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Characterization of promoters in *Escherichia coli* and application for xylitol synthesis☆

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ABSTRACT

Promoters are the most important tools to control and regulate the gene expression in synthetic biology and metabolic engineering. The expression of target genes in *Escherichia coli* is usually controlled by the high-strength inducible promoter with the result that the abnormally high transcription of these genes creates excessive metabolic load on the host, which decreases product formation. The constitutive expression systems are capable of avoiding these defects. In this study, to enrich the application of constitutive promoters in metabolic engineering, four promoters from the glycolytic pathway of *E. coli* were cloned and characterized using the enhanced green fluorescent protein as reporter. Among these promoters, P_{gapA} was determined as the strongest one, the strength of which was about 8.92% of that of the widely used inducible promoter P_{T7}. This promoter was used to control the expression of heterologous xylose reductase in *E. coli* for xylitol synthesis so as to verify its function in pathway engineering. The maximum xylitol titer ($40.6\ g\cdot L^{-1}$) produced by engineered *E. coli* under the control of the constitutive promoter P_{gapA} was obviously higher than that under the control of the inducible promoter P_{T7}, indicating the feasibility and superiority of promoter P_{gapA} in the metabolic engineering of *E. coli*.

1. Introduction

Escherichia coli is the most widely used platform organism not only for the production of a wide range of important high-value compounds, such as taxol precursor [1], isoprene [2,3], terpenoids [4], 5-Aminolevulinic acid [5], coenzyme Q_{10} [6] and plant-specific phenylpropanoids [7], but also for the solution of global energy-related issues such as biofuel production and biomass conversion [8,9]. In these studies, the construction of synthetic pathway frequently involves the expression of multiple genes. The gene expression is regulated by a series of distinct, yet interwoven, levels of regulatory control occurring at the transcriptional, translational and protein levels [10]. One of the fundamental methods to alter the gene expression level is to control transcription at the promoter level. Hence, metabolic engineering application relies on effective promoter discovery and characterization.

The selection of promoters has attracted the attention of the researchers in most pathway engineering studies because selection of appropriate promoters plays a significant role in yield and productivity optimization. This is not only because gene expression level is controlled by the promoter strength, but also because promoters could be regulated and behave differently under different growth conditions [11]. For example, Alper *et al.* constructed a promoter library to achieve precise strength and regulation, and tested the utility of the promoter library by investigating the lycopene production [12]. Duan *et al.* obtained 27% enhancement of riboflavin production by using the vegetative growth promoter P43 [13]. Liu *et al.* utilized the turbulence promoter to improve the permeate flux of membrane in the crossflow microfiltration of calcium carbonate suspension [14]. Others also maximized the production of approximately $1\ g\cdot L^{-1}$ taxadiene with minimal accumulation of toxic intermediate by systematically optimizing the promoters with different strengths and plasmid copy numbers [1]. Therefore, it is imperative to characterize these promoters and evaluate their behavior under different growth conditions.

Among these selected promoters, two very high-strength phage-derived promoter systems based on the T7 RNA polymerase and the P_L temperature-regulated phage promoter systems are frequently used to regulate the gene expression [15–18]. However, the high transcriptional level creates redundant metabolic load for the *E. coli* host. The strong overexpression is not always optimal for a given gene, so a range of promoter strength is necessary. The constitutive expression systems as alternative are able to avoid the problem [19], because they offer several advantages over inducible systems that require a chemical or physical inducer.

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In order to offer more information to appropriate promoter selection for gene expression control, the precise characterization of four constitutive promoters from *E. coli* is completed using enhanced green fluorescent protein (eGFP) as reporter gene at the translational and transcriptional levels. In order to verify the utilization of these promoters, the strongest promoter P_{gapA} is chosen to compare with the widely used inducible promoter P_{T7} in xylitol production.

2. Materials and Methods

2.1. Strains, media and reagents

E. coli BL21 (DE3) pLys was used for promoter characterization and xylitol production. *Candida tropicalis* BIT-Xol-1 used to clone the xylose reductase (XR) gene *xr* was preserved in our laboratory. Luria–Bertani (LB) with 50 $\mu g \cdot ml^{-1}$ kanamycin was used as cultivation medium for *E. coli* transformants. Restriction enzymes, T4 ligase and high-fidelity DNA polymerase were purchased from Fermentas (Burlington, ON). The plasmid pET-28a (+) was obtained from Novagen (Darmstadt, Germany). The primers were purchased from Sangon Biotech (Shanghai, China).

2.2. Plasmid construction

All plasmids for promoter characterization and xylose reductase expression were derivates of plasmid pET-28a (+). Promoters P_{pgi}, P_{gapA}, P_{pykA} and P_{pykF} from the glycolytic pathway were amplified from *E. coli* C600 genomic DNA and their sequences were retrieved from the NCBI database. The expression cassettes of promoters and *egfp* were constructed by overlap extension PCR. *BamH* I and *Bgl* II were employed to digest plasmid pET-28a (+) and promoter P_{T7} was released from the plasmid. The expression cassettes composed of promoters and *egfp* were also digested with the same restriction enzymes and ligated with linearized pET-28a (+).

Gene *xr* was PCR-amplified from the genomic DNA of *C. tropicalis*. The XR expression cassette containing promoter P_{gapA} and gene *xr* was constructed by overlap extension PCR. The XR expression plasmid (pET-28a (+)-*gapA-xr*) under promoter P_{gapA} was constructed by ligating the XR expression cassette with pET-28a (+) digested by *BamH* I and *Bgl* II. The XR expression plasmid (pET-28a (+)-*T7-xr*) under promoter P_{T7} was prepared after ligating linearized pET-28a (+) with gene *xr* digested by *BamH* I and *Xho* I. Strains and plasmids used in this study are listed in Table 1.

2.3. Cultivation and fermentation conditions

For the recombinant strains harboring the four constitutive promoters, 300 μl overnight cultures were inoculated into a fresh 30 ml LB-kan medium and grown at 37 °C, 170 $r \cdot min^{-1}$. The recombinant strain BL21-*T7-egfp* was induced by 0.2 $mmol \cdot L^{-1}$ IPTG at 25 °C when OD_{550} reached 0.8. Then, the cells were harvested to determine the mRNA level and eGFP expression by quantitative PCR and flow cytometry, respectively.

For strain BL21-*gapA-xr*, the overnight cultures were inoculated to an initial OD_{550} of 0.1 and grown at 37 °C, 170 $r \cdot min^{-1}$. For strain BL21-*T7-xr*, the cultures were induced by 0.2 $mmol \cdot L^{-1}$ IPTG at 25 °C when OD_{550} reached 0.8. The cells were harvested for SDS-PAGE and enzyme activity determination.

The fermentation of strain BL21-*gapA-xr* for initial xylose concentration optimization was performed in a 250 ml shake flask with 100 ml LB-kan media containing xylose of different concentrations at 37 °C, 170 $r \cdot min^{-1}$. To determine its optimal feeding concentration, xylose was added at 48, 72, 96, 120, 144 and 168 h. For the strain BL21-*T7-xr*, the optimal lPTG concentration, induction temperature and initial xylose concentration were investigated in a 250 ml shake flask with 100 ml LB medium at 170 $r \cdot min^{-1}$. Each fermentation was carried out in triplicate.

2.4. Measurement of eGFP fluorescence intensity

The eGFP fluorescence intensity of the cells was measured using flow cytometry. Briefly, cells were harvested by centrifugation at 10000 *g* for 2 min, washed and subsequently resuspended in phosphate buffer solution to an OD_{550} of 0.5–0.6. Flow cytometry analysis was performed at Beckman-Coulter CyAn ADP (Dako, Carpinteria, CA). The fluorescence intensity of eGFP was calculated by subtracting the arithmetic mean of auto-fluorescence distribution of control from the fluorescence of sample.

2.5. RNA extraction and quantitative PCR

E. coli cells harvested at the exponential growth phase were used for the total RNA extraction using a High Pure RNA Isolation Kit (Roche, Mannheim, Germany). RNA concentration was quantified by measuring the absorbance at 260 nm using NanoDrop 2000c (Thermo Scientific, Waltham, MA). All RNA samples were stored at −80 °C.

Five hundred nanograms of RNA from each sample was used as a template for the Transcriptor First Strand cDNA Synthesis Kit (Roche). Quantitative PCR analysis was performed with the LightCycler SYBR Green I Master Kit on the LightCycler 480 real-time System using 96

Table 1
Strains and plasmids used in this study

Name	Relevant characteristics	Reference
Plasmids		
pET-28a (+)	*kan*, with promoter P_{T7}	Novagen
pET-28a (+)-*pykA-egfp*	pET-28a (+) carrying promoter P_{pykA} and *egfp*	This study
pET-28a (+)-*pgi-egfp*	pET-28a (+) carrying promoter P_{pgi} and *egfp*	This study
pET-28a (+)-*pykF-egfp*	pET-28a (+) carrying promoter P_{pykF} and *egfp*	This study
pET-28a (+)-*gapA-egfp*	pET-28a (+) carrying promoter P_{gapA} and *egfp*	This study
pET-28a (+)-*T7-egfp*	pET-28a (+) carrying promoter P_{T7} and *egfp*	This study
pET-28a (+)-*gapA-xr*	pET-28a (+) carrying promoter P_{gapA} and *xr*	This study
pET-28a (+)-*T7-xr*	pET-28a (+) carrying promoter P_{T7} and *xr*	This study
Strains		
BL21 (DE3) pLys	Control strain	Novagen
C600	*F^- tonA21 thi-1 thr-1 leuB6 lacY1 glnV44 rfbC1 fhuA1* λ^-	CGSC, Yale
BL21-*pykA-egfp*	BL21 (DE3) pLys carrying plasmid pET-28a (+)-*pykA-egfp*	This study
BL21-*pgi-egfp*	BL21 (DE3) pLys carrying plasmid pET-28a (+)-*pgi-egfp*	This study
BL21-*pykF-egfp*	BL21 (DE3) pLys carrying plasmid pET-28a (+)-*pykF-egfp*	This study
BL21-*gapA-egfp*	BL21 (DE3) pLys carrying plasmid pET-28a (+)-*gapA-egfp*	This study
BL21-*T7-egfp*	BL21 (DE3) pLys carrying plasmid pET-28a (+)-*T7-xr*	This study
BL21-*gapA-xr*	BL21 (DE3) pLys carrying plasmid pET-28a (+)-*gapA-xr*	This study
BL21-*T7-xr*	BL21 (DE3) pLys carrying plasmid pET-28a (+)-*T7-xr*	This study