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Deletion Analysis of *Pichia PGK1* Promoter and Construction of an Episomal Vector for Heterologous Protein Expression in *P. pastoris*

Lee Sung-Jae, In-Pyo Hong, Seon-Yeol Baek, and Shin-Geon Choi*
Department of Bioengineering and Technology, Kangwon National University, Chuncheon 200-701, Korea

Approximately 2.0 kb of the promoter region of the *Pichia pastoris* phosphoglycerate kinase gene (*PGK1*) was reduced to a 266 bp fragment and this minimized portion was used for construction of a new episomal constitutive expression vector in *P. pastoris*. As an approach to developing a constitutive expression vector in *P. pastoris,* the *GAP* promoter region of the *Pichia* expression vector pGAPZB was replaced with sequentially deleted *PGK1* promoter fragments fused to a *beta-galactosidase* gene. When a *lacZ* gene was used as a reporter gene, *PGK1* promoter strength was lower than that of the constitutive *GAP* promoter but it was higher than *TEF1*. We report here the development of the pPGKZ-E vector as a new episomal expression vector for heterologous gene expression by removing non-essential regions of the *PGK1* promoter. This broadens the choice of episomal expression vectors for controlled constitutive expression in *P. pastoris*.

Key words: *Pichia pastoris*, *PGK1*, promoter, expression vector

Introduction

The methylotrophic yeast *Pichia pastoris* has been successfully used for expression of heterologous proteins for the last two decades [5, 10]. This expression system uses relatively simple and inexpensive medium to produce high yields of extracellular proteins mediated by the highly inducible alcohol oxidase (*AOX1*) promoter or the constitutive glyceraldehyde-3-phosphate dehydrogenase (*GAP*) promoter.

Most of the expression vectors for *P. pastoris* use the inducible promoter from the alcohol oxidase gene (*AOX1*), which codes for the first enzyme in the methanol utilization pathway. Although this promoter has been successfully used to direct the expression of numerous foreign genes, there are circumstances in which it may not be suitable, due to the methanol requirement for induction [10].

Strong constitutive promoters have therefore often been chosen as alternatives for efficient foreign protein production in *S. cerevisiae* and *P. pastoris* [14]. Among them, the promoters most commonly used have been those of the glycolytic genes, phosphoglycerate kinase gene (*PGK1*), alcohol dehydrogenase gene (*ADH1*) and glyceraldehyde-3-phosphate dehydrogenase gene (*TDH1*). One of the reasons that the constitutive *GAP* (glyceraldehyde 3-phosphate dehydrogenase) promoter has not been widely used is the belief that constitutive production of foreign proteins in *P. pastoris* may have cytotoxic effects [10]. However, recent studies have found not only that cytotoxic effects are not necessarily observed, but also that production levels of a recombinant exo-levanase (LsdB) using the *GAP* promoter were similar to those using the *AOX1* promoter [11]. The choice of a promoter for heterologous gene constructions should reflect therefore the particular conditions under which the protein production may be conducted.

PGK1 promoters have long been used for constitutive expression of heterologous genes in yeast. Recently, the 3-phosphoglycerate kinase gene (*PGK1*) was cloned from *Pichia pastoris* and its promoter was used for constitutive expression [2]. It was reported that the gene expression level for amylase using the *PGK1* promoter was better than that with the *AOX* promoter [2]. However, it was impractical to use the entire promoter region in gene expression constructs because several restriction sites in the 2.0 kb gene fragment containing the *PGK1* promoter diminished the choice of cloning sites for heterologous expression.

To further characterize the *Pichia PGK1* promoter and

*Corresponding author
Tel: 82-33-250-6277, Fax: 82-33-243-6350
E-mail: choisg@kangwon.ac.kr

construct a convenient constitutive expression vector for *P. pastoris*, we deleted the non-essential region of the *PGK1* promoter without lowering promoter activity and inserted a *Pichia*-specific autonomous replication sequence (PARS1) for episomal expression [4, 8, 9]. We characterized the resultant pPGKZ-E expression vector containing the modified *PGK1* promoter by comparing protein production levels achieved with this vector with those of other constitutive promoters.

Materials and Methods

Strains, media, and DNA works

Escherichia coli bacterial strain XL10-gold (Stratagene, USA) was used as a host for plasmid constructions and was cultured in Luria-Bertani broth (1% tryptone, 0.5% yeast extract, and 1% sodium chloride) supplemented with ampicillin (50 ug/ml) or zeocin (25 ug/ml) when appropriate. The *P. pastoris* yeast strain X33 (Invitrogen, Carlsbad, CA) was cultured on YPD (1% yeast extract, 2% peptone, and 2% glucose) supplemented with zeocin (100 ug/ml) when appropriate. YPD with zeocin was also used when plating yeast that had been transformed by electroporation. For solid media, 1.5% agar was used for the bacterial and yeast media, respectively. All media components were manufactured by Difco Co. (U.S.A.), and all enzymes were purchased from Takara (Japan) unless otherwise noted. *Pichia* expression vectors including pGAPZB and pPIC6lacZ were purchased from Invitrogen (U.S.A.). Restriction enzyme digestions, plasmid engineering, and standard techniques were performed as specified elsewhere [16]. *Escherichia coli* transformation was performed by the $CaCl_2$ method and yeasts were transformed by electroporation with a Mini Gene-Pulser apparatus (Biorad) as specified by the manufacturer.

PCR amplifications of *PGK1* and *TEF1* promoter fragments

Based on the previous reported *Pichia PGK1* gene sequence (GenBank Accession No. AY288296), two PCR primers spanning 3.5 kb of the *PGK1* gene were synthesized and used for PCR gene cloning (see Table 1). The *PGK1* promoter region was cloned from nested-PCR of the entire gene product and its sequence was verified by DNA sequencing. PCR cloning of other regions of the *PGK1* promoter in various expression vector constructs was accomplished by using other sets of forward and reverse primers, as shown in Table 1. The *Pichia TEF-1* promoter region (GenBank Accession No. EF014948) was PCR amplified from *Pichia* chromosomal DNA using the following primers; TEF1-F (*Bgl*II) 5'-GAA GAT CTA TAA CTG TCG CCT CTT TTA TCT GCC-3' and TEF1-

Table 1. Nucleotide sequence of the oligonucleotides used in PCR amplification of different regions of the *PGK1* promoter

Primers	Nucleotide sequences (5'-3')
PGKfull-F	-2006 -1988 AGCGATATGGCACTAGTTG
PGKfull-R	1292 1280 CGAGGCGTCATCAAAATC
PGK2k-F (*Bgl*II)	-2006 -1988 GAAGATCTAGCGATATGGCACTAGTTG
PGK1k-F (*Bgl*II)	-983 -965 GAAGATCTATCACGCCTGCTCTGAGT
PGK0.75k-F (*Bgl*II)	-744 -725 GAAGATCTGCTCTAACTCGAGCAAGTGTC
PGK0.5k-F (*Bgl*II)	-499 -456 GAAGATCTCAGATCCCGTGATGCCACCTCTTG
PGK0.25k-F (*Bgl*II)	-266 -249 GAAGATCTCGGGTCTCTCAGCGAATT
PGK-R (*Bsp*T104I)	-1 -18 GGTAGGCGCGTTCGAATTTCGTAATCAATTGGGC
Promoter-less F (*Bsp*T104I)	CCCTTCGAAGAAACGAGGAATTC
Promoter-less R (*Bsp*T104I)	CCCTTCGAACATGCATGACCAAAATC

R (*Bsp*T104I) 5'-GGT AGG CGC GTT CGA AGT TGG CGA ATA ACT AAA ATG TAT G-3'. After double digestion of the PCR product with *Bgl*II and *Bsp*T104I, the *TFE-1* promoter gene was ligated into the *Bgl*II/*BspT*104I digested pGAPZ-E vector. The resultant plasmid was called pTEFZ-E.

Construction of modified expression vectors using the *Pichia PGK1* promoter

Several episomal constitutive vectors harboring different region of *Pichia PGK1* promotor were constructed by replacing the *GAP* promoter fragment from pGAPZB. PARS1 was PCR amplified from X33 genomic DNA and inserted into recombinant pPGKZ vectors as a replication origin for episomal plasmids. The overall construction strategy for pPGKZ-E is explained in Fig. 2. A promoter-less expression vector fused with the *E. coli beta*-galactosidase gene (pDPGKZ-E/lacZ) was also constructed using inverted PCR.

Beta-galactosidase assay

β-galactosidase activity was determined by the Miller method using O-nitrophenyl glucose as substrate. One β-galactosidase unit is defined as the amount of enzyme that is able to release 1 μmol O-nitrophenol per min at 37°C under the assay conditions.

SDS-PAGE gel analysis of reporter gene analysis

Pichia transformants of X33 were grown at 30°C for 2 days in 20 ml of YPD broth with Zeocin (100 ug/ml). When their optical density reached about 30 at 600 nm, they were centrifuged and resuspended in 0.5 ml of lysis buffer with a protease inhibitor cocktail (Sigma Co.). The cell suspension was transferred into a 2 ml mini bead-beater tube. 0.5 g of acid-washed glass beads (425-600 diameter, Sigma) were added to the cell suspension, and cell breakage was done with a Biospec Mini-Beadbeater (three 30 sec. treatments at 5,000 rpm with 1 min interval cooling on ice). The lysate was centrifuged for 10 min at 9,000 rpm and then the supernatant was further centrifuged for 15 min at 15,000 rpm. 2~3 ul of clear supernatant was subjected to SDS-PAGE gel electrophoresis using a Bio-rad Mini II kit.

Construction of reporter gene fused expression vectors

pPGKZ-E/lacZ and pTEFZ-E/lacZ were constructed by subcloning the *lacZ* gene from pPIC6lacZ (Clontech, U.S.A.). The *lacZ* gene was subcloned from pPIC6lacZ and ligated into *BspT*104I/*Not*I-cut pPGKZ-E. For comparison of promoter strength, PARS1 was inserted into the *BamH*I site of the pGAPZB vector which resulted in pPGKZ-E, pTEFZ-E, and pGAPZ-E vectors. PARS1 in all expression vectors was in the reverse orientation with respect to each promoter. All *Pichia* transformants were verified by colony PCR and the episomal state for each plasmid was verified by back-transformation into *E. coli*.

Results and Discussion

Deletion analysis of *Pichia PGK1* promoter and construction of episomal vectors

A 3.5 kb fragment containing the *PGK1* gene was PCR amplified from X33 genomic DNA and subsequently cloned into the pGEM T-easy vector. The full DNA sequence was verified by restriction analysis and DNA sequencing. The DNA sequence was identical with previously published data. Several forward primers spanning from the -2 kb to -0.25 kb region of the *Pichia PGK1* promoter were designed and used for PCR cloning into *Bgl*II-*Bsp*T104I-cut pGAPZB vector, which resulted in recombinant pPGKZ vector. A 168 bp of PARS1 PCR product was also obtained from X33 genomic DNA and inserted into the *BamH*I site of pPGKZ, yielding pPGKZ-E (Fig. 2). The orientation of the inserted PARS1 in recombinant pPGKZ-E (an episomal form of pPGKZ vector) was determined by DNA sequencing analyses. The gene expression level was slightly higher in reverse orientation than in the forward orientation so that only the reverse PARS1 orientation from all the recombi-

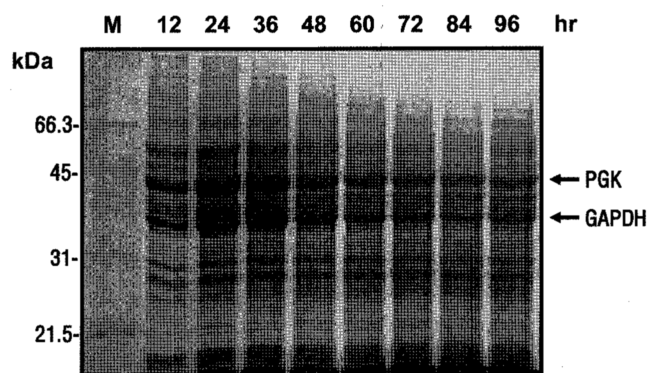

Fig. 1. SDS-PAGE analysis of the total yeast proteins during cell growth. Two major bands represent constitutive expression of PGK (44 kDa) and GAP (35 kDa) proteins, respectively.

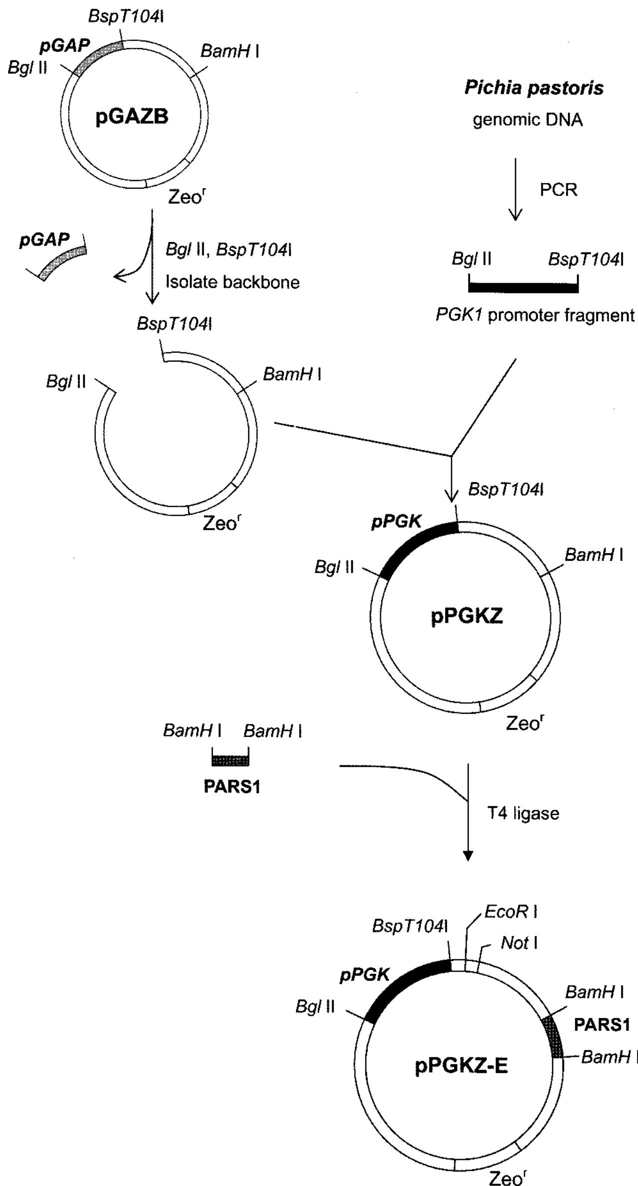

Fig. 2. Schematic representation of the construction of the pPGKZ-E vector.

nant pPGKZ-E vectors was used in further determinations of gene expression of reporter genes. The recombinant pPGKZ-E vectors harboring various deleted regions of the *Pichia PGK1* promoter were electroporated into the X33 strain and Zeocin-resistant transformants were selected and used for a *beta*-galactosidase assay.

The *beta*-galactosidase activities of *Pichia* transformants were initially tested by blue color formation on YPD with Zeocin and X-gal (20 ug/ml) and further analyzed by enzymatic assay using ONPG as a substrate. In case of YPD media used, there were no differences in *beta*-galactosidase activities of *Pichia* transformants between the full 2.0 kbp promoter-harboring clone and the 0.25 kb promoter-harboring clone (Fig.3A & 3B). This fact suggests that the 0.25 kb region of the *PGK1* promoter provides full promoter strength in gene expression (Fig. 3C). The minimum length of *Pichia PGK1* promoter, 0.25 kb, was even shorter than the 0.75 kb of previously reported *PGK1* promoter sequence of *Saccharomyces cerevisiae* [3]. In fact, there are many restriction sites within full 2.0 kb of *PGK1* promoter region which makes it difficult to clone a foreign gene. Therefore, elimination of the non-essential region contributed to construction of a useful multi-cloning site in the constitutive expression vector in *Pichia*. We tried to eliminate the *XbaI* site (box in Fig. 3C) in the 0.25 kb of *PGK1* promoter. The site-directed mutagenesis of a single nucleotide inside the *Xba*I site affected the strength of the promoter activity which means that the region is important for promoter activity (data not shown). So, we could not eliminate the *Xba*I site in the 0.25 kb fragment of the *PGK1* promoter. The episomal state of pPGKZ-E was determined by backtransformation into *E. coli*. The recombinant plasmid isolated from the *E. coli* transformant was identical with pPGKZ-E when it was digested by restriction enzyme analyses (data not shown).

Comparison of *Pichia PGK1* promoter strength with other constitutive promoters

Promoter strength of *Pichia PGK1* was compared with other *Pichia* constitutive promoters such as *GAP* and *TEF1* [1]. In order to compare the *PGK1* promoter's strength with the strong constitutive *GAP* promoter at the same copy number, PARS1 was also inserted into the *BamHI* sites of the pGAPZB and pTEFZ vectors, which resulted in pGAPZ-E and pTEFZ-E, respectively. Even though there was technical problems that we did not know whether these vectors were actually autonomous or has integrated or whether a portion of these vectors was one way and another portion another, it was assumed that the episomal vectors (pPGKZ0.25k-E, pGAPZ-E, and pTEFZ-E) exist at comparable copy numbers inside the X-33 host cell [4]. Then, protein expression levels were determined by subcloning the *lacZ* gene into newly constructed episomal vectors [17] and testing them. First, we monitored the growth of host cells harboring recombinant lacZ expression vectors (Fig. 4). In contrast to the observation of no growth inhibition with control vectors, expression of the lacZ reporter gene hampered the growth of host

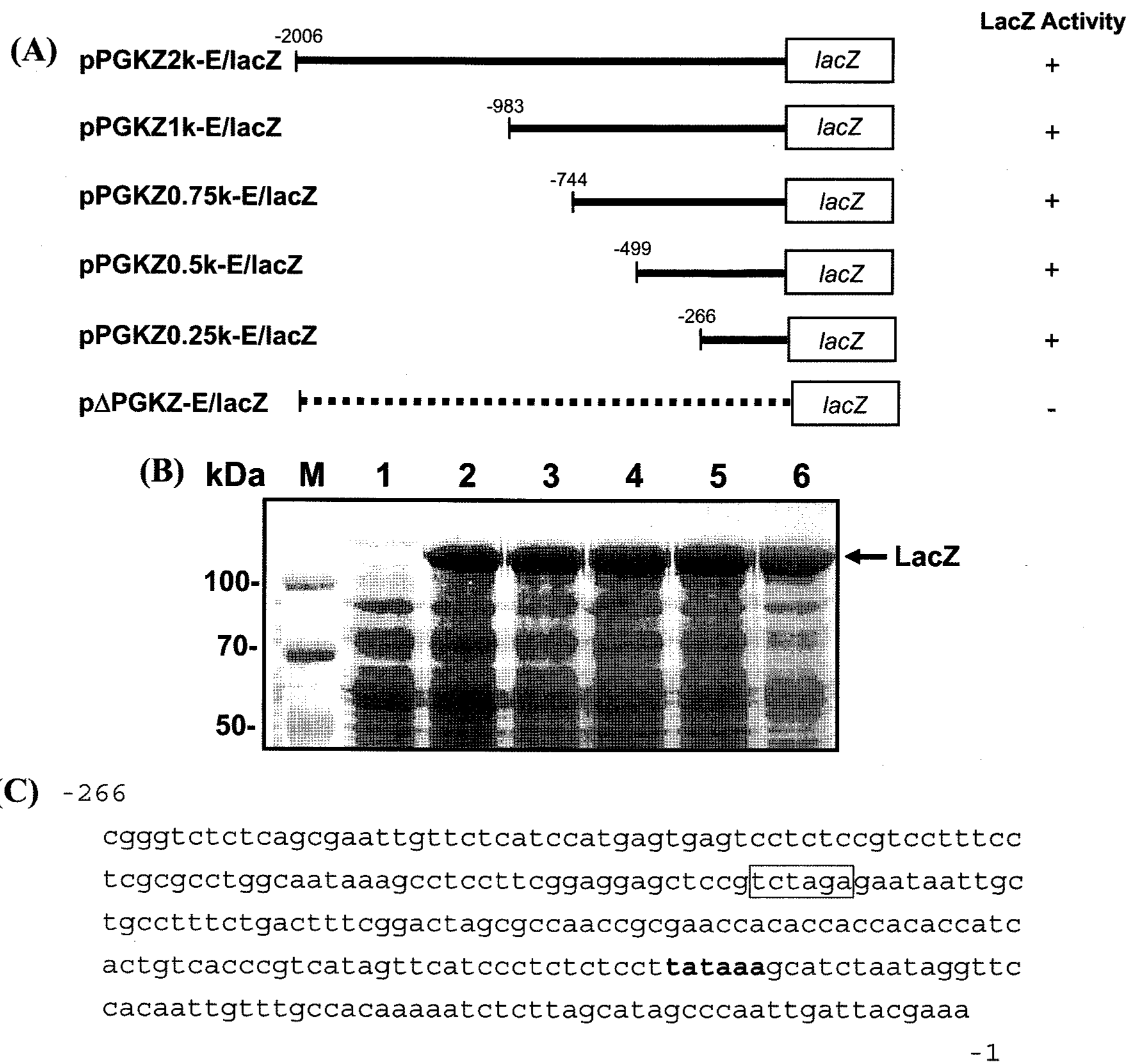

Fig. 3. Characterization of *Pichia PGK1* promoter gene. (A) Deletion analyses of the *PGK1* promoter gene fused with lacZ reporter genes, (B) SDS-PAGE analysis of lacZ reporter genes in yeast cell extracts. Each lane shows Coomassie blue staining of the total yeast proteins from the cells containing the plasmids. Lane 1; pPGKZ2k-E, lane 2; pPGKZ2k-E/lacZ, lane 3; pPGKZ1k-E/lacZ, lane 4; pPGKZ0.75k-E/lacZ, lane 5; pPGKZ0.5k-E/lacZ, lane 6; pPGKZ0.25k-E/lacZ, (C) 266 bp of core region of *Pichia PGK1* promoter. Bold represents TATA box region and box shows an internal *Xba*I site.

cells incorporating the *GAP* promoter. The recombinant yeast cells harboring *PGK1* and *TEF1* promoters did not show any delay in cell growth. This fact suggests that the host cell tolerates the toxic effects of foreign gene expression due to the moderate strengths of the two constitutive gene promoters. Second, the protein expression level of the lacZ protein was checked by analyses using SDS-PAGE protein gels and lacZ activity measurements (Fig. 5). The strong constitutive GAP promoter yielded the highest expression of lacZ reporter gene while 0.25k of *PGK1* and *TEF1* promoters showed slightly lower expression levels. We hypothesized that *Pichia* transformants harboring the *GAP* promoter grew more slowly than transformants with the other promoters due to its higher expression (Fig. 4B & Fig. 5B). The expression level of the lacZ control gene in the pPGKZ0.25k-E vector was lower than that of the pGAPZ-E vector but higher than that of pTEFZ-E (Fig. 5). The modulation in expression level using various constitutive promoters was almost identical with previously reported results for yeast promoters [12,13,14,15]. The cloning and expression of *Pichia TEF-1* promoter has also been reported by another group [1]. Because the expression of both pPGKZ0.25k-E/lacZ and pTEFZ-E/lacZ vectors did not inhibit the growth of host cells, modified expression vectors having the deleted *PGK1* promoter region could

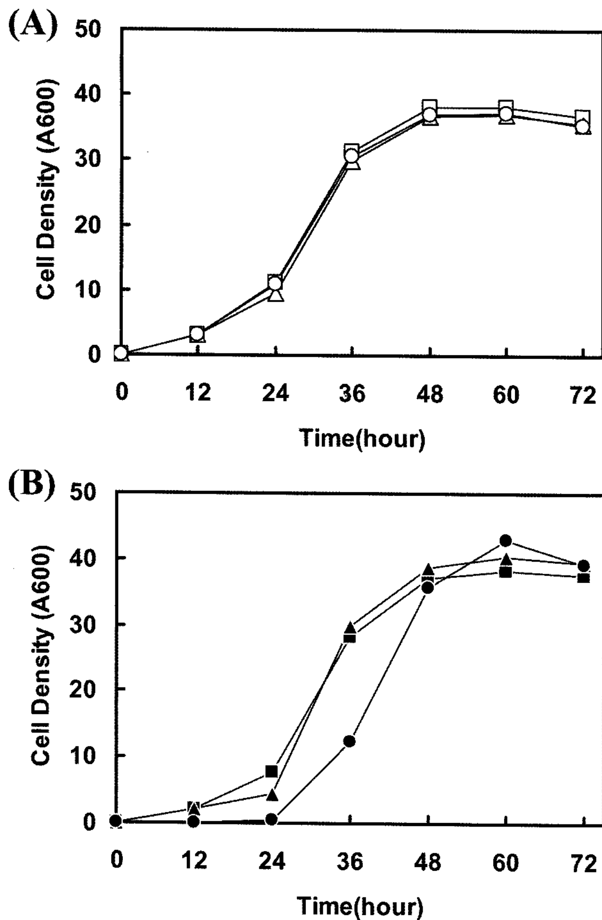

Fig. 4. Cell growth of *P. pastoris* cells with various constitutive promoters. (A) Expression plasmids only pPGKZ-E (–□–), pGAPZ-E (–○–), pTEFZ-E (–△–), (B) Recombinant plasmids containing reporter genes pPGKZ-E/lacZ (–■–), pGAPZ-E/lacZ (–●–), pTEFZ-E/lacZ (–▲–), were used to transform X-33 strains.

be used along with the *TEF1* promoter for expression of putative cytotoxic eukaryotic proteins. We did not measure the modulation of expression level of various promoters by direct comparison of episomal *versus* the integrated form. But, previous studies suggest that the episomal expression based on PARS1 was higher than the integrated expression when *lacZ* gene was used as a reporter gene [6].

In conclusion, it seems that the modified *PGK1* promoter could be an alternative choice for constitutive expression in *Pichia* when expression of heterologous proteins yields cytotoxic effects to the host strain. Furthermore, because it does not cause an instability in cell growth, the new expression vector can be used for controlled constitutive expression in *P. pastoris.*

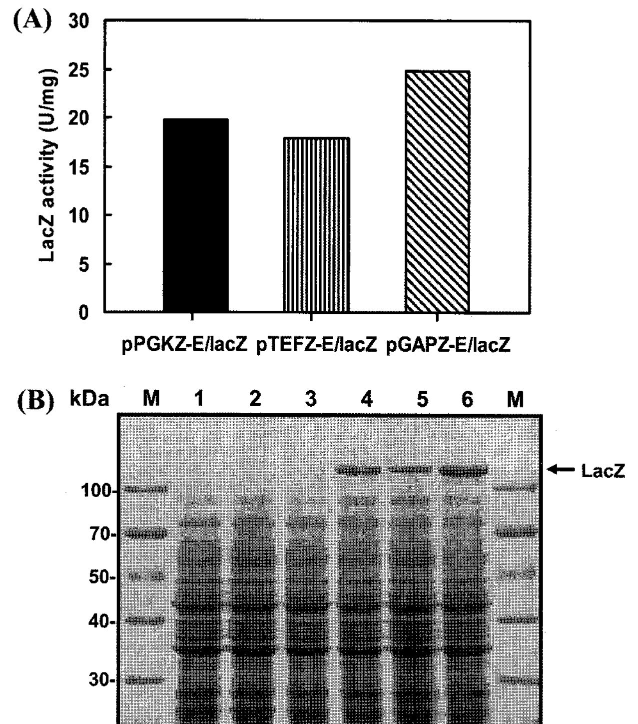

Fig. 5. LacZ acitivities and protein expression levels from various constitutive promoters during cell growth. (A) Specific lacZ activities of yeast cells at 48 hr. Each value represents the averaged lacZ activity measured in three different cell extracts (B) SDS-PAGE gel analyses of yeast cell extracts. Lane 1; pPGKZ-E, lane 2; pTEFZ-E, lane 3; pGAPZ-E, lane 4; pPGKZ-E/lacZ, lane 5; pTEFZ-E/lacZ, lane 6; pGAPZ-E/lacZ.

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국문초록

*Pichia PGK1*프로모터의 분석과 *P. pastoris*에 있어 외래단백질발현을 위한 Episomal벡터의 제조

이성재 · 홍인표 · 백선열 · 최신건*

강원대학교 생물공학과

대략 2 kb의 크기를 가진 *Pichia pastoris* phosphoglycerate kinase gene (*PGK1*)의 프로모터부분을 266bp의 작은 크기로 최소화하여 *P. pastoris*에 있어 episomal형태의 새로운 항시적 발현벡터를 제조하였다. *P. pastoris*의 새로운 항시적 발현벡터를 개발하기 위하여 기존의 *Pichia*발현벡터인 pGABZB의 GAP 프로모터부분을 연속적으로 일정부분이 절단된 *PGK1*프로모터에 beta-galactosidase유전자가 결합된 부분으로 치환하였다. LacZ유전자를 reporter유전자로 사용하였을 때에 *PGK1*프로모터의 발현세기는 다른 항시적 프로모터인 *GAP*프로모터 보다는 낮았지만 *TEF1*프로모터 보다는 높았다. 본 논문에서 *PGK1* 프로모터의 불필요한 부분을 제거함으로서 *Pichia*에서 외래발현을 위한 새로운 episomal발현벡터인 pPGKZ-E를 제조하였으며 이 것은 *P. pastoris*에 있어 발현세기를 선택할 수 있는 발현벡터선택의 폭을 넓게 하였다.