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Bacillus cellulyticus K-12 Crystalline Cellulose-Degrading Avicelase Gene and Expression in *Escherichia coli*

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Abstract

We have cloned the *Bacillus cellulyticus* K-12 avicelase(Avi, E.C.3.2.1.4) gene(*ace* A) in *E.coli*. This was accompanied by using the vector pT7T3U19 and *Hind* Ⅲ-*Hind*Ⅲ libraries of *Bacillus cellulyticus* K-12 chromosomal inserts created in *E.coli*. The libraries were screened for the expression of avicelase by monitoring the immunoreaction of the anti-avicelase(immunoscreening). Positive clones(Ac-3, Ac-5, and Ac-7) contained the identical 3.5kb *Hind*Ⅲ fragment as determined by restriction mapping and Southern hybridization, and expressed avicelase efficiently and constituvely using its own promoter in the heterologous host. From the immunoblotting analysis, a polypeptide which showed a CMCase activity with an Mr of 54000 was detected.

Key words : gene cloning, gene exprsssion, avicelase, localization of recombinant protein, (*Bacillus cellulyticus* K-12).

INTRODUCTION

The cleavage of crystalline cellulose is of great biotechnological and ecological potential, but is still quite poorly understood. It has been mainly studied using microcrystalline cellulose (Avicel), which has a chain length of 300~500 glucose units in comparison with 5,000~15,000 of native cellulose[1]. Up to now the cellulolytic system from the fungus *Trichoderma reesei* has been best characterized. Two endoglucanases and two cellobiohydrolases (EC 3.2.1.91) act synergistically to convert Avicel to cellobiose[2,3]. Only a few bacterial cellulases degrading the crystalline substrate have been studied genetically and biochemically in detail:*Clostridium stercorarium* produces two avicelases which hydrolyse crystalline cellulose synergistically. Its Avicelase I was identified as an endoglucanase[4]. While Avicelase II has been classified as an exoglucanase[5]. In *Streptomyces reticuli* one unusual cellulase (Avicelase) has been identified[1]. The enzyme hydrolyses crystalline and soluble cellulose as well as cellodextrins, predominantly to cellobiose. Recently we have isolated *Bacillus cellulyticus* K-12 which produces powerful cellulases having β-1, 4-glucanases of carboxymethyl cellulase (CMCase)(70~80 U/ml), avicelase (1.0~1.5U/ml), filter paper hydrolase(FPase) (0.5~1.0 U/ml), xylanase (3.5~4.0 U/ml), and β-glucosidase (0.3~0.5 U/ml)[6]. The homogeneous β-glucanase I preparation of *B. cellulyticus* K-12 is a very specifically acting enzyme and shows a specific substrate specificity towards β-1,4-glycosidic linkage of Avicel, microcrystalline artificial cellulose substrate. The most interesting of the enzyme was that the enzyme was acted as not

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only endo β-glucanase, but also exo β-glucanase (Kim et al, unpublished data). It, therefore, appears that the cloning of a single homogeneous gene expressing the enzyme will be useful for elucidation of the enzyme-substrate relation. Here we report the cloning of a cellulase (Avicelase) gene from *B.cellulyticus* K-12 in *E. coli*, expressing Avicelase activity. This is the first report of a gene cloning in *B.cellulyticus* K-12 and endo-exo β-glucanase.

MATERIALS AND METHODS

1. Materials

Avicel SF was supplied by Asahi Chemicals (Tokyo, Japan). Restriction enzymes and modification enzymes were purchased from BM Korea, and were used as recommended by the suppliers. Molecular maker kit for Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and nitrocellulose membrane filter for immunodetection and western blotting analysis, were obtained from Bio-Rad Laboratories (Tokyo, Japan). Nylon membrane filter (Hybond-C) for DNA hybridization was purchased from Amersham Japan (Tokyo, Japan). All other chemicals were commercial products of analytical grade.

2. Bacterial strains and plasmids

B.cellulyticus K-12 was used on the source of the gene that codes for the avicelase. The following bacterial strains, and plasmids were used: *E.coli* MV1184, JM109, and plasmid pT7T3U19 and pBR322.

3. Media and culture conditions

B.cellulyticus K-12 was maintained in induction medium containing of 10g of a filter paper strip ($13\times1cm^2$), 2g of $(NH_4)_2SO_4$, 14g of K_2HPO_4, 0.2g of $MgSO_4\cdot7H_2O$, 0.5g of $CaCl_2\cdot2H_2O$, 1.0g of bactopeptone, 1g of yeast extract and 1ml of trace element in liter of water[6] *E.coil* MV1184, JM109 was maintained in Luria broth(1% peptone, 0.5% yeast extract and 0.5% NaCl, pH 7.2), while JM109, MV1184/olasmid was maintained in Luria broth containing 40μg/ml ampicillin. Cultures were preserved in 25% glycerol at -70℃.

4. DNA isolation

Chromosomal DNA was extracted from *B. cellulyticus* K-12, according to the method of Saito and Miura[7]. Large scale preparation of plasmid DNA was carried out by the method of Birnboim and Doly[8]. Restriction endonucleases (BRL. Gaithersberg, MD, USA) were used under the assay conditions described by the manufacturers. Agarose gel electrophoresis of DNA fragments was carried out Tris-acetate buffer, pH 7.8, containing EDTA as described[9]. DNA from the gel was transferred onto Hybond C membrane(Amersham) and used for hybridization. The DNA probe was nick translated with [α-^{32}P]dCTP as described by Maniatis et al.[9]

5. Gene library construction and cloning of avicelase gene

Chromosomal DNA from B. cellulyticus K-12 was partially digested with *Hind* Ⅲ. After removal of proteins, the resulting fragments were ligated to *Hind*Ⅲ-digested pT7T3U19 DNA using T4 DNA ligase (Sugino et al. 1982)[10]. *E. coli* MV1184 was transformed using these recombinant plasmids according to Mandel and Higa[11]. The transformants were transferred to nitrocellulose membrane plate for avicelase genes by immunoreacting with anti-avicelase

antiserum[12], affinity purified goat anti-mouce IgG-alkaline phosphatase conjugate and substrate of alkaline phosphatase(Vector Laboratories, OH, USA). Original colonies showing immunoreactivity on the nitrocellulose membrane were selected and screened for their enzyme activity.

6. Enzyme assays, purification and preparation of polyclonal antibody

Avicelase activity was determined by incubating the appropriately diluted enzyme with 2% Avicel SF (Asahi Chem. Tokyo, Japan) with shaking for 30min at 50℃. After incubation, the remaining sugars released were estimated as cellobiose equivalents by the modified dinitrosalicylic acid method described previously[13]. One international unit(IU) was defined as micro moles of product formed per minute under the given temperature and pH conditions. For preparation of the substrate used for avicelase, 2% avicel suspension in 50mM phosphate buffer, pH6.5, was left standing room temperature for 20min. and then centrifuged. After the supernatant was discarded, the pellet resuspended in buffer was used as substrate.

7. SDS-PAGE and western blotting

SDS-PAGE of the cell extract was carried out on 12.5% slab gels(Laemmli, 1970)[14]. This was then transblotted onto hybond sheets and processed according to Towbin et al(1979).[15] using antiavicelase antibodies raised in mice. The polyclonal antiavicelase antibodies used were raised against avicelase which was purified from *B. cellulyticus* K-12. Purification of extracellular avicelase from *B. cellulyticus* K-12 was succesfully carried out by avicel adsorption, DEAE-Toyopearl M650 ion exchange chromatography, Phenyl Toyoperal M650 hydrophobic chromatography and TSK gel HW50S chromatography(Kim et al., unpublished data). For immunization, the purified avicelase(100 μg) was periodically boosted for three times to 4 mice(BALB/c). After four boosting, the antibodies were titrated by double diffusion assay. Details of enzyme purification and production of polyclonal antibody will be reported elsewhere.

RESULTS AND DISCUSSION

1. Cloning of avicelase gene

Of 25,000 recombinats derived from a library, 3 were found to produce avicelase. These recombinant plasmids with avicelase protein were analysed by *Hin*dIII disestion, and classified into one category according to distinct DNA. Pattern of *Hin*dIII site in the insert *Hin*dIII fragment is common. Restriction site analysis revealed that 3.5-kb fragment is the same, indicating that 3.5-kb DNA fragmet is responsible for avicelase expression.

A physical map of 3.5-kb insert DNA showed that the insert has 2 different restricion sites for *Eco*RI, DraI, *Pvu*II and one site for *Sma*I and *Bam*HI. No sites for the enzymes PstI, SstI, and XhoI could be seen within this site(Fig. 1).

2. Southern hybridization

The cloned avicelase gene was used as a probe to carry out Southern hybridization analysis of genomic DNA from *Bacillus cellulyticus* K-12. The 3.5kb insert fragment of pAV-3. The pAV-3 insert probe was found to hybridize with a single 3.5kb *Hin*dIII fragment, with two *Bam*HI fragments of 4.4 and 9.6 kb, and a single 4.6kb *Pst* I fragment(Fig. 2). DNA sequencing of the 3.5-kb fragment is now underway in our laboratory to compare amino acid sequences among endoglucanase and avicelase genes in *Bacillus*

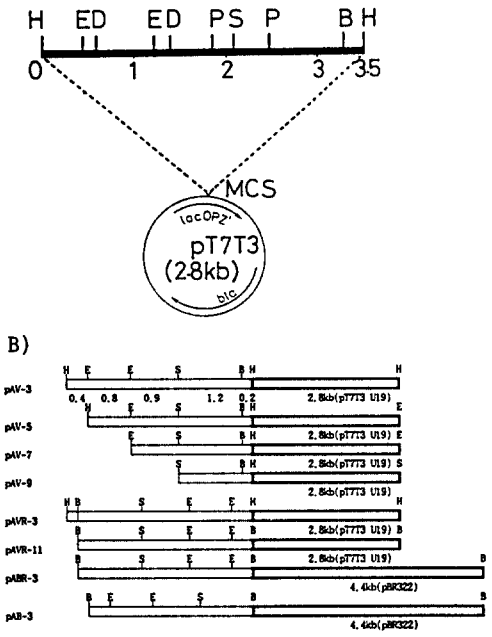

Fig. 1. Restriction enzyme maps of the fragments clone in recombinant plasmid pAV-3 and their derivative fragments.

The solid bars represent *B.sellulyticus* K-12 DNAs. The arrow indicates a functional orientation of its own promoter. The *bracket* indicates the fragment necessary for avicelase expression. Plasmid pABR-3 was constructed by subcloning of the 3.3-kb *Bam* HI fragment to pBR322. There is on PstI site in these inserts. Abbreviations:H, *Hin*dIII; E, *Eco*RI; B, *Bam* HI; S, *Sma*I. A) Restriction map of pAV-3. B) Subclones of the exzyme gene. Shmbols:+, avicelase activity was detected on Congo red plates; −, avicelase activity was not detected on Congo red plates.

cellulyticus K-12 and also to determine the precise homologous region to the gene to other *Hin*DIII fragments.

3. Expression of the avicelase gene

To localize the structural gene in a 3.5-kb insert fragment of pAV-3(6.3kb), *Eco*RI-or *Sma*I-digested DNA fragments were religated and avicelase-positive clones were selectively screened. Plasmids of pAV-5(5.9 kb), pAV-7(5.1 kb), and pAV-9(4.5 kb) were newly created as shown in Fig. 1. *E.coli* carrying plasmids of pAV-3 and pAV-5 showed their high enzyme activities(1.7 U/ml for pAV-3 and 1.8 U/ml for pAV-5) while *E. coli*(pAV-7) produced only 0.7 U/ml of avicelase, Furthermore, pAV-9 did not conferred any avicelase activity. These results cleary indicated that plasmid pAV-7 carried the DNA fragment responsible for the avicelaseencoding structural gene without its own promoter and a 0.8-kb DNA fragment of *Eco*RI-*Eco*RI site

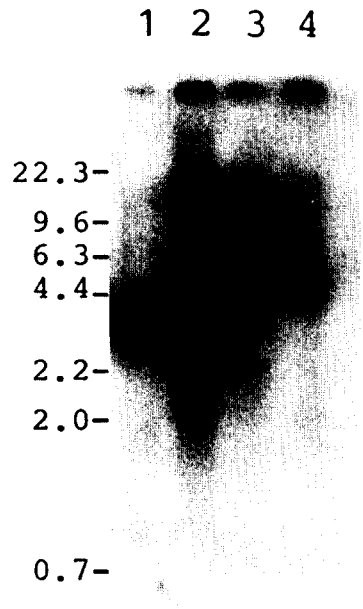

Fig. 2. Southern hybridization analysis using the cloned plasmids as probes against avidelase-digested *B.celluyticus* K-12 genomic DNA.

Lane 1, *Hind* III-digested DNA of *B.cellulyicus* K-12(5μg); 2, *Eco*RI-digested DNA of *B.celluylticus* K-12(10μg); 3, *Eco*RI-digested DNA of *B.cellulyticus* K-12(5μg); 4, *Pst* I-digested DNA of *B.cellulyticus* K-12(5μg).

might be its own 5'-promoter region for the gene transcription. Since the pT7T3U19 as a plasmid vector has T7 and T3 transcriptional promoter in both side, there is no any difference between the enzyme activities produced by the two plasmids of pAV-3 and pAV-5. *E.coli* JM109 carrying the derivative plasmid pAV-7 expressed 2.4 fold lower avicelase activity than the original plasmid pAV-3(Table 1). The most reasonable explanation is that the deletion of the 1.2-kb fragment caused less-efficient read-throughtranscription. Addition of IPTG, an inducer of the *lac* promoter, into the cell extract stimulated expression of the avicelase gene in pAV-7, PAV-5 and pAV-3, indicating that the functional orientations of the avicelase and *lac* promoter of the vector are the same in this plasmid and that the *lac* promoter also participates in expressing the gene(Table 1). On the other hand, a 3.5kb insert DNA of plasmid pAV-3 was subcloned into pT7T3U19 in reverse orientaion and then *Bam*HI-digested DNA fragment was religated. The newly designated plasmid pAVR-3 and pAVR-11 conferred the same avicelase activities as those produced by pAV-3 to *E.coli*, indicating that transcription of the gene arose in part from a *B.cellulyticus* K-12 promoter. For further characterization of the gene and promoter localization for avicelase, a *Bam*HI-digested 3.3 kb DNA fragment of pAV-3 was subcloned to promoter-negative pBR-322. Two derivative plasmids, pABR-3 and pAB-3 in which the pAV-3 insert was in direct and reverse orientation, revealing that a promoter was present in this fragment.

Table 1. Avicelase acvities expressed in *E. coli* carrying pAV-3 and its derivative plasmids.

Plasmid	Activity (U/ml culture)	
	+IPTG	−IPTG
pAV-3	6.8	1.7
pAV-5	8.3	1.8
pAV-7	2.4	0.7
pAVR-3	1.7	1.6
pAVR-11	1.7	1.9
pAB-3	0.56	0.57
pABR-3	0.65	0.53

The culture supernatant of *B.cellulydus* K-12 contained multiple forms of cellulase, which probably act synergistically, whereas, the *E.coli* (pAV-3) made only one of these active forms (Fig. 3). This could be one of the possible reasons for relatively low avicelase activity in the pAV-3 cell extract. It is well known that multiple cellulases are produced by some microorganisms[16-18], which are mainly due to either post-translational modification such as proteolysis /glycosylation, or multiple genes for these enzymes[19, 23]. In the case of *B. cellulyticus* K-12, in the present study, eventhough multiple activity bands corresponding to slow migrating and fast migrating proteins were observed on zymogram staining after PAGE; but the pAV-3 coded avicelase was a fast migrating protein. The size of the insert also indicated the presence of only one gene in the clone.

4. Cross-reactivity of the cloned avicelase with anti-avicelase produce by *B.cellulyticus* K-12

To determine the chemical relation between the cloned avicelase component and the avicelase produced by *B.cellulyticus* K-12, the cell extracts of *E.coli* were subjected to the immunodiffusion experiment. *E.coli* JM109 with and without pAV-3 was cultured on L-broth medium. Each cell exteact of *E.coli* transformed was tested for reactivity against antiserum.

Avicelases of the cell extracts of all the strains except *E. coil*; JM109 without plasmids cross-reacted with the antiserum, and the precipitin lines fused completely with each other (not shown). Additionally, cell extracts produced by *E. coli* JM109(pAV-3) or without plasmid were subjected to SDS-PAGE, blotted to nitrocellulose, and the cross-reactivity toward antiserum was examined by Western methods (Fig. 3). Results shown in Fig. 3 indicate that *E. coli* producing avicelase has a common antigenity to *B.cellulyticus* K-12 avicelase, Furthermore, from the result of SDS-PAGE, the molecular weight of the cloned avicelase was calculated to be 64,000. This value is similar to the avicelase(Mr about 64,000) of *B.cellulyticus* K-12.

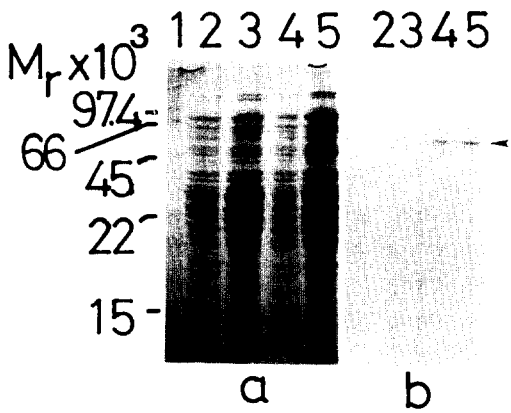

Fig. 3. SDS-Western blotting analysis of the cloned avicelase samples expressed in *E.Coli* JM109(pAV-3).

The cell extracts were separated 12.5% SDS-PAGE, transferred and treated with anti-avicelase. Reaction was carrie out by the method described in Materials and Methods. a: Cell extracts of *E.coli* JM109 with pT7T3 U19 or pAV-3 were stained with Coomassie Blue R250. Lane 1, molecular sizes of standard marker(in kilodal tons) are indicated at the left; lane 2, *E.coli* JM109(pT7T3 U19) as a negative control(6μg); lane 3; *E.coli* JM109(pT7T3 U19) as a negative control(12μg); lane 4; *E.colo* JM109(pAV-3) as a positive control(6μg); lane 5, *E. coli* JM109 (pAV-3) as a positive control(12μg). b:Immunoreacted samples of the corresponding to the lanes of a.

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