

Isolation of Fully Synthetic Promoters for High-Level Gene Expression in *Corynebacterium glutamicum*

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ABSTRACT: *Corynebacterium glutamicum* is an important industrial organism that is widely used in the production of amino acids, nucleotides and vitamins. To extend its product spectrum and improve productivity, *C. glutamicum* needs to undergo further engineering, including the development of applicable promoter system. Here, we isolated new promoters from the fully synthetic promoter library consisting of 70-bp random sequences in *C. glutamicum*. Using green fluorescent protein (GFP) as a reporter, highly fluorescent cells were screened from the library by fluorescent activated cell sorting (FACS). Twenty potential promoters of various strengths were isolated and characterized through extensive analysis of DNA sequences and mRNA transcripts. Among 20 promoters, 6 promoters which have different strengths were selected and their activities were successfully demonstrated using two model proteins (antibody fragment and endoxylanase). Finally, the strongest promoter (P_{H36}) was employed for the secretory production of endoxylanase in fed-batch cultivation, achieving production levels of 746 mg/L in culture supernatant. This is the first report of synthetic promoters constructed in *C. glutamicum*, and our screening strategy together with the use of synthetic promoters of various strengths will contribute to the future engineering of *C. glutamicum*.

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KEYWORDS: *Corynebacterium glutamicum*; synthetic promoter; FACS; secretion

Introduction

Corynebacterium glutamicum, a non-pathogenic, non-sporulating, Gram-positive bacterium, has been traditionally used for the industrial production of various L-form amino acids, nucleotides and vitamins (Diesveld et al., 2009; Lv et al., 2012). In recent years, far beyond traditional amino acid production, there has been impressive progress in engineering *C. glutamicum* toward a broad product spectrum, including various chemicals (diamines, diols, polymers, etc.) and biofuels (ethanol, advanced alcohols, etc.) (Becker and Wittmann, 2012). Due to numerous ideal intrinsic attributes, the potential of *C. glutamicum* as a microbial factory for the production of various biomolecules and chemicals continues to grow.

In the post-genome era, systems and synthetic biology became big trends in biotechnology (Khalil and Collins, 2010; Kim et al., 2012), and many bacteria were engineered by reprogramming metabolic pathways based on genomic databases and omics studies. *C. glutamicum* was also engineered to extend its product spectrum and improve its production yields (Becker and Wittmann, 2012; Brinkrolf et al., 2007; van Ooyen et al., 2012). In cellular engineering, the expression levels of many related genes need to be tightly regulated and these controls and regulations require efficient gene expression systems. In the development of these, the choice of promoter is particularly important as it is closely related to gene expression level and their regulation. For the expression of genes in *C. glutamicum*, the well-known *E. coli* inducible promoters such as *lacUV5*, *tac*, *araBAD*, and phage λ P_R/P_L promoters have been used (Park et al., 2008; Vasicová et al., 1999). However, the activities of these promoters in a *C. glutamicum* were lower than in an *E. coli* and, due to less inducer permeability (e.g., arabinose) or the lack of repressors, the regulation/deregulation of gene expression was less efficient in *C. glutamicum* than in *E. coli* (Pátek et al., 2003; Salim et al., 1997). The cost of the inducer (particularly

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IPTG) is a further consideration. Expression systems based on gene expression after the addition of IPTG are very useful for gene analysis in the laboratory. However, their use at an industrial scale is limited due to the high cost of the inducer compound. As alternatives, several endogenous inducible promoters have been described in *C. glutamicum* (Pátek et al., 2003). For example, promoters for *aceA* and *aceB* gene expression by acetate induction (Gerstmeir et al., 2004) and for *gntP* and *gntK* gene expression by gluconate induction (Letek et al., 2006) have been reported, but these promoters require the addition of a large amount of inducer to regulate gene expression, which can affect the physiology of the hosts.

Instead of inducible promoter, constitutive promoters have also been considered, as these offer the advantages of not requiring an expensive inducer or the need to optimize induction conditions. To date, *E. coli*-originated promoters such as the *trc* promoter have been used for constitutive expression without induction (Liu et al., 2007; Suzuki et al., 2009). In addition, a limited number of endogenous promoters have been developed for constitutive gene expression in *C. glutamicum* hosts (Nešvera and Pátek, 2011). However, the relative strength of each promoter was not compared directly. Also the use of strong promoters does not always guarantee the higher expression of target genes due to effect of other genetic elements such as 5' UTR (untranslated region) and TIR (transcription initiation region) (Teramoto et al., 2011) and so, it is difficult to choose the promoter with optimal strength when different gene expression levels are required. Thus, for the more extensive engineering of *C. glutamicum*, it is necessary to develop new promoters of various strengths.

In this study, we isolated fully synthetic promoters capable of mediating the constitutive expression of heterologous genes in *C. glutamicum*. First, we constructed synthetic promoter libraries using green fluorescent protein (GFP) as a reporter protein. The potential promoters of various strengths were isolated by FACS-based high-throughput screening. The usefulness of the synthetic promoters was verified with two model proteins namely endoxylanase and an antibody fragment (scFv). Finally, fed-batch cultivation was performed in a 5 L bioreactor to demonstrate the potential of synthetic promoters in large-scale production.

Materials and Methods

Bacterial Strains and Plasmid Manipulation

All bacterial strains and plasmids used in this work are listed in Table I. *E. coli* Jude1 and JM110 were used as hosts for cloning and library construction. *C. glutamicum* ATCC 13032 was used as the main host for gene expression and screening. *E. coli* XL1-Blue and BL21(DE3) were also used as hosts for gene expression. The polymerase chain reaction (PCR) was performed using a C1000TM Thermal Cycler (Bio-Rad, Richmond, CA) and PrimeSTAR HS Polymerase (TAKARA

BIO, Inc., Shiga, Japan). Primer nucleotide sequences are listed in Supporting Information (Table S1). As a positive control, we constructed a GFP expression system under the *trc* promoter (P_{trc}). A 74-bp P_{trc} DNA fragment was amplified from the pTrc99a vector by PCR with primers Ptrc-F and Ptrc-R. The PCR product was digested with *KpnI* and *BamHI* restriction enzymes, and cloned into the same restriction enzyme sites of pCES208 to yield pCES-Trc. The GFP gene was amplified from the pEGFP plasmid by PCR with primers GFP-F and GFP-R, digested with *BamHI* and *NotI* and cloned into the pCES-Trc vector to yield pCES-Trc-GFP. In this cloning, a FLAG tag (DYKDDDDK) was fused to the C-terminus of GFP for easy detection of expression. For the expression of the antibody fragment, the M18 scFv gene with porB signal sequence was amplified from pMoPac16-M18 by PCR with primers M18-F and M18-R, and digested with *BamHI* and *NotI*. The digested products were then cloned into pCES-GFP series which contain the synthetic promoters (P_{L10} , P_{L26} , P_{I16} , P_{I51} , P_{H30} , and P_{H36}) and *trc* promoter, respectively, to yield pCES-L10-M18, pCES-L26-M18, pCES-I16-M18, pCES-I51-M18, pCES-H30-M18, pCES-H36-M18, and pCES-Trc-M18, respectively. For the expression of the endoxylanase (*xynA*) gene, *xynA* was amplified from chromosomal DNA of *Streptomyces coelicolor* A3(2) by PCR with primers XynA-F and XynA-R. The PCR products containing *xynA* as well as the PorB signal sequence were digested with *BamHI* and *NdeI*, then cloned into pCES-GFP series to yield pCES-L10-XynA, pCES-L26-XynA, pCES-I16-XynA, pCES-I51-XynA, pCES-H30-XynA, and pCES-H36-XynA. To confirm the activity of synthetic promoters in *E. coli*, GFP gene was amplified from pEGFP plasmid by PCR with primers pETGFP-F and pETGFP-R, digested with *NdeI* and *HindIII*, and cloned into pET22b to yield pETGFP. P_{H30} and P_{H36} were amplified from pCES-H30-GFP and pCES-H36-GFP by PCR with primers H30-F and H30-R, H36-F and H36-R, respectively. The PCR products containing P_{H30} and P_{H36} sequences were digested with *BglII* and *XbaI*, then cloned into pETGFP to yield pET-H30-GFP and pET-H36-GFP, respectively. All plasmid constructions were carried out in the *E. coli* Jude1, and each plasmid was transformed into *C. glutamicum* by electroporation using a Gene Pulser (Bio-Rad, Hercules, CA). All DNA manipulations including restriction digestion, ligation, and agarose gel electrophoresis were carried out using standard procedures (Sambrook and Russell, 2001).

Construction of Synthetic Promoter Library in *C. glutamicum*

Randomization of the promoter region and 5' untranslated region (5'UTR) was enabled by the use of a long forward primer, Synpro-F, containing 70 bp of the degenerate oligonucleotide N (with a 25% possibility chance of each of the A, G, C, and T bases). Using this primer with Synpro-R, PCR synthesized the randomized promoter region and the N-terminal of GFP. The PCR product was digested with *KpnI* and *NdeI* restriction enzymes, and cloned into the same

Table I. Bacterial strains and plasmids used in this study.

Strain	Relevant characteristics	Refs. or source
Jude 1	<i>DH10B F'::Tn10 (Tet^r)</i>	Jeong et al. (2007)
JM110	<i>rpsL thr leu thi lacY galK galT ara tonA tsx dam dcm glnV44 Δ(lac-proAB) e14- [F' traD36 proAB⁺ lacI^q lacZΔM15] hsdR17(r_K⁻ m_K⁺)</i>	New England Biolabs ^a
XL1-Blue	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F' proAB lacI^qZΔM15 Tn10 (Tet^r)]</i>	Stratagene ^b
BL21(DE3)	<i>E. coli B F⁻ dcm ompT hsdS(r_B⁻ m_B⁻) gal λ(DE3)</i>	Stratagene ^b
<i>C. glutamicum</i>	Wild type	ATCC 13032
Plasmids		
pCES208	<i>E. coli</i> - <i>C. glutamicum</i> shuttle vector, Km ^r	Park et al. (2008)
pTrc99a	4.2 kb, P _{trc} , β-lactamase gene (Ap ^r)	Pharmacia ^c
pMoPac16	7.4 kb, pelB signal peptide, skp co-expression	Hayhurst et al. (2003)
pET22b	5.5 kb, P _{T7} , β-lactamase gene (Ap ^r)	Novagen ^d
pEGFP	3.4 kb, eGFP gene	Clontech ^e
pMoPac16-M18	pMoPac16 containing M18 scFv gene	Harvey et al. (2004)
pCES-Trc	6.1 kb, pCES208 derivative; P _{trc}	This study
pCES-Trc-GFP	6.7 kb, pCES208 derivative; P _{trc} , eGFP	This study
pCES-Trc-M18	6.8 kb, pCES208 derivative; P _{trc} , M18 scFv	This study
pCES-PorB-XynA	7.9 kb, pCES208 derivative; P _{porB} , xynA	This study
pCES-H30-GFP	6.7 kb, pCES208 derivative; P _{H30} , eGFP	This study
pCES-H36-GFP	6.7 kb, pCES208 derivative; P _{H36} , eGFP	This study
pCES-L10-M18	6.8 kb, pCES208 derivative; P _{L10} , M18 scFv	This study
pCES-L26-M18	6.8 kb, pCES208 derivative; P _{L26} , M18 scFv	This study
pCES-I16-M18	6.8 kb, pCES208 derivative; P _{I16} , M18 scFv	This study
pCES-I51-M18	6.8 kb, pCES208 derivative; P _{I51} , M18 scFv	This study
pCES-H30-M18	6.8 kb, pCES208 derivative; P _{H30} , M18 scFv	This study
pCES-H36-M18	6.8 kb, pCES208 derivative; P _{H36} , M18 scFv	This study
pCES-L10-XynA	7.9 kb, pCES208 derivative; P _{L10} , xynA	This study
pCES-L26-XynA	7.9 kb, pCES208 derivative; P _{L26} , xynA	This study
pCES-I16-XynA	7.9 kb, pCES208 derivative; P _{I16} , xynA	This study
pCES-I51-XynA	7.9 kb, pCES208 derivative; P _{I51} , xynA	This study
pCES-H30-XynA	7.9 kb, pCES208 derivative; P _{H30} , xynA	This study
pCES-H36-XynA	7.9 kb, pCES208 derivative; P _{H36} , xynA	This study
pET-GFP	6.1 kb, pET22b derivative; P _{T7} , eGFP	This study
pET-H30-GFP	6.1 kb, pET22b derivative; P _{H30} , eGFP	This study
pET-H36-GFP	6.1 kb, pET22b derivative; P _{H36} , eGFP	This study

^aNew England Biolabs (Beverly, MA).^bStratagene (La Jolla, CA).^cPharmacia Biotech (Uppsala, Sweden).^dNovagen (Darmstadt, Germany).^eBD Clontech (Palo Alto, CA).

restriction sites of pCES-Trc-GFP. The ligated plasmids were introduced into *E. coli* Jude1 by electroporation, and the transformed cells were cultured in 200 mL of Super Optimal broth with Catabolite repression (SOC) media consisting of 20 g tryptone, 5 g yeast extract, 0.5 g NaCl, 0.19 g KCl, 10 mL of 1 M MgCl₂, 10 mL of 1 M MgSO₄, and 20 mL of 1 M glucose per liter with kanamycin (25 μg/L) at 37°C for 12 h. The synthetic promoter library was purified with Nucleo Bond[®] Xtra Midi Plus EF (Macherey-Nagel GmbH & Co. KG, Düren, Germany) and then retransformed into *E. coli* JM110 by electroporation. After cell cultivation and plasmid preparation as described above, purified plasmid DNA was finally transformed into *C. glutamicum*. Transformed cells were cultivated on recovery glucose (RG) (40 g brain heart infusion (BHI), 10 g glucose, 10 g beef extract, 30 g sorbitol per liter) agar square plates containing kanamycin (25 μg/L) at 30°C for 24 h. All grown colonies were collected and stored at -80°C as 15% glycerol stocks.

Library Screening by FACS

The synthetic promoter library of *C. glutamicum* was inoculated into BHI media containing kanamycin (25 μg/L), and cultivated at 30°C for 24 h with agitation at 200 rpm. Then, the fraction of cells (1/100) was transferred into fresh BHI media, and further cultivated at 30°C for 24 h with 200 rpm shaking. Cells were then harvested by centrifugation at 6,000 rpm for 10 min at 4°C. The cells were washed twice with phosphate-buffered saline (PBS) and resuspended in the same buffer. They were then sorted on a fluorescent activated cell sorter (FACS; MoFlo XDP, Beckman Coulter, Inc., Miami, FL) based on high fluorescence intensity detection through a 530/40 band-pass filter for the GFP emission spectrum. For the FACS sort mode, a purify mode that sorts drops containing only positive cells was used. Sorted cells were directly poured into fresh BHI media, cultivated overnight and transferred into fresh BHI media in a flask for the next round of screening. All

cultivation and sorting procedures were then repeated as described above.

Quantitative Reverse Transcription PCR (qRT-PCR)

Cells were grown in BHI medium until the mid-exponential growth phase, and total RNA was extracted from the cells using the Qiagen RNeasy Mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. RNA was then stored at -80°C until use. qRT-PCR was performed using a Bio-Rad CFX Connect (Bio-Rad) and One Step SYBR PrimeScript RT-PCR kit (TAKARA BIO, Inc.) according to the manufacturer's instructions. For each PCR reaction, $5\ \mu\text{L}$ of $2\times$ One Step SYBR RT-PCR buffer III, $0.2\ \mu\text{L}$ of TaKaRa Ex Taq HS, $0.2\ \mu\text{L}$ of PrimeScript RT enzyme Mix II, $0.2\ \mu\text{M}$ of forward and reverse primers (qPCR-F and qPCR-R), $2\ \mu\text{L}$ of total RNA samples and $3.2\ \mu\text{L}$ of RNase-Free dH_2O were mixed, and PCR conditions were as follows: 50°C for 31 min, 95°C for 15 min, then 44 cycles of 94°C for 30 s, 53°C for 30 s, and 72°C for 30 s, then 95°C for 10 min, 65°C for 5 s, and 95°C for 10 min. The value of the quantification cycle (C_q), which is defined as the cycle when the reporter fluorescence is distinguishable from the background in the extension phase of the PCR reaction, was averaged with triplicates.

5'-Rapid Amplification of cDNA Ends (5'-RACE)

The transcription start positions (TSPs) were determined using the SMARTer RACE cDNA Amplification Kit (Clontech, Mountain View, CA) according to the manufacturer's instructions with minor modifications: for reverse transcription, the primer RACE-RT-R was used and the 5'-cDNA end was PCR-amplified with gene-specific primers RACE-UP-F and RACE-GSP-R. The PCR product was digested with *Sfi*I restriction enzyme and cloned into the same restriction site of the pMoPac16 vector for sequence analysis.

Protein Preparation and Analysis

After flask cultivation, cells were harvested by centrifugation at 6,000 rpm for 10 min at 4°C . Cytoplasmic proteins were prepared by sonication (10 min at 50% pulse and 20% amplitude). For the preparation of extracellular proteins, culture supernatants were prepared by centrifugation at 13,000 rpm for 10 min at 4°C , and used for activity assays. For SDS-polyacrylamide gel electrophoresis (PAGE) and Western blotting, culture supernatants were concentrated by acetone precipitation (Jiang et al., 2004). All protein samples were analyzed by electrophoresis on a 12% (w/v) SDS-PAGE gel. For the immunodetection of the FLAG-tag fused protein, a monoclonal ANTI-FLAG M2 antibody-horseradish peroxidase (HRP) conjugate (Sigma-Aldrich, St. Louis, MO) was used. The ECL kit (Amersham ECL Prime Western Blotting Detection Reagent, GE Healthcare Bio-Science AB, Uppsala, Sweden) was used for signal detection. The binding activity of the antibody fragment (M18 scFv) secreted into the culture

medium was assayed by ELISA as described previously (Jeong and Rani, 2011). The signals were quantified by measuring the absorbance at 450 nm with the TECAN Infinite M200 Pro (Tecan Group Ltd, Männedorf, Switzerland). The activity of XynA secreted into the extracellular medium was assayed by the 3,5-dinitrosalicylic acid (DNS) method as described previously (Jeong et al., 1998).

Protein Production in *E. coli*

E. coli XL1-Blue harboring pCES208 derivatives with 20 synthetic promoters and GFP were inoculated in Luria-Bertani (LB) medium consisting of 10 g tryptone, 5 g yeast extract, 5 g NaCl per liter with kanamycin ($25\ \mu\text{g}/\text{L}$) at 37°C for 12 h with agitation at 200 rpm. Cells were transferred into fresh LB media, and further cultivated at 37°C for 12 h with agitation at 200 rpm. Cells were then harvested by centrifugation at 6,000 rpm for 10 min at 4°C for further analysis. *E. coli* BL21(DE3) harboring recombinant plasmids were cultivated in LB medium with ampicillin ($50\ \mu\text{g}/\text{L}$) at 37°C and 200 rpm. When cell density (optical density at 600 nm, OD_{600}) reached to 0.6, cells were induced with 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) and further cultivated at 37°C for 4 h at 200 rpm. Cells were then harvested by centrifugation at 6,000 rpm for 10 min at 4°C for further analysis.

Fed-Batch Cultivation

C. glutamicum harboring pCES-H36-XynA was inoculated into 200 mL of defined medium containing 20 g/L glucose in a 1 L baffle flask and cultivated at 30°C for 20 h with shaking at 200 rpm. The defined media consisted of 3 g K_2HPO_4 , 1 g KH_2PO_4 , 2 g urea, 10 g $(\text{NH}_4)_2\text{SO}_4$, 2 g MgSO_4 , 200 μg biotin, 5 mg thiamine, 10 mg CPN, 10 mg FeSO_4 , 1 mg MnSO_4 , 1 mg ZnSO_4 , 200 μg CuSO_4 , and 10 mg CaCl_2 per liter with 25 mg/L kanamycin. The seed culture (200 mL) was inoculated into 1.8 L of fresh defined media in a Marado 5 L jar bioreactor (BioCNS, Daejeon, South Korea). The temperature was maintained at 30°C throughout the cultivation. The pH and dissolved oxygen (DO) concentration were controlled at set points by online monitoring. The DO concentration was kept at 30% (v/v) by automatically increasing the agitation speed up to 1,200 rpm and then by mixing pure oxygen through a gas mixer. The pH was maintained at 7.0 by supplementing with 5 N ammonia solution. During the cultivation, the glucose concentration was monitored by a glucose analyzer (YSI 2700 SELECT™ Biochemistry Analyzer, YSI Life Science, Yellow Springs, OH) in the supernatant collected after centrifugation of an aliquot culture. To prevent glucose starvation, glucose solution (90 g in 150 mL) was added to the cultures when glucose levels were lower than 0.5% (w/v). Cell growth was monitored by measuring the optical density at 600 nm (OD_{600}) with a spectrophotometer (Optizen POP, Mecasys, Daejeon, South Korea).

Results

Construction of Synthetic Promoter Library

The 70 bp promoter region and 5'UTR sequence was designed to have fully random sequences, with the exception of the ribosome binding site (RBS) "AGGA" and *Bam*HI site "GGATCC" (Fig. 1). The ligated synthetic promoter library plasmids were first introduced into *E. coli* Jude1 to obtain a high number of transformants. After recovery of plasmid, they were retransformed into JM110 in which plasmids were demethylated. Demethylated plasmids are not susceptible to DNase activity in *C. glutamicum*, so efficiency of transformation into *C. glutamicum* can be significantly improved. Consequently, using demethylated plasmids, a large-size library (*ca.* 1×10^5 cells) could be constructed in *C. glutamicum* ATCC 13032. To confirm the diversity of the promoter region sequences, 40 clones were randomly picked from the *C. glutamicum* synthetic promoter library, and all showed fully randomized sequences in the promoter region (Supporting Information, Fig S1a).

Library Screening by FACS

After cultivation of the *C. glutamicum* library, cells were screened by flow cytometry. As shown in Figure 2, the fluorescence intensity of original library cells was slightly higher than that of negative control cells harboring pCES208 only. In the first round of sorting, 8.7×10^7 library cells were screened and the population with a high fluorescent signal (the top 0.41% of all screened cells) was selectively sorted. In this FACS sorting, we used the purify mode in which only single droplet containing all fluorescent cells without any negative cell could be collected. With this purify mode, the quality of sorted cells (ratio of positive cells) could be much improved, however, there was much loss of positive cells compared to other sorting modes (enrich mode, etc.). Therefore, in the first round of sorting, we did aggressive oversampling (8.7×10^7) than original library size (10^5 cells) to collect the enough amounts of cells for next round. These sorted cells were directly poured into fresh BHI media and,

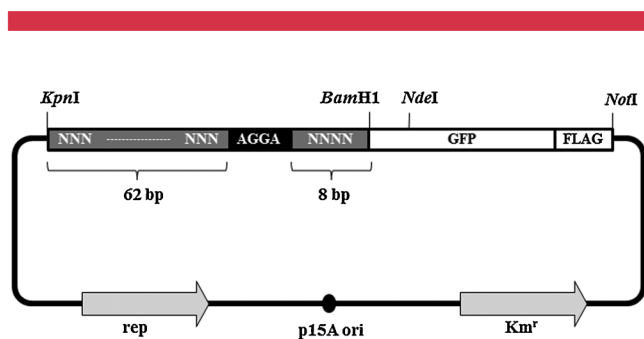


Figure 1. Schematic diagram of plasmids used for library construction. The 70 bp promoter region and 5' UTR were fully randomized with N except for the ribosome binding site (AGGA). (*rep*, DNA replicase gene for replication in *C. glutamicum*. *Km^r*, kanamycin resistance gene).

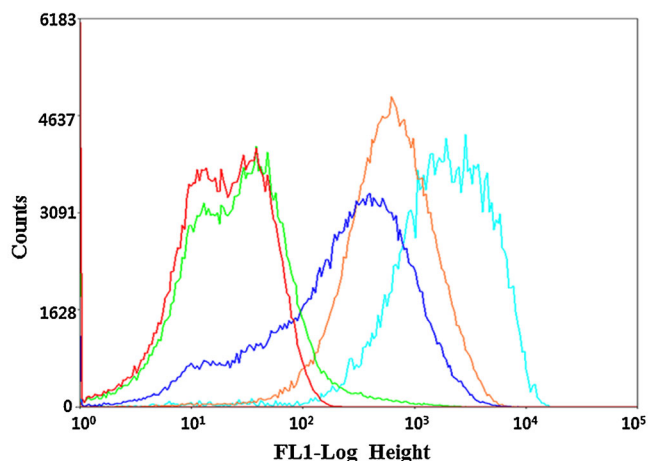


Figure 2. FACS screening of synthetic promoter library. *C. glutamicum* (pCES208) (red curve) was used as a negative control. The histograms of original library, 1st round sorted cells, 2nd round sorted cells, and 3rd round sorted cells are represented by green, blue, orange, and cyan curves, respectively. The 4th round sorted cells were used for individual cell analysis and its histogram was not shown here.

after overnight cultivation, underwent second round of sorting. This screening was repeated two more times (so, total four times), and the positive cells were fully enriched (Fig. 2). After fourth round of sorting, the sorted cells (50,031 cells) were cultivated on BHI agar plates for individual analysis. All sorting results were summarized in Supporting Information (Table S2).

Promoter Strength Analysis with GFP Fluorescence Intensity

A total of 80 colonies were randomly picked and the GFP expression level in each cell was investigated by FACS. Most sorted clones showed higher signal intensities than the positive control containing the *P_{trc}* promoter and a wide range of GFP expression levels from 100 to 1,200 fluorescence intensity values could be obtained (Supporting Information, Fig. S2). Out of 80 clones, 20 clones were selected and classified according to the level of fluorescent intensity into three groups: high (H), intermediate (I) and low (L) strength (Fig. 3a). The GFP expression of each clone was also confirmed by Western blotting following SDS-PAGE. All 20 clones showed higher GFP expression levels than the control clone (*P_{trc}* promoter) and expression levels coincided well with FACS analysis (Fig. 3b). All clones in the H-group showed relatively higher GFP expression levels than those of the I-group, which also showed higher expression levels than those of the L-group.

Transcriptional Analysis of Synthetic Promoters

To compare the strength of isolated synthetic promoters more accurately, transcription levels of the GFP gene in

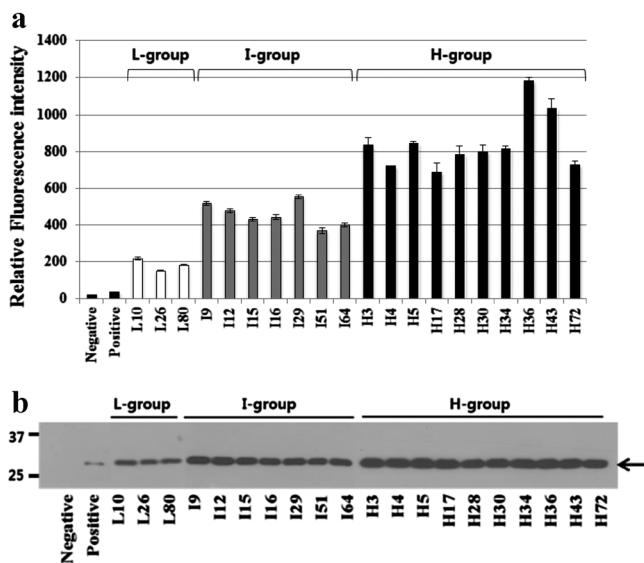


Figure 3. Promoter strength analysis of synthetic promoters. **a:** GFP fluorescence intensities of the isolated *C. glutamicum* clones were analyzed by flow cytometry. *C. glutamicum* (pCES-Trc-GFP) and *C. glutamicum* (pCES208) were used as positive and negative controls, respectively. White, gray and black bars represent the low (L), intermediate (I), and high (H)-strength promoter groups, respectively. All error bars represent the value of standard deviation which were calculated from three times repeated experiments. **b:** The level of GFP expression in each clone was analyzed by Western blotting. In each lane, protein sample was prepared from the same cell concentration which was normalized to OD₆₀₀ of 4. Arrow indicates GFP (~30 kDa).

20 clones were analyzed by qRT-PCR. Quantification cycles for each sample were calculated from the fluorescence data using CFX Manager Software (Bio-Rad). In this analysis, a low value in the quantification cycle indicates a higher transcription level and higher promoter strength. With the exception of a few clones, most showed similar values to previous Western blotting and FACS analysis data (Fig. 4). Average quantification cycles (10.5) of the H-group were relatively lower than those of the I-group (12.4 cycles), which were also lower than the L-group (13.8 cycles). Thus, average transcription levels by H-group promoters are $2^{1.9}$ (≈ 4) times and $2^{3.3}$ (≈ 9.8) times higher than those of I- and L-group promoters, respectively. Of the 20 clones, clone H36 containing the P_{H36} promoter exhibited the lowest value of quantification cycles (8.08 cycles), which means that the transcription level under P_{H36} was $2^{4.01}$ (≈ 16) times higher than that under the P_{trc} promoter (12.09 cycles). For 20 synthetic promoters, we constructed a correlation matrix with all experimental data (mean fluorescence by FACS, GFP level by Western blot, and mRNA transcript levels by qRT-PCR) and in 3-D plot, we found significance in correlation between mean fluorescence, GFP expression level and mRNA transcript levels (Supporting Information, Table S3 and Fig S3). In all experiments, clone H36 showed the highest activities, which suggest that the P_{H36} promoter is the strongest among all synthetic promoters.

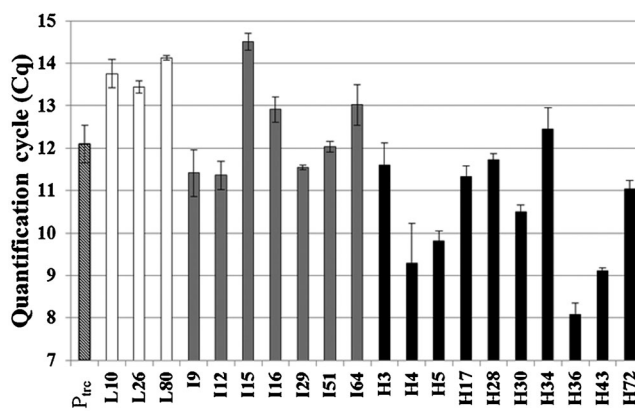


Figure 4. Evaluation of promoter strength by quantitative reverse transcription PCR (qRT-PCR). White, gray, and black bars represent the low, intermediate, and high-strength promoter groups, respectively. *C. glutamicum* (pCES-Trc-GFP) was used as a control (oblique-line bar). All error bars represent the value of standard deviation which were calculated from three times repeated experiments.

Sequence Analysis of Synthetic Promoters

The promoter regions were sequenced for all 20 clones. All clones were shown to contain very different sequences, with the exception of the RBS, which was not randomized in library construction (Fig. 5). No homologous sequences could be identified for any clone in a BLAST search (<http://blast.ncbi.nlm.nih.gov>). All clones were 74 bp long, except for clone I29 that contained one more base in the promoter region, which might have been inserted during synthesis with the random primer (Synpro-F). BPROM software (<http://www.softberry.com/berry.phtml>) successfully predicted the promoter region (−35 and −10 region) in all clones except L80. All promoters contained various sequences in both regions and various distances (11–20 bp) between the −35 and −10 regions (Fig. 5). In each clone, the transcription starting position (TSP, +1 position in the mRNA transcript) was also determined by 5'-RACE. In 5'-RACE experiment, only a single TSP was found in each clone (boxed letters in Fig. 5), and this suggested that the isolated sequences do not form any overlapping promoters. However, surprisingly, we found that most promoters, except for P_{H4}, P_{I9}, P_{I16}, and P_{I64}, produced leaderless mRNA transcripts in which the TSP was identical or very close to the first nucleotide of the start codon (AUG). The P_{L80} promoter also produced a leaderless transcript even though the prediction of the promoter region failed. P_{H4}, P_{I9}, P_{I16}, and P_{I64} promoters produced relatively longer mRNA transcripts including the RBS. In P_{I16}, the TSP determined by 5'-RACE was located upstream of the predicted promoter region which might be predicted wrong. Although several softwares are now available for promoter prediction and they provide highly reliable prediction results, still it is not easy to say those predictions are perfect. Particularly, promoters of *C. glutamicum* might differ in some features from those of other bacteria (Pátek and

be easily produced into culture media just by crossing single membrane. For secretory production into culture media, target proteins require the fusion with proper signal peptides at N-terminus and in this work, we used the signal peptide (27 amino acids) of *C. glutamicum* PorB protein (An et al., 2013). After cultivation of *C. glutamicum*, the secretion of M18 scFv and XynA into culture supernatants was analyzed. In the case of M18 scFv, well-known P_{trc} promoter was used as a control. In each promoter system, production yield of M18 scFv into culture supernatant was analyzed by Western blotting. All synthetic promoters showed much higher expression levels than expression under P_{trc} promoter (pCES-Trc-M18) and negative control (pCES208 only) (Fig. 6a). Also, the expression levels of M18 scFv under the six promoters were highly correlated with the strengths of promoters determined with GFP (Fig. 3). Two H-group promoters (P_{H30} and P_{H36}) exhibited higher expression levels of M18 scFv than I-group promoters (P_{I16} and P_{I51}) which also exhibited relatively higher expression levels than those of L-group promoters (P_{L10} and P_{L26}) (Fig. 6a). The production of M18 scFv under the synthetic promoters was also analyzed by ELISA, and the high correlation of promoter strength and production of M18 scFv was also clearly confirmed (Fig. 6b). Two promoters in the same strength group also exhibited a little different expression levels which were well matched with

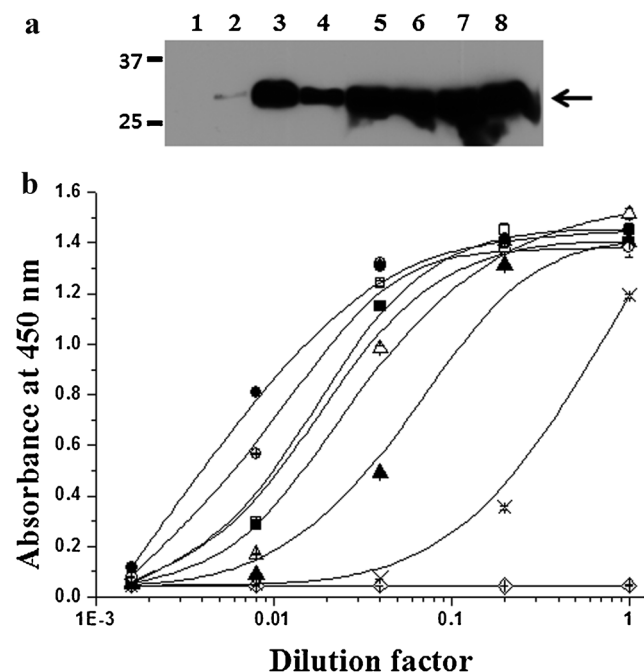


Figure 6. Secretory production of M18 scFv. **a:** Western blot analysis of culture supernatant. Lane 1, pCES208; Lane 2, pCES-Trc-M18; Lane 3, pCES-L10-M18; Lane 4, pCES-L26-M18; Lane 5, pCES-I16-M18; Lane 6, pCES-I51-M18; Lane 7, pCES-H30-M18; Lane 8, pCES-H36-M18. Same volume (10 μ L) of 30 times concentrated culture supernatant was loaded on each lane. Arrow indicates M18 scFv (\sim 28 kDa). **b:** ELISA of the culture supernatant. *C. glutamicum* (pCES208) was used as a negative control. Symbols: \diamond , pCES208; \times , pCES-Trc-M18; Δ , pCES-L10-M18; \blacktriangle , pCES-L26-M18; \square , pCES-I16-M18; \blacksquare , pCES-I51-M18; \circ , pCES-H30-M18; \bullet , pCES-H36-M18.

the promoter strength: $P_{H36} > P_{H30}$, $P_{I16} > P_{I51}$, and $P_{L10} > P_{L26}$ (Fig. 6).

In the case of XynA, the endogenous *C. glutamicum* PorB promoter (P_{porB}) (An et al., 2013) was used as a control and production levels of XynA in culture medium under the six synthetic promoters were compared. As for M18 scFv, both H-group promoters (P_{H30} and P_{H36}) also gave higher levels productions of XynA than other synthetic promoters and control P_{porB} promoter (Fig. 7a). Two I-group promoters (P_{I16} and P_{I51}) exhibited similar level as control (P_{porB} promoter) but higher production levels than those of L-group promoters (P_{L10} and P_{L26}). The biological activities of XynA produced in culture medium were assayed by DNS method and the results of the activity assay also confirmed

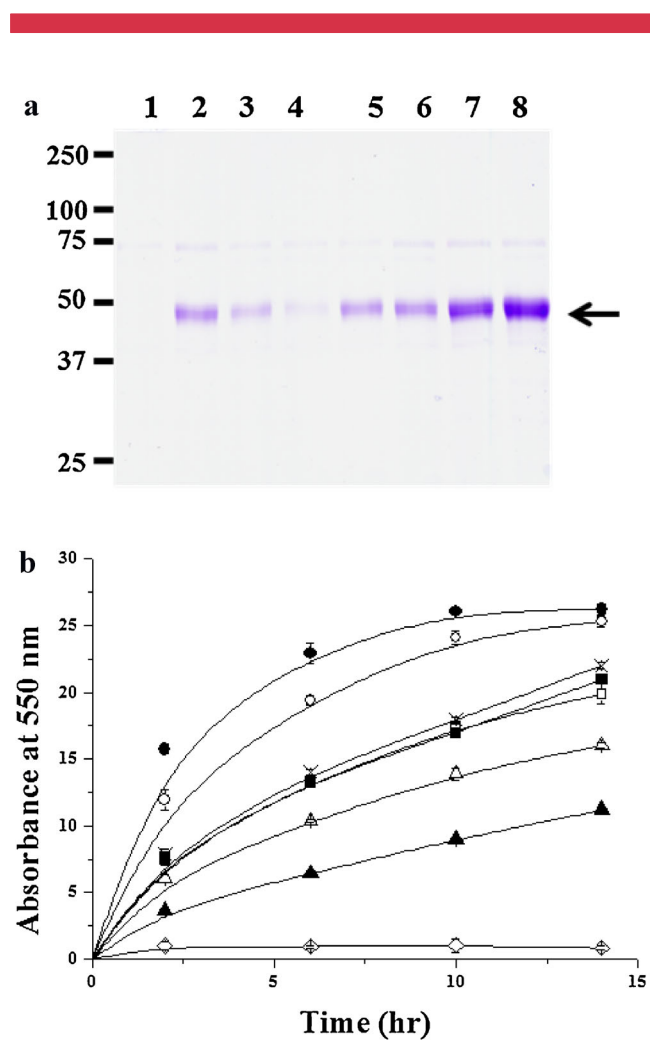


Figure 7. Secretory production of endoxylanase (XynA). **a:** SDS-PAGE analysis of culture supernatant. The gels were stained with Coomassie brilliant blue G-250. Same volume (10 μ L) of 30 times concentrated culture supernatant was loaded on each lane. Lane 1, pCES208; Lane 2, pCES-PorB-XynA; Lane 3, pCES-L10-XynA; Lane 4, pCES-L26-XynA; Lane 5, pCES-I16-XynA; Lane 6, pCES-I51-XynA; Lane 7, pCES-H30-XynA; Lane 8, pCES-H36-XynA. Arrow indicates XynA (\sim 49 kDa). **b:** Endoxylanase activity assay (DNS method) of the culture supernatant. *C. glutamicum* (pCES208) was used as a negative control. Symbols: \diamond , pCES208; \times , pCES-PorB-XynA; Δ , pCES-L10-XynA; \blacktriangle , pCES-L26-XynA; \square , pCES-I16-XynA; \blacksquare , pCES-I51-XynA; \circ , pCES-H30-XynA; \bullet , pCES-H36-XynA.

that the production yields of XynA under the synthetic promoters were well correlated with the promoter strengths ($P_{H36} > P_{H30} > P_{I16} \approx P_{I51} > P_{L10} > P_{L26}$) (Fig. 7b).

High-Level Production of XynA by Fed-Batch Cultivation

To demonstrate the use of synthetic promoters in large-scale cultivation and to achieve high-level secretory production of XynA, fed-batch cultivations of *C. glutamicum* harboring pCES-H36-XynA were carried out in a 5 L lab-scale bioreactor system. Cells were cultivated in defined media containing glucose as a sole carbon source, and new glucose solutions (90 g per feeding) were added according to glucose levels in the media. Cells continued to grow to an OD_{600} of 113.6 for 35.6 h and the cell density then decreased suddenly (Fig. 8a). During the exponential growth phase, the specific growth rate (μ) was approximately 0.22 h^{-1} . Supernatant samples were taken periodically during cultivation and analyzed by SDS-PAGE. At 14 h after inoculation, the endoxylanase band appeared first and increased in density (Fig. 8b). The endoxylanase content of the culture supernatant was determined by a densitometer and the maximum level (approx. 75.8% of total extracellular proteins) was

obtained at 35.6 h, after which the content decreased gradually to 54.6% by the end of cultivation. Based on densitometric analysis and protein quantification, the maximum production yield was approximately 746 mg/L at 35.6 h and the productivity of endoxylanase was about 21 mg/L/h.

Discussion

In current metabolic engineering, the importance of tuning gene expression and modulation increase more and more for the application to metabolic optimization and control analysis. For this purpose, there is an urgent need for a continuous set of promoters with various strengths, and this need can be met by using synthetic promoter libraries (Hammer et al., 2006; Jensen and Hammer, 1998). In various organisms including *E. coli*, *Lactobacillus plantarum*, *Lactococcus lactis*, etc., the potential promoters set have been successfully isolated through screening of synthetic promoter libraries, and they were utilized for the fine control of expression of various genes (Alper et al., 2005; Rud et al., 2006; Solem and Jensen, 2002). From the synthetic promoter libraries which employ several reporter proteins such as GFP, β -glucuronidase (GusA), β -galactosidase, etc., the potential promoters which exhibit different phenotypic activities of reporter could be screened. Here, we constructed a fully synthetic promoter library in the *C. glutamicum*, and potential promoters of various strengths were successfully isolated by FACS-based high-throughput screening. In this study, total 70 bases were randomized for the construction of promoter library. Actually, to cover all 70-bases, theoretical library size should be around 1.4×10^{42} which is impossible to achieve in even high efficiency library formats (e.g., phage or in vitro compartmentalization). However, although 70 bases are too long to be achieved in current library format, we could get much higher diversity in 70 bases-length library than short-length library (Supporting Information, Fig. S1a). Also our initial efforts focused to isolate new synthetic promoters which do not have any similarity to natural promoters in *C. glutamicum*. As shown here, although the size of library (10^5 cells) used for screening was much smaller than theoretical size, we could isolate useful and potential promoters which have no homology with any sequences in BLAST search.

During transcription, most RNA polymerases require sigma factors for promoter binding. Indeed, *C. glutamicum* contains seven sigma factors, of which SigA (σ^A) is considered to be the principal factor responsible for the transcription of housekeeping genes (Pátek and Nešvera, 2011). Recently, the putative sequences of these σ^A -dependent promoters have been analyzed and the resulting statistical consensus sequences of the -35 region and the extended -10 region shown to be ttgnca and gnTAnAnTng (bold and capital letters represent bases present in more than 80% of sequences; core hexamers are underlined), respectively (Pátek and Nešvera, 2011; Pátek et al., 2003). With DNA sequences of 20 synthetic promoters, multiple sequence alignment was performed and, we found the one conserved sequence “GTAnnAnTng” flanking two invariable regions, “AGGA” and “GGATCC” (Supporting

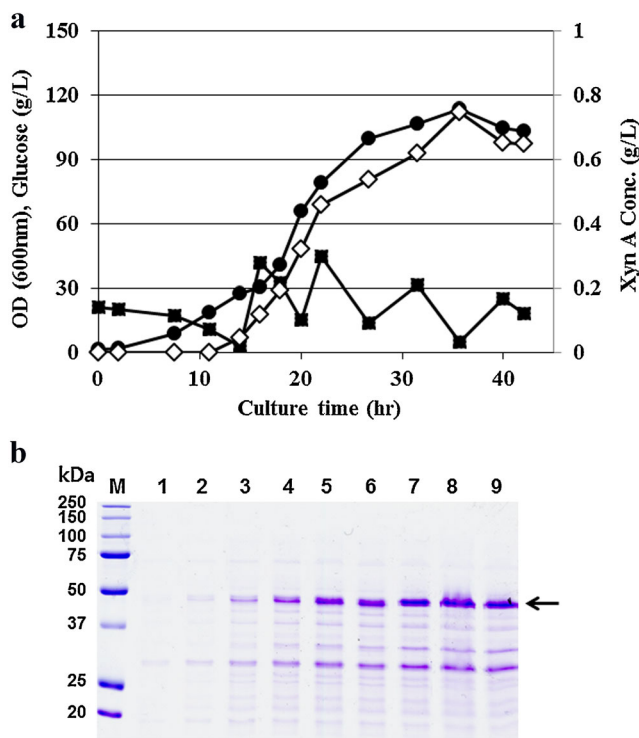


Figure 8. Fed-batch cultivation of *C. glutamicum* (pCES-H36-XynA) for the secretory production of XynA. **a:** Profiles of cell growth (●), glucose concentration (■), and XynA concentration (◇) in culture supernatant. **b:** SDS-PAGE analysis of culture supernatant from fed-batch fermentation. The gels were stained with Coomassie brilliant blue G-250. Lane M, molecular weight markers (kDa). Lanes 1–9, samples at 14, 16, 18, 20, 22, 26.6, 31.5, 35.6, and 40 h, respectively. At each time point, 8 μ L of culture supernatant (without concentration) was loaded onto SDS-PAGE. Arrow indicates XynA (~49 kDa).

Information, Fig. S1b). Interestingly, this conserved sequence was very similar to the extended -10 region sequence (gnTAnAnTng) of *C. glutamicum*. This suggested that the synthetic promoters are probably σ^A dependent and mediate constitutive gene expression. More interestingly, this conserved sequence was found in most H-group promoters (P_{H5} , P_{H17} , P_{H28} , P_{H30} , P_{H36} , P_{H72}) and in one I-group promoter (P_{I12}). However, it is difficult to conclude that the conserved sequences are critical for the strength of promoters yet, and we need more studies to elucidate the effect of this conserved sequence on strength of promoters.

The most surprising point of the synthetic promoters is the formation of a leaderless transcript. For most synthetic promoters, 5'-RACE clearly confirmed that TSP was identical or very close to the first nucleotide of the start codon (AUG) (Fig. 5). In such cases, the mRNA lacks a 5' UTR and hence has no RBS, thus the start codon (AUG) itself serves as the most important signal for translation initiation. Although the formation of leaderless transcripts is not common in *E. coli*, many living organisms from bacteria to eukaryotes have this alternative gene expression system, and it is also well known that leaderless transcripts appear more frequently in Gram-positive and high G + C content bacteria including *Actinobacteria* sp., *Streptomyces* sp., and *C. glutamicum* genes (about 20% in each genome) (Janssen, 1993; Zheng et al., 2011). However, the mechanism of translation and the role of the leaderless transcript remain unclear. Also, in our screening, it is still questionable why promoters producing leaderless transcripts were isolated dominantly. Interestingly, the consensus sequence "GTAnnaTnG" were present between two invariable sequences ("AGGA" and "GGATCC"), and it is supposed that two invariable sequences might affect the biased selection of the -10 promoter sequences producing leaderless transcript. This may be also one possible reason for successful isolation of potential promoters from much smaller library (10^5 cells) than theoretical library size (1.4×10^{42} cells). In addition, it remains to be determined whether the strong activity of the synthetic promoters is related to the formation of leaderless transcripts, and this deserves further investigation.

The usefulness of the synthetic promoters was successfully demonstrated with two recombinant proteins models (antibody fragment and endoxylanase) which were secreted into culture medium. Compared to *E. coli* as a host for protein production, *C. glutamicum* has several important features that facilitate the secretion of proteins into the culture medium: (i) significantly lower contamination of endogenous proteins in culture medium, and (ii) the lack of detectable extracellular hydrolytic enzyme activity (Vertes, 2013). Among two model proteins (XynA and M18 scFv), we recently reported the secretory production of M18 scFv in *E. coli* (Lee et al. accepted for publication in *Biotechnol Bioproc Eng*). In that report, M18 scFv were produced in periplasm of *E. coli* (not in culture supernatant) and under the optimized expression condition, the final production yield of M18 scFv in flask cultivation was about 2.9 mg/L which was much lower than the present data (13 mg/L) in *C. glutamicum* (under the P_{H36} promoter). If we consider the downstream process, the production yields in *C.*

glutamicum would be much higher than that in *E. coli* which requires high-cost, labor-intensive purification procedures. In addition, we successfully demonstrated the usability of the synthetic promoter (P_{H36}) in large-scale fed-batch cultivation. During cultivation, the secretory production of XynA began in the early stages of growth and production levels increased continuously with good association with cell growth, indicating the strong and constitutive activities of synthetic promoter during the exponential growth phase. Within 36 h of cultivation, approximately 746 mg/L of XynA was produced in the extracellular medium, which is equivalent to almost 2 g of XynA in 2.6 L of culture supernatant (final volume). Recently, Dr. Yukawa's research group (RITE, Japan) reported two great secretory production results using *tac* promoter in which GFPs were produced as high as 1.36 g/L (Teramoto et al., 2011) and 2.8 g/L (Watanabe et al., 2013) in 48 h culture. However, those records could be achieved by additional modifications such as addition of Ca^{2+} into the media (Teramoto et al., 2011) or *sigB* disruption in host cell (Watanabe et al., 2013). When we compared the productivity without modification, our system employing a synthetic promoter (P_{H36}) was quite competitive to others (Vertes, 2013), and this indicates that synthetic promoters have high potential for the production of heterologous proteins in *C. glutamicum*.

In conclusion, we performed FACS screening to successfully isolate synthetic promoters with various strengths in the *C. glutamicum*. The strong activities of the synthetic promoters were clearly verified using three model proteins (GFP, M18 scFv, and XynA). In particular, the high-level secretory production of XynA was successfully demonstrated in large-scale fed-batch cultivation, which will be beneficial in the use of xylan and will be an important tool in current white biotechnology (Zheng et al., 2012). Further, as shown in Figures 6 and 7, the synthetic promoters with various strengths allowed the variable levels of gene expression and this means the synthetic promoters with broad range of strength can be very useful tools for fine tuning of gene expression in *C. glutamicum*. To the best of our knowledge, this is the first report of synthetic promoters constructed in *C. glutamicum*. Using a similar screening strategy shown here, much stronger or regulatable promoters could be isolated and use of those synthetic promoters will contribute to the extensive future engineering of *C. glutamicum*.

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References

- Alper H, Fischer C, Nevoigt E, Stephanopoulos G. 2005. Tuning genetic control through promoter engineering. *Proc Natl Acad Sci USA* 102 (36):12678–12683.

- An SJ, Yim SS, Jeong KJ. 2013. Development of a secretion system for the production of heterologous proteins in *Corynebacterium glutamicum* using PorB signal peptide. *Protein Expr Purif* 89(2):251–257.
- Becker J, Wittmann C. 2012. Bio-based production of chemicals, materials and fuels—*Corynebacterium glutamicum* as versatile cell factory. *Curr Opin Biotechnol* 23(4):631–640.
- Brinkrolf K, Brune I, Tauch A. 2007. The transcriptional regulatory network of the amino acid producer *Corynebacterium glutamicum*. *J Biotechnol* 129(2):191–211.
- Diesveld R, Tietze N, Reth A, Bathe B, Sahn H, Eggeling L. 2009. Activity of exporters of *Escherichia coli* in *Corynebacterium glutamicum*, and their use to increase L-threonine production. *J Mol Microbiol Biotechnol* 16:198–207.
- Gerstmeir R, Cramer A, Dangel P, Schaffer S, Eikmanns BJ. 2004. RamB a novel transcriptional regulator of genes involved in acetate metabolism of *Corynebacterium glutamicum*. *J Bacteriol* 186(9):2798–2809.
- Hammer K, Mijakovic I, Jensen PR. 2006. Synthetic promoter libraries—Tuning of gene expression. *Trends Biotechnol* 24(2):53–55.
- Harvey BR, Georgiou G, Hayhurst A, Jeong KJ, Iverson BL, Rogers GK. 2004. Anchored periplasmic expression, a versatile technology for the isolation of high-affinity antibodies from *Escherichia coli*-expressed libraries. *Proc Natl Acad Sci USA* 101:9193–9198.
- Hayhurst A, Happe S, Mabry R, Koch Z, Iverson BL, Georgiou G. 2003. Isolation and expression of recombinant antibody fragments to the biological warfare pathogen *Brucella melitensis*. *J Immunol Methods* 276:185–196.
- Janssen GR. 1993. Eubacterial, archaeobacterial, and eukaryotic genes that encode leaderless mRNA. In: Baltz RH, Hegeman GD, Skatrud PL, editors. *Industrial microorganisms: Basic and applied molecular genetics*. Washington, DC: American Society for Microbiology Press. p 59–67.
- Jensen PR, Hammer K. 1998. Artificial promoters for metabolic optimization. *Biotechnol Bioeng* 58(2–3):191–195.
- Jeong KJ, Rani M. 2011. High-level production of a single chain antibody against anthrax toxin in *Escherichia coli* by high cell density cultivation. *Bioprocess Biosyst Eng* 34(7):811–817.
- Jeong KJ, Lee PC, Park IY, Kim MS, Kim SC. 1998. Molecular cloning and characterization of an endoxylanase gene of *Bacillus* sp. in *Escherichia coli*. *Enzyme Microb Technol* 22(7):599–605.
- Jeong KJ, Seo M, Iverson BL, Georgiou G. 2007. APEX 2-hybrid, a quantitative protein-protein interaction assay for antibody discovery and engineering. *Proc Natl Acad Sci USA* 104:8247–8252.
- Jiang L, He L, Fountoulakis M. 2004. Comparison of protein precipitation methods for sample preparation prior to proteomic analysis. *J Chromatogr A* 1023(2):317–320.
- Khalil AS, Collins JJ. 2010. Synthetic biology: Applications come of age. *Nat Rev Genet* 11(5):367–379.
- Kim TY, Sohn SB, Kim YB, Kim WJ, Lee SY. 2012. Recent advances in reconstruction and applications of genome-scale metabolic models. *Curr Opin Biotechnol* 23(4):617–623.
- Letek M, Valbuena N, Ramos A, Ordóñez E, Gil JA, Mateos LM. 2006. Characterization and use of catabolite-repressed promoters from gluconate genes in *Corynebacterium glutamicum*. *J Bacteriol* 188(2):409–423.
- Liu Q, Ouyang SP, Kim J, Chen GQ. 2007. The impact of PHB accumulation on L-glutamate production by recombinant *Corynebacterium glutamicum*. *J Biotechnol* 132(3):273–279.
- Lv Y, Wu Z, Han S, Lin Y, Zheng S. 2012. Construction of recombinant *Corynebacterium glutamicum* for L-threonine production. *Biotechnol Bioprocess Eng* 17(1):16–21.
- Nešvera J, Pátek M. 2011. Tools for genetic manipulations in *Corynebacterium glutamicum* and their applications. *Appl Microbiol Biotechnol* 90(5):1641–1654.
- Park JU, Jo JH, Kim YJ, Chung SS, Lee JH, Lee HH. 2008. Construction of heat-inducible expression vector of *Corynebacterium glutamicum* and *C. ammoniagenes*: Fusion of lambda operator with promoters isolated from *C. ammoniagenes*. *J Microbiol Biotechnol* 18(4):639–647.
- Pátek M, Nešvera J. 2011. Sigma factors and promoters in *Corynebacterium glutamicum*. *J Biotechnol* 154(2–3):101–113.
- Pátek M, Nešvera J, Guyonvarch A, Reyes O, Leblon G. 2003. Promoters of *Corynebacterium glutamicum*. *J Biotechnol* 104(1–3):311–323.
- Rud I, Jensen PR, Naterstad K, Axelsson L. 2006. A synthetic promoter library for constitutive gene expression in *Lactobacillus plantarum*. *Microbiology* 152(4):1011–1019.
- Salim K, Haedens V, Content J, Leblon G, Huygen K. 1997. Heterologous expression of the *Mycobacterium tuberculosis* gene encoding antigen 85A in *Corynebacterium glutamicum*. *Appl Environ Microbiol* 63(11):4392–4400.
- Sambrook J, Russell D. 2001. *Molecular cloning: A laboratory manual*, 3rd edn. New York: Cold Spring Harbor Laboratory Press.
- Solem C, Jensen PR. 2002. Modulation of gene expression made easy. *Appl Environ Microbiol* 68(5):2397–2403.
- Suzuki N, Watanabe K, Okibe N, Tsuchida Y, Inui M, Yukawa H. 2009. Identification of new secreted proteins and secretion of heterologous amylase by *C. glutamicum*. *Appl Microbiol Biotechnol* 82(3):491–500.
- Teramoto H, Watanabe K, Suzuki N, Inui M, Yukawa H. 2011. High yield secretion of heterologous proteins in *Corynebacterium glutamicum* using its own Tat-type signal sequence. *Appl Microbiol Biotechnol* 91(3):677–687.
- van Ooyen J, Noack S, Bott M, Reth A, Eggeling L. 2012. Improved L-lysine production with *Corynebacterium glutamicum* and systemic insight into citrate synthase flux and activity. *Biotechnol Bioeng* 109:2070–2081.
- Vasicová P, Pátek M, Nešvera J, Sahn H, Eikmanns B. 1999. Analysis of the *Corynebacterium glutamicum* dapA promoter. *J Bacteriol* 181(19):6188–6191.
- Vertes AA. 2013. Protein secretion systems of *Corynebacterium glutamicum*. In: Yukawa H, Inui M, editors. *Corynebacterium glutamicum; biology and biotechnology*. Heidelberg: Springer. p 351–389.
- Watanabe K, Teramoto H, Suzuki N, Inui M, Yukawa H. 2013. Influence of SigB inactivation on *Corynebacterium glutamicum* protein secretion. *Appl Microbiol Biotechnol* 97(11):4917–4926.
- Zheng X, Hu GQ, She ZS, Zhu H. 2011. Leaderless genes in bacteria: Clue to the evolution of translation initiation mechanisms in prokaryotes. *BMC Genomics* 12:361.
- Zheng Z, Chen T, Zhao M, Wang Z, Zhao X. 2012. Engineering *Escherichia coli* for succinate production from hemicellulose via consolidated bioprocessing. *Microb Cell Fact* 11:37.

Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site.