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Development of a Novel Vector System for Programmed Cell Lysis in *Escherichia coli*

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Abstract Although widely used as a host for recombinant protein production, *Escherichia coli* is unsuitable for massive screening of recombinant clones, owing to its poor secretion of proteins. A vector system containing T4 holin and T7 lysozyme genes under the control of the *ptsG* promoter derivative that is inducible in the absence of glucose was developed for programmed cell lysis of *E. coli*. Because *E. coli* harboring the vector grows well in the presence of glucose, but is lysed upon glucose exhaustion, the activity of the foreign gene expressed in *E. coli* can be monitored easily without an additional step for cell disruption after the foreign gene is expressed sufficiently with an appropriate concentration of glucose. The effectiveness of the vector was demonstrated by efficient screening of the amylase gene from a *Bacillus subtilis* genomic library. This vector system is expected to provide a more efficient and economic screening of bioactive products from DNA libraries in large quantities.

Keywords: *Escherichia coli*, programmed cell lysis, *ptsG* promoter, glucose, library screening

Escherichia coli, owing to its simplicity and well-known physiological and genetic properties, is one of the most widely used organisms in biotechnology [1, 2]. However, as often cited, *E. coli*, with a few exceptions, does not secrete proteins out of the cells. Secretion of bioactive proteins into extracellular media prevents protein aggregation, misformation of disulfide bonds, and growth inhibition owing to the accumulation of toxic products [10, 12]. It also provides a significant economical advantage, particularly in high throughput screening, thanks to the omission of the costly cell disruption step [5]. Various methods such as active transport of recombinant protein by fusion with signal peptides have been used to make bacteria secrete foreign proteins [1, 3, 6, 22]. This system can guarantee higher stability and easier purification of the target protein in *E. coli*. Yet, the availability and efficiency of active transport systems are not universal; the system is applicable only when target genes are cloned in frame, which is not suitable for construction of genomic libraries.

Host lysis systems of bacteriophages have also been suggested as a solution to this problem [5, 12, 15, 18]. Holins and endolysins play key roles in bacterial cell lysis by bacteriophage. Holins are small bacteriophage-encoded proteins that form a lesion to permeabilize the host cell membranes, and endolysins are soluble proteins with muralytic activities on the peptidoglycan of the bacterial cell wall [4, 24]. In order to prevent cell lysis before sufficient cell growth, lytic proteins are expressed with various inducible promoters known to provide tight regulation such as a temperature-sensitive promoter, T7 promoter, or xylose-inducible expression system [5, 15, 18]. However, these systems require additional induction steps, which make screening of large numbers of clones inefficient. From this viewpoint, we tried to find an inducible promoter that does not require additional steps for induction. We found that the P1 promoter of *ptsG* was suitable to control the expression of the lytic proteins because the promoter is induced upon glucose exhaustion when the promoter exists on a multicopy plasmid. The gene product of *ptsG* is the membrane-bound glucose permease, enzyme $IICB^{Glc}$, which is a component of the glucose-specific phosphoenolpyruvate:sugar phosphotransferase system. The *ptsG* gene is transcribed from two promoters, P1 and P2 [20]. In single copy in the chromosome, P1 is a major promoter and induced by glucose through the regulation of two global systems, positively by CRP-cAMP and negatively by Mlc [7–9, 16, 17, 20, 21]. In multiple copy on a plasmid, however, the regulation is reversed and the P1 promoter is induced by the absence of glucose.

In this study, a pBlueLysis+vector, in which the expressions of T4 holin and T7 lysozyme are controlled by a P1 promoter derivative of *ptsG*, was constructed for programmed cell lysis of *E. coli*. To facilitate the library construction, multicloning sites and α-fragment of the *lacZ*

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gene were inserted into pBlueLysis+, and *E. coli* cell lysis was evaluated by examining the secretion of GFPuv and amylase that were cloned in the vector. In addition, the utility of the vector was tested for screening of an amylase gene from a genomic library of *Bacillus subtilis*.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

E. coli DH5α was used as a host. *Bacillus subtilis* 168 was used to construct a genomic library [11]. Plasmids pACYC184, pLysT, pUC19, pBR322, pMW10, and pGFPuv (Clontech) were used as vectors and templates to amplify the target genes [15, 23]. Bacteria were cultured in either LB or Tryptone broth (TB; Tryptone 1%, NaCl 0.8%).

DNA Manipulations and Protein Methods

All DNA manipulations including cloning, transformation into *E. coli*, DNA isolation from agarose gel, polymerase chain reactions, and DNA sequencing were performed according to standard techniques and manufacturers' instructions, unless otherwise indicated [19]. Oligonucleotide primers used in PCRs are listed in Table 1. DNA sequencing was performed using a BigDye terminator cycle sequencing kit (PE Applied Biosystems, U.S.A.) and an ABI Prism 3730 XL DNA analyzer (PE Applied Biosystems, Foster City, CA, U.S.A.) at the National Instrumentation Center for Environmental Management (Seoul, Korea).

Construction of the Vector for Programmed Cell Lysis

Plasmid pACYC184 was used as the backbone of the lysis vector. The promoter region of *ptsG* was amplified with the primers of P*ptsG*-F and P*ptsF*-R (Table 1), and the CRP-binding site of *ptsG* was mutated through site-directed mutagenesis [18]. The holin and T7 lysozyme genes were amplified from pLysT using the primers of Lys-F and Lys-R. These PCR products were sequentially cloned into pACYC184 using restriction enzymes to obtain pGlys (Table 1). To remove the T7 terminator located between gene *t* (holin gene) and the T7 lysozyme gene, pGlys was partially digested with BamHI and completely digested with Bpu1102I, treated with Klenow fragment, and ligated.

To assure blue-white selection on X-gal plates and to add multicloning sites (MCS) to pGlys, *lacZ'* with MCS and its promoter was amplified from pUC19. In this step, the BamHI site was replaced with a BglII site through PCRs. First, *lacZ'* with MCS was amplified from pUC19 using one primer set of Lac-F0 and Lac-R0, and another set of Lac-F0 and Lac-R1. The PCR product from each reaction was digested with AvaI, and both fragments were ligated. PCR was performed on the ligate using Lac-F0 and Lac-R0, and only the DNA fragment of 572 bp was isolated from 1% agarose gel using a MinElute Gel extraction kit (Qiagen, Germany). The DNA was digested with EcoRV and ligated into PvuII-digested pGlys. The resultant plasmid was designated as pBlueLysis+ (Fig. 1). To acquire a negative control, pBlueLysis+ was digested with NcoI and BalI, treated with the Klenow fragment, and self-ligated. The resultant plasmid was designated as pBlueLysis−, which lacks the *ptsG* promoter and does not express lysis genes.

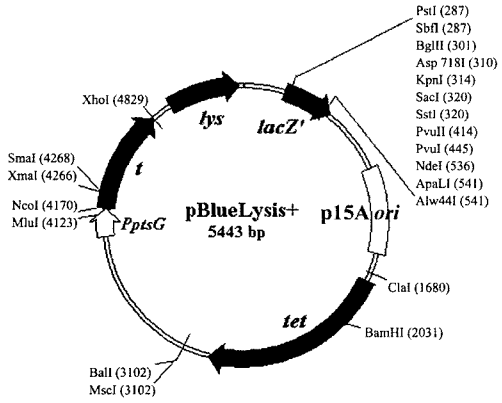
Construction of the Plasmids used to Verify pBlueLysis+ System

To examine the expression by the *ptsG* promoter derivative under various conditions, *lacZ* was amplified from pMW10 using primers LacZ-F and LacZ-R (Table 1). Then, it was ligated into pGly03 digested by EcoRI and SmaI, and then treated with Klenow fragment. The resultant plasmid in

Table 1. Oligonucleotide primers.

Primer designation[a]	Nucleotide sequence[b]	Description
PptsG-F (ScaI)	5'TGT**AGTACT**TCTCCAATGATCTGAA3'	For amplification of *ptsG* promoter
PptsG-R (NcoI)	5'ATGCATTCTTAA**CCATGG**TTGAGAGTGCTC3'	
Lys-F (NcoI)	5'GAAGGAGATATA**CCATGG**CACCTAGAATATCA3'	For amplification of genes of holin and T7 lysozyme
Lys-R (EcoRI)	5'TGCGAACAAAGG**GAATTC**GCTGTGGTCTCC3'	
Lac-F0 (EcoRV)	5'CCTCTGACACATG**GATATC**CGG3'	For amplification of P*lac*, *LacZ'*, and MCS from pUC19
Lac-R0 (EcoRV)	5'GCACGGACA**GATATC**CCGACTGG3'	
Lac-R1 (BglII)	5'ACTCTAGA**AGATCT**CCGGGTACCG3'	To replace BamHI site with BglII site in MCS
LacZ-F	5'CATCGTAGAGGGTATTAATAATG3'	For amplification of *lacZ* from pMW10
LacZ-R	5'AATACGGGCAGACATGGCC3'	
GFP-F (SmaI)	5'GGATC**CCCGGG**TACAAGGAGAAAAAATGAG3'	For amplification of *gfpuv* from pGFPuv
GFP-R (PstI)	5'CCTATTATTTTTGA**CTGCAG**ACAAGTTGG3'	
AmyE-F (BamHI)	5'CTTTTTTTATA**GGATCC**TTGATTTG 3'	For amplification of *amyE* from *Bacillus subtilis* 168
AmyE-R (BamHI)	5'GGTAAGTCCCGT**GGATCC**TTG 3'	

[a]The sites of restriction enzyme in blanks were inserted in the primer for cloning.
[b]The sequences in bold letters are the restriction enzyme sites.



B

-60 -40 -20

TCTGAAGTTGAAACGTGATAGCCGTCAAACAAATTGGCACTGA **Wild Type**

TCTGAAGTTGAAACATGATAGCCGTTAAACAAATTGGCACTGA **Mutated**

Fig. 1. Description of the constructed vector pBlueLysis+.
A. The map of pBlueLysis+. **B**. The mutated sequences in the CRP-binding site of the *ptsG* promoter. The underlined sequences are CRP-binding sites, and the sequences with asterisks are the mutated sequences. Numbers indicate distance from the transcriptional start point.

which *lacZ* was controlled by the *ptsG* promoter derivative was named pJH03.

To evaluate the lysis efficiencies of holin and T7 lysozyme, *gfpuv* controlled by the *bla* promoter or *amyE* with its native promoter was inserted into the MCS of pBlueLysis+ and pBlueLysis−. The *gfpuv* gene from pGFPuv was amplified by PCR using primers GFP-F and GFP-R (Table 1). The amplified product was digested with SmaI and PstI, and was ligated into pBR322 digested with SspI and PstI. The resultant plasmid (pBR-GFP) was digested with EcoRV and PstI, and the fragments including the *bla* promoter flanked by *gfpuv* was isolated from 1% agarose gel using a MinElute Gel extraction kit (Qiagen, Germany). The extracted fragments were ligated into pBlueLysis+ and pBlueLysis− digested with both PvuII and PstI. The acquired plasmids were designated pBL+GFPuv and pBL-GFPuv. The *amyE* with its native promoter was amplified from *B. subtilis* 168 using AmyE-F and AmyE-R (Table 1), and then cloned in pBlueLysis+ and pBlueLysis- treated with BglII. The resultant plasmids were designated as pBL+AmyE and pBL-AmyE.

β-Galactosidase Assay

The expression of β-galactosidase by pJH03 harboring *E. coli* was measured by the method of Miller [14].

Assay of GFPuv Activity

The culture medium was sampled periodically (1 ml). Cell-free supernatant was separated by centrifugation (12,000 ×*g* for 5 min at 4°C). The fluorescence was measured on a fluorescence spectrophotometer (Hitachi F4500, Tokyo, Japan) in a 1-cm quartz cuvette at excitation and emission wavelengths of 385 and 509 nm, respectively.

Screening of Amylase Activity on Plates

LB agar plates containing glucose, tetracycline (10 μg/ml), and 2% soluble starch was used to screen the amylase activity. Seed culture of *E. coli*, which harbored a plasmid containing the amylase gene, was prepared, and 10 μl of seed culture was dropped onto the surface of the agar plates. After an appropriate time, 10 ml of iodine solution (0.203 g I_2 and 5.2 g KI in 100 ml aqueous solution) was added to the plates, and degradation of the starch by the secreted amylase was detected as bright halos upon light illumination.

Genomic Library Construction of *B. subtilis* 168

Genomic DNA of *Bacillus subtilis* 168 was extracted according to the standard protocol with some modifications. *B. subtilis* cells were harvested from 3-ml overnight culture, and the pellet was resuspended in 400 μl of TE. After incubation at room temperature for 5 min, 50 μl of 10% SDS, 50 μl protease K (20 mg/ml in 50 mM Tris-HCl, pH 8.0), and 50 μl lysozyme (10 mg/ml in 10 mM Tris-HCl, pH 8.0) were added to the cell suspension, and incubated at 37°C for 1 h. The cell debris was extracted with an equal volume of phenol/chloroform/isoamylalcohol (25:24:1) and the supernatant was transferred into a fresh tube. After 5 μl of RNase (5 mg/ml) was mixed, the mixture was incubated for 30 min at 37°C. DNA was precipitated with 2 volumes of ethanol and 1/10 volumes of 5 M NaCl. The pellet was resuspended in 40 μl of TE. Purified genomic DNA was partially digested with Sau3AI, and DNA ranging from 3 kb to 6 kb was extracted from 1% agarose gel. Then, it was ligated into BglII-digested pBlueLysis+ or pBlueLysis−, and transformed into *E. coli* DH5α. Only white colonies on X-gal plates were picked, and inoculated into LB broth containing 10 μg/ml of tetracycline in 96-well plates. The constructed libraries were stored in a deep-freezer until screening. Screening on agar plates was performed using a 48-replica pin (Sigma, U.S.A.).

Quantification of Glucose Concentration in the Media

The glucose concentration remaining in the cell-free supernatant was determined by the dinitrosalicylic acid (DNS) method [13]. After 24-h incubation, cell-free supernatant was acquired by centrifugation (12,000 ×g, 5 min), and 500 μl of the cell-free supernatant was mixed with an equal volume of DNS solution (10.6 g 3,5-dinitrosalicylic acid, 19.8 g NaOH, 306 g potassium sodium tartrate, 7.6 ml phenol, 8.3 g sodium metabisulfate, and 1,416 ml distilled water). The reaction mixture was boiled for 5 min and cooled by placing the tubes on ice. Absorbance was measured at 575 nm in a 1-cm polystyrene cuvette using a spectrophotometer (Hitachi U-1100, Tokyo, Japan). Glucose

concentration was calculated by comparing against that of the standard curve.

Computer Programs

Sequence manipulation was conducted with DNASTAR software (DNASTAR Inc.). The plasmid map was drawn using Vector NTI 8 (InforMax, Inc.). The sequences of clones of genomic libraries were searched from the *B. subtilis* 168 genome using BLAST provided by the National Center for Biotechnology Information.

RESULTS

Description of pBlueLysis+

We tested the *ptsG* P1 promoter for controlled expression of lytic proteins in developing a programmed cell lysis system in *E. coli* because the promoter can be induced upon glucose exhaustion without any additional induction step. We mutated the CRP-cAMP binding site of the *ptsG* P1 promoter in order to make the basal promoter activity lower in the presence of glucose. The two nucleotides replaced in the CRP-cAMP binding site are shown on Fig. 1 and the new promoter was designated as *ptsG*PL. The constructed vector, pBlueLysis+ (GenBank Accession No. AY796342, Fig. 1), is a 5.4 kb low-copy number plasmid that has a p15A origin of replication and Tet marker. The control vector, pBlueLysis−, was also constructed to compare the effect of the lysis by the pBlueLysis+ system by removing the *ptsG*PL promoter from pBlueLysis+.

Optimization of Glucose Concentration for Lytic Protein Expression

Since the transcription of the *ptsG*PL promoter is dependent on glucose concentration, the expression pattern of the promoter was examined in the presence of various amounts of glucose. *E. coli* DH5α harboring pJH03 that has the *lacZ* gene under the control of the *ptsG*PL promoter was inoculated in LB containing various concentrations of glucose, and the expression of β-galactosidase was assayed. As shown in Fig. 2, the overall *lacZ* expression level was reduced by an increased glucose level in the media. Kinetics of the *lacZ* expression revealed that the promoter activity was increased as the glucose in the media was consumed by *E. coli*. The expression level of the *ptsG*PL promoter was very low in either LB or TB containing glucose higher than 0.1% (data not shown). LB containing 0.05% glucose was chosen as the optimum lysis condition in broth because the expression of the *ptsG*PL promoter was low enough in the presence of glucose in log phase, and induced high as glucose concentration was reduced at stationary phase.

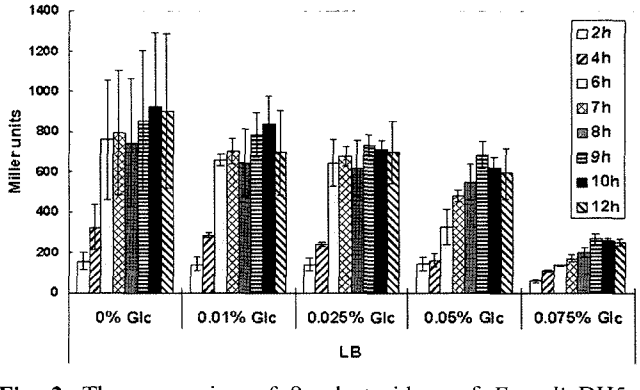


Fig. 2. The expression of β-galactosidase of *E. coli* DH5α harboring pJH03 in LB broth containing various concentrations of glucose.

GFPuv Release

With the expressions of holin and lysozyme, GFPuv, although located in the cytoplasm of *E. coli*, was detected in the culture supernatant (Fig. 3). The intensity of released GFPuv by holin and lysozyme was approximately twice as strong as that without holin and lysozyme. In the presence of 0.05% glucose in LB, GFPuv was detected in the cell-free supernatant after 8 h incubation. When 1% glucose was added, GFPuv release was not detected within 24 h after incubation. The delay in the release was mainly caused by the relatively high glucose concentration (0.56%) remaining after 24 h of incubation. This result suggested that 0.05% glucose is suitable for cell disruption and protein release within 12 h in broth culture.

Amylase Release

The effect of glucose concentration on secretion of recombinant protein on agar plates and the efficiency of

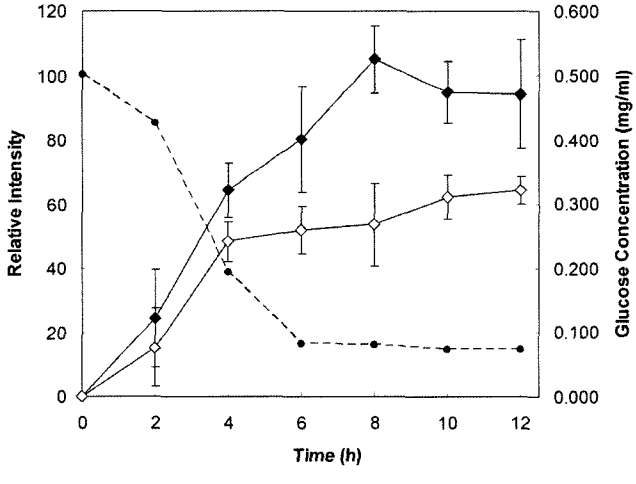


Fig. 3. GFPuv release resulting from the expression of cell lysis genes (holin and lysozyme).

■, GFPuv in cell-free supernatant with the expression of holin and lysozyme (pBL+GFPuv); □, GFPuv in cell-free supernatant without the expression of holin and lysozyme (pBL-GFPuv). The dotted line indicates the glucose concentration of cell-free supernatant.

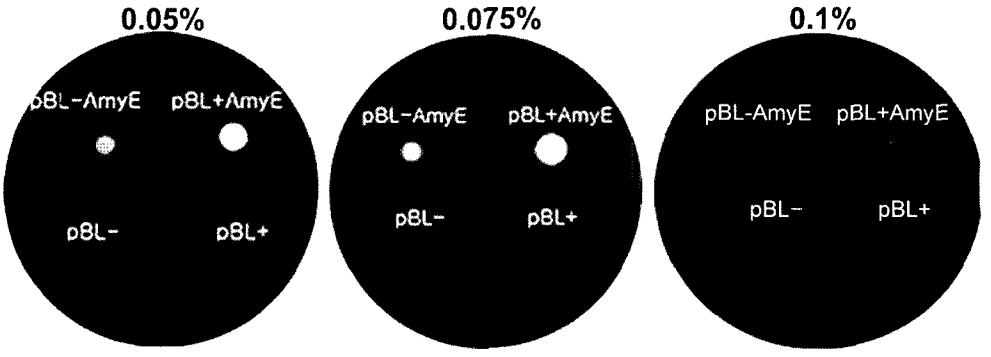


Fig. 4. Amylase release on LB agar plates containing 2% starch and 0.05%, 0.075%, or 0.1% glucose.
Holin and lysozyme are expressed from pBL+AmyE, but not from pBL-AmyE. Bright halos were formed around pBL+AmyE, due to starch hydrolysis, which were compared with *E. coli* harboring pBL-AmyE, pBL+ (pBlueLysis+), and pBL- (pBlueLysis-).

the constructed lysis system were studied with a cytoplasmic bacterial protein, AmyE, from *Bacillus subtilis* 168. *E. coli* DH5α harboring pBL+AmyE or pBL-AmyE, which has *amyE* with its native promoter in pBlueLysis+ or pBlueLysis-, was cultivated on LB agar plates containing 2% soluble starch and various concentrations of glucose. After incubation for 6 h to 10 h, iodine was stained on plates to see whether the clear zone was formed. The release of amylase was shown from 8 h after inoculation. At 8 h after inoculation, the degree of amylase release on LB agar plates containing 0.05% glucose was similar to that on the plates with 0.075% glucose (Fig. 4). However, the release of AmyE was not clearly detected at 8 h after inoculation in the presence of 0.1% glucose in the media. LB agar plates containing 0.075% glucose was selected as the optimum condition of the programmed lysis system on agar plates.

Secretion and Cell Viability

The changes in OD_{600} values were not significantly different for 12 h after inoculation between the cultures of *E. coli* DH5α harboring pBlueLysis+ and the negative control, pBlueLysis-, even though the secretion of intracellular protein was increased in the presence of pBlueLysis+ as described above (data not shown). However, we could observe the biggest difference in viable cell count in 8-9 h after inoculation (Fig. 5). The CFU of *E. coli* harboring pBlueLysis+ was decreased about one log scale after 8 h of growth compared with *E. coli* harboring pBlueLysis- (Fig. 5).

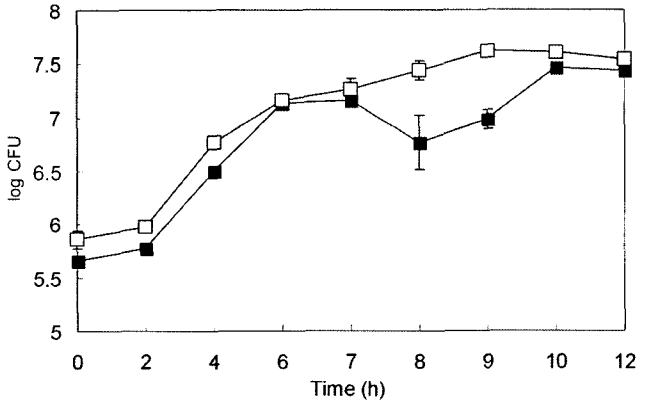


Fig. 5. Plate cell counting result of *E. coli* DH5α harboring pBlueLysis+ (■) or pBlueLysis- (□).

Application of pBlueLysis+

The genomic library of *B. subtilis* strain 168 was constructed using pBlueLysis+ and pBlueLysis- as described in Materials and Methods. Each genomic library of *B. subtilis* consisted of 3,648 clones. The average insert size of 60 randomly chosen clones from the genomic library was about 4.5 kb, and the percentage of the plasmids containing insert was about 78%.

Amylase was screened as the target enzyme. The screening was carried out on LB agar plates containing 0.075% glucose and 2% soluble starch by replica-plating of libraries arrayed in 96-well plates. After incubation at 37°C for 8 h, the plates were stained with the iodine solution. Two clones were found to have amylolytic activity on plates from the library using the pBlueLysis+ vector. Sequencing of the clones revealed that the two clones contained the *amyE* gene in the pBlueLysis+. No clone was found to have amylolytic activity from the library using pBlueLysis- in the same conditions.

Discussion

When a *ptsG* P1 promoter exists on a chromosome, repression of the promoter by Mlc is dominant over activation by CRP-cAMP such that the P1 promoter is activated in the presence of glucose. However, we found that the effect of glucose was reversed when the P1 promoter existed on a multicopy plasmid; that is, the *ptsG* P1 promoter was

repressed in the presence of glucose but activated in the absence of glucose (Fig. 3). This is probably because the intracellular concentration of Mlc is not high enough to bind to every P1 promoter that the activity of P1 promoter on a multicopy plasmid is more dependent on CRP-cAMP.

Using holin and lysozyme genes under the control of the *ptsG*PL promoter that is inducible in the absence of glucose, the pBlueLysis+ vector was developed to enable *E. coli* to release recombinant proteins upon glucose exhaustion without any extra step for cell disruption. pBlueLysis+ has several merits as a cloning vector. Firstly, insertion of *lacZ'* makes blue-white selection on the X-gal plate possible, allowing easy discrimination of the self-ligated clones. Secondly, a multicloning site inserted in *lacZ'* provides convenience in cloning of heterologous DNA in pBlueLysis+. In particular, BglII and PvuII sites are considered very useful, because partial digestion of DNA with Sau3AI or physical fragmentation of DNA that makes blunt ends are often used for library construction. Thirdly, pBlueLysis+ has a p15A origin of replication, which is compatible with the ColE1 compatibility group. Thus, pBlueLysis+ is able to coexit with pBR322 or pUC-derived plasmids, which facilitates the programmed cell lysis without further cloning of the target gene into pBlueLysis+.

pBlueLysis+ was very effective for rapid screening of bioactive molecules from recombinant clones. When pBlueLysis+ was used as a vector, *E. coli* successfully released a detectable amount of GFPuv in broth or amylase on agar plates in only 8 hours after inoculation. The utility of pBlueLysis+ in the construction and screening of genomic library was tested with DNA extracted from *B. subtilis*. Two clones harboring *amyE* were detected from a library made with pBlueLysis+ using iodine staining 8 h after spreading the library on plates, but none with pBlueLysis−. After 24-h incubation, four additional clones from library constructed with pBlueLysis+ and four clones from library constructed with pBlueLysis− showed clear halos on plates upon iodine staining (data not shown). However, all these clones were found to be false-positive. We do not know the exact cause of the high appearance of false-positive clones after extended incubation of the plates, but these results demonstrated that rapid activity screening enabled by using pBlueLysis+ can help reduce the appearance of false-positive clones in the screening of amylase activity using iodine staining.

Although this vector system enables enough lysis of *E. coli* for activity screening, application of this system did not lead to a huge reduction in the viability of *E. coli* after glucose exhaustion. Viable cell counting of *E. coli* harboring pBlueLysis+ revealed that the number of viable cells decreased by about one log scale (Fig. 5). This result indicates that the lysis gene induction may not be strong enough to kill *E. coli* cells because of low promoter activity. The low expression of holin and lysozyme may result in lower extracellular secretion of the recombinant proteins. However, a low level of cell lysis is better for efficient DNA library construction and the following high throughput screening of bioactive molecules. Because the pBlueLysis+ derived plasmids do not reduce the viability of its host *E. coli* much, the growth, maintenance, and storage of *E. coli* libraries made with pBlueLysis+ are easier and more stable.

The most significant merit of the pBlueLysis+ system is that neither inducers nor additional induction steps are necessary since the expression of lysis genes are induced automatically upon glucose exhaustion. This is advantageous for high throughput screening of DNA libraries, in particular. Addition of 0.05% to 0.075% glucose in LB media is sufficient for autoinduction of lysis genes after sufficient growth of *E. coli* cells. The autoinduction of lysis genes saves cost as well as effort, and discriminates pBlueLysis+ from previously developed systems such as the pLysT system [5, 15]. Taken together, pBlueLysis+ can provide a more efficient and economic screening of bioactive products from DNA libraries in large quantities.

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