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Cloning and Expression in *Escherichia coli* of Cellulase Genes from a Mesophilic *Clostridium* sp.

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A genomic library of a mesophilic cellulolytic anaerobe, *Clostridium* sp. KCTC 8440 DNA was constructed in *Escherichia coli* using plasmid pUC9. Clones of *E. coli* exhibiting carboxymethyl cellulose-hydrolyzing activity (CMCase) were isolated and divided into seven types based on the restriction enzyme patterns of recombinant plasmids. *E. coli* strains carrying type A genes showed activity on carboxymethyl cellulose about 7-8 times greater than clones carrying genes of other types. Restriction maps of the cloned DNA fragments were determined, and homologies between them were investigated. The results suggest that *Clostridium* sp. KCTC 8440 has seven distinct CMCase genes.

Cellulose is the most abundant organic material on the earth. Since a cellulose molecule is a linear polymer of β-1,4-glucoside, extensive hydrogen bonds are formed between adjacent molecules. Due to the hydrogen bonds, natural cellulose is highly crystallized. For the mineralization of the crystalline polysaccharide, cellulolytic microbes produce a number of enzymes with different functions (12, 14). This polysaccharide is degraded mainly by microorganisms in the aerobic as well as anaerobic ecosystems. Most of the studies on microbial cellulolysis have been done using aerobic bacteria, fungi, and thermophilic anaerobic bacteria.

Based on the biochemical studies on various microbial enzymes, the cellulolytic enzymes have been classified into three basic groups; endo-1,4-β-glucanase (EC 3.2.1.4), exo-1,4-β-glucanase (cellobiohydrolase, EC 3.2.1.91), and β-1,4-glucosidase (EC 3.2.1.21) (20). Cellulolytic enzymes were further characterized by cloning the genes from an aerobic bacterium, *Cellulomonas fimi* (10), and a thermophilic anaerobe, *Clostridium thermocellum* (3, 9, 19, 32, 34). Cellulolytic enzymes of *C. thermocellum* are known to form a high-molecular mass, cellulose binding, multicellulase-containing protein complex, termed the cellulosome (15). Biochemical studies have shown that the cellulosome of *C. thermocellum* is composed of more than 14 polypeptides having endo- or exo-glucanase activities (15). In another study fifteen *cel* genes of *C. thermocellum* were cloned and expressed into *E. coli* (9). The reason for such a high number of endoglucanase gene is puzzling and awaits further studies. Genes for endoglucanse (*cenA*, *cenB* and *cenC*), and for exoglucanase (*ceX*) were cloned and characterized from *Cellulomonas fimi* (4, 11, 22, 33).

Though a large part of cellulose is mineralized in anaerobic environment at ambient temperature, few attempts have been made to characterize the cellulolytic enzymes of mesophilic anaerobes. Cellulolytic mesophilic anaerobes have been isolated from various sources (23, 25), and enzymes were purified from some of the isolates. There have been several reports on the cloning of cellulase genes from mesophilic *Clostridium* species (6, 13, 21, 28, 35). Several endoglucanase genes (*engA*, *engB*, *engC* and *engD*) have been cloned from *Clostridium cellulovorans*. Enzymes coded by *engA* and *engD*

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genes showed β-glucosidase and cellobiosidase activity, respectively, in addition to CMCase activity. Two CMCase genes of *Clostridium cellulolyticum* ATCC 3519 were cloned and characterized (6).

Clostridium sp. KCTC 8440 was used in this study for the charaterization of its genes for the cellulolytic enzymes. The strain has been isolated and screened to use as a feed additive for ruminants(Korean Patent Application number; 89-3676). This mesophilic bacterium is able to grow on native cellulose and apparently possesses a complete enzymatic system for the utilization of crystalline cellulose. In the present investigation several cellulase genes of *Clostridium* sp. KCTC 8440 were cloned and characterized.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

Clostridium sp. KCTC 8440 was used as a source of cellulase genes. This mesophilic spore-forming cellulolytic bacterium was isolated from soil in this laboratory. *E. coli* JM83(*ara*, Δ(*lac pro* A, B), *rspL*, φ80, *lacZ*ΔM15, (r^+, m^+)) served as the host for transformation. Plasmid pUC9 was used as a vector for the cloning of genes.

Media and Culture Conditions

Clostridium sp. KCTC 8440 was cultured at 35℃ in a DSM broth (27) supplemented with 1% of filter paper (Whatman No. 1) as a carbon source. The medium was anaerobically prepared and dispensed into a serum vial under constant flow of nitrogen gas. *E. coli* was cultured in LB broth(tryptone, 10g/*l*; yeast extract, 5g/*l*; NaCl, 10g/*l* in distilled water, pH 7.0). *E. coli* transformants were grown in LB medium containing 50 μg of ampicillin/m*l*.

Preparation of DNA and Gel Electrophoresis

The pUC9 DNA was prepared from *E. coli* by cesium chloride-ethidium bromide centrifugation of cleared lysate (17). For rapid isolation of plasmids from the *E. coli* transformants, the alkaline lysis method described by Birnboim and Doly (2) was employed. Clostridial chromosomal DNA was isolated from cells of mid-exponential phase according to the preparative method described by Rodriquez and Tait (26). DNA and their restriction digests were analyzed by electrophoresis using 0.7% to 1% agarose gels.

Construction of the Gene Library

Fifty μg of chromosomal DNA purified from *Clostridium* sp. KCTC 8440 was partially digested with *Hin*dIII, and DNA fragments ranging from 2 to 10 kb were isolated by sucrose gradient centrifugation for 20 hr at 25, 000 rpm in a Beckman SW40 rotor. The *Hin*dIII-digested chromosomal DNA fragments(3 μg) were ligated to pUC9 (1 μg) which was digested with *Hin*dIII and dephosphorylated using calf intestinal alkaline phosphatase (Promega). The ligation mixture was used to transform *E. coli* JM83.

Southern Hybridization

The insert DNA of the cloned plasmid was isolated and dephosphorylated using calf intestinal alkaline phosphatase. End- labelling of the dephosphorylated DNA fragment was done using T4 polynucleotide kinase in the presence of [γ-^{32}P]dATP(150 μCi). For blotting and hybridization of DNA digests, the method described by Southern (29) was used.

Enzyme Assay and Analytical Method

Enzyme activities were determined using reaction mixture containing 1 m*l* of 1% carboxymethylcellulose (No. C-4888 Sigma) or oat spelts xylan (No. X0627 Sigma) and 0.5 m*l* of crude enzyme solution in 50 mM phosphate buffer (pH 6.8).

The mixture was incubated at 45℃ for 30 min before the enzyme reaction was stopped by boiling for 5 min. After boiling, insoluble materials were removed by centrifugation. The reducing sugar released by the enzyme was measured by dinitrosalicylic acid (DNS) method (18). One unit of enzyme activity was defined as the amount of protein which produces 1 μmole of reducing sugar in one minute. Enzymatic hydrolysis of *p*-nitro-phenyl-β-D-cellobioside(pNPC) was measured by the amount of released *p*-nitrophenol as described by Deshpande et al (5). One unit of activity corresponded the release of 1μmole *p*-nitrophenol in one minute. Enzymatic activities on methylumbelliferyl-β-D-cellobiopyranoside (MUC) were determined by the method described by Faure et al (6).

Protein concentration was determined by the method of Lowry et al (16).

Preparation of Cell-Free Extract

Cells grown for 15-16hrs were harvested and washed with 50 mM potassium phosphate buffer (pH 6.8), and resuspended in the same buffer. The cell suspension was sonicated for 2 min using the Branson Sonifier followed by centrifugation for 20 min at 10,000×g. The supernatants were used in enzyme assays.

RESULTS AND DISCUSSION

Cloning of the DNA Fragments Carrying Cellulolytic Enzyme Genes

The genomic library of *Clostridium* sp. KCTC 8440 was constructed in *E. coli* JM 83 using pUC9 as the vector. In order to detect *E. coli* clones exhibiting CMCase activity, *E. coli* transformants were screened for degradation of CMC by Congo red dye method (30). Approximately 3,800 white colonies obtained on MacConkey agar containing ampicillin were picked onto LB agar

Table 1. Recombinant plasmids containing CMCase genes of *Clostridium* sp. KCTC8440

Type	Plasmid (pNO number)	Insert DNA (kb)		CMCase activity
		Total size	Fragment Size digested by *Hin*dIII	
A	3, 5	3.0	3.0	+++
	21, 30-2	8.2	3.0, 1.4, 1.0, 0.8, 0.7, 0.6, 0.4, 0.3	+++
	22, 45	4.2	3.0, 0.8, 0.4	+++
	14, 20	4.3	3.0, 0.9, 0.4	+++
	8	4.8	3.0, 1.2, 0.6	+++
B	27, 43, 30-1	3.6	3.6	++
C	42	3.3	1.6, 1.1, 0.6	++
	31	1.6	1.6	++
D	12, 13	6.3	2.0, 1.8, 1.3, 0.7, 0.5	++
	44	9.2	3.5, 1.8, 1.3, 0.8, 0.7, 0.6, 0.5	++
E	2	2.6	2.1, 0.5	+
F	17-1, 18-1	4.1	2.2, 1.9	+
G	22-1	8.7	4.2, 1.9, 1.0, 0.9, 0.5, 0.2	+
	25	7.7	4.2, 1.9, 0.9, 0.5, 0.2	+
	27-1	5.8	4.2, 0.9, 0.5, 0.2	+
	35	5.3	4.2, 0.9, 0.2	+

Plasmids are classfied based on their digestion patterns by *Hin*dIII. CMCase activity was determined on plates by Congo Red method.

supplemented with 0.5% CMC. The overnight grown colonies were stained with 0.2% Congo red solution. After washing with 1M NaCl solution, 24 CMCase positive clones surrounded by yellow halo were obtained as shown in Table 1.

Restriction Enzyme Patterns of the Cloned DNA Fragments Carrying CMCase Genes

In order to investigate the cloned genes, plasmid DNAs were isolated from 24 CMCase positive clones and digested with *Hin*dIII. The plasmid digests were analyzed by agarose gel electrophoresis. The cloned genes were different in total size ranging from 1.6 kb to 9.2 kb. The cloned genes were divided into seven types based on the *Hin*dIII restriction patterns as shown in Table 1. Inserted DNA fragments of seven clones representing each group were digested by several restriction enzymes. The restriction maps obtained are shown in Fig. 1. It is worthy to note that the division of cloned genes based on their restriction enzyme patterns coincided with cellulolytic activities of *E. coli* clones carrying them.

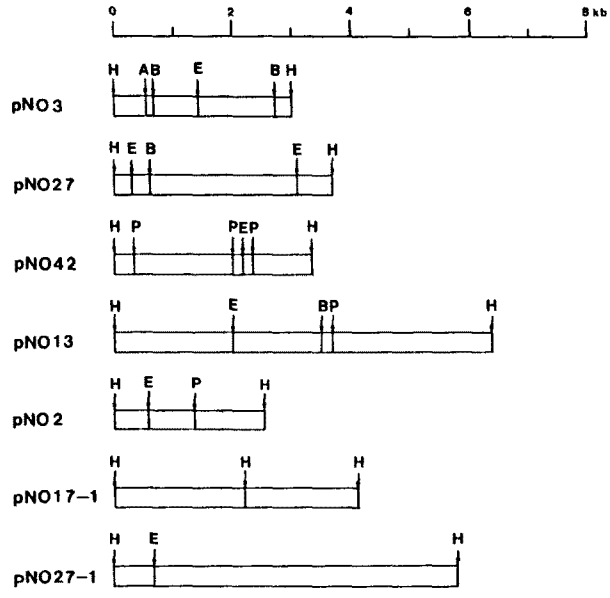

Fig. 1. Restriction endonuclease map of the CMCase genes isolated from mosophilic *Clostridium* sp. KCTC 8440.
Restriction enzymes used are: H; *Hin*dIII, A; *Acc*I, E; *Eco*RI, B; *Bam*HI, P; *Pst*I

Relationships Among the Cloned DNA Fragments

To discover whether there is a homology among the cloned genes or not, Southern blot experiments were performed using 3.0 kb *Hin*dIII fragment of clostridial insert from pNO3 as the probe. As shown in Fig. 2, hybridization occurred only with the inserted DNA fragments prepared from the type A clones. These results probably show that homology between cellulase genes of *Clostridium* sp. KCTC 8440 is very low even though the further analysis is needed. This finding differs from that obtained using *Bacillus* sp. strain N-4, whose two cellulase genes are highly homologous and located tandemly on the chromosome (7, 8).

Enzyme Activity Expressed in *E. coli*

One clone from each group shown in Table 1 was cultivated, and cell-free extracts were prepared to measure enzyme activities on CMC, xylan, pNPC, and MUC (Table 2). As shown in the table, all clones tested showed activities on CMC and xylan. Among them, the clone carrying type A gene pNO3 showed the highest CMCase activity. This result was consistant with the observation made on CMC plate stained by Congo red (Table 1).

In addition to the high CMCase activity, *E. coli* transformant carrying pNO3 showed pNPC and MUC degrading activity. Because of the high CMCase activity,

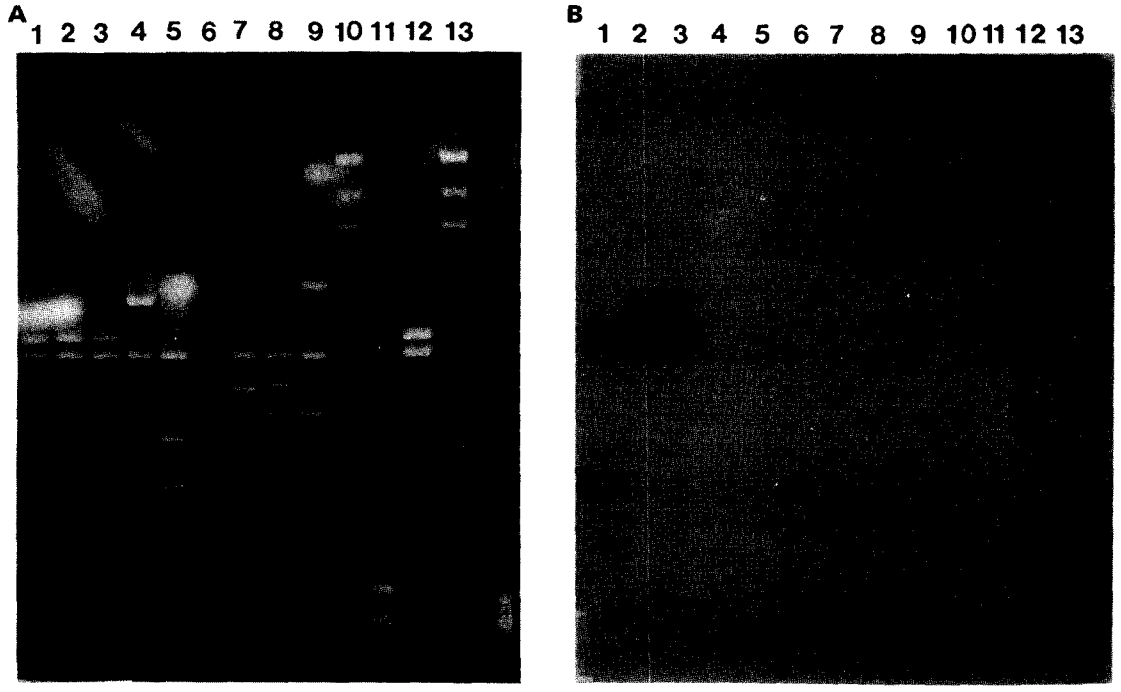

Fig. 2. Southern hybridization of the inserted DNA fragments of recombinant plasmids with pNO3 insert as the probe.

A, Agarose gel electrophoresis of *Hin*dIII digests of recombinant plasmids. Lanes: 1; pNO21, 2; pNO14, 3; pNO8, 4; pNO27, 5; pNO42, 6; pNO13, 7; pNO2, 8; pNO17-1, 9; pNO25, 12; pNO3. Molecular size standards: lane 10 and 13; λ-DNA digested with *Hin*dIII, lane 11; pUC19 digested with *Hae*III

B, Autoradiograph of the gel (shown in A) hybridized with probe DNA.

Table 2. Enzymatic activities expressed by *E. coli* clones carrying recombinant plasmids.

Recombinant Plasmid	Specific activity (mU) on			MUC degrading activity[3]	CMCase / Xylanase
	CMC[1]	Xylan[1]	pNPC[2]		
pNO3	176	46	10	+	3.83
pNO27	38	37	<1	−	1.03
pNO42	18	21	<1	−	0.86
pNO13	15	22	<1	−	0.68
pNO2	26	22	<1	−	1.18
pNO17-1	15	22	<1	−	0.62
pNO22-1	24	46	<1	−	0.52

1) Specific activity is expressed as μmole of reducing sugar liberated per min per mg of protein.

2) Specific activity is expressed as μmole of *p*-nitrophenol liberated per min per mg of protein.

3) Methylumbelliferyl-cellobiopyranoside degrading activity is expressed as positive(+) or negative(−).

the cellulolytic enzyme produced from pNO3 seems to be an endoglucanase. Both pNPC and MUC are considered as the substrates for exoglucanase, but these chromogenic substrates were degraded by certain endoglucanases (1, 6, 24). Another possibility is that pNO3 carries genes for exoglucanase activity as well as endoglucanase.

Differences in CMCase activities of cell free extracts prepared from the *E. coli* strains carrying different types may be attributable to the expression efficiency of the cloned genes in *E. coli* or specific activity of enzyme. As suggested earlier all clones tested showed CMCase and xylanase activities. They could be divided into three groups according to the ratios for activity of CMCase to that of xylanase. The transformant carrying pNO3 showed the ratio of about 3.83 whilst the value of about 0.52 was obtained in the transformant carrying pNO22-1. Others showed the ratio of about 1. From these values it is concluded that the differences in CMCase activities among the clones are due to the nature of the enzymes. Because the size of the cloned DNA in some of the plasmids are bigger than for one protein, further characterizations are needed.

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