Production of Minicellulosomes from *Clostridium cellulovorans* in *Bacillus subtilis* WB800

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Two genes encoding EngB endoglucanase and mini-CbpA1 scaffolding protein of *Clostridium cellulovorans* were constructed and coexpressed in *Bacillus subtilis* WB800. The resulting minicellulosomes were isolated by gel filtration chromatography and characterized. Biochemical and immunological evidence indicated that fraction II contained minicellulosomes consisting of mini-CbpA1 and EngB. The in vivo synthesis of minicellulosomes suggests that it will be possible in the future to insert into *B. subtilis* cellulosomal genes that will allow growth on cellulosic materials and the production of various designer cellulosomes with specific functions.

Clostridium cellulovorans, an anaerobic, mesophilic, spore-forming bacterium (14), produces a cellulosome with a molecular weight of about 1 million and in which several cellulases are bound tightly to a scaffolding protein, CbpA (3). Dockerin domains of cellulosomal enzyme subunits bind to hydrophobic domains termed "cohesins" (20, 27), which are repeated nine times in CbpA. So far, eight cellulosomal cellulase genes (*engB*, *engE*, *engH*, *engK*, *engL*, *engM*, *engY*, and *exgS*) (19) and the gene for the scaffolding protein CbpA (13) have been cloned and sequenced from *C. cellulovorans*.

It is very important to make recombinant cellulosomes, or designer cellulosomes, with specific functions for understanding the hydrolytic mechanism of cellulosomes and for industrial processes. There has been interest in constructing designer minicellulosomes of *C. cellulovorans* for several purposes (2, 8, 9, 10), such as for synergy studies between various cellulosomal enzymes and for improving the efficiency of cellulosomes. Production of intact *C. cellulovorans* endoglucanase, EngB, in *Bacillus subtilis* WB800 and the interaction with mini-CbpA produced in *Escherichia coli* allowed the successful construction of minicellulosomes in vitro (10). These studies indicated that a minicellulosome containing mini-scaffolding protein and cellulosomal enzyme subunits could be created and could be functional in vitro (4, 10). Also, there have been reports of the production of heterologous and chimeric scaffoldins in *Clostridium acetobutylicum* (11) and the in vivo production of recombinant minicellulosomes in *C. acetobutylicum* (12). These works showed the possibility of assembling in vivo a recombinant minicellulosome by overexpressing a mini-scaffolding protein.

We have used *B. subtilis*, which is an aerobic, mesophilic, bacterium and an attractive host for the heterologous production of secretory proteins, as an expression host (22, 24). *B. subtilis* strain WB800, which is a strain deficient in eight extra-

cellular proteases, was constructed by Wong and colleagues (25, 26, 28) to overcome the problem of protein degradation by extracellular *B. subtilis* proteases (1). To prepare recombinant cellulosomes, it is necessary to express intact cellulosomal subunits which retain their dockerin domains.

In this study, we report the construction of expression plasmids carrying genes encoding EngB endoglucanase and mini-CbpA1 scaffolding protein of *C. cellulovorans* and demonstrate the expression of these genes and the in vivo assembly of minicellulosomes in *B. subtilis*.

Construction of pWB980–mini-*cbpA1*–*engB* expression plasmid. We have expressed *C. cellulovorans* cellulosomal enzyme subunit genes in *E. coli* successfully; however, these expressed subunits were partially degraded by *E. coli* proteases during the culture period. Therefore, the use of *B. subtilis* WB800, deficient in eight extracellular proteases, was essential for the production of intact EngB and minicellulosomes (26, 28). For *B. subtilis* expression, we chose the construction of a small mini-CbpA (i.e., mini-CbpA1), which consisted of a cellulose binding domain, a hydrophilic domain (HLD1), and one cohesin domain (Coh1) of the scaffolding protein, CbpA (17). The DNA fragment coding for mini-CbpA1 was amplified from pET–mini-*cbpA1*, which was designed for *E. coli* expression, by using primers T7 promoter (5′-TAATACGACTCAC TATAGGG) and Hisph2 (5′-CGGGCTTTGTTAGCAGCGC ATGCTCAGTGGTG) (Fig. 1). The Hisph2 primer was designed to allow an in-frame fusion at the C-terminal end with a His tag from pET-22b (Novagen). This amplified 1.3-kb fragment was introduced between the BamHI and SphI sites of the pWB980 *Bacillus* expression vector that carried the constitutively expressed P43 promoter (21); the engineered levansucrase signal sequence, *sacB* signal peptide (SP) (23); and the kanamycin resistance marker to generate pWB980–mini-*cbpA1*. The P43 promoter is a strong and constitutively expressed promoter and was used to direct the transcription of mini-*cbpA1* and *engB* during growth. The *sacB* signal sequence allowed the recombinant proteins to be secreted into the culture broth. *B. subtilis* WB800 harboring pWB980–mini-*cbpA1*

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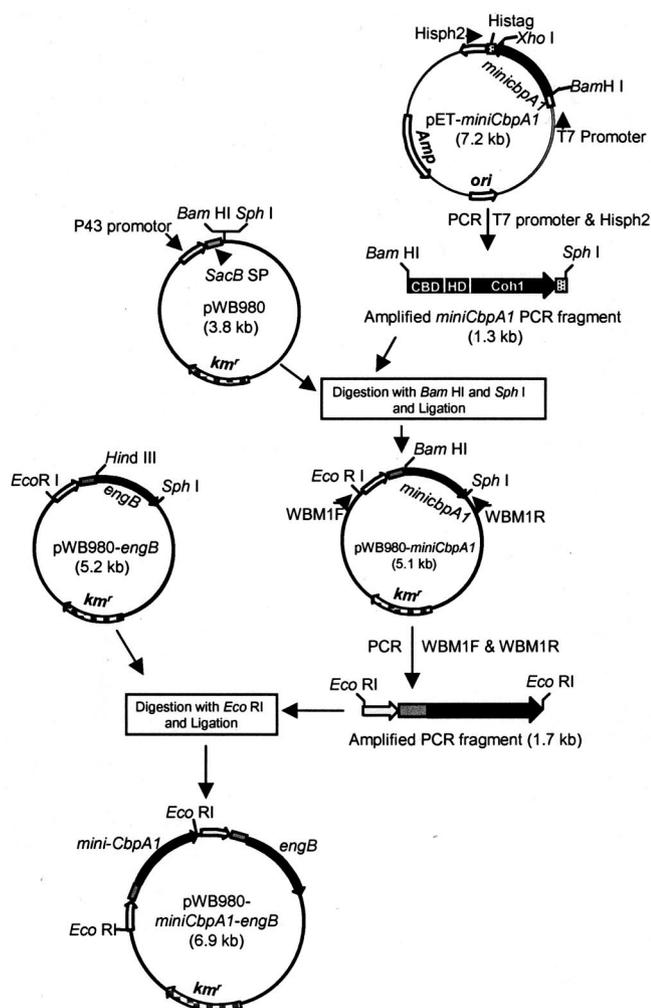
FIG. 1. Construction of the pWB980–mini-*cbpA*1–*engB* expression plasmid. For *B. subtilis* expression, we chose a small mini-CbpA, i.e., mini-CbpA1 consisting of a cellulose binding domain, a hydrophilic domain (HLD1), and one cohesin domain (Coh1). Plasmid pWB980, which is a *Bacillus* expression vector, carries both the constitutively expressed P43 promoter and *sacB* SP, the engineered levansucrase signal sequence for heterologous protein expression in *B. subtilis* (23). The sequences coding for the kanamycin resistance marker and levansucrase signal sequence are represented by *km^r* and *sacB* SP, respectively. Arrows show the transcription directions for the genes.

FIG. 2. Purification procedure for minicellulosomes from *B. subtilis* WB800 harboring pWB980–mini-*cbpA1*–*engB*. SR, superrich; NTA, nitrilotriacetic acid.

produced intact mini-CbpA1 in *B. subtilis* and could be purified by a Ni^{2+}-nitrilotriacetic acid agarose column (QIAGEN). For the two-gene construct of *engB* and mini-*cbpA1*, the pWB980–mini-*cbpA1* plasmid was amplified with a pair of primers, WBM1F (5′-GGTTTTATGGTTTTGGTCGGCACTGAATTC) and WBM1R (5′-CGGGGCGAATTCGCATGCTCAGTGG TGGTG), which allowed the amplification of the mini-*cbpA1* gene containing a promoter and signal sequence. The amplified 1.7-kb PCR fragment was digested with EcoRI, inserted into pWB980-*engB* (10), and digested with the same restriction enzyme to generate pWB980–mini-*cbpA1*–*engB* (Fig. 1). We chose *engB* for this experiment because it was the smallest cellulosomal cellulase gene of *C. cellulovorans* (19). Standard DNA transformation was performed with *B. subtilis* by the compet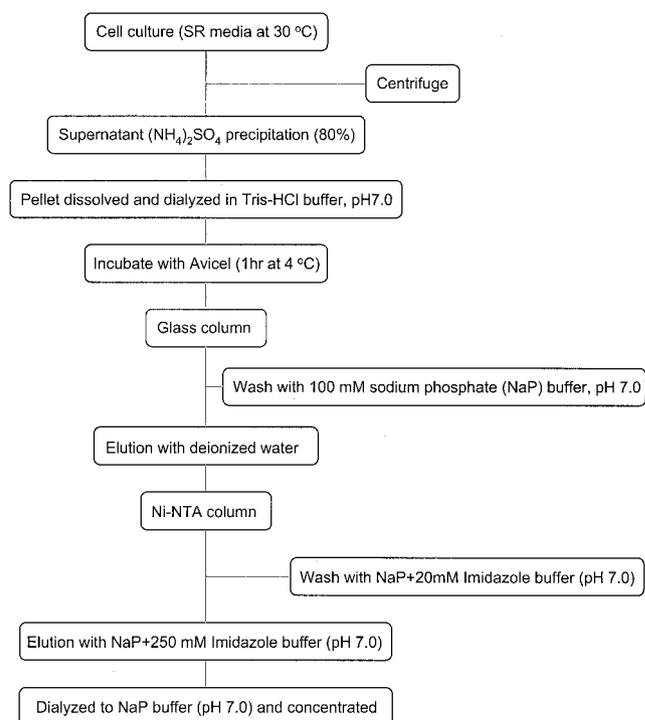ent-cell method (15). The clone was selected on an agar plate containing, per liter, 10 g of peptone, 5 g of tryptone, and 5 g of NaCl supplemented with kanamycin (50 μg/ml).

Expression and purification of minicellulosomes from *B. subtilis*. *B. subtilis* WB800 harboring pWB980–mini-*cbpA1*–*engB* was cultivated in 2,000 ml of superrich medium (containing, per liter, 25 g of yeast extract, 15 g of tryptone, and 3 g of K_2HPO_4 supplemented with kanamycin [50 μg/ml]) for 20 h at 30°C. Figure 2 shows the purification procedure for minicellulosomes. The culture was centrifuged to remove cells. Culture supernatant was collected, precipitated with ammonium sulfate at 80% saturation, and dialyzed. This enzyme solution was mixed with Avicel, which resulted in the binding of the minicellulosomes and free mini-CbpA1. After incubation for 1 h at 4°C, the suspension was poured into a glass column and the column was washed with 3 volumes of 100 mM sodium phosphate buffer (pH 7.0). The Avicel-bound fraction was eluted with deionized water and concentrated. This Avicel-bound enzyme solution was then applied to a Ni^{2+}-nitrilotriacetic acid agarose column (QIAGEN) and washed with 2 column volumes of 50 mM sodium phosphate buffer with 20 mM imidazole (pH 7.0). The bound fraction was eluted with 250 mM imidazole in 50 mM sodium phosphate buffer (pH 7.0), dialyzed against 50 mM sodium phosphate buffer (pH 7.0), and concentrated with Vivaspin 20 mini-spin column (10-kDa cutoff) (Viva Science). The apparent size and homogeneity of the cellulosome preparation were determined by gel filtration on a Superdex 75 preparation-grade column (Amersham Pharmacia Biotech) with 50 mM sodium phosphate buffer as the elution buffer (Fig. 3). Three fractions (I, II, and III) were isolated. Fraction I corresponded to an apparent molecular mass
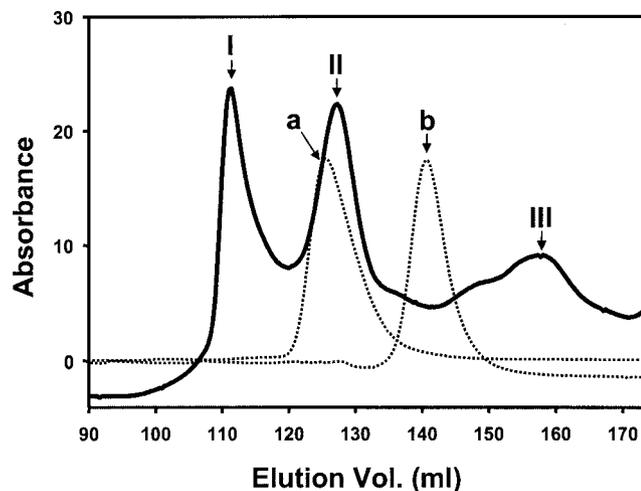
FIG. 3. Gel filtration chromatography of the water-eluted and Ni^{2+}-nitrilotriacetic acid agarose-purified cellulosome fraction on a Superdex 75 preparation-grade column. Fractions I, II, and III are shown as solid lines, and standards are shown as dotted lines; (a, aldolase [158 kDa]; b, bovine serum albumin [67 kDa]).
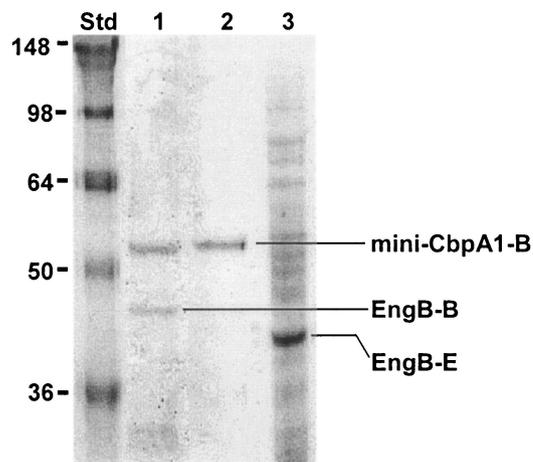

FIG. 4. SDS-PAGE analysis of fraction II stained with Coomassie blue. Lane 1, fraction II (mini-CbpA1-B and EngB-B); lane 2, purified mini-CbpA1 from *B. subtilis* WB800 (mini-CbpA1-B); lane 3, crude extract from *E. coli* harboring the *engB* gene (EngB-E). Lane Std, molecular weight standards (indicated in thousands).

of about 300 kDa, fraction II corresponded to an apparent molecular mass of about 120 kDa, and fraction III corresponded to an apparent molecular mass of about 50 kDa. These values were calculated from the calibration curve obtained with the standards used, i.e., aldolase (158 kDa) and bovine serum albumin (67 kDa). Based on the molecular mass, fraction II appeared to be the minicellulosome fraction. However, the molecular mass from the gel filtration result (120 kDa) was larger than the theoretical molecular mass of 94 kDa (mini-CbpA1, 46 kDa; EngB, 48 kDa). The difference in size seemed likely to be due to the resulting mini-CbpA1–EngB complex being an elongated molecule rather than a globular molecule. Since both the shape and the mass of the molecule determine the apparent molecular mass of this molecule by the gel filtration method, the deviation from the expected molecular mass in this case may be attributed to the nonglobular shape of the complex.

Characterization of minicellulosomes assembled in *B. subtilis*. Two fractions from the gel filtration analysis were collected and characterized. Based on the estimated molecular mass from gel filtration analysis, fraction II was considered to be the minicellulosome fraction. We subjected fraction II to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to identify the subunit composition (Fig. 4, lane 1). Purified mini-CbpA1 from *B. subtilis* (Fig. 4, lane 2) and the crude extract from *E. coli* harboring the *engB* gene (Fig. 4, lane 3) were also analyzed as standards. From the SDS-PAGE gel, fraction II (lane 1) showed two bands. The upper band exactly matched the purified mini-CbpA1 from *B. subtilis* (lane 2), although the molecular mass was larger than the theoretical value (46 kDa). This result showed the difference between the theoretical and experimental molecular mass determinations from SDS-PAGE and gel filtration chromatography. The lower band visible in Fig. 4, lane 1, is EngB, as demonstrated later by Western blotting and zymogram analysis. The band visible in Fig. 4, lane 3, was larger than the EngB produced from *E. coli*,

because *E. coli* produces a truncated EngB without dockerin (5). From these SDS-PAGE results, we could see that fraction II (Fig. 4, lane 1) consisted mainly of mini-CbpA1 and EngB.

To verify the SDS-PAGE results, we performed a Western blot analysis. The samples were subjected to SDS–10% PAGE and blotted onto nitrocellulose membranes for Western blot analysis (Fig. 5A). We used two kinds of antibodies for detection. We have defined anti-HLD as the antibody made against one of the hydrophilic domains of CbpA, and thus anti-HLD1 is specific for hydrophilic domain 1 of CbpA. Anti-EngB is the specific antibody for EngB. The membrane incubated with the HLD1-specific antibody revealed bands corresponding to mini-CbpA1, showing that both fractions I and II contained mini-CbpA1. The membrane incubated with EngB-specific antibody revealed that only fraction II contained a band corresponding
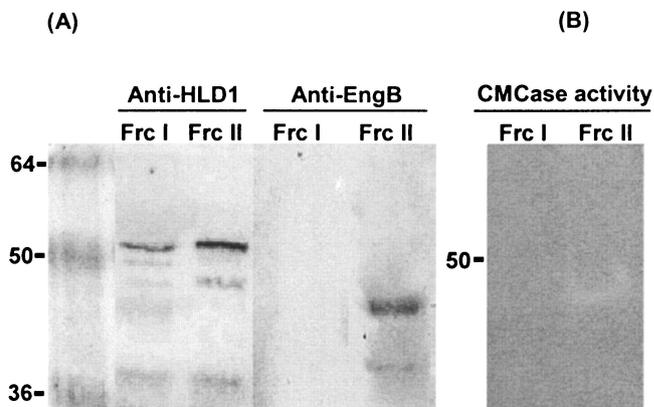

FIG. 5. Characterization of fractions I and II. (A) Western blot showing detection with antisera raised against HLD1 and EngB, respectively. Anti-HLD1 was made against the hydrophilic domain 1 of CbpA, and anti-EngB was made against EngB. (B) Zymogram analysis with 0.1% carboxymethyl cellulase (CMCase) incorporated into the polyacrylamide. Lane Frc I, fraction I; lane Frc II, fraction II. Molecular weights are indicated on the left in thousands.

to EngB. These results confirmed that fraction II consisted of mini-CbpA1 and EngB, which had been assembled into mini-cellulosomes in vivo. Fraction III contained only free mini-CbpA and a small amount of free EngB, as shown by Western blot analysis (data not shown).

As an additional method for confirming the nature of the minicellulosome, zymograms were created by using a 0.1% (wt/vol) concentration of carboxymethyl cellulose incorporated into the polyacrylamide gel. After electrophoresis, the gel was incubated at 30°C for 2 h in 50 mM sodium acetate buffer (pH 6.0). The enzymatic activity was visualized after staining with 0.25% Congo red and destaining with 1 M NaCl (6). As a result, strong EngB activity was detected in fraction II, which corresponded to a molecular weight of about 48 kDa, and no activity was detected in fraction I. However, this minicellulo-some could not degrade Avicel. From the immunological and biochemical evidence, we concluded that fraction II contained minicellulosomes consisting of mini-CbpA1 and EngB. The yield of minicellulosomes in fraction II was approximately 0.2 mg/liter of culture.

Until now, the *C. cellulovorans* cellulosomal *engB* and mini-*cbpA* genes had been expressed in *E. coli* (5, 7, 16, 17, 18, 19) and an in vitro interaction had been shown between EngB and mini-CbpA. This is the first report that a cellulosomal cellulase gene and a scaffolding gene could be coexpressed and could form a minicellulosome in *B. subtilis* in vivo. Our goal is to express minicellulosomes and a variety of designer cellulo-somes in vivo in high yields. The in vivo construction of mini-cellulosomes suggests that it will be possible in the future to insert into *B. subtilis* cellulosomal genes that will produce var-ious designer cellulosomes with specific functions. Moreover, with further development of this system, *B. subtilis* may be able to grow on cellulose and on plant cell wall materials and produce valuable products from inexpensive substrates.

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