952–4958.
22. Young, R. A., and R. W. Davis. 1983. Efficient isolation of genes by using antibody probes. *Proc. Natl. Acad. Sci. USA* **80**:1194–1198.