Biotechnology and Bioprocess Engineering **2004**, 9: 389-392

Development of an *E. coli* Expression Cassette for the Efficient Production of a Harmful Protein

Ok Soo Kim[1], Hwan Jong Kwak[2], Jae-Hwa Lee[1], Jong Myung Ha[1], Bae-Jin Ha[1], and Sang-Hyeon Lee[1]*

[1] Major in Bioscience and Biotechnology, Division of Environmental Engineering and Biotechnology, Silla University, Busan 617-736, Korea
[2] Department of Pathology, College of Medicine, The Catholic University of Korea, Seoul 137-701, Korea

Abstract In order to produce a harmful protein more efficiently, this expression cassette, dubbed pCol-MICT, is directed by the colicin promoter, and was constructed by the insertion of a rrnBT_1T_2 fragment of pEXP7, and a MxeInteinCBD fragment of pTXB3, into pSH375. To test whether harmful proteins, including proteolytic enzymes, could be effectively produced by this cassette, the carboxypeptidase (CPase) *Taq* gene was inserted into the pCol-MICT cassette to yield pCol-CPase*Taq*-MICT. *E. coli* W3110 cells harboring pCol-CPase*Taq*-MICT produced a large quantity of this enzyme, as much as 47.2 mg of purified from per liter of culture, when cultured in the presence of mitomycin C (0.4 μg/mL). This indicates that the colicin promoter-controlled *E. coli* expression cassette was able to produce almost 8 times of protein than the conventional *tac* promoter-based system, and that this cassette may be useful in the synthesis of other harmful proteins.

Keywords: colicin promoter, expression cassette, *E. coli* W3110 cells, carboxypeptidase *Taq*, mitomycin C

INTRODUCTION

Many industrially useful genes have recently been cloned and expressed in the *Escherichia coli* host cells. For the industrial use of gene products, the efficient large-scale production system is required and several expression vectors have been developed to effectively express those proteins. The strongest *E. coli* expression systems developed thus far are the pET series of Invitrogen (CA, USA) and the pBluescript series of Stratagene (CA, USA). Because these series employ the powerful T7 promoter, it can efficiently express many genes, and the system generally produces large amounts of proteins [1]. However, when researchers try to express some harmful genes, most notably protease genes, using the T7 promoter, inclusion bodies are often formed to make protease study difficult, and industrial use of such harmful proteins is all but impossible. Clearly, a novel gene expression system, which can successfully produce such harmful proteins, is required to be developed.

Carboxypeptidase (CPase) *Taq* is a thermostable, zinc-dependent, metallo-carboxypeptidase, which has been successfully cloned from *Thermus aquaticus* YT-1 in previous research [2]. The molecular weight of CPase *Taq* is about 56 kDa [2]. As CPase *Taq* is a kind of proteolytic enzyme, its production in *E. coli* cells would be inimical to bacterial growth. When we attempted to express the CPase *Taq* gene using the T7 promoter in the conventional expression systems, we met with utter failure because it was not suitable to express this proteolytic enzymes.

Plasmid pSH357, the backbone of our cassette, is a pSH350-derivative, and possesses the wild-type colicin E3 operon [3]. Colicin synthesis in *E. coli* cells directed by the colicin plasmid is repressed under ordinary conditions, but it can be induced by treatment with UV light or mitomycin C [4]. Colicin induction involves the SOS response [5-8], and is controlled coordinately by two regulatory elements, the *recA* and *lexA* proteins [4]. The *lexA* protein operates exclusively as a SOS gene repressor whereas the *recA* protein activated as a protease by signals occurring after SOS-inducing treatment, deactivates the *lexA* protein. Because the *E. coli* W3110 cells used for an expression host are *recA* positive, mitomycin C treatment of colicin plasmid results in significantly increased transcription of the gene [9]. Expression cassette which contains the structural genes encoding β-thromboglobulin, and the regulatory sequences from the colicin E1 operon, was revealed to have high-level expression capability, producing recombinant protein up to 30% of total cellular protein [9]. Chaperone proteins have also been produced by the SOS response, and have functioned to promote proper folding of the target proteins [10].

***Corresponding author**
Tel: +82-51-999-5624 Fax: +82-51-999-5636
e-mail: slee@silla.ac.kr

In this paper, we describe the development of the expression cassette using the colicin plasmid as a backbone, and the chitin-binding domain (CBD) as an affinity tag. We also tested the expression capabilities of this cassette, using the CPase *Taq* gene as a model harmful gene. Because gene expression in this cassette is regulated by the SOS response, the formation of inclusion bodies can be theoretically avoided, and hence this cassette may be quite useful in the expression of harmful proteins, such as proteolytic enzymes in *E. coli* host cells.

MATERIALS AND METHODS

Enzymes and Reagents Used for Gene Engineering

Taq DNA polymerase, restriction enzymes, DNA ligation kit, and agarose powder were all purchased from TaKaRa Korea (Seoul, Korea). Mitomycin C was purchased from Sigma-Aldrich (MO, USA).

Cells, Plasmid Vectors, and Culture Conditions

E. coli W3110 (F⁻ *mcrA mcrB* IN(*rrnD-rrnE*)1 λ⁻) was obtained from KCTC (Daejeon, Korea). Host cells were grown in LB medium (1% Bacto tryptone, 0.5% yeast extract, 0.5% NaCl) at 37°C. When necessary, ampicillin (100 μg/mL) was added for selection and 1.5% agar for plates preparation. The pSH375 plasmid [3] was an appreciated gift from Dr. Masaki (Department of Biotechnology, The University of Tokyo, Japan). Litmus28 and pTXB3 plasmids were supplied from New England Biolabs (MA, USA).

Colicin Promoter Sequential Analysis

The pSH375 plasmid was partially sequenced by the dideoxy-chain-termination procedure [11].

Expression of the CPase *Taq* Gene in *E. coli* and Purification of the Enzyme

E. coli W3110 cells transformed by pCol-CPaseTaq-MICT plasmid harboring the CPase *Taq* gene were grown in 1 L of LB medium containing ampicillin (100 μg/mL) at 37°C for 30 min, and mitomycin C (0.4 μg/mL) was added in order to induce the formation of the colicin promoter. Nine hours after mitomycin C induction, the cells were collected by centrifugation and suspended in 30 mL of ice-cold column buffer (20 mM Tris (pH 7.4), 0.5 M NaCl, 0.2% Triton X-100, 2 mM EDTA). After cell disruption by sonication, the sample was centrifuged at 20,000 × *g* for 20 min, and the supernatant was put on a chitin bead column (20 mL of set volumes) (New England Biolabs, MA, USA) equilibrated with column buffer. The column was washed with the same buffer, and then equilibrated with a cleavage buffer (column buffer with 30 mM DTT) at 4°C overnight. Proteins were eluted with column buffer to a total volume of 50 mL. The amount of protein was measured using BCA protein assay reagent (Pierce Biotechnology, IL, USA), utilizing bovine serum albumin as the standard protein.

```
ColE3   TTTGTGGCCCGCTCTGCGTTTT-CTAAGTGTTATCCCTCCTGATTTCTAA 199
ColPro  -----GGCCCGCTATGCGTTTTGCTAAGTGTTATCCCTCCTGATTTCTAA  45
        ******** ******** ****************************

ColE3   AAAATTTTCCACCTGAACTTGACAGAAAAAACGATGACGAGTACTTTTTG 249
ColPro  AAAATTTTCCACCTGAACTTGACAGAAAAAACGATGACGAGTACTTTTTG  95
        **************************************************

ColE3   ATCTGTACATAAACCCAGTGGTTTTATGTACAGTATTAATCGTGTAATCA 299
ColPro  ATCTGTACATAAACCCAGTGGTTTTATGTACAGTATTAATCGTGTAATCA 145
        **************************************************

ColE3   ATTGTTTTAACGCTTAAAAGAGGGAATTTTTATGAGCGGTGGCGATGGAC 349
ColPro  ATTGTTTTAACGCTTAAAAGAGGGAATTTCCATGACTCCGGAAGCCGCTT 195
        ****************************** ****     * *  *

ColE3   GCGGCCATAACACGGGCGCGCATAGCACAAGTGGTAACATTAATGGTGGC 399
ColPro  A---TCAGAACCTG                                   206
          ** ***  *
```

Fig. 1. Sequencing alignment of the colicin promoter. The colicin promoter sequence (ColPro) of pSH357 (6) is aligned with the ColE3-CA38 plasmid sequence (ColE3) (7). Asterisks indicate identical sequences.

Enzyme Activity Assay

Enzyme activity was assayed at 70°C for 30 min by Rosen's ninhydrin method [12] with 0.5 mM Cbz-Phe-Tyr (Protein Research Foundation, Osaka, Japan) as the substrate, in 50 mM N-2-hydroxyethylpiperazine-N-3-propanesulfonic acid (HEPPS)-NaOH buffer (pH 8.5). One unit of enzyme activity was defined as the amount of enzyme produing a ninhydrin-positive substance corresponding to 1 mmol of tyrosine per minute. Specific activity was expressed as units (U) per milligram of protein.

SDS-PAGE

SDS-PAGE was performed by the Laemmli method [13] with an 11% polyacrylamide gel. The enzyme solution was mixed with the sample buffer, and then boiled for 5 min, before being placed on the gel.

RESULTS AND DISCUSSION

Sequencing Analysis of the Colicin Promoter of the pSH375 Plasmid

To confirm positions of the ribosome binding site (SD sequence) and the translation start site (first ATG), a ~200 bp nucleotide sequence of the colicin promoter region in the pSH375 plasmid was determined. Sequence analysis of this region revealed 89% homology with that in the ColE3-CA38 plasmid, which is a notable colicin plasmid [14] (Fig. 1).

Construction of an Expression Cassette Using Colicin Plasmid pSH375

Since the pSH375 plasmid does not possess its own transcriptional terminator, an rrnBT_1T_2 terminator fragment from pEXP7 plasmid [2] was introduced (Fig. 2).

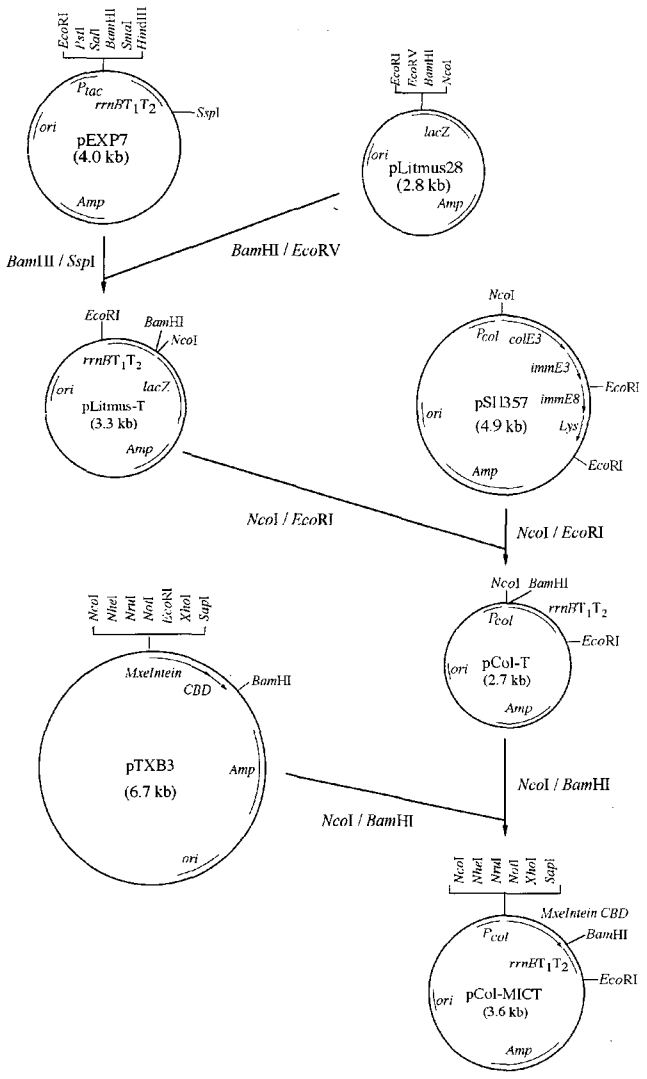

Fig. 2. Construction of the expression cassette. Amp, ampicillin resistance gene; P*tac*, tac promoter; P*col*, colicin promoter; rrnBT1T2, ribosomal RNA transcription terminators 1 and 2; colE3, gene encoding colicin E3; immE3, gene encoding immunity protein, which protects the host from colicin E3; immE8 gene encoding immunity protein, which protects the host from colicin E8; Lys, gene encoding lysozyme; lacZ, *E. coli* β-galactosidase gene; MxeIntein, *M. xenopi* GyrA intein gene; CBD, *B. circulans* chitin-binding domain gene; ori, replication origin of the plasmid.

Firstly, pEXP7 was digested by *Bam*HI and *Ssp*I, and a 524 bp fragment including $rrnBT_1T_2$ terminator was inserted into the *Bam*HI and *Eco*RV sites of Litmus28, resulting in pLitmus-T plasmid. A 530 bp *Nco*I-*Eco*RI fragment of $rrnBT_1T_2$ terminator in pLitmus-T was then isolated and inserted into corresponding sites of pSH375 plasmid, resulting in pCol-T plasmid (Fig. 2). Finally, an 850 bp fragment of CBD tag generated by digestion of pTXB3 plasmid with *Nco*I and *Bam*HI was introduced into pCol-T to construct pCol-MICT plasmid (Fig. 2).

```
-176       -166       -156       -146       -136       -126       -116
   G GCCCGCTATG CGTTTTGCTA AGTGTTATCC CTCCTGATTT CTAAAAAATT TTCCACCTGA

-106       -96        -86        -76        -66        -56        -46
ACTTGACAGA AAAAACGATG ACGAGTACTT TTTGATCTGT ACATAAACCC AGTGGTTTTA TGTACAGTAT
                                                Colicin Promoter

-36        -26        -16        -6       Mat Ala His |Met Thr Pro Glu---
TAATCGTGTA ATCAATTGTT TTAACGCTTA AAAGAGGGAA TTTCCATG GCT CAT |ATG ACG CCG GAA---
                                  SD          NcoI       NdeI   CPase Taq →

---Arg Tyr Leu Glu Ala Lys Tyr Gly Ala Leu Tyr Gly Phe|Arg Gly Arg Glu Phe Leu
---AGG TAC CTG GAG GCC AAG TAC GGG GCC CTT TAC GGC TTC|CGC GGC CGC GAA TTC CTC
   KpnI                          ApaI    ← CPase Taq SacII NotI          XhoI

Glu Gly Ser Ser | Cys
GAG GGC TCT TCC | TGC → MxeIntein
     SapI
```

Fig. 3. Structure and restriction map of pCol-CPaseTaq-MICT. DNA sequence is numbered by an adenosine of first ATG on an *Nco*I site as +1. The colicin promoter region and Shine-Dalgarno (SD) sequence are underlined. Some restriction enzyme recognition sites are shown. The position of the CPase *Taq* gene is indicated as arrows.

Table 1. Purification of CPase *Taq* from *E. coli* cells harboring pCol-CPase*Taq*-MICT

Purification step	Total protein (mg/L)	Total activity (U)	Specific activity (U/mg)	Yield (%)
Cell-free extract	192.0	251,000	1,307	100
Affinity chromatography	47.2	182,000	3,856	73

Construction of an Expression Plasmid for CPase *Taq* Using the Expression Cassette

The carboxypeptidase *Taq* gene was amplified using primers CPaseTaq2-F (5'-CCATGGCTCATATGACGCC GGAAGCCGC-3' (inserted *Nco*I and *Nde*I sites underlined)) and CPaseTaq4-R (5'-GCGGCCGCGGAAGCC GTAAAGGGCCC-3' (*Not*I and *Sac*II sites underlined)), using pCP1-7 plasmid [2] as a template. The PCR product was ligated with pGEMT vector (Promega, USA), and a 1600 bp *Nco*I-*Not*I fragment was inserted into pCol-MICT, resulting in pCol-CPaseTaq-MICT plasmid. In the course of gene manipulations, glucose (final concentration: 2~3%) should be added to LB medium to repress the basal promoter activity of pCol-CPaseTaq-MICT plasmid. Otherwise, when *E. coli* cells were transformed by pCol-CPaseTaq-MICT plasmid, no any colony was obtained. The structure and restriction map of pCol-CPaseTaq-MICT is shown in Fig. 3.

Expression of the CPase *Taq* Gene in *E. coli* and Purification of the Enzyme

E. coli W3110 cells harboring pCol-CPaseTaq-MICT plasmid produced a large quantity of CPase *Taq* (Table 1 and Fig. 4). CPase *Taq* was effectively purified by affinity chromatography, using chitin beads, with an extremely high yield of 73% (Table 1 and Fig. 4). The mobility of the purified CPase *Taq* produced in *E. coli* (Fig. 4, lane 2) was identical with that of the CPase *Taq* purified from *T. aquaticus* YT-1 (Fig. 4, lane 3).

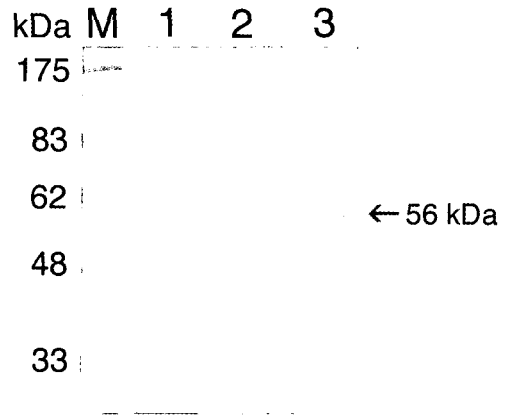

Fig. 4. SDS-PAGE of CPase *Taq* produced in *E. coli* cells harboring pCol-CPaseTaq-MICT plasmid. Lane M. size standard marker: lane 1 supernatant obtained on centrifugation after disruption of the cells by sonication: lane 2 sample after affinity chromatography: lane 3 purified CPase *Taq* from *T. aquaticus* TY-1. The arrow indicates the position of CPase *Taq*.

Table 2. Comparison of CPase *Taq* production by three expression systems

Expression Plasmid	Vector used	Promoter used	Purified protein (mg/L)	Specific activity (U/mg)
pCol-CPaseTaq-MICT[1)]	pCol-MICT	colicin	47.2	3,856
pCP1-7[2)]	pEXP7	*tac*	6.0	3,000
pET-CPaseTaq	pET15b	T7	N. D.[3)]	-

1) This work, 2) Reference 2, 3) N. D. indicates not detected.

The amount of finally purified enzyme produced by pCol-CPaseTaq-MICT plasmid was 47.2 mg/L of culture, which is approximately 8 times higher yield than that by pCP1-7 plasmid [2], a *tac* promoter-directed system (Table 2). On the other hand, no soluble form of CPase *Taq* was obtained using pET-CPaseTaq plasmid (Table 2). These data suggest that our colicin promoter-directed system could avoid the formation of inclusion bodies. The specific activity of the purified enzyme produced by pCol-CPaseTaq-MICT was also 30% higher than that produced by pCP1-7 (Table 1). Since mitomycin C is a DNA synthesis inhibitor, the colicin promoter is effectively induced by mitomycin C through the SOS response [5-8]. No protein was produced by pCol-CPaseTaq-MICT in the absence of mitomycin C (data not shown). Another advantage of our cassette is that the enzymes were purified homogeneously, by one-step affinity chromatography using chitin beads (Table 1 and Fig. 4).

Since no expression of CPase *Taq* was observed using the T7 promoter-based expression system (Table 2), our expression cassette would appear to be the clear choice for the expression of harmful proteins, especially for proteolytic enzymes.

Acknowledgement This work was supported by the 2002 grant from the Research Foundation of Silla University.

REFERENCES

[1] Hong, J.-K., M.-S. Park, F. M. Raushel, and Y.-H. Khang (2003) Expression and characterization of *Escherichia coli* prolidase with organophosphorus compounds. *Biotechnol. Bioprocess Eng.* 8: 126-129.

[2] Lee, S.-H., H. Taguchi, E. Yoshimura, E. Minagawa, S. Kaminogawa, T. Ohta, and H. Matsuzawa (1994) Carboxypeptidase *Taq*, a thermostable zinc enzyme, from *Thermus aquaticus* YT-1: Molecular cloning, sequencing, and expression of the encoding gene in *Escherichia coli*. *Biosci. Biotech. Biochem.* 58: 1490-1495.

[3] Masaki, H., A. Akutsu, T. Uozumi, and T. Ohta (1991) Identification of a unique specificity determinant of the colicin E3 immunity protein. *Gene* 107: 133-138.

[4] Ebina, Y., Y. Takahara, K. Shirabe, M. Yamada, T. Nakazawa, and A. Nakazawa (1983) Plasmid-encoded regulation of colicin E1 gene expression. *J. Bacteriol.* 156: 487-492.

[5] Cavard, D., S. P. Howard, R. Lloubes, and C. Lazdunski (1989) High-level expression of the colicin A lysis protein. *Mol. Gen. Genet.* 217: 511-519.

[6] Parker, R. C. (1986) Mitomycin C-induced bidirectional transcription from the colicin E1 promoter region in plasmid ColE1. *Biochim. Biophys. Acta* 868: 39-44.

[7] Waleh, N. S., I. Sohel, J. B. Lazar, D. V. Hudson, P. Sze, P. A. Underhill, and P. H. Johnson (1992) Expression of synthetic genes encoding fused proteins under tight control of modified regulatory regions of the colicin operon. *Gene* 117: 7-14.

[8] Waleh, N. S. and P. H. Johnson (1985) Structural and functional organization of the colicin E1 operon. *Proc. Natl. Acad. Sci. USA* 82: 8389-8393.

[9] Van den Elzen, P. J., M. J. Maat, H. H. B., Walters, E. Veltkamp, and H. J. Nijkamp (1982) The nucleotide sequence of the bacteriocin promoters of plasmids Clo DF13 and Col E1: Role of lexA repressor and cAmp in the regulation of promoter activity. *Nucleic Acids Res.* 10: 1913-1928.

[10] Petit, M. A., W. Bedale, J. Osipiuk, C. Lu, M. Rajagopalan, P. McInerney, M. F. Goodman, H. Echols (1994) Sequential folding of UmuC by the Hsp70 and Hsp60 chaperone complexes of *Escherichia coli*. *J. Biol. Chem.* 269: 23824-23829.

[11] Sanger, F., S. Nicklen, and A. R. Coulson (1977) DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* 74: 5463-5467.

[12] Rosen, H. (1957) A modified ninhydrin colorimetric analysis for amino acids. *Arch. Biochem. Biophys.* 67: 10-15.

[13] Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680-685.

[14] Masaki, H. and T. Ohta (1985) Colicin E3 and its immunity genes. *J. Mol. Biol.* 182: 217-227.

[Received June 5, 2004; accepted September 28, 2004]