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Cloning and DNA Sequence of Carboxymethylcellulase (CMCase) Gene from *Cellulomonas* sp. YE-5

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CMCase positive clones were screened from *Cellulomonas* sp. YE-5 and named pCE1, pCE2 and pCE3. Among the positive clones pCE1 was used for this study, because it has the smallest insert and the highest CMCase activity among the 3 clones, and its nucleotide sequence was determined. The CMCase gene in pCE1 was composed of 1071 bp of nucleotides coding 357 amino acids. Computer analysis showed that the pCE1 has 65% sequence homology with the endoglucanase from *Cellulomonas fimi*.

Cellulosic material is a reproducible carbon source, and is the most abundant naturally occurring biopolymer. Therefore, many studies were done using cellulosic material as a carbon source to produce bioenergy and other useful products. In order to use cellulose as a carbon source, cellulosic materials need to be decomposed back into its components.

Generally, there are two methods of hydrolysing cellulose to glucose. One is a chemical treatment using acid or base, and the other is enzymatic degradation using cellulase. The problems of using chemical treatment are the difficulty in eliminating acid or base after treatment, and the mass production of waste-water during treatment. On the other hand, enzymatic degradation has some advantages such as the facility in enzyme recovery, substrate specificity, reduction of energy consumption and waste-water production (10). But, still the high cost of enzymes and the low yield of cellulose hydrolysis are limiting factors.

Studies on cellulase originated from studies on fungi such as *Trichoderma* (18), *Penicillium* (21), *Phanrochaete, Fusarium* (12), and *Sporoticum* (11). However, practical use of cellulase from fungi has been restricted, because of the low productivity, the difficulty in gene manipulation and the long culture time and so on. Therefore, the use of cellulolytic bacteria is more concerned lately. In this study, we cloned cellulase gene from *Cellulomonas* sp. YE-5 (6) which has high cellulase activity, and determined its DNA sequence.

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MATERIALS AND METHODS

Bacteria and Plasmids

Cellulomonas sp. YE-5 (6), isolated from soil, was used as the cellulase gene donor strain. *Eschrichia coli* JM83 was used as the cloning host, and *E. coli* JM109 as the sequencing host. The plasmid vector pUC19 was used for cloning and subcloning, and M13mp19 for sequencing.

Media

Luria broth(LB) and YT medium were used for the transformation of *E. coli*, and M9 agar medium was used for the preservation of *E. coli* JM109, and 2YT medium for the preparation of a single strand DNA. LB medium containing 0.5% (W/V) CMC and ampicillin (100μg/ml) was used for the production of CMCase from the recombinants.

Isolation of Chromosomal DNA

Modified Chaster's method (5) was used for isolating chromosomal DNA. *Cellulomonas* sp. YE-5 was cultured on LB medium at 37℃ for 48 hrs, and the supernatant was removed by centrifugation, and 2 g of the cells were washed with 50 ml of TE buffer. The cells were suspended with 10 ml of TE buffer, and incubated at 37℃ for 30 min with 20 mg of lysozyme. Then EDTA and SDS were added to the mixture at the final concentration of 2.4 ml of 0.5 M EDTA and 1.4 ml of 10% SDS respectively. The mixture was further incubated at 37℃ for 2 hrs. After incubation, 0.25 volume of 5 M NaCl was added to the lysed solution. Phenol/chloroform extraction was done three times and the upper

phase was collected. The Chromosomal DNA was precipitated by addition of 2.5 volume of 100% ethanol to the upper phase, and resuspended with 20 m*l* of TE buffer. The DNA solution was treated with 0.1 m*l* of RNase A (10 mg/m*l*) at 37℃ for 2 hrs, then with 0.1 m*l* of proteinase K (20 mg/m*l*) at 37℃ for 1 hr, and precipitated with 2.5 volume of ethanol after extraction with phenol/chloroform. The precipitated DNA was resuspended with 5 m*l* of TE buffer and used for this study.

Isolation of Plasmid DNA

Plasmid DNA of *E. coli* JM83 was isolated by the alkali lysis method of Maniatis *et al* (12).

Isolation of CMCase Recombinants

Transformed white colonies were screened on LB agar medium containing X-gal (50 μg/m*l*) and ampicillin (100 μg/m*l*), and the selected recombinant colonies were transferred by a toothpick onto LB agar medium containing 0.5% (W/V) of CMC, and cultured overnight at 37℃. The CMCase positive clones were detected by Congo-red staining.

DNA Sequencing

The DNA sequence was determined by Sanger's dideoxy chain termination sequencing method (15).

Preparation of Deleted Subclones

Deleted subclones were prepared to determine the DNA sequence by Erase-A-Base system (2, 9, 22) deviced by Henikoff (8).

RESULTS AND DISCUSSION

Cloning of CMCase Gene

In order to clone CMCase gene of *Cellulomonas* sp. YE-5, chromosomal DNA was partially digested with *Pst*I, and pUC19 was digested with same enzyme, then transformed into *E. coli* JM83 after ligation using T4 DNA ligase. For the primary screening, transformants were cultured on LB agar medium contaning X-gal (50 μg/m*l*), and white colonies were selected and cultured on LB agar medium containing 0.5% (W/V) CMC and ampicillin (100 μg/m*l*) at 37℃ for 16 hrs. The transformants were lysed by chloroform vapor and D-cycloserine, and the CMCase positive clones were detected by Congo-red staining.

Among fifteen thousand transformants of the primary screening, 3 transformants showed the CMCase activity. When plasmids were isolated from the CMCase positive clones and retransformed into *E. coli* JM83, all retransformants have showed CMCase activity (data about pCE2 and pCE3 are not shown), indicating that the recombinant plasmids are stable (Fig. 1).

When the three CMCase positive clones were digested with *Pst*I and run on the agarose gel, the three clones showed 5.3 kb, 12.7 kb and 6.2 kb inserts, and were named pCE1, pCE2 and pCE3, respectively (Fig. 2). Because of the smallest insert and the highest CMCase activity among the three clones (unpublished data), pCE1 was chosen for this study.

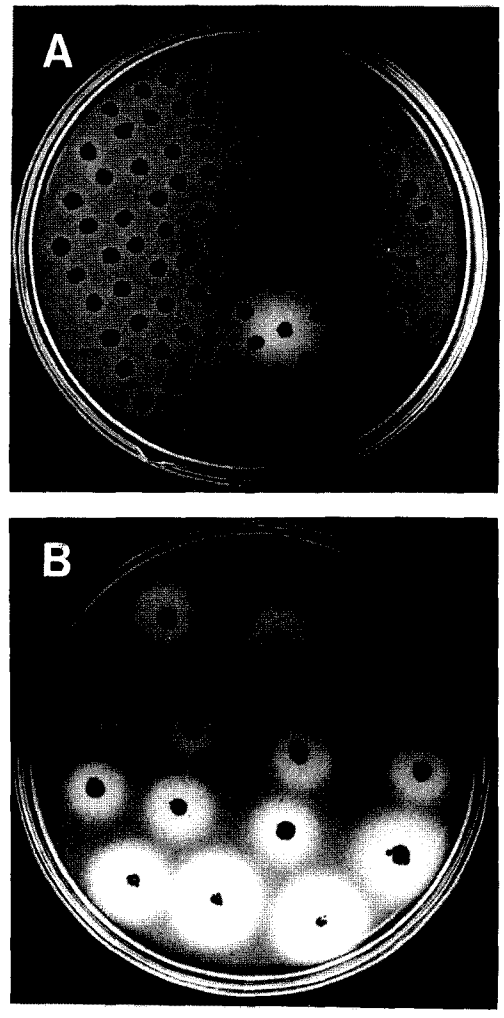


Fig. 1. Detection of recombinant *E. coli* JM83 containing CMCase gene by Congo-red staining.
Panel A, CMCase positive clone pCE1; panel B, retransformed clones of pCE1.

Subcloning of pCE1

Because the insert of pCE1 carring CMCase gene had no *EcoR*I, *Kpn*I, *BamH*I, *Xba*I and *Hind*III site, and had more than 3 recognition sites for *Sac*I, *Sma*I, *Sal*I and *Xho*I, the pCE1 was digested with *Sac*I, *Sma*I, *Sal*I and *Xho*I, and self-ligated, then transformed into *E. coli* JM 83. Among the transformants treated with *Sac*I, more than 100 transformants showed halo zone on the CMC-plate. One of the *Sac*I treated CMCase positive clones,

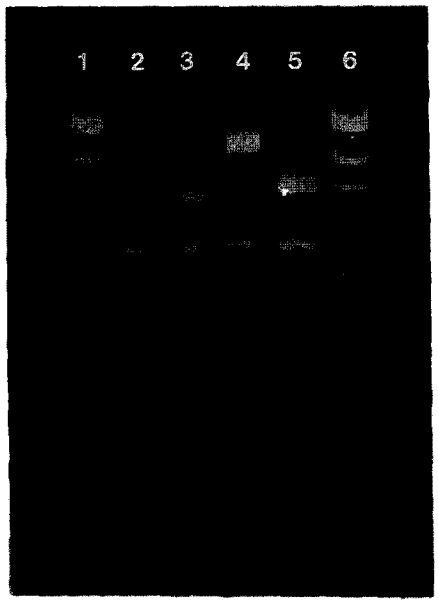


Fig. 2. EtBr staining gel pattern of CMCase positive clones after digestion with *PstI*.
Lane 1 and 6, λ DNA digested with *Hin*dIII; lane 2, pUC19; lane 3, pCE1; lane 4, pCE2; lane 5, pCE3.

which had the smallest 2 kb insert and relatively high CMCase activity, was selected and named pCE101. According to the electrophoresis band pattern, the fragment of pCE1 between *Pst*I and *Kpn*I was missing (Fig. 3).

Preparation of Deleted Subclones

In order to make serially deleted suclones, pCE101 was digested with *Eco*RI and *Hin*dIII. The insert was electroeluted and modified into a blunt-end fragment by the treatment of T4 DNA polymerase in the presence of dNTP, and subcloned into M13mp19 vector digested with *Hin*cII.

Using the Erase-A-Base system, 15 clones, containing different sizes of inserts, were isolated and named M13 mp19CEH1-M13mp19CEH15, according to the size of the inserts starting from the biggest one. The M13mp19 CEH series were digested with *Eco*RI and *Hin*dIII, and the inserts were resubcloned into pUC19 and named pCEHE1-pCEHE15, respectively. And the CMCase activity of the pCEHE series were checked on the CMC-plate using Congo-red staining (Fig. 4).

According to the result in Figure 4, at least 1,100 bp of nucleotides from *Hin*dIII site were required to show CMCase activity in *Cellulomonas* sp. YE-5 (6).

Determination of DNA Sequence of CMCase Gene

We confirmed that each 100-150 bp of the inserts of M13mpCHEH1 were deleted in M13mpCHEH series (Fig. 4), and the DNA sequence and amino acids coding

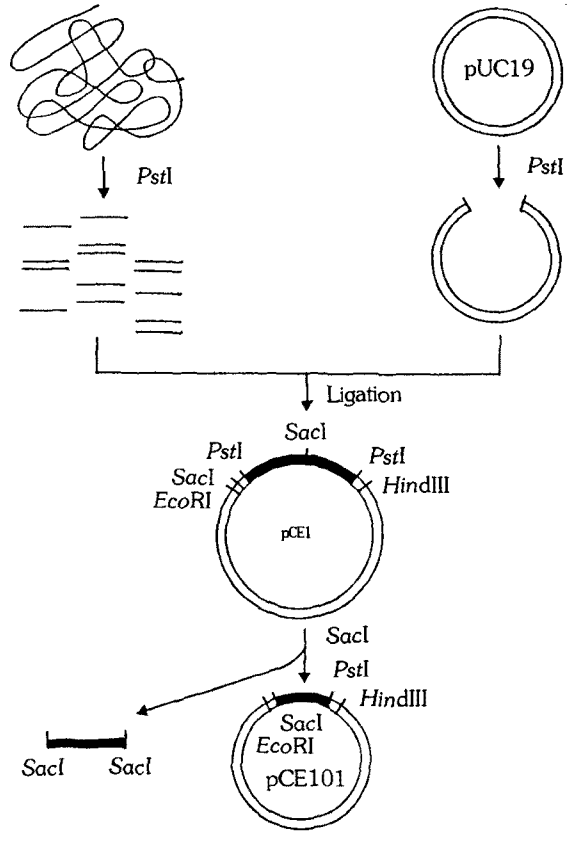


Fig. 3. Preparation of pCE1 and pCE101.

region of CMCase gene were determined by single DNA sequencing of M13mpCHEH series and double strand DNA sequencing of pCHEH series using universal primer and reverse primer (Fig. 5).

The CMCase gene was composed of 1071 nucleotides coding 357 amino acids, which can organize a protein of approximately 39 KDa of molecular weight.

These results coincided with the previous publication (6), and the size of CMCase gene of *Cellulomonas* sp. YE-5 was simillar to those of *Prevotella ruminicola* (7), *Clostridium thermocellum* (4), *Ruminococcus flavefaciens* (16) and the catalytic domain of *Clostridium thermocellum* (1, 20) and *Clostridium cellulolyticum* (3). The sizes of these enzymes were very small compared to most of the published cellulases, so the CMCase gene of *Cellulomonas* sp. YE-5 is supposed to be in charge of one of the catalytic domain of cellulosome.

The pCE101 had whole CMCase structural gene of *Cellulomonas* sp. YE-5, 860 bp of 3'-untranslated region, and 60 bp of 5'-upstream region from the translation start site. The entire promoter region for CMCase gene was not found on the PCE101. The putative Shine-Dalgarno-type rebosome binding site, however, was lo-

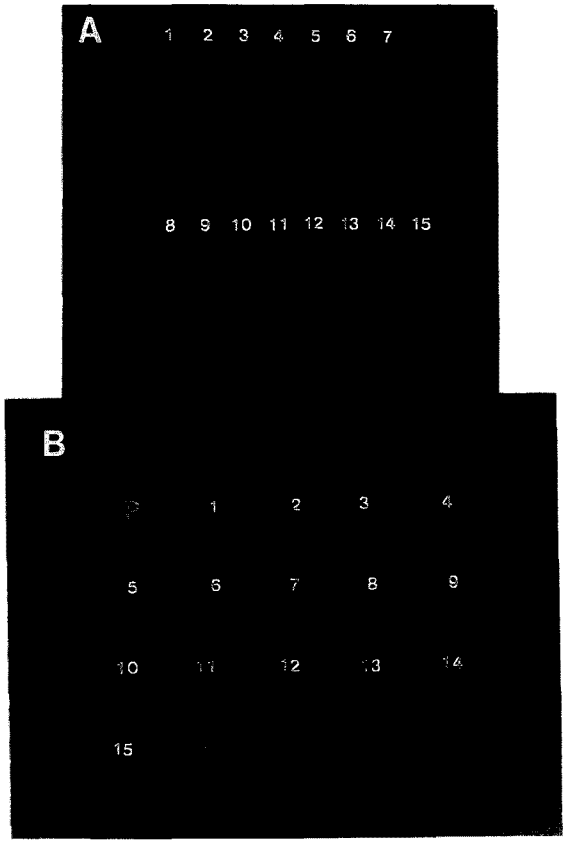
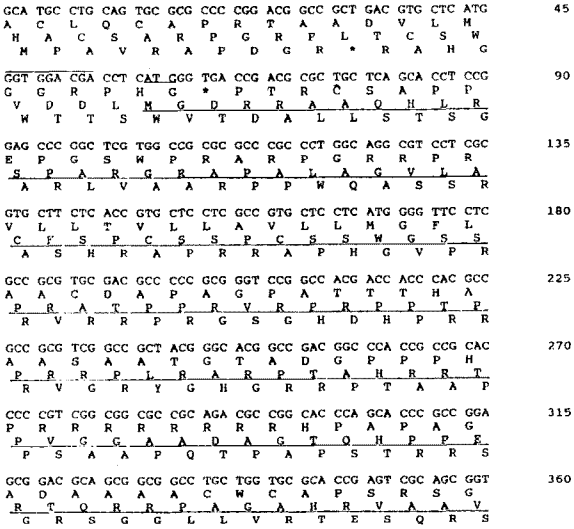


Fig. 4. Deleted mp19 CEH series and its Congo-red staining pattern.

Panel A, EtBr staining gel pattern of deleted mp19 CEH series; panel B, Congo-red staining pattern of corresponding clone in panel A, P and N represent positive contro (pCE101) and negative control, respectively.



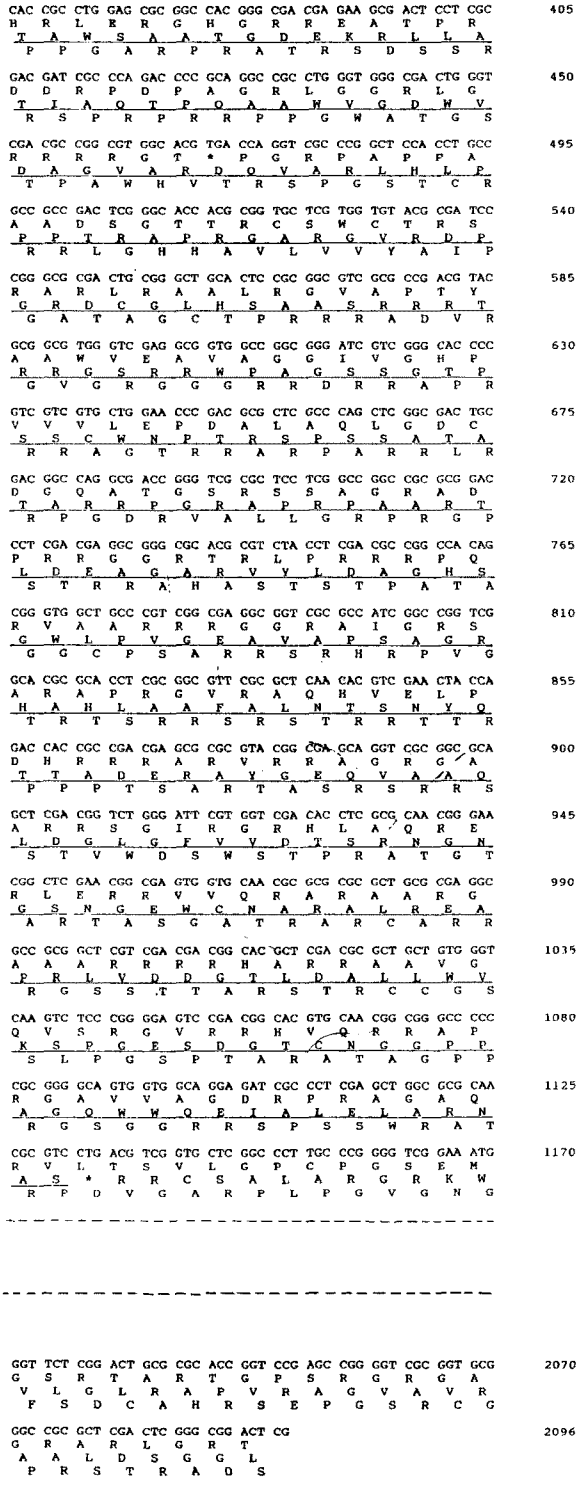


Fig. 5. Nucleotide and deduced amino acid sequence of CMCase gene from pCE101.

Translation start site and deduced acids are underlined, and putative Shine-Dalgarno-type ribosome-binding site is overlined.

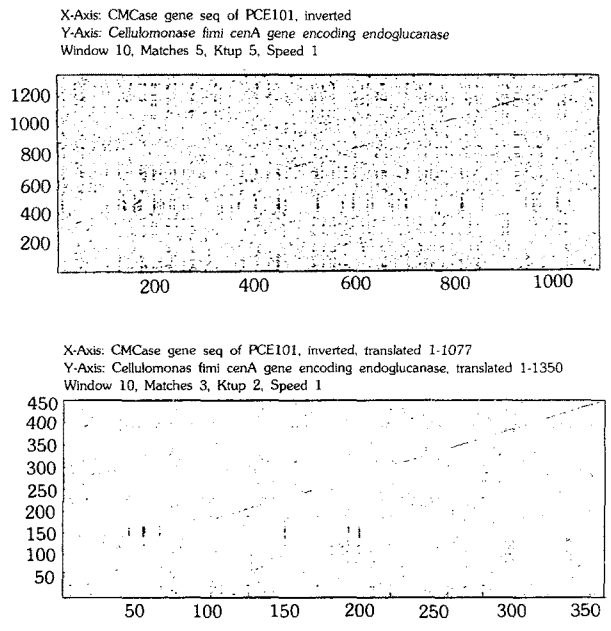


Fig. 6. Comparison of nucleotide and amino acid sequence of CMCase gene from *Cellulomonas* sp. YE-5 and endoglucanase gene from *Cellulomonas fimi*.
Panel A, DNA sequence comparison; panel B, deduced amino acid sequence comparison.

cated before translation start site (Fig. 5).

Comparison of DNA Sequence Homology

The DNA sequence of CMCase gene from *Cellulomonas* sp. YE-5 was compared with the data in Gene Bank. As a result, the endo-β-1,4-glucanase of *Cellulomonas fimi* showed a homologous region of DNA and deduced amino acid sequence with 65% homology (Fig. 6). But, we could not get any other homologous sequence from Gene Bank. So, the CMCase gene of *Cellulomonas* sp. YE-5 is supposed to be one of the isoenzyme of endo-β-1,4-glucanase *cenA* of *Cellulomonas fimi* (14, 19).

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