J. Microbiol. Biotechnol. (2001), **11**(1), 85–91

JOURNAL OF
MICROBIOLOGY
AND
BIOTECHNOLOGY

Isolation of a Promoter Element that is Functional in *Bacillus subtilis* for Heterologous Gene Expression

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Received: August 23, 2000
Accepted: October 23, 2000

Abstract To construct an efficient *Bacillus subtilis* expression vector, strong promoters were isolated from the chromosomal DNA libraries of *Clostridium acetobutylicum* ATCC 4259, *Thermoactinomyces* sp. E79, and *Bacillus thermoglucosidasius* KCTC 3400. The P_{C27} promoter cloned from the clostridial chromosomal DNA showed a 5-fold higher promoter strength than the P_{SPO2} promoter in the expression of the *cat* gene, and its sequence was estimated as an upstream region of the predicted hypothetical gene (tet-R family bacterial transcription regulator gene) in *C. acetobutylicum*. As a promoter element, P_{C27} exhibited putative nucleotide sequences that can bind with bacterial RNAP and the 3' end of the 16S rRNA just upstream of the start codon. In addition, the promoter activity of P_{C27} was distinctively repressed in the presence of glucose. Using P_{C27} as the promoter element, a glucose controllable *B. subtilis* expression vector was constructed and the lipase gene from *Staphylococcus haemolyticus* KCTC 8957P was expressed in *B. subtilis*. When compared with the lipase expression by the T7 promoter induced by IPTG in *E. coli*, the P_{C27} promoter showed about a 1.5-fold higher expression level in *B. subtilis* than that without induction.

Key words: Strong promoter, *Bacillus subtilis* expression vector, lipase, overexpression

The production of heterologous proteins by bacteria at high levels is commonly achieved using *Escherichia coli* as the host [10, 18]. However, there are occasions in which the product of heterologous proteins produced are expressed as insoluble aggregates in *E. coli*. The overexpression of a protein in *E. coli* usually has toxic effects such as growth retardation or death of the bacterial host [13]. *Bacillus subtilis* is another efficient host strain for protein production because of its high secretion capability, well-studied genetic system, and extended fermentation technology [19]. Various foreign proteins including human interleukin-1 [1] and the antidigoxin single-chain antibody [27] have been industrially expressed in *B. subtilis* as extracellular soluble forms. There have been many attempts to improve the expression efficiency of heterologous proteins in *B. subtilis*, such as the development of protease-deficient strains [26] and the elucidation of efficient regulatory elements in transcription, translation, vector systems, and protein secretion [24].

Recently, various expression systems have been developed for controllable, high-level protein production in *B. subtilis*. Commonly used systems are based on the P_{spac} promoter [28], *Xyl*R-controlled promoter [6], and *sacB* promoter [26]. These promoters have advantages of controllable expression and a low cost for the inducer, yet also have certain disadvantages such as low activity or the need for further vector development.

The initiation of eubacterial gene transcription requires RNA polymerase (RNAP) and specific proteins, known as σ factors. $E\sigma^A$, the major form of *B. subtilis* RNAP, recognizes the $E\sigma^A$ consensus promoter by matching at both the -35 (TTGACA) and -10 regions (TATAAT) with an optimal spacing of 17 bp [8, 20]. The translation initiation of *B. subtilis* needs a higher stringency between the ribosome binding site of the promoter and the 3' end of 16S rRNA than in *E. coli* due to the absence of the S1 protein implicated in translation initiation [7]. In general, the strength of a promoter is determined based on similarity to the consensus sequence of the -35 and -10 regions and by the spacing between them [4, 25].

In this study, strong promoters were isolated and an efficient, glucose-controllable *B. subtilis* expression vector was constructed. Using this expression vector, heterologous

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lipase was then produced in *B. subtilis* with a higher expression level.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Media

E. coli DH5α [*supE44, ΔlacU169 (φ80 lacZΔM15), hsdR17, recA1, endA1, gyrA96, thi-1, relA1*] and *B. subtilis* DB104 (*his*$^-$, *nprR2, nprE18, aprEΔ3*) were used as the host strains for the DNA manipulation and expression, respectively. For the expression in *E. coli*, *E. coli* BL21(DE3) [F-*ompT, hsdS*$_B$(*r*^-_B*M*^-_B), *gal, dcm*] was used. The bacteria were usually grown in Luria-Bertani broth (LB) and the *E. coli* and *B. subtilis* transformants were selected on LB medium containing appropriate antibiotics. The DM3 regeneration medium used for the *B. subtilis* protoplast transformation [2] contained (per liter): 500 ml sodium succinate (1 M, pH 7.3), 100 ml casamino acid (5% w/v), 50 ml yeast extract (10% w/v), 100 ml phosphate buffer (3.5 g K_2HPO_4; 1.5 g KH_2PO_4 per 100 ml), 30 ml glucose (20% w/v), 20 ml $MgCl_2$ (20.3 g $MgCl_2 \cdot 6H_2O$ per 100 ml), 5 ml bovine serum albumin (BSA) (2% w/v), 8 g agar, and 5 mg chloramphenicol. To examine the effects of glucose on the expression level, the *B. subtilis* transformants were grown in Schaeffer's medium (2× SG) [12] with either 0.1% or 1% (w/v) of glucose.

For the isolation of the promoter, the chromosomal DNAs of *Clostridium acetobutylicum* ATCC 4259, *Thermoactinomyces* sp. E79, and *Bacillus thermoglucosidasius* KCTC 3400 were used. *Staphylococcus haemolyticus* L62 was used as the lipase producer [15]. Plasmid pPL703, a *Bacillus* promoter-probe vector, was used for the isolation of the promoters, and pTrc99A (Amersham Pharmacia Biotech. Ltd.) was used for the *B. subtilis-E. coli* shuttle vector construction. Plasmid pPL708 contained a promoter fragment derived from *Bacillus* phage SPO2 DNA in front of the CAT (chloramphenicol acetyltransferase) gene of pPL703 [23].

Chromosomal DNA Isolation and DNA Manipulation

The chromosomal DNAs from *C. acetobutylicum* ATCC 4259, *Thermoactinomyces* sp. E79, and *B. thermoglucosidasius* KCTC 3400 were isolated as described by Zahler [29]. To construct libraries, the chromosomal DNA was first digested with *Sau*3AI. The digested fragment mixture was then ligated with pPL703, which had been digested with *Bam*HI and dephosphorylated with calf intestinal alkaline phosphatase (Promega, Madison, U.S.A.). The library was obtained after the transformation of the ligation mixture into *B. subtilis* DB104 using the protoplast transformation method [2]. After the regeneration of the *B. subtilis* protoplast on DM3 agar plates, the transformants were transferred to LB agar plates supplemented with 500 μg/ml of chloramphenicol.

The transformation of *E. coli* was performed by the eletroporation method. Eletroporation-competent *E. coli* cells were prepared and used for electroporation with a GenePulser apparatus (Bio-Rad Lab., Hercules, U.S.A.) using the method of Dower *et al.* [5].

The general DNA manipulations were performed as described by Sambrook *et al.* [16].

The enzymes used for the DNA manipulation were obtained from commercial sources and used under the conditions recommended by the manufacturer.

Nucleotide Sequencing

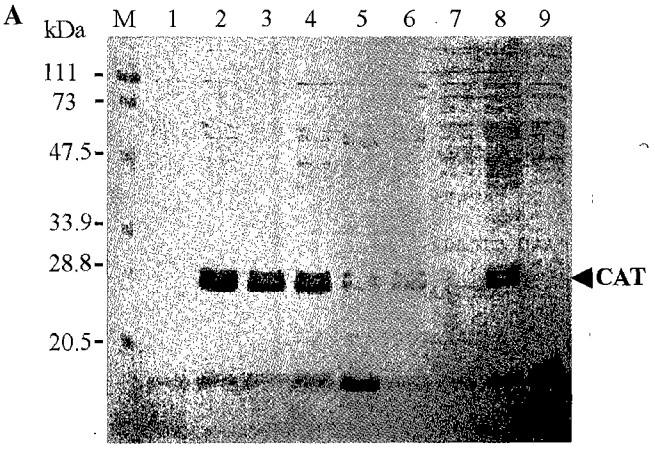
The plasmids from the *B. subtilis* transformants were isolated by the Qiagen plasmid isolation kit. The sequences of the cloned DNA in pPL703 were determined by the dideoxy sequencing reaction with the ABI Prism dye terminator cycle sequencing ready reaction kit (Perkin-Elmer Cetus, Foster City, U.S.A.), and the sequences were read on an automatic DNA sequencer (model 373A; Applied Biosystems, Foster City, U.S.A.) using primer PLN1 (5'-CACCACCACAATCATCCTTTCAAGAG-3') and PLN2 (5'-TGTGATTTACTGTATTCAGGAGGAG-3').

Assay of Enzyme Activity

To assay the enzyme activity, a 1 ml culture of transformed *B. subtilis* grown for 18 h in LB medium, containing 10 μg/ml of chloramphenicol for chloramphenicol acetyltransferase (CAT) production or 10 μg/ml of kanamycin for lipase production, was harvested by centrifugation at 12,000 rpm for 1 min. The cell pellet was resuspended in 0.4 ml of buffer [100 mM Tris/HCl (pH 8.0), 1 mM EDTA, 5 mM dithiothreitol, and 2 mg/ml of lysozyme] and incubated at 30°C for 20 min with slow shaking. Next, the cells were disintegrated with an ultrasonic homogenizer for 40 sec. The samples were then centrifuged at 12,000 rpm for 15 min and the supernatant was used for the enzyme activity assay. The CAT activity was measured spectrophotometrically [17]. One unit of CAT activity was defined as the amount of enzyme required to acetylate 1 μmol of chloramphenicol for 1 min at 37°C.

The lipase activity was measured with the pH-stat method by titrating the free fatty acids released by the hydrolysis of olive oil. The olive oil emulsion was prepared by emulsifying 5 ml of olive oil in 495 ml of 20 mM NaCl, 1 mM $CaCl_2$, and 0.5% (w/v) gum arabic solution for 2 min at maximum speed in a Waring blender. After the pH of the substrate emulsion (20 ml) was adjusted to 8.5 by the addition of 10 mM NaOH solution, an appropriate amount (10-50 μl) of the enzyme solution was added. The release rate of the fatty acid was measured using a pH titrator (718 Stat Titrino, Metrohm, Switzerland) for 5 min at 28°C. One lipase unit was defined as the amount of enzyme liberating 1 μmol of fatty acid per minute [9].

A



B

Promoter in pPL703	CAT activity (units/mg)
no promoter	not determined
P_{SPO2}	9.48
P_{C27}	50.21
P_{C71}	36.08
P_{T1}	32.95
P_{T19}	19.42
P_{T30}	17.57
P_{T38}	6.13
P_{B6}	19.53
P_{B18}	7.19

Fig. 1. SDS-PAGE analysis (A) of *cat-86* gene expression (B).
The cell extract of the *B. subtilis* transformant harboring each plasmid cultured in LB medium containing 10 μg/ml of chloramphenicol for 18 h at 37°C was electrophoresed on a 12% SDS polyacrylamide gel. Eighty μg of protein was loaded into each lane. The arrow designates the 26 kDa of CAT protein. Lane M: molecular weight marker; lane 1: pPL708 (pPL703-P_{SPO2}); lane 2: pPL703-P_{C27}; lane 3: pPL703-P_{C71}; lane 4: pPL703-P_{T1}; lane 5: pPL703-P_{T19}; lane 6: pPL703-P_{T30}; lane 7: pPL703-P_{T38}; lane 8: pPL703-P_{B6}; and lane 9: pPL703-P_{B18}.

The protein concentration was measured by the Bradford method using the protein assay kit (Bio-Rad Lab., Hercules, U.S.A.) with BSA as the standard protein.

Sequence Analysis

Sequence comparisons with the GenBank database were performed using the Advanced BLAST search of the BLAST 2.0 network service of the National Center for Biotechnology Information with the default parameter value provided. The unfinished *C. acetobutylicum* complete genome sequences were obtained from http://www.cric.com.

RESULTS AND DISCUSSION

Screening of a Strong Promoter for *B. subtilis*

To construct an effective expression vector for *B. subtilis*, strong promoters were screened from the chromosomal DNA of *C. acetobutylicum* ATCC 4259, *Thermoactinomyces* sp. E79, and *B. thermoglucosidasius* KCTC 3400 using the promoter probe vector, pPL703, which contained a promoterless *cat-86* gene as the reporter. Libraries were established by the insertion of *Sau*3AI digested fragments of the chromosomal DNAs into the *Bam*HI site of pPL703, which was then transformed into *B. subtilis* DB104. To isolate the strong promoter, LB agar medium containing a high concentration of chloramphenicol (500 μg/ml) was used as the selection medium. Only a *Bacillus* transformant harboring a strong promoter for *cat* gene expression could form a colony with this concentration of chloramphenicol. After plate selection, the promoter strengths of all the transformants were evaluated by the CAT activity assay and SDS-PAGE analysis. P_{SPO2}, already known as a strong promoter in *Bacillus* expression [22, 23], was used as the control promoter. Among the 103 colonies grown on LB agar medium containing 500 μg/ml of chloramphenicol, eight colonies (C27 and C71 from *C. acetobutylicum*, T1, T19, T30, and T38 from *Thermoactinomyces* sp., and B6 and B18 from *B. thermoglucosidasius*) showed higher CAT activities than that of the control strain. In particular, 4 transformants (C27, C71, T1, and B6) showed a distinct protein band of CAT on the SDS-PAGE analysis. Among the selected promoters, P_{C27}, the strongest, exhibited more than 5-fold higher promoter activities than P_{SPO2} (Fig. 1). The size of the cloned DNA fragment of these promoters was about 450 bp in the case of P_{C27}, P_{C71}, and P_{T1}, and about 1 kb in the case of P_{B6} (data not shown).

Analysis of Promoter P_{C27}

P_{C27} cloned from the chromosomal DNA of *C. acetobutylicum* ATCC 4259 was 468 bp and the DNA sequence completely matched with the *C. acetobutylicum* unfinished fragment of the complete genome from nt 3622126 to 3622593. It was also found that the P_{C27} promoter was followed by a predicted gene consisting of 187 amino acids. The ORF (open reading frame) of this gene is predicted to be a hypothetical tet-R family transcription regulator protein. The translation initiation site of P_{C27} was determined experimentally by N-terminal sequencing and this coincided with the predicted start site of the hypothetical gene. The promoter sequence analysis using the prediction program of the Berkely Drosophila genome project (http://www.fruitfly.org/~nomi/) predicted two transcription initiation sites located at -51 and -41 nucleotides upstream from the translation initiation site (+1), with prediction scores of 0.98 and 0.97, respectively. This prediction suggested that P_{C27} might include the elements needed for the transcription initiation of the predicted gene and could be functional as a promoter

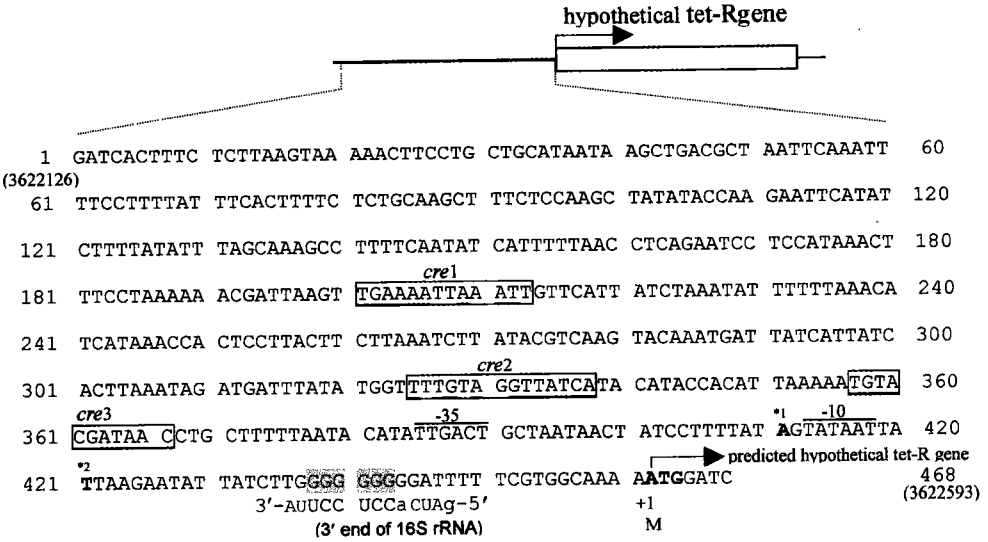
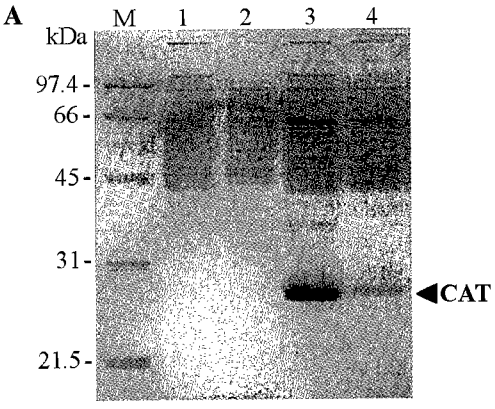


Fig. 2. Nucleotide sequence of the P_{C27} promoter and putative regions.
*1, Predicted transcription initiation site with score of 0.98; *2, Predicted transcription initiation site with score of 0.97.

in *C. acetobutylicum*. As a promoter element, P_{C27} had sequences similar to the -35 (TTGACA) and -10 regions (TATAAT), which could be recognized by the major RNAP σ factor of *B. subtilis*, σ^A. It also had a sequence complementary to the 3'-end of 16S rRNA, 15 nucleotides upstream of the translation initiation site, which might be functional as a ribosome binding site (Fig. 2). In addition, there were putative catabolite responsive elements (*cre*) upstream of the -35 region. These putative *cre* sequences of P_{C27} were predicted, based on their similarity to the *cre* consensus sequence (TGWNANCGNTNWCA) of Gram-positive bacteria [3, 14, 21]. Therefore, the effects of glucose on the promoter activity of P_{C27} were examined using *cat* gene expression in *B. subtilis*. When the *B. subtilis* transformant harboring pPL703- P_{C27} was grown in a 2× SG medium supplemented with glucose as the carbon source, the CAT activity of the culture containing 1% glucose was 19% of the activity obtained from the culture containing 0.1% glucose. This indicates that the promoter activity of P_{C27} in *B. subtilis* was repressed about 5.3-fold, probably by glucose-mediated catabolite repression (Fig. 3), and this result also suggests that there may be at least one *cre* sequence in P_{C27}. However, the level of glucose repression was quite low, which might have been caused by the existence of multiple copies of the *cat* gene in the host transformant or by a low similarity of the P_{C27} *cre* sequences to the *cre* consensus sequence in *B. subtilis*. Accordingly, the activity of P_{C27} can be controlled by the



B

Plasmid in strain DB104	Activity of CAT (units/mg)		
	0.1% glucose	1% glucose	Ratio[a] (1 - b/a)×100
pPL708	2	0.6	70 (%)
pPL703-P_{C27}	12.2	2.3	81.1 (%)

Fig. 3. Effect of glucose on the expression of the *cat-86* gene in *B. subtilis* bearing pPL703- P_{C27}.
Cells bearing each plasmid were grown in the 2× SG medium containing 10 μg/ml of chloramphenicol and either 0.1 or 1% glucose at 37°C for 18 h. A: SDS-PAGE analysis of CAT expression level in *B. subtilis* transformants. Lane M: molecular weight marker; lanes 1, 2: pPL708; lanes 3, 4: pPL703-P_{C27}; and lanes 1, 3: with 0.1% glucose; lanes 2, 4: with 1% glucose. B: CAT activities expressed by P_{SPO2} and P_{C27} promoters. [a](1 - b/a)×100 means the ratio of catabolite repression with the addition of glucose.

addition of glucose to the culture medium, and the metabolic burden on the host can also be reduced when P_{C27} is used as the promoter in the expression of foreign genes.

Construction of *B. subtilis* Expression Vector

To make a *B. subtilis* expression vector, a *B. subtilis-E. coli* shuttle vector was constructed and the P_{C27} promoter was then inserted into that plasmid. To make a *B. subtilis-E. coli* shuttle vector, a 3,513 bp fragment of pPL703 digested with *Eco*RI and *Pvu*II was ligated with a 2,430 bp fragment of pTrc99A digested with *Eco*RI and *Nde*I. The digested ends of the *Pvu*II and *Nde*I site was blunt-ended by a Klenow fragment (Boehringer Mannheim, Germany). The resulting vector pCJ1 (5,946 bp) contained two replication origins, the pBR322 origin for replication in *E. coli* and the pUB110 origin for the replication in *B. subtilis*, antibiotic resistant genes (Amp^r and Km^r) able to function in *E. coli* and *B. subtilis*, the rrnBT$_1$T$_2$ transcription terminator for plasmid stability, and a multiple cloning site. To use the *Nde*I restriction enzyme site as an insertion site for a foreign gene, an additional *Nde*I site in the pCJ1 was made blunt using the Klenow fragment. The resulting vector was designated as pCJ2 (5,949 bp). The promoter P_{C27} was then inserted into pCJ2 to make the *B. subtilis* expression vector pCJ227 (6,462 bp) (Fig. 4).

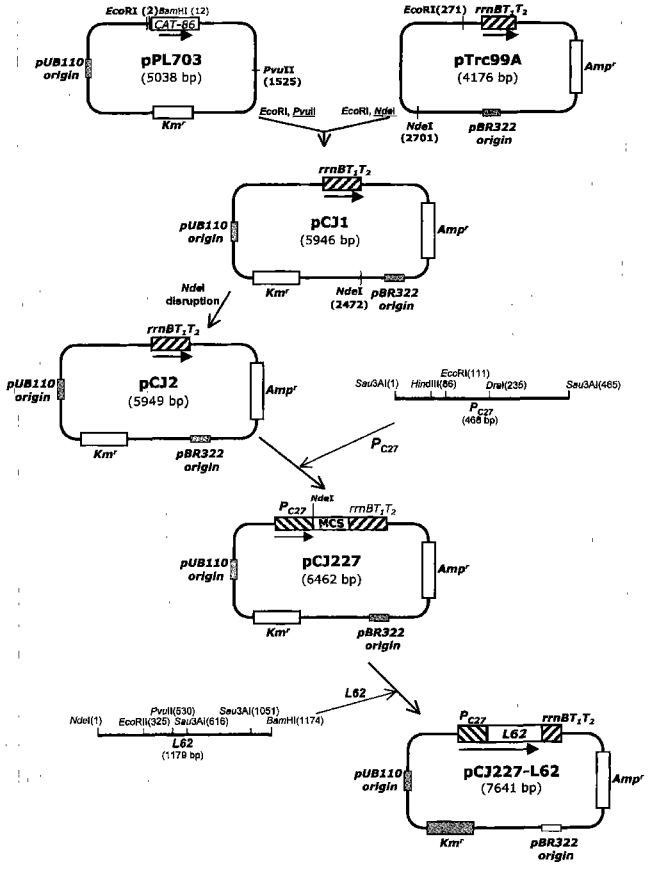


Fig. 4. Construction of the *B. subtilis-E. coli* shuttle vector and L62 lipase expression vector.
Underline: The restriction enzyme site was made blunt using the Klenow fragment.

Expression of Heterologous Lipase Genes in *B. subtilis*

Previously, the L62 lipase gene (1,179 bp) cloned from *Staphylococcus haemolyticus* KCTC 8957P has been expressed in *E. coli* in the pET22b(+) (Novagen) expression vector [15]. To establish P_{C27} as an efficient promoter able to function in *B. subtilis*, the L62 lipase gene was translationally fused to the P_{C27} promoter in pCJ227 (Fig. 4). *B. subtilis* DB104 was then transformed with the resulting plasmid pCJ227-L62. After 18 h of culture in LB medium containing 10 μg/ml of kanamycin at 37°C, the transformants were analyzed for their lipase production. *B. subtilis* containing the plasmid pCJ227-L62 expressed about 263 U/mg of L62 lipase, which accounted for about 44% of the total intracellular proteins, and L62 lipase (43 kDa) was observed as the major protein of the cellular extract on SDS-PAGE analysis (Fig. 5). When compared with the T7 promoter in *E. coli*, which was induced by IPTG, the P_{C27} promoter showed a 1.5-fold higher expression of L62 lipase than that without induction in *B. subtilis* (Table 1).

In this study, strong promoters were isolated that were able to function in *B. subtilis*, and an efficient *B. subtilis* expression vector was constructed. This expression system proved to have a higher expression activity than the T7 promoter. In addition, since the promoter activity of P_{C27} could be controlled by the addition of glucose in the culture medium, the P_{C27} promoter would be useful to control the expression of heterologous genes. For the further improvement

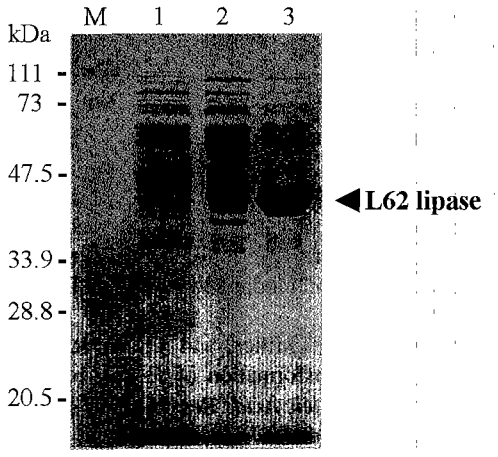


Fig. 5. SDS-PAGE analysis of L62 lipase expression.
Cells bearing the plasmid were cultured in LB medium containing 10 μg/ml of kanamycin at 30°C or 37°C for 18 h. Lane M: molecular weight marker; lane 1: cell extract of *B. subtilis* bearing pCJ227 cultured at 37°C; lanes 2 and 3: cell extract of *B. subtilis* bearing pCJ227-L62 cultured at 30°C and 37°C, respectively.

Table 1. Comparison of the L62 lipase expression levels of *Staphylococcus heamolyticus* L62 and various transformants.

Strain (plasmid)	Enzyme preparation	L62 lipase activity[a]		
		units/ml	units/mg	Ratio[b] (%)
S. heamolyticus KCTC 8957P	Culture sup	9.8	82.8	13.9
E. coli BL21 (pCJ227)	Sup[c]	ND[d]	ND	ND
E. coli BL21 (pET22b(+)-L62)[e]	Sup	70.8	180.0	30.3
B. subtilis DB104 (pCJ227)	Sup	ND	ND	ND
B. subtilis DB104 (pCJ227-L62)	Sup	743.0	263.4	44.3

[a]L62 lipase activity was measured by the method described in Materials and Methods.
[b]Ratio means the portion of L62 lipase in the total protein of each preparation. The specific activity of L62 lipase was estimated as 596 units/mg [15].
[c]Sup was the soluble fraction of whole cell lysate obtained after ultrasonic treatment.
[d]ND: not determined.
[e]Expression of the lipase gene fused with the T7 promoter was induced by IPTG for 4 h.

of the *B. subtilis* expression vector constructed in this study, certain sequences of the P_{C27} promoter need to be modified and the use of a signal peptide should be tested in order to achieve a more efficient extracellular production of heterologous proteins.

Acknowledgments

This work was supported by a research grant NT2030 from the Ministry of Science and Technology of Korea.

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