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High-Level Expression of *Pseudomonas sp.* LBC505 Endoglucanase Gene in *Escherichia coli*

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Endoglucanase gene of *Pseudomonas* sp. LBC505 was previously cloned in pUC19 to yield plasmid pLC1. The *Pseudomonas sp.* LBC505 endoglucanase gene was subcloned in a temperature-regulated *Escherichia coli* expression vector, pAS1, containing the leftward promoter P_L of bacteriophage lambda. The level of gene expression was controlled by the thermal inactivation of the heat-sensitive lambda *cI857* repressor. Best yield of endoglucanase was obtained by lowering the incubation temperature to 37°C after induction at 42°C for 1h. Under these conditions enzyme production continued for about 5h at a gradually decreasing rate. *E.coli* harboring recombinant plasmid pASC10 expressed 4.3 times as much CMCase activity as *E.coli* containing pLC1. To enhance the expression level of endoglucanase gene, we have also changed the presumptive Shine-Dalgarno sequence (AGAGGT) of the gene to consensus sequence (AGGAGGT) by site-directed mutagenesis. The genes mutated were subcloned in pAS1 resulting in the formation of recombinant plasmid pASS50. *E.coli* harboring the plasmid pASS50 expressed 6.2-fold higher levels of CMCase activity than that of *E.coli* harboring pLC1.

Complete hydrolysis of cellulose by cellulolytic microorganisms is usually dependent upon the presence of three enzymes: an endo-β-1.4-glucanase (carboxymethylcellulase, CMCase) which randomly cleaves glucan chains, an exo-β-1.4-glucanase (cellobiohydrolase) which removes either glucose or cellobiose from the exposed glucan chains, and a β-1.4-glucosidase (cellobiase) which cleaves cellobiose units (6, 15). Endoglucanases have been reported to participate in synergistic action for the hydrolysis of insoluble cellulose with cellobiohydrolases (2, 8, 12, 20). We have previously cloned a *Pseudomonas sp.* LBC505 endoglucanase gene in *E.coli* (4) and determined the complete nucleotide sequence (5).

In general, *Pseudomonas* genes function inefficiently in *E.coli* because of poor recognition of *Pseudomonas* promoter regions by *E.coli* RNA polymerase (1). Endoglucanase gene of *Pseudomonas sp.* LBC505 cloned was also poorly expressed in *E.coli.*

Considering the effects of a high level of transcription and gene dosage, strong promoters and multicopy plasmid vectors have often been empolyed for the production of a desired protein by the recombinant DNA technique. Since the extensive intracellular production of a protein is often harmful to the cell, an inducible promoter is much more suitable than a constitutive one; using this type of promoter, the expression is repressed during the growth of cells, and is induced under specified conditions.

The most common 'bottle neck' in the expression of reading-frames borne on DNA fragments inserted into plasmids is the translational initiation. An important approach toward the optimization of translational initiation is to recombine the reading frame to be expressed with a Shine-Dalgarno (SD) sequence that, in its normal environment, contributes to the efficient translation of a gene that follows it (10, 11, 18).

For studying the structure and function of cellulase components and for evaluating their biotechnological potential, it is necessary to produce active enzyme in large amounts. *E.coli* expression vectors have been developed using the powerful promoters, such as the P_L (13) and P_R of lambda phage, *trp, lac* UV5, *lpp, lpp-lacO, tac* (3, 14), *rac* and *pac* (19).

We describe here the application of a temperature-controlled lambda P_L expression system for the over-

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production of *Pseudomonas sp.* LBC505 endoglucanase and the high-level expression of its gene by site-directed mutagenesis of the presumptive ribosome binding site.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

The bacterial strains used for genetic manipulation were *E.coli* JM109 (*end*A1, *rec*A1, *gyr*A96, *thi*, *hsd*R17 (r_k^-,m_k^+), *rel*A1, *sup*E44, △(*lac-pro*AB), [F^-, *tra*D36, *pro*AB, *lacI*^{q}Z△M15]). *E.coli* N4830-1(F^-, *su*, *his*, *ilv*, galK8, (*chl*D-*pgl*), [λ, *Bam*, N^+, *cI*857]) and *E.coli* TG1 (*sup*E, *hsd*△5, *thi*, △(*lac-pro*B), [F^-, *tra*D36, *pro*AB, *lacI*^{q}Z△M 15]). The vectors empolyed were pUC19, pAS1 and M13mp8.

Media

E.coli strains were grown in either 2YT medium(16g tryptone, 10g yeast extract and 5g NaCl per liter, pH 7.2) supplemented with 0.5% (w/v) carboxymethylcellulose (CMC) or M9 medium (9) with appropriate supplements. Media were solidified by the addition of 1.5% (w/v) Bacto-agar (Difco). Ampicillin (100 μg/ml) and 5-bromo-4-chloro-indolyl-β-D-galactoside(X-gal, 2 μg/ml) were used in media for the selection and growth of transformants.

Site-Directed Mutagenesis

Oligonucleotides for mutagenesis were synthesized on DNA synthesizer (Millipore 8909, U.S.A). The oligonucleotides were designed to change the presumptive SD sequence (AGAGGT) to *E.coli* consensus SD sequence (AGGAGGT). Mutagenesis was performed by the specifications of mutagenesis kit (Amersham, U.S.A).

Nucleotide Sequencing

The mutations were identified by DNA sequence analysis. The DNA sequencing was performed according to the method described by Sanger (16) using the dye primer sequencing kit (Promega, U.S.A) with autosequencer (ABI 373A, U.S.A).

General Recombinant DNA Procedures

Agarose gel electrophoresis, transformation of *E.coli* and the manipulation of DNA using restriction enzymes and T4 DNA ligase were carried out according to the methods described by Maniatis *et al.* (9).

Preparation of Subcellular Fractions and Enzyme Assay

Cell fractionation and CMCase assay were carried out according to the procedure of Horikoshi *et al.* (17). CMCase activity was assayed by incubating the fractionated sample for 10min at 50℃ in a 1% (w/v) solution of CMC in 20 mM citrate-phosphate buffer pH 6.5. Reducing sugars released from the substrate were determined with the 3.5-dinitrosalicylic acid reagent. One unit of enzyme activity corresponds to the amount of the enzyme to release 1 mg glucose per min.

RESULTS AND DISCUSSION

Subcloning of Endoglucanase Gene

A 0.7kb *Hin*dIII fragment carrying the endoglucanase gene from *Pseudomonas sp.* LBC505 was previously cloned in pUC19 to yield plasmid pLC1 (4). Since the presumptive ribosome binding site of the endoglucanase

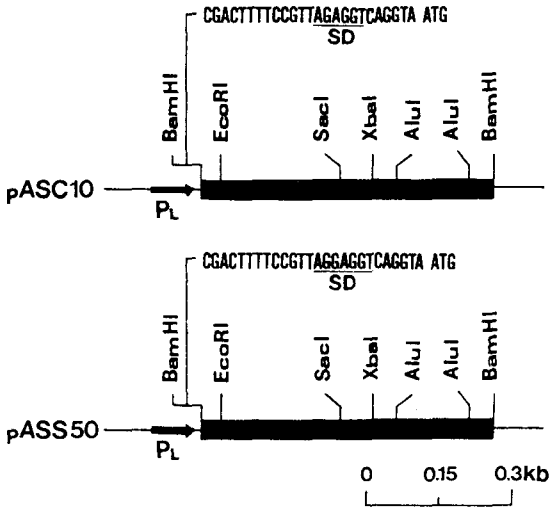

Fig. 1. Physical map of subcloned endoglucanase gene.
The heavy line corresponds to the endoglucanase gene region of *Pseudomonas* sp.LBC 505 DNA. The thin line represents the insertion region of the vector pAS1.

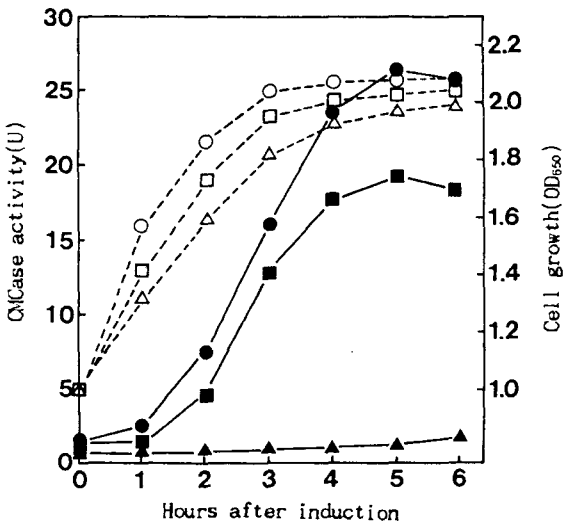

Fig. 2. Heat induction of endolgucanase gene.
Cultures of *E.coli* N4830-1 carrying the plasmids. pASC10 and pASS50 were grown at 30C to an OD_{650}=1. The cultures were heat-induced for 1h at 42C and further incubated at 37C. Uninduced control (*E. coli* JM109 carrying the plasmid pLC1) was incubated at 37℃. Samples were taken at the times indicated. Solid lines represent CMCase activities of induced(■:pASC10, ●:pASS50) or uninduced (▲:pLC1) cultures. Dashes lines represent optical densities at 650nm of induced (□:pASC10, ○:pASS50) or uninduced (△:pLC1) cultures.

Table 1. Subcellular distribution of CMCase activity

E. coli strains	CMCase activity(U)[a]			
	Total	Cytoplasm	Periplasm	Supernatant
JM109(pLC1)	4.28	0.71(16.6)	2.56(59.6)	1.02(23.8)
N4830-1(pASC10)	18.40	3.01(16.3)	11.19(60.9)	4.20(22.8)
N4830-1(pASS50)	26.54	4.54(17.0)	16.00(60.4)	6.00(22.6)

[a]Samples from cultures (*E.coli* N4830-1 carrying pASC10 and pASS50) were taken 5 hours after induction. Samples from uninduced culture (*E. coli* JM109 carrying pLC1) were taken after incubation for 36 hours at 37C. The parentheses represent % CMCase activity.

gene is immediately preceded by an *Taq*I recognition site, it is possible to remove the promoter region by cleavage with *Taq*I. The 0.6kb *Taq*I-*Hin*dIII fragment containing the coding sequence of the endoglucanase gene was filled in with a Klenow fragment of DNA polymeraseI to make a blunt end. The blunt-ended fragment was ligated with *Bam*HI linker, dCGGATCCG. After digesting it with *Bam*HI and eluting on agarose gel (1%), the DNA fragment obtained was inserted to *Bam*HI site of the expression vector pAS1. A 0.6kb *Bam*HI fragment containing the coding sequence of the endoglucanase gene was thereby placed under the control of the lambda P_L promoter (Fig. 1). The resulting plasmid pASC10 was transformed into *E.coli* N4830-1, a cryptic lambda lysogen that carries the thermolabile *cI857* repressor. Transformants exhibited the expected phenotype of expressing CMCase activity only upon inactivation of the lambda repressor (Fig. 2).

Overexpression of Endoglucanase Gene by Lambda P_L Promoter

Inactivation of the thermosensitive lambda repressor by heat induction resulted in a burst of CMCase synthesis (Fig. 2). Best yield of endoglucanase were obtained by lowering the incubation temperature to 37℃ after induction at 42℃ for 1h. Under these conditions, the enzyme production continued for about 5h at a gradually decreasing rate (Fig. 2). The time course of endoglucanase production correlated with the increase in cell density. The recombinant strain expressed 4.3-fold higher levels of CMCase than *E.coli* containing pLC1.

Overexpression of Endoglucanase Gene by Site-directed Mutagenesis

We have attempted to change the presumptive SD sequence (AGAGGT) of the endoglucanase gene (5) to *E.coli* consensus SD sequence (AGGAGGT) for the optimization of endoglucanase gene in *E.coli*. A 0.6-kb *Bam*HI fragment from the plasmid pASC10 was ligated into the same recognition site of M13mp8.

The presumptive SD sequence was changed by site-directed mutagenesis using oligonucleotides synthesized by a DNA synthesizer. The nucleotide sequence mutated was identified by DNA sequencing. A 0.6-kb *Bam*HI fragment mutated was inserted into the *Bam*HI site of pAS1 (Fig. 1). *E.coli* transformed by the resulting plasmid pASS50 expressed 6.2-fold higher levels of CMCase than *E.coli* harboring pLC1 (Fig. 2). Expression efficiency of the endoglucanase gene of which SD sequence was mutated was higher than that of the intact gene after heat induction. The results suggest that SD sequence together with strong bacteriophage lambda P_L promoter plays an important role in an efficient expression of the endoglucanase gene with a manner of synergistic action(18).

Localization of Endoglucanase Gene Product

The cellular localization of the pseudomonad endoglucanase produced in *E.coli* were examined (Table 1). It can be seen that about 60% of the endoglucanase constitutively expressed by pLC1 is exported into the periplasm. High-level expression of the endoglucanase gene product under the control of the P_L promoter had a little effect on the localization of the enzyme. Upon induction of pASC10 and pASS50, nearly 61% of the total CMCase activity was found in periplasmic space and the secretion of into the extracellular fraction was slightly decreased. However, the fact that, with iirrespective to the level of gene expression, around 23% of the total enzyme activity was always found in the extracellular fraction could be supported by the presence of signal peptide (7) deduced from the data of nucleotide sequence of endoglucanase gene (5).

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