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## **Molecular Cloning of Nucleotide Sequence of a Neutral Cellulase Gene of Thermophilic *Clostridium* sp. F-3**

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A 1.7-kb HindIII fragment of *Clostridium* DNA cloned in *Escherichia coli* was shown to direct the synthesis of a neutral cellulase. The optimum pH was 6.5 for the enzyme encoded on the 1.7-kb DNA fragment and 7.5 for the enzyme produced by transformant was slightly decreased from 70 °C to 65 °C. The neutral cellulase gene was located on a 1.2-kb DNA fragment as a functional state, derived from the 1.7-kb DNA fragment, and the nucleotide sequence was determined. The coding sequence showed an open reading frame encoding 552 bp corresponding to 184 amino acid residues with molecular weight of 20,378 daltons. However, the regulatory sequences and/or N-terminal region of the encoded polypeptide were not detected in the sequence encoded the neutral cellulase gene. The enzyme encoded on the 1.7-kb fragment might consist of a half region (C-terminal region) of the mature enzyme, and be expressed by *lacZ* promoter of vector, pUC9.

### **INTRODUCTION**

Cellulase (1, 4- $\beta$ -D-glucan Glucanohydrolase, EC 3. 2. 1. 4) extracellularly produced by various microorganisms has been the subject of many enzymological studies as well as being applied for the biodegradation of cellulose materials. Cloning of the cellulase genes from these organisms would be useful not only for increasing the enzyme productivity but also for obtaining the informations on structure/function relationship of these enzymes.

The cellulase genes of *Clostridium thermocellum* (Cornet et al., 1983a and 1983b), *Cellulomonas fimi* (Whittle et al., 1982 ; Gilkes et al., 1984), *Trichoderma reesei* (Shoemaker et al., 1983), *Thermoanaerobacter cellulolyticus* (Honda et al., 1987), *Bacillus subtilis* (Koide et al., 1986) and alkalophilic *Bacillus* strains (Sashihara et al., 1984 ; Fukumori et al., 1986b) have already been cloned, and recently, the nucleotide sequences of the cellulase gene of *C. thermocellum* (Beguín et al., 1985 ; Grepinet and Beguín, 1986 ; Joliff et al., 1986), *Cellulomonas uda* (Nakamura et al., 1986) and *Pseudomonas fluorescens* subsp. *cellulosa* (Hall and Gilbert, 1986), the  $\beta$ -glucanase gene of *C. acetobutylicum* (Zappe et al., 1988) and *B. subtilis* (Murphy et al., 1984 ; Nakamura et al., 1987) and the alkaline cellulase gene of the alkalophilic *Bacillus* sp. (Fukumori et al., 1986a and 1986b) and *Streptomyces* (Nakai et al., 1988) were determined.

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However, nothing is known about the structure of DNA regions controlling gene expression or about signal sequences governing protein transport in gram-positive thermophiles. In order to increase cellulase production and understand the structure and function of cellulase(s) of this strain, we cloned a neutral cellulase gene of thermophilic *Clostridium* sp. F-3 in *Escherichia coli* JM83. The present paper deals with the cloning and expression of a neutral cellulase gene in *E. coli* and the nucleotide and deduced amino acid sequences of the cloned cellulase gene derived from thermophilic *Clostridium*.

## MATERIALS AND METHODS

### Bacterial strains and plasmid

*Clostridium* sp. F-3, a producer of alkaline and neutral cellulases, isolated from sewage sludge (Kume and Fujio, 1986). After complete consumption of cellulose in the medium, cells were harvested for the DNA preparation. *Escherichia coli* K12 strain JM83 and recombinant clones were cultivated in Luria-Bertani (LB) medium at 37 °C. When necessary, 50 µg/ml ampicillin (Amp) was added to the medium. Plasmid pUC9 was used as a vector for the cloning and in DNA sequencing.

### DNA preparation and construction of recombinant DNA

Chromosomal DNA was prepared from the isolated *Clostridium* strain F-3 by the method of Saito and Miura (1963). Plasmid DNAs were extracted from *E. coli* transformants by the alkaline lysis procedure (Birnboim and Doly, 1979). Chromosomal DNA of the *Clostridium* strain F-3 was partially digested with *Hind*III and ligated to dephosphorylated-*Hind*III-cut pUC9. The ligated DNA was used to transform competent cells of strain JM83 (Cohen *et al.*, 1972). Cellulase-producing transformants were selected by the Congo-red method as described by Cornet *et al.* (1983a).

### Enzyme assay

For the cellulase assay, the amount of reducing sugars produced by the enzyme reaction was measured. Reaction mixture contained 0.1 M potassium phosphate buffer (pH 7.0), 1.0% carboxymethyl cellulose (CMC) and suitable amount of crude enzyme solution. The mixture was incubated at 60 °C for 10 min, then enzyme reaction was stopped by boiling for 5 min. The reducing sugars were determined by the method as described by Sumner and Somer (1944). One unit of enzyme activity was defined as 1 µmol glucose-equivalent reducing sugars released per minute.

### DNA labeling and hybridization of DNA digests

DNA was labeled, as nonradioactive system, by random primed incorporation of digoxigenin-labeled deoxyuridine-triphosphate (Feinberg and Vogelstein, 1983). The hybridization technique used was essentially the same that described previously (Southern, 1975). After hybridization, the hybrids were detected with enzyme immunoassay by using DIG-ELISA method under the conditions recommended by the supplier (Boehringer Mannheim GmbH, West Germany).

### DNA sequencing

This was done by the dideoxy chain termination method (Sanger et al., 1977), specific restriction fragments being cloned into pUC9 vector for dideoxy sequencing by using  $\alpha$ -<sup>32</sup>PdCTP.

### Reagents

All restriction enzymes used were obtained from commercial suppliers and were used according to manufactures' recommendations. T4 DNA ligase and M13 Sequencing kit were from Takara Shuzo Co., Ltd.  $\alpha$ -<sup>32</sup>PdCTP was obtained from Amersham International Plc., UK. *E. coli* DNA polymerase I and nonradioactive DNA labeling and detection kit were purchased from Boehringer Mannheim GbmH, West Germany. The CMC, with a degree of substitution of 0.65 and an average molecular weight of 110,000 was obtained from Nakarai Chemical Co., Kyoto, Japan. Congo-red was purchased from Merck Co., Ltd.

## RESULTS

### Cloning of a cellulase gene in *E. coli*

The chromosomal DNA of the isolated *Clostridium* strain F-3 and plasmid pUC9 DNA were digested with *Hind*III restriction endonuclease and ligated with T4 DNA ligase. The ligation mixture were used to transform into *E. coli* JM83 and about 10<sup>4</sup> Amp<sup>r</sup> transformants per  $\mu$ g of DNA were obtained. Only one transformant produced a shallow crater around colonies on CMC-containing LB plate. The cellulase-producing transformant was identified by a clear zone around the colonies on CMC-Congo red plates. The plasmid DNA was isolated from this transformant and designated pIK1. The pIK1 contained 1.7-kilobases (kb) fragment and could transform *E. coli* to Amp<sup>r</sup> Cel<sup>+</sup> at high frequency. Therefore, we concluded that this fragment contained the cellulase gene. Figure 1 shows the restriction map of pIK1. The 1.7-kb DNA fragment of pIK1 was found to possess two sites with *Hinc*II and three sites with *Eco*RI and *Hind*III.

### Some enzymatic properties of *Clostridium* cellulase produced by *E. coli*

The authentic enzyme prepared from *Clostridium* sp. F-3 indicated cellulolytic activity on various substrates, while the enzyme produced by transformant only on a CMC as a substrate (Table 1). The effects of pH and temperature on the cellulase activity were determined by measuring the reducing sugars derived from CMC (Fig. 2). The optimum pH was 7.5 and 10.0 for the authentic enzyme prepared from *Clostridium* sp. F-3, and 6.5 for the enzyme encoded by the plasmid pIK1 (Fig. 2A). The thermal stability of the enzyme prepared from *Clostridium* sp. F-3 was quite stable up to 70 C, while that of transformant up to 65 C (Fig. 2B). Both enzymes produced by *Clostridium* sp. F-3 and the transformant were rapidly inactivated at 75 C with similar profile. These results support that the neutral cellulase gene derived from the strain *Clostridium* sp. F-3 was cloned and expressed in *E. coli* JM83.

### Homology of plasmid pIK1 with chromosomal DNA

Location of the cellulase gene in the 1.7-kb fragment was studied by constructing

smaller derivatives as shown in Fig. 3. The transformant carrying either pIKH1 or pIKH2 exhibited a level of cellulase activity similar to that of the original clone. The transformant carrying pIKH5, which deleted the *Hinc*II-*Hind*III fragment from the pIK1, did not, however, show the enzyme activity. These results suggest that the gene might be located on the fragment between *Hinc*II and *Hind*III sites (Fig. 3). To confirm that the 1.7-kb DNA fragment was derived from *Clostridium* sp. F-3, the 1.2-kb *Hinc*II-*Hind*III fragment of pIK1 was isolated, labeled by random primed incorporation of digoxigenin-labeled deoxyuridine-triphosphate and hybridized to chromosomal DNAs by the method of Southern (1975). As shown in Fig. 4, the labeled probe

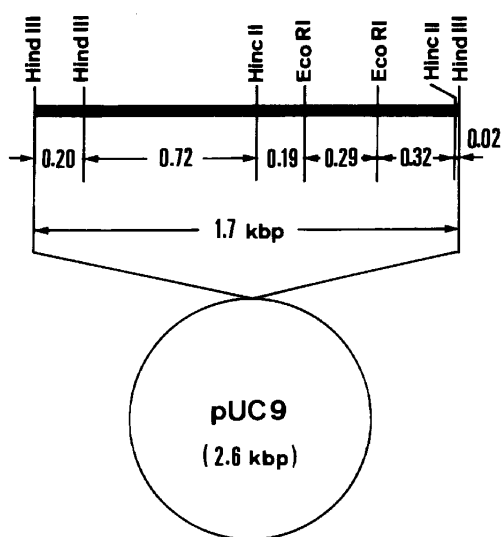


Fig. 1. Restriction map of plasmid pIK1.

Restriction sites are indicated, and their coordinates are given in kb. The thick line shows the cloned 1.7-kb fragment from *Clostridium* sp. F-3, and the thin line the vector, pUC9.

Table 1. Comparison of cellulase productivity on various substrates between *Clostridium* sp. F-3 and *E. coli* JM83 (pIK1)

Substrate	<i>Clostridium</i> sp. F-3	<i>E. coli</i> JM83 (pIK1)
CM-cellulose <sup>a</sup>	1.16	0.64
Filter paper <sup>b</sup>	0.27	ND <sub>c</sub>
Avicel <sup>b</sup>	0.16	ND <sub>c</sub>
Cotton <sup>b</sup>	0.03	ND <sub>c</sub>
Laminarin <sup>a</sup>	0.07	ND <sub>c</sub>

The values are expressed as units per milliliter of enzyme solution.

<sup>a</sup>1 unit=1  $\mu$ mole of glucose released per min.

<sup>b</sup>1 unit=1  $\mu$ mole of glucose released per hr.

<sup>c</sup>Not detected.

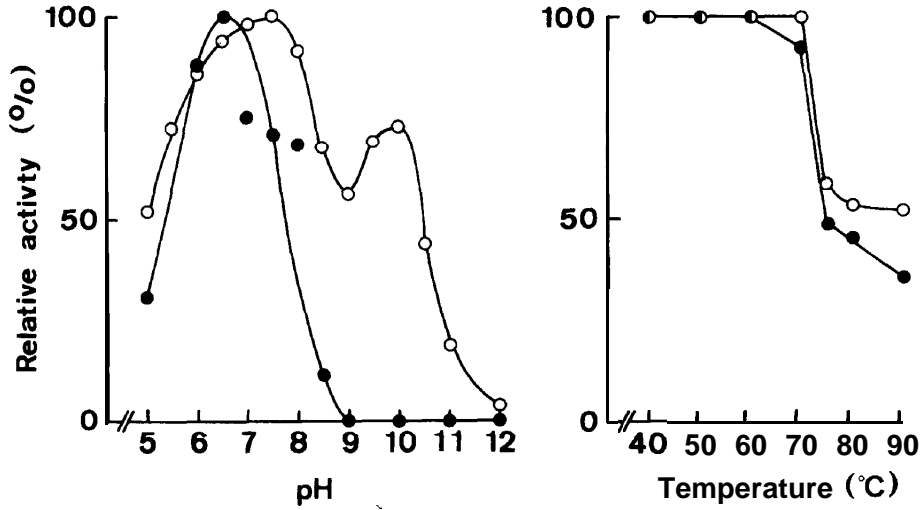


Fig. 2. Comparison of optimum pH and thermal stability produced by *Clostridium* sp. F-3 (●) and *E. coli* JM83 (pIK1) (○).

(A) The optimum pH was measured by using CMC as a substrate at 60 °C for 10 min and the following buffer systems were used: 0.05M  $\text{CH}_3\text{COOH}-\text{CH}_3\text{COONa}$  (pH 4-6), 0.05M  $\text{KH}_2\text{PO}_4-\text{K}_2\text{HPO}_4$  (pH 6-8), 0.05M  $\text{KH}_2\text{PO}_4-\text{Na}_2\text{B}_4\text{O}_7$  (pH 8-9) and 0.05M  $\text{Na}_2\text{B}_4\text{O}_7-\text{NaOH}$  (pH 9-12). (B) The thermal stability was measured after incubation at indicated temperature for 10 min, and then enzyme activity was measured by using CMC as a substrate.

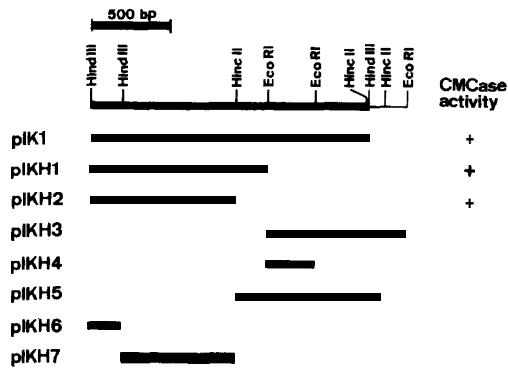
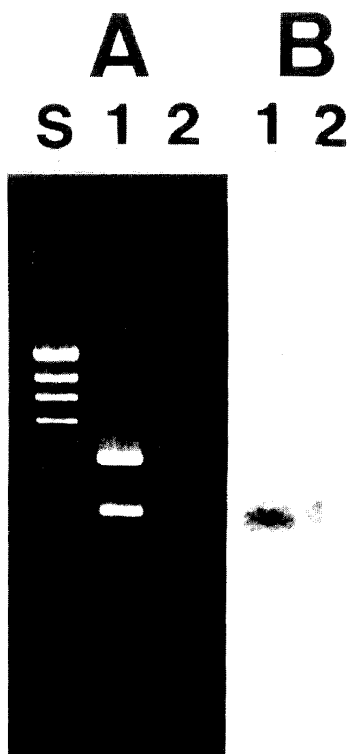


Fig. 3. Deletion analysis of cellulase gene encoding on 1.7-kb fragment. Thick line indicates DNA insert derived from 1.7-kb DNA fragment.



**Fig. 4.** (A) Agarose gel electrophoresis of digests of pIK1 (lane 1) and *Clostridium* sp. F-3 chromosomal DNA with Hind III (lane 3).

The DNA was denatured, transferred to a nitrocellulose filter and then hybridized with the 1.7-kb insert DNA fragment labeled by random primed incorporation of digoxigenin-labeled deoxyuridine-triphosphate. Hind III fragments of phage  $\lambda$  DNA (lane S) were used as molecular size markers.

Hybridization patterns with digoxigenin-labeled probe (B).

hybridized strongly to the *Hind*III digests derived from chromosomal DNA prepared from the isolated strain *Clostridium* sp. F-3.

#### DNA sequence

Subcloning of the 1.7-kb insert of pIK1 revealed that the cloned cellulase gene was located on a 1.2-kb *Hinc*II-*Hind*III fragment. As shown in Fig. 5, the nucleotide sequence of the 1.2-kb insert DNA of pIKH2 was determined by the chain-terminating dideoxy method (1977). Though the strategy is not shown, the nucleotide sequence was determined for both strands using numerous restriction fragments to give enough overlapping regions. The pIKH2 contained a single open reading frame of 552 base pairs (bp), which encoded 184 amino acid residues (Fig. 5). The relative molecular mass calculated from the DNA sequence was 20,378 daltons. On this coding frame,

there were two initiation codon (ATG at positions 8-10 and 11-13), but we could not detect the potential initiation codon preceded by a ribosome binding site. However, a palindromic repeat sequence of 5 bp was found 44 bp downstream of the termination codon, which seemed to resemble a p-independent terminator. We therefore concluded that only C-terminal region of the mature cellulase gene located on the 1.7-kb *Hind*III DNA fragment derived from *Clostridium* sp. F-3 was cloned, and only a limited segment of the authentic enzyme was expressed by *lacZ* promoter of pUC9 in *E. coli* JM83.

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5'  AAGCTTAATGATGAATTCATGTTTGAACCGCTACCGAAGTATCAGATTGAGGGTGGGGA    60
    SerLeuMetMetAsnSerCysLeuGluProLeuProLysTyrGlnIleGluGlyGlyAs

    TACGGGAAATACATGGTATAAGTGGTGCCAGGCAGGACGTATCAAGGACTCCAGCAGCTG    120
    pThrGlyAsnThrTrpTyrLysTrpCysGlnAlaGlyArgIleLysAspSerSerSerCy

    TATCACTGCATGTGACCATTGGACAGGTGAGGAGGATACGGAGCTGTTGAAGAACTTGGG    180
    sIleThrAlaCysAspHisTrpThrGlyGluGluAspThrGluLeuLeuLysAsnLeuGl

    AGTTCAAACCCACAGAATGAGTCTTGAGTGGAGCAGAATAGAGCCTTCCAGGGGCAAATT    240
    yValGlnThrHisArgMetSerLeuGluTrpSerArgIleGluProSerArgGlyLysPh

    TTCCGATGACGCAATGAAACATTACAGAGATGAGATTAAGCTTTTGGATGCAAGCGGAAA    300
    eSerAspAspAlaMetLysHisTyrArgAspGluIleLysLeuLeuAspAlaSerGlyAs

    CGAGCTTGTAATGAGGGGCATGCGTGATATTTTCAGCAATAGATTTGGTTAAAGAAATAAA    360
    nGluLeuValMetArgGlyMetArgAspIleSerAlaIleAspLeuValLysGluIleLy

    AATCGGATGGAATTTGGGAAATACTTTGGATGCTCCTACAGAGACTGCCTGGGGAAATCC    420
    sIleGlyTrpAsnLeuGlyAsnThrLeuAspAlaProThrGluThrAlaTrpGlyAsnPr

    AAGGACAGCCAAGGCAATGATAGAAAAGGTAAGGGAAATGGGCTTTAATGCCGTCAGAGT    480
    oArgThrAlaLysAlaMetIleGluLysValArgGluMetGlyPheAsnAlaValArgVa

    GCCTGTTACCTGGGATACGCACATCGGACCTTCTCCGACTATAAAATTGACGAAGCATG    540
    lProValThrTrpAspThrHisIleGlyProSerProAspTyrLysIleAspGluAlaTr

    GCTGACACAGAGTTGAGGAAGTGGTAAACTATGTTCTTGACTGCGGATCATAAATGTTCA    600
    pLeuThrGlnSer***

    CCATGACAATAATGGATTATACCTACATATGCCAATGAGCAAAGGAGTAAAGAAAAACT    660
    TGTA AAAAGTTTGGGAACAAATAGGCAACCCCGTTTTAAAGATTATGACGACCATTTGTT    720
    GTTTGAGACAATGAACCGGAACCGAGAGAAGCTAGGTTACCTATGGAATGGATGGGCGG    780
    ACCGTATGAAAACCGAGATGCGATAAACAGATTTAATTTGGCGGCTGTTAATACCATCAG    840
    AGCAAGCGGCGGAAATAACGATAAAAGATTCTACTGGTTCGACCAATCGGCAACCGGCC    900
    TGGATGTTGCATTAACGACCTTGTCAATCCGAACAATCGGACAGGAGATCATAGTATC    960
    CATACATGCTTATTCACCGTATTTCTTGCTATGGATGTC    3'

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**Fig. 5.** Nucleotide sequence of the cellulase gene from *Clostridium* sp. F-3.

The DNA sequence of the coding strand is given from 5' to 3', numbered from nucleotide 1 at the cloning site. The predicted amino acid sequence is given below the DNA sequence.



Amino acid sequence of the predicted cellulase gene was compared with a number of protein sequences registered in Genbank with use of the homology search system of GENAS (Kuhara *et al.*, 1984). No convincing homology could be found among various cellulases ; endoglucanases A, B and D from *C. thermocellum* and alkaline cellulase and  $\beta$ -glucanase from *B. subtilis*, with the neutral cellulase of *Clostridium* sp. F-3, these cellulases for which complete sequence data are available.

## DISCUSSION

We have cloned a neutral cellulase gene of *Clostridium* sp. F-3, produced two kinds of cellulase with the optimum pH at 7.5 and 10.0 in the process of fermentative solubilization of sewage sludge by mix culture (Kume and Fujio, 1986). The authentic enzyme prepared from *Clostridium* sp. F-3 released reducing sugars from various substrates, while the enzyme produced by transformant degraded only CMC as a substrate (Table 1). The optimum pH and thermal stability of the enzyme produced by transformant were slightly decreased as compared with the authentic enzyme prepared from *Clostridium* sp. F-3 (Fig. 2). These results, therefore, indicate that the cloned cellulase gene is corresponding to a neutral CMCase, and it is very interesting to reveal the different features of both enzymes. The nucleotide sequence of subcloned 1.2-kb DNA fragment of pIKH2 contained a single open reading frame of 552 bp, which was corresponding to 20,378 as a relative molecular mass. However, we could not detect any regulatory sequences of promoter on the upstream of the open reading frame encoding the neutral cellulase gene of *Clostridium* sp. F-3 (Fig 5). On the other hand, Kume and Fujio (1986) reported that the molecular mass of the purified neutral cellulase of *Clostridium* sp. F-3 was about 40,000 by SDS-PAGE. Based on these results, we concluded that the region of the cloned cellulase gene is corresponding to a half size containing the C-terminal region of the matured enzyme and that the cellulolytic enzyme activity was expressed by *lacZ* promoter of vector pUC9. It, furthermore, contained 4 cystein residues to be formable disulfide bridges in a polypeptide (Fig. 5), so that the enzyme produced by transformant might indicate the thermal stability up to at 65 C. In fact, the enzyme had been shown to be insensitive to oxidation or to SH-modifying reagents (Kume and Fujio, data not shown). It must, therefore, be assumed that free SH groups either are not required for enzymatic activity or are protected against chemical modification.

No convincing homology could be found among the complete sequences of the cloned cellulase genes by GENAS, but only a Ala-Ser-Gly-Asn-Glu-Leu-Val-Met segment was detected as homologous region with endo-glucanase A of *C. thermocellum* (Beguin *et al.*, 1985) and endo- $\beta$ -1, 4-glucanase of *B. subtilis* (Murphy *et al.*, 1984). However, we have no information concerning with the relationship between structure and function of cellulolytic enzymes because of the insoluble crystalline nature of cellulose. Further comparison of the amino acid sequence of the *Clostridium* cellulase with those of *Clostridium* cellulases and amino acid residue conversion by in site mutagenesis should provide information on the structure determining the optimum pH and thermal stability for the enzyme activity.

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