Vector promoters used in *Klebsiella pneumoniae*

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Abstract

Much effort has been devoted to the metabolic engineering of *Klebsiella pneumoniae*; however, our knowledge of the actual expression level of promoters used in *K. pneumoniae* is limited. In this study, the expression levels of three promoters were compared systematically by using the *lacZ* reporter gene with different carbon sources in *K. pneumoniae*. The results showed that, although promoters P*T5* and P*tac* designed for *Escherichia coli* were functional, P*T5* appeared more efficient and the induction/repression ratio of P*tac* was decreased extremely in *K. pneumoniae.* The basal level of P*tac* for *lacZ* expression reached 396.5 U/mg, which was 9.5-fold higher compared with P*T5* in LB medium, indicating P*tac* can be used as an efficient "constitutive" promoter as well as an efficient induced promoter in *K. pneumoniae*. In different carbon sources medium, a newly constructed endogenous constitutive P*bud* proved to be a stable and weak promoter. On the basis of our data, a set of P*bud* and P*tac* promoters could meet the broad range (about 1,000 orders of magnitude) of gene expression needed for engineered *K. pneumoniae* in glycerol-based medium. © 2015 International Union of Biochemistry and Molecular Biology, Inc. Volume 00, Number 0, Pages 1–6, 2015

Keywords: *gene expression, Klebsiella pneumoniae, LacZ reporter gene, metabolic engineering, promoter*

1. Introduction

The common Gram-negative bacterium *Klebsiella pneumoniae* is a fast growing, metabolically versatile microbe with the ability to produce 1,3-propanediol [1], 2,3-butanediol [2], 3-hydroxypropionic acid [3], pyrroloquinoline quinine [4], and other chemicals; many of its products are bulk platform chemicals and *K. pneumoniae* has demonstrated considerable capacity for their biological production [5, 6]. Although *K. pneumoniae* is a hazard group 2 facultative pathogen, its importance as an industrial microorganism has attracted worldwide interest [7–10].

In order to achieve strains of *K. pneumoniae* suitable for industrial use, cloning and overexpression of genes involved in product synthesis pathway is the most common method [11–14]. In engineered cell construction, the expression levels of many related genes need to be tightly regulated [15]. Ma et al. [16] found 1,3-propanediol yield was failed to improve in *K. pneumoniae*, whereas the *dhaB* and *dhaT* genes displayed a high level of expression with a strong promoter. Sun et al. [4] reported a moderate rather than strong promoter was efficient for production of pyrroloquinoline quinone by *K. pneumoniae*. Consequently, the genes for specific production biosynthesis should be expressed by appropriate promoters for effective engineering of a *K. pneumoniae* strain.

Usually, regulated promoters are constructed for high-level expression of target genes [17, 18]. To date, only one temperature-regulated promoter has been constructed directly for *K. pneumoniae* [19]. Owing to changes of temperature, this kind of promoter has been used only rarely in metabolic engineering applications. Some heterogeneous inducible promoters derived from *Escherichia coli*, including P*lac* and P*tac*, were normally considered as strong promoters for engineered *K. pneumoniae* [13, 16, 20–22]. Nevertheless, the actual

Abbreviations: *β-Gal, β-galactosidase; DCW, cell dry weight; IPTG, isopropyl β-D-1-thiogalactopyranoside; LB, Luria-Bertani medium; ONPG, O-nitrophenyl-β-D-galactopyranoside; Pbud, bud promoter; Ptac, tac promoter; PT5, T5 promoter.*

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activities of these promoters in *K. pneumoniae* are not known; further, with the change of repressor, the regulation/deregulation of gene expression would be different in *K. pneumoniae* compared with *E. coli*. Apart from inducible promoters, some endogenous and heterogeneous constitutive promoters have been used in *K. pneumoniae* and the relative strength of each promoter was not compared directly [4, 11]. Therefore, unlike *E. coli* or other well-studied microorganisms, our knowledge of the actual expression levels of promoters used for engineering of *K. pneumoniae* is limited.

In this study, the expression levels of promoters were compared systematically by using the *lacZ* reporter gene with different carbon sources in *K. pneumoniae*. Two heterogeneous promoters (P*tac* and P*T5*) and one endogenous promoter (P*bud*) were selected. P*tac* was known as a strongly induced promoter and P*T5* was also reported as a strong constitutive promoter in *E. coli* [23]. Because of the ability of *K. pneumoniae* to produce 2,3-butanodiol in various conditions, the promoter of P*bud*, responsible for 2,3-butanediol biosynthesis, seems to be an efficient native constitutive promoter. All comparisons were done using an identical vector backbone; we believe the results can be used as guidance for effective engineering of *K. pneumoniae*.

2. Materials and Methods

2.1. Strains, media, and growth conditions

E. coli strain DH5α was used for cloning and expression. *K. pneumoniae* strain KG1, which was a high 1,3-propanediol producer [24], was used to compare the expression of different promoters in *K. pneumoniae*.

For expression of the *lacZ* reporter gene, cell culture was with a 5% (v/v) inoculation in shaker flasks at 200 rpm at 37 °C for 24 H. Normally, Luria-Bertani (LB) medium was used for both seeding and expression processes. During the investigation of promoter characteristics with different carbon sources, the expression medium (per liter) contained 20 g glucose (or glycerol), 1 g KCl, 2.2 g $KH_2PO_4 \cdot 2H_2O$, 4 g $(NH_4)_2SO_4$, 0.4 g $MgSO_4 \cdot 7H_2O$, 2 g yeast extract, and 0.4 mL trace element solution. Kanamycin (50 μg/mL), ampicillin (50 μg/mL), or isopropyl β-D-1-thiogalactopyranoside (IPTG) (0.1 mmol/L) was added to the medium when required.

2.2. Construction of expression vectors

General genetic manipulations followed standard procedures. Sequences of all the oligonucleotides used for PCR in this study are given in Table 1. A 3,075 bp fragment of *lacZ* was amplified by PCR from *E. coli* BL21 and then inserted into the plasmid pMD18-T. The resulting plasmid pMD18::lacZ was used as donator of the *lacZ* reporter gene with *Hin*dIII–*Xho*I digestion.

Three expression vectors with different promoters were all constructed from the vector backbone of pET28a, which is a broad-host-range plasmid for Gram-negative bacteria. The sequences containing P*tac* (260 bp) and P*bud* (121 bp) were amplified from the vector of pGEX-4T-1 and the genome of *K. pneumoniae* KG1, respectively. The amplified fragments were inserted into pET28a at the *Bgl*II–*Eco*RI site with replacement of the native T7 promoter and the ribosome binding site to generate two vectors named pETtac and pETbu. The sequence of the T5 promoter (75 bp) [23] was synthesized (Invitrogen, Shanghai, People's Republic of China) and then inserted into pET28a at the *Bgl*II–*Xba*I site with replacement of the native T7 promoter to constructed the vector named pETT5.

The *lacZ* reporter gene was inserted downstream of the promoter sequence of the three vectors mentioned above at the *Hin*dIII–*Xho*I site. The positive recombinants were screened by application to an LB kanamycin plate and identified by sequencing. The recombinant plasmids were transformed into *K. pneumoniae* KG1 and *E. coli* DH5α by electroporation for further experiments. All the plasmids used and constructed in this study are given in Table 2.

TABLE 1 *Oligonucleotides used for PCR*

Primer	*Sequence(5′–3′)*
lacZ-F	CACaagcttGCGTTTTACAACGTCGTGAC
lacZ-R	CCGctcgagTTATTTTTGACACCAGACCA
P*bud*-F	GGAagatctATCGAAAACGTCTCA
P*bud*-R	CCGgaattcCATATACCACTTCCT
P*tac*-F	GGAagatctACGTTATCGACTGCACGG
P*tac*-R	GGCgaattcCATGAATACTGTTTCCTGT
P*T5*-F	GGAagatatAAAAAACTGCAAAAAAT
P*T5*-R	GGCttcgaaGTTATCGCTCTCATCGAA

2.3. Analytical methods

Cell concentration was measured as the optical density at 620 nm (OD_{620}) after growth for the appropriate length of time. The cell dry weight (DCW) was related to the cell optical density with an experimentally determined calibration curve. β-Galactosidase (β-Gal) activities were measured as described by Macgregor et al. [25]. The cell harvested from 1.5 mL broth by centrifugation (8,000*g* for 20 Min at 4 °C) were washed with cold PBS buffer, and then resuspended in the same volume of lysis buffer (50 mmol/L Tris–HCl, 1 mmol/L EDTA, 100 mmol/L NaCl, 8 mmol/L $MgCl_2$, and pH 8.0). After sonication, the cell lysate was centrifuged at 8,000*g* for 20 Min at 4 °C, and then the supernatant was diluted with cold deionized water at a suitable concentration for detection the activity of β-Gal. O-nitrophenyl-β-D-galactopyranoside (ONPG) was used as substrate during the reaction. One unit (1 U) of β-Gal activity is defined as the amount of enzyme converting 1.0 nmol ONPG/Min at 37 °C. Here, U/mg of β-Gal activity means the β-Gal activity (U) per

TABLE 2 *Plasmids used in this study*

Plasmid	*Relevant characteristics*	*Source or reference*
pET28a	Broad-host-range vector; P*T7*	Novagen
pMD18-T	P*lac*; *Amp*^{r}	Takara
pMD18::lacZ	P*lac*; *lacZ*	This study
pGEX-4T-1	P*tac*;	Amersham
pETbu	pET28a derivative; P*bud*	This study
pETtac	pET28a derivative; P*tac*	This study
pETT5	pET28a derivative; P*T5*	This study
pETbu::lacZ	pET28a derivative; P*bud*; *lacZ*	This study
pETtac::lacZ	pET28a derivative; P*tac*; *lacZ*	This study
pETT5::lacZ	pET28a derivative; P*T5*; *lacZ*	This study

TABLE 3 *Cell growth and β-Gal activity in strains*

Strain/plasmid	*Inducer (IPTG)*	*Cell concentration (mg/L)*	*β-Gal activity (U/mg)*
DH5α/ pET28a	–	1.42	–
DH5α/pETbu::lacZ	–	1.35	10.3
DH5α/pETT5::lacZ	–	1.49	22.2
DH5α/pETtac::lacZ	–	1.40	152.6
DH5α/pETtac::lacZ	+	1.59	37,544.2
KG1/pET28a	–	2.10	1.5
KG1/pETbu::lacZ	–	2.09	26.6
KG1/pETT5::lacZ	–	2.09	41.9
KG1/pETtac::lacZ	–	1.87	396.5
KG1/pET28a	+	1.86	400.7
KG1/pETtac::lacZ	+	2.22	30,321.3

"–", without IPTG; "+", with 0.1 mmol/L IPTG. Results are averages of three identical cultures.

mg DCW. Expression of the β-Gal protein was quantified by SDS-PAGE electrophoresis.

3. Results

3.1. Quantitative evaluation of promoters in *K. pneumoniae*

The expression of three promoters in *K. pneumoniae* was evaluated by the *lacZ* reporter gene. The cell growth and β-Gal activity of different *K. pneumoniae* transformants after 12 H of flask-scale cultivation and the expression properties of the promoters in *E. coli* are given in Table 3. Under normal culture conditions in the absence of the inducer IPTG, the β-Gal activity in all the transformants with these three different promoters was significantly greater than that of KG1 containing the blank vector pET28a, which means these promoters for *lacZ* expression were valid. As constitutive promoters of P*bud* and P*T5*, the β-Gal activity in their relevant transformants was 26.6 and 41.9 U/mg, respectively. It is interesting that the leaky expression of P*tac* was very high, the β-Gal activity of the strain with P*tac* without inducer reached 396.5 U/mg, which was 9.5-fold higher compared with P*T5*. The order of strength of the promoters in the case of DH5α transformants and KG1 was the same; that is, P*tac* > P*T5* > P*bud*. Our data indicated the native promoter of P*bud* and the heterogeneous promoters of P*tac* and P*T5* were more efficient for *lacZ* expression in KG1 compared with DH5α (about twofold).

There is a native lac operon in the KG1 genome and the level of β-Gal activity in KG1 containing the vector of pET28a as a negative control was 400.7 U/mg under IPTG-induced conditions. It was found that the β-Gal activity in the KG1 transformant from P*tac* as induced by IPTG and was very high at 30,321.3 U/mg, which was almost the same level as that in DH5α. Consequently, P*tac* was confirmed as a strongly induced promoter in *K. pneumoniae*; however, owing to the high level of basal expression, the induction/repression ratios of LacZ expression under P*tac* decreased from 246-fold in DH5α to 76.5-fold in KG1. The expression profiles of β-Gal protein in *K. pneumoniae* transformants are shown in Fig. 1. Although the β-Gal activity in all transformants was detectable, only in the transformant with P*tac* under induced conditions, the β-Gal protein (116 kDa) was expressed at sufficient levels to be detected by SDS-PAGE (Fig. 1, band 5).

3.2. Promoter stability in *K. pneumoniae* during culture

To test the stability of promoters in *K. pneumoniae* during the culture process, cell growth and β-Gal expression by P*tac*, P*bud*, or P*T5* were compared under flask-scale cultivation with LB medium. As shown in Fig. 2a, either containing different promoters or under different induced conditions, the cell growth properties of all these transformants were similar to that of parent strain. During the first 8 H, cells grew faster and reached maximum concentration (~2.32 g/L), after which the cell concentration decreased slowly.

Variations of β-Gal activity in transformants with P*T5* or P*bud* are shown in Fig. 2b. The β-Gal activity with both P*T5* and P*bud* increased with increased cell growth. When cell

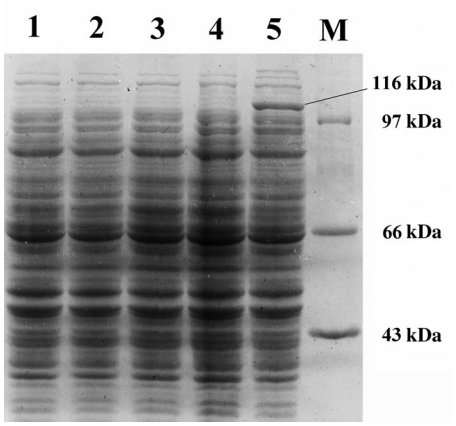

FIG. 1 *Expression profiles of β-Gal protein in K. pneumoniae strains. 1, KG1/pET28 as control. 2, KG1/ pETbu::lacZ. 3, KG1/pETT5::lacZ. 4, KG1/ pETtac::lacZ without inducer. 5, KG1/pETtac::lacZ with induced. 6, Marker.*

growth ceased, the β-Gal activity continued to increase until 12 H of culture and then decreased. The order of peak values was the same as indicated above (P*T5* > P*bud*). The decline of β-Gal activity with these two promoters was different. The β-Gal activity with P*T5* was only 33% of its peak value, whereas it was 66% with P*bud* after 24 H of culture. The variation of β-Gal with P*tac* without inducer was similar to those with the two constitutive promoters; β-Gal activity reached maximum at 12 H and then decreased (Fig. 2c). It was interesting that the β-Gal activity with P*tac* was of 89% maximum at the end of culture, which was much higher compared with the two constitutive promoters. With the inducer added at time 0 H, the *β-Gal* activity with P*tac* increased rapidly and reached maximum earlier, at 8 H, and then decreased (Fig. 2c). Good stability of P*tac* under induced conditions was confirmed; β-Gal activity was 75% of maximum at the end of culture.

3.3. Promoter characteristics of *K. pneumoniae* in different carbon source media

The effects of glucose and glycerol, the two most frequently used carbon sources, on β-gal expression by the three promoters were investigated. Cell growth in glycerol (or glucose) medium and in LB medium was very similar (data not shown). The promoter strength of β-gal expression differed among carbon sources; for the three promoters tested, the β-Gal activity of transformants in glycerol medium was generally higher compared with glucose medium (Fig. 3). Compared with that in LB medium, the peak value of β-Gal activity expressed with P*bud* increased 8%, whereas β-Gal activity expressed with P*T5* decreased 68% in glycerol medium (Fig. 3a), resulting in a change of the strength order of these promoters (from P*T5* > P*bud* to P*bud* > P*T5*). The strength of P*bud* in glucose medium was greater compared with P*T5* (Fig. 3a). It was found that the promoter of P*tac* induced or did not express more protein in glycerol medium compared with LB medium.

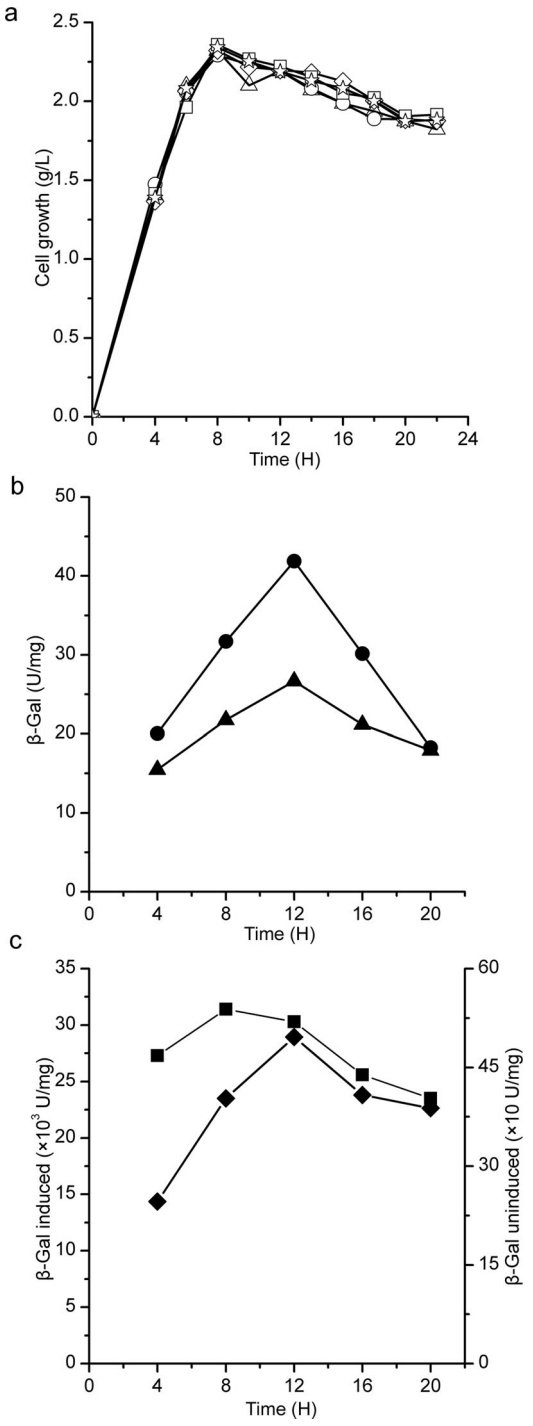

FIG. 2 *Cell concentrations and β-Gal activities during culture in KG1/pETbu::lacZ (triangle), KG1/pETT5::lacZ (circle), KG1/pETtac::lacZ (square or diamond) with LB medium. (a) Cell concentrations (open), parent strain of KG1 used as control (open star). (b) β-Gal activities in KG1/pETbu::lacZ (solid) and KG1/pETT5::lacZ (solid). (c) β-Gal activities in KG1/pETtac::lacZ with inducer (solid square) and without inducer (solid diamond). Data points are averages of three identical cultures.*

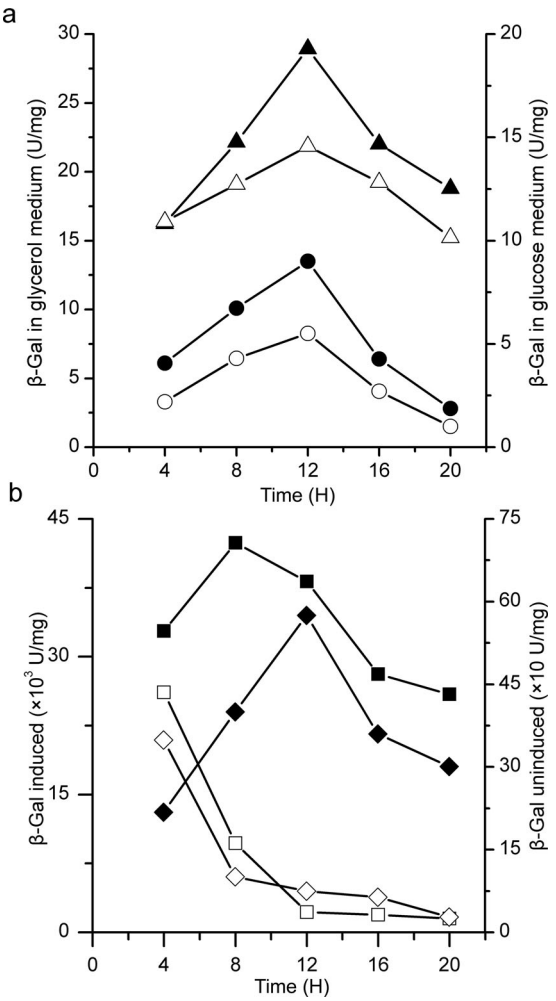

FIG. 3 *β-Gal activities during culture in KG1/pETbu::lacZ (triangle), KG1/pETT5::lacZ (circle), KG1/pETtac::lacZ (square or diamond) with glycerol (solid) or glucose (open) medium. (a) β-Gal in KG1/pETbu::lacZ and KG1/pETT5::lacZ. (b) β-Gal in KG1/pETtac::lacZ with inducer (square) and without inducer (diamond). Data points are averages of three identical cultures.*

The stability of promoters was affected by the identity of the carbon source. Although the change of trends by the three promoters of β-Gal activity in glycerol medium and in LB medium was similar, the curves of β-Gal activity expressed by P*tac* were different in glucose medium. In induced and uninduced conditions, β-Gal activity reached a peak value rapidly (at ~4 H) and then decreased continuously until the end of the culture time (Fig. 3b).

4. Discussion

K. pneumoniae is well known as the best natural producer of some important platform chemicals, such as 1,3-propanediol and 2,3-butanediol [26, 27]. Much effort has been devoted to the metabolic engineering of *K. pneumoniae* for improvement of the biosynthesis of platform chemicals. Unlike *E. coli* or other well-studied microorganisms, knowledge of the actual expression levels of promoters to use for effective engineering of *K. pneumoniae* is quite limited. In this study, the properties of promoters P*bud*, P*T5*, and P*tac* in *K. pneumoniae* were analyzed using the *lacZ* reporter gene. The results indicated β-Gal activity of different promoters ranged from 26.6 to 30,321.3 U/mg (Table 1), a difference of more than 1,000-fold. Consequently, the selection of promoter for effective engineering of *K. pneumoniae* must be made with care.

K. pneumoniae belongs to the Enterobacteriaceae family and has relatively close genetic relatedness to *E. coli*. Many genetic engineering tools designed for *E. coli* are available for *K. pneumoniae* [5]. This study indicated the heterogeneous promoters of P*tac* and P*T5* were functional but some properties of the two promoters were changed in *K. pneumoniae*. P*T5* appeared more efficient and the induction/repression ratio of P*tac* was decreased extremely in *K. pneumoniae*. P*tac* is one of the most frequently used strongly induced promoters and P*T5* is reported as a strong constitutive promoter, both of which have been used in *K. pneumoniae* [16, 23, 28, 29]. Here, the high degree of strength of P*tac* with inducer and the basal expression from P*tac* was very high in *K. pneumoniae*. The β-Gal activity of P*tac* without inducer was 9.5-fold higher compared with P*T5*. The high-level leaky expression of P*tac* might be caused by the change of repressor in *K. pneumoniae*. The species specificity of lac repressor on P*tac* was reported also for Anabaena [30]. The basal level expression of P*tac*-based vectors is usually considered as a problem for high-producing recombinant proteins in *E. coli* or other organisms, which can be overcome by introducing lac repressor gene in P*tac*-based vectors [31–33]. Nevertheless, owing to no demand for extra induced conditions, the constitutive promoters are convenient for metabolic engineering [34]. On the basis of our data, P*tac* can be used as a highly efficient "constitutive" promoter in *K. pneumoniae*, which was shown for the first time. The stability of P*tac* and P*T5* in LB medium was further assessed and P*tac* showed good characteristics for use in *K. pneumoniae* (Fig. 2).

A new native promoter of P*bud* was constructed in this study. The sequence of P*bud* was selected from upstream of the first gene (*budA*) in the *bud* operon, which is responsible for 2,3-butanediol biosynthesis in *K. pneumoniae* [24]. The 121 bp fragment obtained was sequenced and verified to be the same as it is in the genome (GenBank: CP002910) of *K. pneumoniae* KCTC 2242 [35]. Although *K. pneumoniae* was capable of high levels of 2,3-butanediol production, P*bud* did not show great strength in this study. By contrast, expression of *lacZ* by P*bud* was the lowest among the three promoters tested in LB medium. Such low strength of P*bud* may be because of its source from the *K. pneumoniae* strain of KG1, which is a high 1,3-propanediol (not 2,3-butanediol) producer. Nevertheless, P*bud* proved to be the most stable constitutive promoter with adaptation to glucose (or glycerol) medium. In glycerol (or

glucose) medium, the strength of P*bud* was greater compared with P*T5*. Optimization of complex networks of metabolic pathways does not always require the highest activity of all genes of interest. So, stable and weak promoters like P*bud* are needed.

As mentioned above, our results showed clearly the strength and stability of a promoter can be affected by the identity of the carbon source. In glucose medium, the stability of P*tac* decreased rapidly, which might be caused by catabolite repression. So, it is necessary to consider the rapid decrease of expression from P*tac* in glucose medium. According to our data, P*bud* and P*tac* were more stable and expressed more protein in glycerol medium, the peak value of β-Gal expressed by P*bud*, P*tac* without, and with inducer were 28.9, 575.4, and 42,411.2 U/mg respectively. Consequently, a set of P*bud* and P*tac* promoters could meet the broad range (about 1,000 orders of magnitude) of gene expression needed for engineered *K. pneumoniae* in glycerol-based medium. Recently, because of a large amount of low-cost raw glycerol produced during the biodiesel production process, the bio-conversion of valued products, such as 1,3-propanediol, from glycerol by *K. pneumoniae* has attracted significant interest [1, 36].

In summary, the properties of three promoters in *K. pneumoniae* were analyzed using the *lacZ* reporter gene. The results indicated promoters designed for *E. coli* were functional in *K. pneumoniae* but some properties of those promoters can be changed. In *K. pneumoniae*, a heterogeneous promoter of P*tac* was found with a high level of basal expression as well as a high level of expression under induced conditions; the endogenous promoter of P*bud* proved to be a stable and weak promoter. According to our data, these two promoters can meet the broad range of gene expression required for effective engineering of *K. pneumoniae* in glycerol-based medium.

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