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Molecular Cloning and Expression of Cellulase of Gene of *Pseudomonas* sp. in *Escherichia coli*

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Pseudomonas sp.의 Cellulase 유전자의 대장균에의 클로닝 및 발현

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The genes for cellulases of *Pseudomonas* sp. LBC505 and CYC10, potent cellulase complex-producing strains, were cloned in *Escherichia coli* with pUC19. Recombinant plasmids pLC1 and pLC2 were isolated from transformants producing cellulase by congo red staining, and their genes cloned were 0.7 kb and 4.6 kb *Hin*dIII fragments, respectively. The inserts of pLC1 and pLC2 were hybridized to chromosomal DNAs digested with *Hin*dIII from *Pseudomonas* sp. LBC505 and CYC10, respectively. Immunodiffusion assays revealed that pLC1-and pLC2-encoded cellulase showed similarity with that of host strains. About 24% of cellulase activity was observed in the extracellular fraction of *E. coli* carrying pLC1, and its activity was higher about 1.4 times than that of LBC505. The enzymatic properties of pLC1 and pLC2 encoded cellulase were the same as those of cellulase from host strains. HPLC analysis and substrate specificity showed that cellulases were the same as those of cellulase from host strains. HPLC analysis and substrate specificity showed that cellulases cloned were endocellulase.

Microorganisms capable of degrading cellulose have a great economic potential for converting cellulosic biomasses and industrial wastes into fermentation substrates such as glucose, xylose or oligosaccharides. Cellulase is multienzyme consisting of endo-1,4- β-glucanase, exo-1,4- β-glucanase and β -1,4-glucosidase, and their coponents act synergistically and completely to solubilize crystaline cellulose to glucose and/or cellulobiose (1,2). However, for the production of useful product from cellulose, enzymatic degradation is not especially cast effective at present, since large quantities of active cellulase preparations are required, and insoluble crystalline cellulose makes it difficult to degrade by cellulase. To overcome these shortcoming, high-yielding cellulase-producing strains (3, 4) as well as mutants that are resistant to catabolite repression (5, 6) and endo product inhibition (7) have been isolated. Recently, one approach to increasing cellulase production by cellulolytic organisms would be to isolate the genes coding for cellulase on a recombinant DNA plasmid and then to modify the expression of these genes by current molecular genetic techniques (8-13). These could include coupling the genes to strong promoters (14), eliminating operators sensitive to repression, and increasing the efficiency of translation (15, 16). In our laboratory, many cellulolytic bacteria (17, 18), fungi (19) and actinomycetes (20) have been isolated and breeded by mutation and protoplast fusion. Among these strains isolated, *Pseudomonas* sp. CYC10 and LBC505 (21) had endo- and exo-1,4- β-glucanase, β -1,4-glucosidase and xylanase

Key words: *Pseudomonas* sp., cellulase gene, cloning
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activities. We have started to clone cellulase gene in order to analyze genetic information for the multicomponents of cellulase and finally improve the production of cellulases. This paper deals with molecular cloning and expression of the cellulase genes of *Pseudomonas* sp. in *E. coli,* and some enzymatic properties were also discussed.

Materials and Methods

Strains and plasmids

The bacterial strains and plasmids used in this experiment are listed in Table 1. *E. coli* JM109 was used as the host strain. *Pseudomonas* sp. CYC10 and LBC505 used as gene source strains were a producer of high-yielding cellulase complex and were isolated in our laboratory (21). The pUC19 was used as a cloning vector, and the plasmid pKK223-3 containing potent tac-promoter was used as a expression vector to increase enzyme activity.

Media and growth conditions

E. coli was incubated aerobically at 37°C in LB medium (10g of Bacto-tryptone, 5g of Bacto-yeast extract, 5g of NaCl, per 1*l* of deionized water, pH 7.0). *Pseudomonas* sp. was grown at 42°C with shaking in modified PY-CMC (CMC, 1%; yeast extract, 0.5%; polypeptone, 0.3%; polypeptone, 0.3%; $(NH_4)_2SO_4$, 0.2%; NaCl, 0.5%; K_2HPO_4 0.2%; $MgSO_4 \cdot 7H_2O$, 0.02%; $CaCl_2$, 0.04%; $FeSO_4 \cdot 7H_2O$, 0.02%; $MnSO_4 \cdot H_2O$, 0.001%) medium (22). MacConkey agar was used as a selective medium for *E. coli* JM 109 transformants, and LB agar containing ampicillin and 0.5% CMC was used as a selective medium for *E. coli* and *B. subtilis* transformants producing cellulase.

Recombinant DNA techniques

Chromosomal DNA of *Pseudomonas* sp. was isolated by the method of Miura (23), modified to include treatment with proteinase K (1 mg/m*l*; Sigma).

Large scale preperations of plasmid DNA were made by alkaline lysis and CsCl-EtBr density gradient ultracentrifugation method (24). The method of Birnboim and Doly (25) was used for small-scale extraction and rapid screening of plasmid-containing clones.

The chromosomal DNA was partially digested with *Hin*dIII and fractionated in a 15-30% sucrose gradient. The fractions 10 Kb were pooled and were ligated to the *Hin*dIII cleaved and dephosphorylated pUC19 plasmid. Ligation of *Pseudomonas* sp. DNA fragment (5μg) to linearized pUC19 (2.5μg) was carried out at 12.5°C for 36 hr under the buffering conditions specified for T_4DNA ligase by the supplier (TaKaRa).

Table 1. Bacterial strains and plasmids used

Strains and plasmids	Relevant genotype or phenotype
Strains	
E. coli JM109	(*lac pro*), *thi*, *str*A, *sup*E *end*A, *sbc*B, *Had*R, F*tra*D36 *pro*AB, *lac*I^q, ZM15,r^-,m^+
Pseudomonas sp. CYC10 and LBC505	Hyper-cellulase complex producing-strain isolated in our lab.
Plasmids	
pUC19	Amp. *lac*Z
pKK223-3	Amp. tac promoter

The selection of clones with cellulase activity was performed by the congo red technique as follows: white colonies of *E. coli* transformants appearing on MacConkey plates were toothpiked onto LB plates containing ampicillin and 0.5% CMC. After overnight growth, cellulase-positive transformants were detected directly on the plates by yellow color formed around colony when stained with 0.1% congo red.

Southern hybridization

The chromosomal fragments contained in the constructed plasmid pLC1 and pLC2 (see Results) were isolated and labelled by the nick translation according to the procedures recommended by Boehringer Mannheim (Nonradioactive immunoassay kit), with labelled DNAs as probes. Hybridization of chromosomal DNA digests was performed following procedures described by Maniatis *et al* (24).

Immunological method

Pseudomonas sp. CYC10 and LBC505 were grown in modified PY-CMC medium for 36 hrs at 37°C. The cells were removed by centrifugation and the supernatant was precipitated by the addition of 4 volumes of cold ethanol suspended in a small volume of buffer.

One m*l* of the sample (Ca. 5.0 U of carboxymethylcellulase activity) was mixed with 1 m*l* of the

complete Fruend adjuvant, and the mixture was injected into matured white rabbits. Booster injections of the samples were given in the same manner after 4 and 6 weeks, and the sera were collected after the last booster injection. Double immunodiffusion test was performed by the method described by Johnstone *et al* (26).

Enzyme assay and localization

The CMCase assays were performed according to the method of Horikoshi *et al* (8, 22). The enzyme solution (0.3 m*l*) was mixed with 0.7 m*l* of 1% CMC solution (made up with 0.02 M citrate-phosphate buffer, pH 6.5). After 10 min of incubation at 50°C, 1 m*l* of dinitrosalicylic acid solution (27) was added, and the mixture was heated in a boiling water bath for 5 min. Then, 4 m*l* of water was added, and the absorbance of the sample was measured at 510 nm. One unit of enzyme activity is defined as the amount of the enzyme which liberates 1 mg of reducing sugar expressed glucose per min under the above conditions. The enzyme localization was carried out by a modified method (8) of Cornelis *et al* (3).

HPLC analysis

Enzyme hydrolysis of the soluble cellooligosaccharides was followed by HPLC (waters) analysis. Before applying the sugar mixtures to an HPLC column (water, μ-Bondapak carbohydrate column, 3.9×300 mm) the protein and pigments in the mixtures were removed by the treatment of 5% trichloroacetic acid, Sep-Pak (C18, waters) and 0.45 μ membrane filter.

The mixture (10 μl) was injected into column using acetonitrile/water mixtures (65%, v/v) as elution solvent, a flow rate of 0.5 m*l*/min being maintained. Cellooligosaccharide standards were purchased from Seikagaku Kogyo Co.

Results

Cloning of cellulase genes in *E. coli*

The chromosomal DNAs of *Pseudomonas* sp. CYC-10, LBC-505 and pUC19 were digested with *Hin*dIII and ligated with T_4DNA ligase. The ligation mixtures were transformed *E. coli* JM109, and transformants were selected on MacConkey agar plates containing ampicillin (50 μg/m*l*). Ampicillin resistant and white color forming colonies were toothpicked onto LB plates containing ampicillin (50 μg/m*l*) and 0.5% CMC.

The plates were incubated at 37°C for 36 hr and then 1 mg/m*l* of congo red solution was added. After 1 hr, the dye solution was discarded and the plates were washed with 1 M NaCl for 1 hr. Cellulase activity was detected as clear zones around colonies.

Among 2.3×10^4 ampicillin resistant and white color foming colonies checked, 2 colonis had distinct cellulase activity on the CMC-congo red plate. Recombinant plasmids of these transformants were named pLC1 and pLC2, and their inserts was 0.7 kb and 4.6 kb *Hin*dIII fragments.

The *Hin*dIII fragments of pLC1 and pLC2 were originated from *Pseudomonas* sp. LBC505 and CYC 10, respectively. The pLC1 was digested with several restriction enzymes and analyzed by agarose gel electrophoresis.

The DNA fragment contained neither *Hinc*II, *Pst*I, *Sma*I, *Kpn*I, *Sac*I, *Sph*I, *Bgl*II, *Xma*I, nor *Sal*I sites, while it was digested with *Eco*RI and *Bam*HI sites (Fig. 1).

Homology among the fragments and chromosomal DNA

To analyze the origin of the DNA fragment inserted in pUC19 digoxigenin-deoxyuridine-triphosphate-labeled fragments of pLC1 and pLC2 were hybridized to the *Hin*dIII-digested chromosomal DNAs of *Pseudomonas* CYC10, LBC505 and *E. coli* JM109.

As shown in Fig. 2., the probe of pLC1 was hybridized to the 0.7 kb *Hin*dIII fragment from *Pseudomonas* sp. CYC10 as well as LBC505. This indicates that there is partial homology between CYC10 and LBC505. The probe of pLC2 was hybridized only to the 4.6 kb *Hin*dIII fragment from *Pseudomonas* sp. CYC10 (Fig. 3).

Immunological studies

The crude enzyme of *E. coli* JM109 carrying pLC1 and pLC2 plasmid were checked for the presence of antigenic material reacting with antiserum prepared against the crude cellulase of *Pseudomonas* sp. LBC505 and CYC10.

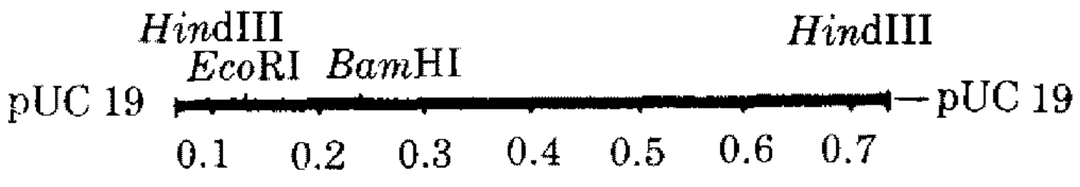

Fig. 1. Restriction map of plasmid pLC 1

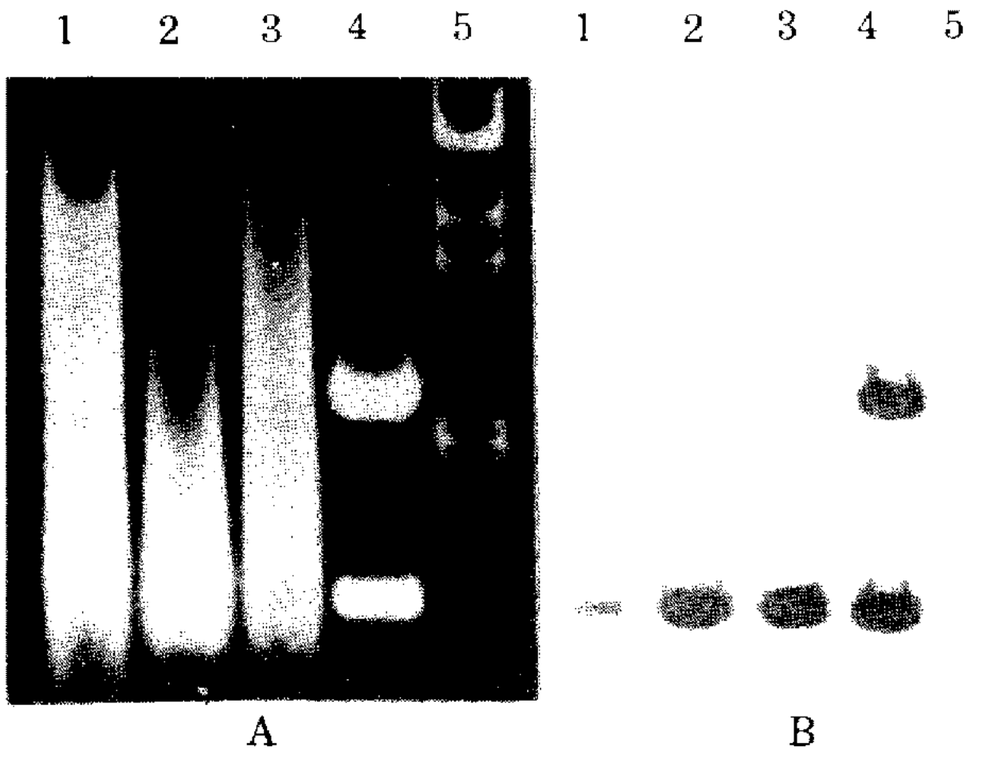

Fig. 2. Homology between pLC1 and chromosomal DNAs .

A: Agarose (0.8%) gel electrophoresis of digests of various DNAs
lane 1: *Pseudomonas* sp. CYC10 DNA (*Hin*dIII) lane 2: *Pseudomonas* sp. LBC505 DNA (2μg) (*Hin*dIII) lane 3: *Pseudomonas* sp. LBC505 DNA (4μg) (*Hin*dIII) lane 4: pLC1 (*Hin*dIII) lane 5: DNA (*Hin*dIII)
B: Hybridization analysis of the Southern transfer from the gel A

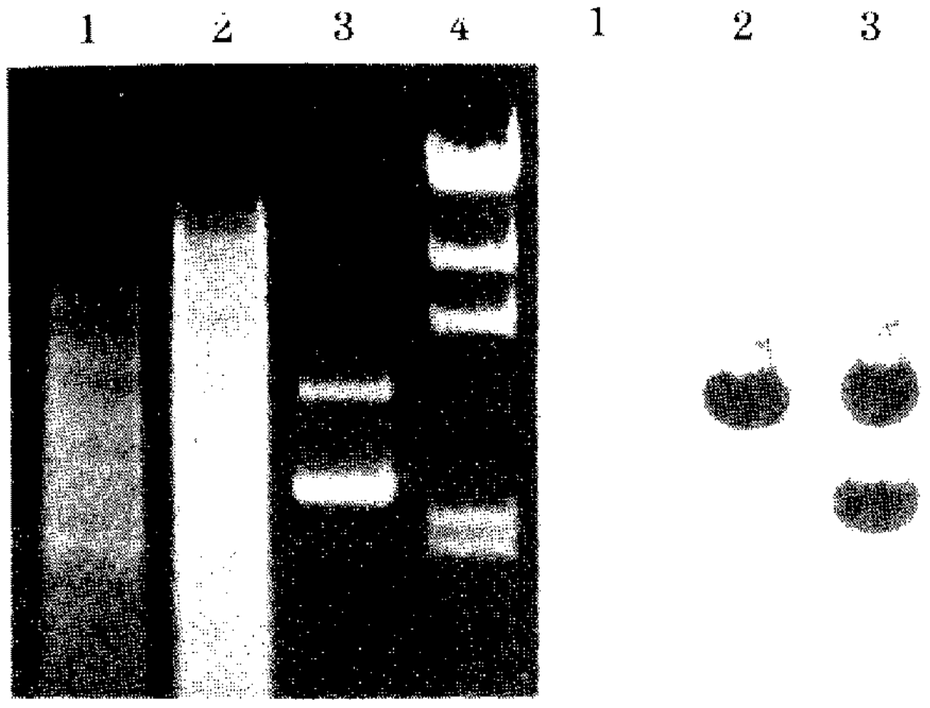

Fig. 3. Homology between pLC2 and chromosomal DNAs .

A: Agarose (0.8%) gel electrophoresis of digests of various DNAs
lane 1: *E. coli* JM109 DNA (*Hin*dIII) lane 2: *Pseudomonas* sp. CYC10 (*Hin*dIII) lane 3: pLC2 (*Hin*dIII) lane 4: DNA (*Hin*dIII)
B: Hybridization analysis of the Southern transfer from the gel A

As shown in Fig. 4., the enzymes of *E. coli* JM109 carrying pLC1 and pLC2 gave lines of precipitation which fused with that formed by cellulase of *Pseudomonas* sp. LBC505 and CYC10, respectively.

No reaction was observed with extracts of strains

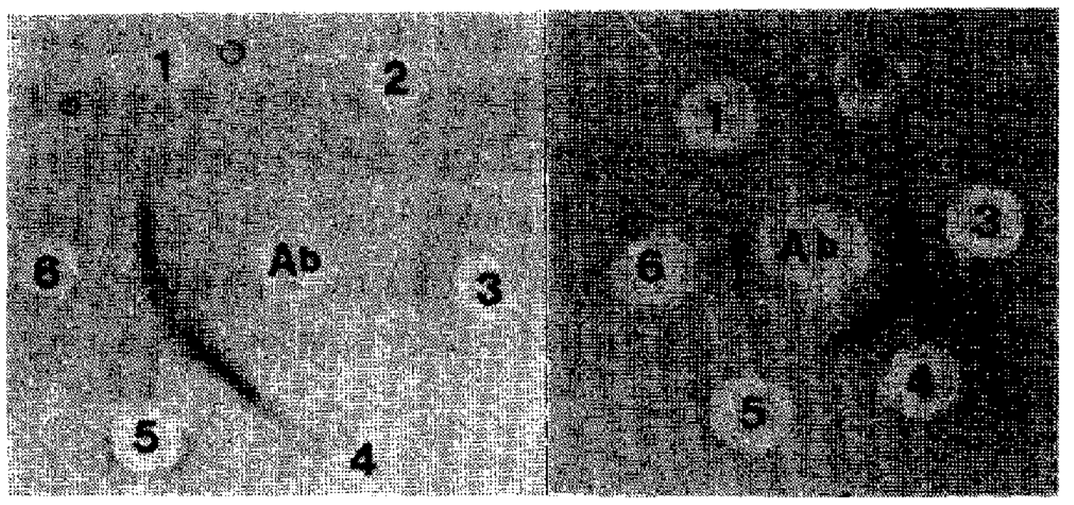

Fig. 4. Oucherlony double diffusion assay. Samples were added to the outer well as indicated below.

A: Ab, antiserum to the purified enzyme of *Pseudomonas* sp. LBC 505: well 1, periplasm fraction of *E. coli* JM 109 carrying pUC19: well 2, *Trichoderma viride* cellulase (Sigma C2274: well 3, *Aspergillus niger* cellulase (Sigma C7377): well 4, crude enzyme of *Cellulomonas fimi*: well 5, crude enzyme of *E. coli* JM 109 carrying pLC1: well 6, the purified enzyme of *Pseudomonas* sp. LBC505
B: Ab, antiserum to the purified enzyme of *Pseudomonas* sp. CYC10: well 1, periplasm fraction of *E. coli* JM109 carrying pUC19: well 2, *Trichoderma viride* cellulase (Sigma C2274: well 3, pLC2 crude enzyme: well 4, crude enzyme of *Cellulomonas fimi*: well 5,*A. niger* cellulase: well 6, pLC1 crude enzyme

carrying pUC19 and commercial cellulase.

These results clearly indicate that the cellulase of *E. coli* JM109 carrying pLC1 and pLC2 could be shown to similanity with that of *Pseudomonas* sp. LBC505 and CYC10, respectively. In the cellulase activity assay system, the addition of 30 μl of antiserum caused 95% inhibition of cellulase activity of *E. coli* JM 109 carrying pLC 1 or pLC 2.

Activity and localization of cellulase in *E. coli*

E. coli JM109 carrying pLC1 and pLC2 plasmids was aerobically cultured in LB containing 1% CMC and ampicillin for 36 hr at 37°C and the extracellular, periplasmic, and cytoplasmic CMCase activities were assayed (Table 2). A significant amount of the enzyme activity was found in periplasmic and cytoplasmic fraction in *E. coli* carrying pLC2, but about 24% of the total activity was found in extracellular fraction in *E. coli* carrying pLC1. pLC1 produced 1.4 times as much total activity as the host strain, LBC505.

We attempted to clone the 0.7 kb *Hin*dIII fragment of pLC1 to the expression vector, pKK223-3 containing tac-promoter, to increase the expression efficiency of cellulase gene. This recombinant plasmid was named pKC10 and the CMCase activity of *E. coli* harboring pKC10 was increased about 2 times and 2.7

Table 2. Activity and distribution of CMCase in *E. coli*[a] JM109 carrying plasmids

Strains and plasmids	CMCase activity (%) (U)[b]			
	Total	Extracellular (%)	Periplasmic (%)	Cytoplasmic (%)
E. coli JM 109				
pLC1	4.28	1.02 (23.8)	2.56 (59.6)	0.71 (16.6)
pLC2	3.34	0.44 (13.2)	1.46 (43.7)	1.44 (43.1)
pLC10[c]	8.60	1.89 (22.0)	4.73 (55.0)	1.12 (13.0)
Pseudomonas sp.[d]				
CYC10	3.04	2.83 (90.4)	0.21 (9.6)	ND[e]
LBC505	3.21	3.03 (94.5)	0.18 (5.5)	ND[e]

[a] Strains were aerobically grown in LB for 36 hrs at 37°C
[b] One unit of enzyme activity is defined as the amount of enzyme which liberates 1 mg of reducing sugar expressed as glucose per min
[c] pKC 10 is pKK223-3 containing 0.7 kb *Hin*dIII fragment of pLC1
[d] Strains were aerobically grown in PY-CMC medium for 36 hrs at 37°C
ND: Not determined

times than that of *E. coli* (pLC1) and LBC 505, respectively.

The synthesis of CMCase in *E. coli* was constitutive, and no effect of CMC supplement was observed (data not shown).

Substrate specificity of cellulases encoded by recombinant plasmids

Cellulolytic bacteria usually contain multiple cellulass. Therefore, characterization of their properties has been impossible without separating them from each other. Thus, we characterized the enzyme encoded by pLC1 and pLC2 (Table 3). The cellulase encoded by pLC1 and pLC2 could hydrolyze CMC, but could not hydrolyze xylan, cellobiose, laminarin, pNPG, pNPX and avicel.

E. coli carrying pLC 2 hydrolyzed avicel (0.2 U), cellobiose (0.8 U) as well as CMC (3.34 U).

Enzymatic properties of cellulase produced by *E. coli* carrying pLC1

Cellulase encoded by pLC1 was stable from pH 4.5 4.0 to 9.0 at 40°C for 10 min, and their optimum pH value was pH 6.0. The enzyme produced by *E. coli* carrying pLC1 were stable up to 70°C for 30 min. The molecular weight of pLC1 encoding cellulase was about 14,000. The enzymatic propertis of pLC1 encoded cellulase was the same as those of CMCase produced by *Pseudomonas* sp. LBC505 (28).

Hydrolysis products from cellooligosaccharides and CMC

Table 3. Substrate specificity of cellulase encoded by pLC1 and pLC2

Substrate	Enzyme activity (unit)	
	pLC1	pLC2
CMC	4.28	3.34
Laminarin	–	–
Avicel	–	–
Xylan	–	0.2
pNPG	–	–
pNPX	–	–
Cellobiose	–	0.81

pNPG: p-nitrophenyl-β-D-glucopyranoside
pNPX: p-nitrophenyl-β-D-xylopyranoside
E. coli JM109 was cultured in LB for 36 hrs at 37°C

HPLC analysis of the products released from cellopentaose, cellohexaose and CMC by pLC1 and pLC2 encoded cellulase was performed. pLC1 and pLC2 encoded cellulase released glucose, cellobiose, cellotriose, cellotetraose, and cellopentaose from cellohexaose during 5, 30 and 60 min of incubation, but could not hydrolyze cellobiose. When cellohexaose was incubated, at 50°C for 1 hr, a great amount of glucose and cellobiose were detected. When CMC was incubated with pLC1 and pLC2 encoded cellulase at 50°C, glucose and cellohexaose were firstly released, and later cellopentaose, cellotetraose, cellotriose, and cellobiose were released (data not shown). This results rev-

eal that pLC1 and pLC2 encoded cellulases are endocellulase.

Discussion

pLC1 and pLC2 were easily constructed by using the insertional inactivation of *lacZ* gene in pUC19, the selection of white colony on MacConkey agar medium, and then the congo red staining of *E. coli* carrying recombinant DNA on LB plate containg CMC and ampicillin. In spite of originating from different gene sources, pLC1 and pLC2 were partial homology on the basis of results of DNA hybridization experiment. From results of immunodiffusion experiment, we can expect that pLC1 and pLC2 encoded cellulases are different from those of *T. viride, A. niger* and *C. fimi,* since their enzymes did not appear precipitation line against antiserum. *E. coli* JM109 containing pLC1 produced 1.4 times as much total cellulase activity as gene source strain, *Pseudomonas* sp. LBC505. Cellulase activity of *E. coli* containing pKC10, recombinant DNA constructed by inserting 0.7 kb *Hin*dIII fragment of pLC1 in pKK223-3 was higher about 2 times than that of *E. coli* carrying pLC1. It seems that pKK223-3 containing tac promoter facilitated expression efficiency in *E. coli.* 83% of cellulase produced by *E. coli* JM109 carrying pLC 1 was secreted through inner cell membrane of *E. coli.* Recently, secretion of the cloned gene through the outer membrane of *E. coli.* was reported by Kudo *et al.* (29), Yanagida *et al.* (30), Yoshinano *et al.* (31), Immanaka *et al.* (32). According to these reports, there are two possibilities that cellulase gene cloned possesses signal sequence coding hydrophobic amino acid, and that cellulase produced from transformants is facilitated excretion by fusing with cell membrane protein such as Omp A, Omp C, PhoC and Lam B. The mechanism for the secretion of cloned cellulase will be elucidated after more precise experiments. Investigation of excretion mechanism will be useful for development of secretion vector for Gram negative bacteria. Thus, we are investigating about DNA base sequence. In addition, the *E. coli* JM 109/pLC1 and 2 produced cellulase under the presence of 1% glucose while *Pseudomonas* sp. was repressed by the addition of glucose (21). This result indicates that the cloned gene was eliminated operators sensitive to catabolite repression.

요 약

Cellulase 복합체와 xylanase를 동시에 분비하는 *Pseudomonas* sp. LBC 505와 CYC 10의 cellulase 유전자를 pUC19를 사용하여 *E. coli*에 클로닝시켰다. Congo red 염색시 노란색 환을 형성하는 대장균 형질전환체에서 0.7 Kb-와 4.6 Kb-*Hin*dIII 단편을 함유한 재조합 플라스미드 pLC1과 pLC2를 각각 분리하였다.

DNA hybridization 실험에서 pLC1과 pLC2는 *Pseudomonas* sp. LBC 505와 CYC 10 유래임이 각각 밝혀졌고, Immunoassay 실험에서도 유사성이 인정되었다. pLC1을 함유하고 있는 대장균은 cellulase의 24%를 세포외로 분비하였고, 효소활성은 모균주에 비해 1.4배 증가하였다. pLC1과 pLC2의 효소학적 성질도 모균주와 동일하였으며, 기질특이성과 HPLC로 유리당을 분석한 결과, 클로닝된 유전자는 endo type인 것으로 나타났다.

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