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Development and Application of an Arabinose-Inducible Expression System by Facilitating Inducer Uptake in *Corynebacterium glutamicum*

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***Corynebacterium glutamicum* is currently used for the industrial production of a variety of biological materials. Many available inducible expression systems in this species use *lac*-derived promoters from *Escherichia coli* that exhibit much lower levels of inducible expression and leaky basal expression. We developed an arabinose-inducible expression system that contains the L-arabinose regulator AraC, the P_{BAD} promoter from the *araBAD* operon, and the L-arabinose transporter AraE, all of which are derived from *E. coli*. The level of inducible P_{BAD}-based expression could be modulated over a wide concentration range from 0.001 to 0.4% L-arabinose. This system tightly controlled the expression of the uracil phosphoribosyltransferase without leaky expression. When the gene encoding green fluorescent protein (GFP) was under the control of P_{BAD} promoter, flow cytometry analysis showed that GFP was expressed in a highly homogeneous profile throughout the cell population. In contrast to the case in *E. coli*, P_{BAD} induction was not significantly affected in the presence of different carbon sources in *C. glutamicum*, which makes it useful in fermentation applications. We used this system to regulate the expression of the *odhI* gene from *C. glutamicum*, which encodes an inhibitor of α-oxoglutarate dehydrogenase, resulting in high levels of glutamate production (up to 13.7 mM) under biotin nonlimiting conditions. This system provides an efficient tool available for molecular biology and metabolic engineering of *C. glutamicum*.**

Corynebacterium glutamicum is one of the most important microorganisms for producing bulk amino acids and organic acids (18, 44). The development of genetic tools has made it convenient to metabolically engineer specific traits in this bacterium (16, 27). Through expressing exogenous gene clusters to construct new metabolic pathways, *C. glutamicum* has been engineered to produce a variety of biological materials, such as D-pantothenate, xylitol, trehalose, and polyhydroxybutyrate (2, 15, 19, 32).

As an important tool for molecular biology and metabolic engineering, an efficient inducible expression system should have several characteristics that include sensitivity to a nontoxic and inexpensive inducer, a wide dynamic rang regulation, and little or no leaky basal expression. To date, the P_{lac}-derived promoter systems from *Escherichia coli* have been the most widely used controllable expression systems in corynebacteria; however, these expression systems exhibit a lower level of inducible expression in *C. glutamicum* and high basal expression under noninducing conditions (26). Although many attempts have been made to increase the expression and tight regulation of the P_{tac} promoter, which is a hybrid promoter of P_{trp} and P_{lacUV5} (45, 46), the inducibility of these promoters remains relatively low as a result of the low isopropyl-β-D-thiogalactopyranoside (IPTG) permeability of *C. glutamicum* strains (30). Moreover, the high cost and potential toxicity of IPTG are not ideal for industrial-scale protein expression or production of biological materials. As an alternative, a heat-inducible expression system and the high constitutive expression promoter (HCE) have been used for protein expression in *C. glutamicum* (29, 40, 41). Despite the fact that the regulatory mechanisms of many promoters in *C. glutamicum* are well understood (30, 31, 38), a strong, reliably regulated promoter that is tightly repressed and efficiently induced is still not available for use in corynebacteria (26).

The P_{BAD} promoter from the arabinose operon fulfills all of the criteria of inducible expression systems. This promoter displays tighter control of gene expression, which is attributed to the dual regulatory role of AraC (i.e., AraC functions both as an inducer and as a repressor [20]). Although the level of P_{BAD}-based expression can be modulated over a wide range of L-arabinose concentrations (8), the cell population exposed to subsaturating L-arabinose concentrations is divided into two subpopulations of induced and uninduced cells for the differences between individual cells in the availability of L-arabinose transporter (13, 37). Due to carbon catabolite repression, the *araC*-P_{BAD} promoter system could provide a broader range of regulation by the addition of glucose (8, 25). This system is now available in many Gram-negative bacteria, such as *E. coli*, *Salmonella enterica* serovar Typhimurium, and *Xanthomonas* (21, 28, 39).

In the present study, we developed an arabinose-inducible expression system that allows for control over a wide range of inducer concentrations, tight regulation, and homogeneous high-level expression in *C. glutamicum*. This inducible expression system will facilitate the molecular biology and metabolic engineering of *C. glutamicum*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids used in the present study are listed in Table 1. *E. coli* DH5α was used for vector construction. *C. glutamicum* strain ATCC 13032 was used for genetic disruption and expression using plasmid pK18*mobsacB* and

Received 10 April 2012 Accepted 3 June 2012
Published ahead of print 8 June 2012
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Supplemental material for this article may be found at http://aem.asm.org/.

doi:10.1128/AEM.01147-12

TABLE 1 Bacterial strains and plasmids

Strain or plasmid	Characteristics[a]	Source or reference[b]
Strains		
E. coli		
DH5α	F$^-$ ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17*(r_K^- m_K^+) *phoA supE44* λ$^-$ *thi-1 gyrA96 relA1*	Invitrogen
W3110	λ$^-$ IN(*rrnD-rrnE*)*1 rpb-1*	EGSC
C. glutamicum		
ATCC 13032	Wild type, biotin auxotrophic	ATCC
ATCC 13032Δ*upp*	*upp* gene was deleted, derived from strain ATCC 13032	This study
Plasmids		
pMD19	T vector; Amp^r	TaKaRa
pKD46	pSC101 (Ts$^-$); Amp^r *araC*$^+$ P_{BAD}-Red	4
pAD123	Kan^r; *gfpmut3a*	5
pK18*mobsacB*	Mobilizable vector, allows for selection of double crossover in *C. glutamicum*; Kan^r	35
pXMJ19	Shuttle vector (Cm^r; P_{tac} *lacI*^{q} pBL1 *oriV*$_{C.\ glutamicum}$ pK18 *oriV*$_{E.\ coli}$)	10
pWYE1067	pXMJ19 derivative carrying the *araC*-P_{BAD}	This study
pWYE1088	pXMJ19 derivative carrying the *araC*-P_{BAD} and P_{hom}-*araE*	This study
pXMJ19-*lacZ*	pXMJ19 carrying *lacZ* from *E. coli* W3110	This study
pWYE1067-*lacZ*	pWYE1067 derivative carrying the *lacZ* gene	This study
pWYE1088-*lacZ*	pWYE1088 derivative carrying the *lacZ* gene	This study
pWYE1088-*upp*	pWYE1088 derivative carrying the *upp* gene	This study
pWYE1067-*gfpmut3a*	pWYE1067 derivative carrying the *gfpmut3a* gene	This study
pWYE1088-*gfpmut3a*	pWYE1088 derivative carrying the *gfpmut3a* gene	This study
pWYE1088-*odhI*	pWYE1088 derivative carrying the *odhI* gene	This study

[a] Cm^r, chloramphenicol resistance; Amp^r, ampicillin resistance; Kan^r, kanamycin resistance.
[b] EGSC, *E. coli* Genetic Stock Center; ATCC, American Type Culture Collection.

pXMJ19 derivatives (10, 35). *E. coli* was grown aerobically on a rotary shaker (180 rpm) at 37°C in Luria-Bertani (LB) broth or on LB plates with 1.5% (wt/vol) agar. *C. glutamicum* was routinely grown at 30°C in LB or CGIII medium (23). For the generation of mutants and maintenance of *C. glutamicum*, brain heart infusion broth with 0.5 M sorbitol was used (43). When needed, antibiotics were used at the following concentrations: ampicillin, 100 μg/ml for *E. coli*; kanamycin, 50 μg/ml for *E. coli* and 25 μg/ml for *C. glutamicum*; and chloramphenicol, 20 μg/ml for *E. coli* and 10 μg/ml for *C. glutamicum*.

DNA isolation and manipulation. The genomic DNA of *C. glutamicum* was isolated as described by Tauch et al. (42). DNA restriction enzymes, ligase, and DNA polymerase (TaKaRa, Dalian, China) were used as recommended by the manufacturer's instructions. PCR products were separated by agarose gel electrophoresis and purified using a gel extraction kit (Omega Bio-Tek, Norcross, GA). Plasmid DNA from *E. coli* was prepared using a plasmid isolation kit (Tiangen, Beijing, China). *C. glutamicum* was transformed by electroporation according to previously described methods (43).

Vector constructions. All primers are listed in Table 2. To compare the strengths of different constitutive promoters in *C. glutamicum*, the promoter-less *lacZ* gene containing the open reading frame from the start codon was amplified from *E. coli* W3110 chromosome and then ligated into the PstI and SmaI sites of pXMJ19 to generate the *E. coli-C. glutamicum* shuttle vector pXMJ19-*lacZ*. Constitutive promoters, including P_{hom}, P_{45}, P_{fda}, P_{eno}, and P_{glyA} (30, 36), were amplified from *C. glutamicum* using the different sets of primers listed in Table 2. The P_{hom} and P_{45} PCR products were ligated into the EcoRV and HindIII sites of pXMJ19-*lacZ*, and the P_{fda}, P_{eno}, and P_{glyA} fragments were ligated into the NarI and PstI sites of pXMJ19-*lacZ*. The resulting vectors were transformed into *C. glutamicum* cells to measure β-galactosidase activity.

The fragment containing the *araC* gene under the control of the native P_{araC} promoter and P_{BAD} promoter was amplified from the *E. coli* vector pKD46 (4). The PCR product was digested with NarI and PstI and ligated into the vector pXMJ19 to generate the vector pWYE1067. To abolish the L-arabinose-dependent regulation of *araE* gene encoding L-arabinose transporter under its native promoter, the P_{hom} promoter from *C. glutamicum* was fused to the *araE* gene from *E. coli* by overlap extension PCR. The P_{hom}-*araE* fragment was ligated into the pMD19 T vector and inserted into the dephosphorylated ClaI site of pWYE1067 to generate the vector pWYE1088.

Genetic disruption and complementation in *C. glutamicum*. The pK18*mobsacB* derivative used for *upp* gene (encoding uracil phosphoribosyltransferase) disruption and pWYE1088 derivative used for *upp* gene expression were constructed in the present study (Table 1) and transformed into *C. glutamicum* cells by electroporation (43). Screening for the first and second recombination events and confirmation of the chromosomal deletion was performed as described previously (35). Expression of the *upp* gene from pWYE1088 in *C. glutamicum* was induced by the addition of 0.02% L-arabinose to the culture broth.

β-Galactosidase assay. For the synthesis of β-galactosidase, the cells were grown to an optical density at 600 nm (OD_{600}) of 0.4, and then L-arabinose or IPTG was added to the indicated final concentrations. The cells were harvested at different cultivation times and resuspended in 1 ml of Z-buffer (40 mM NaH_2PO_4, 60 mM Na_2HPO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol [pH 7.0]). The β-galactosidase activity was determined by using a Miller assay based on the degradation of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) (24). One unit of β-galactosidase activity is defined as the amount that hydrolyzes 1 μmol of ONPG to *o*-nitrophenol and D-galactose per min per cell.

Flow cytometry analysis. The *gfpmut3a* gene encoding the green fluorescent protein (GFP), which has more intense fluorescence, a maximum excitation wavelength at 488 nm, and maximum emission at 511 nm, was used as a reporter gene to investigate the population homogeneity after L-arabinose induction. The *gfpmut3a* gene was amplified from the vector pAD123 and ligated into the HindIII and EcoRI sites of pWYE1067 and pWYE1088, respectively. The *C. glutamicum* ATCC 13032 harboring either pWYE1067-*gfpmut3a* or pWYE1088-*gfpmut3a* was cultivated in LB medium and harvested after 2 h of induction with different concentrations of L-arabinose.

Flow cytometry was performed on a BD FACSCalibur flow cytometer

TABLE 2 Primers used in this study

Primer	Sequences (5′–3′)[a]	Function
WZ279	AGTCATGGCGCCCATCGATTTATTATGACAAC (NarI)	*araC*-P_{BAD} amplification
WZ280	CGAACTGCAGGCATGCAAGCTTTTATAACCTCCTTAG (HindIII, PstI)	
WZ291	CCATCGATCCGTTGAAAACTAAAAAGCTGG (ClaI)	P_{hom} amplification
WZ292	**TTTCCTGCCA**TACTTTGTTTCGGCCACCC	
WZ293	**AAACAAAGTA**TGGCAGGAAAAAATGGT	*araE* amplification
WZ294	CCATCGATGGCCCGTGAAATCAGA (ClaI)	
WZ259	CCGGATATCCCGTTGAAAACTAAAAAGCTGG (EcoRV)	P_{hom} amplification
WZ260	GATAAGCTTTACTTTGTTTCGGCCACCC (HindIII)	
WZ255	CCGGATATCGTGTTTTTCTGTGATCCTC (EcoRV)	P_{45} amplification
WZ256	GATAAGCTTGCTTTTAAAACCATGCA (HindIII)	
WZ720	AGTCATGGCGCCCCCCGATAGTGTATGTGC (NarI)	P_{eno} amplification
WZ721	CGACCTGCAG GCATGCAAGCTTAAGGTGTCTCCTCCAAAAG (PstI)	
WZ724	AGTCATGGCGCCCTTAACAAGCGCAACCC (NarI)	P_{fba} amplification
WZ725	CGACCTGCAGGCATGCAAGCTTGCCTCCTATGCCAACTT (PstI)	
WZ421	AGTCATGGCGCCAGCTACTCCACTAGTGTGATCG (NarI)	P_{glyA} amplification
WZ422	GCCCTGCAGGCGTAAGACCTCACTCGC (PstI)	
WZ231	GCCCTGCAGATGACCATGATTACGGA (PstI)	*lacZ* amplification
WZ232	GGGATCCCGGGGAAATACGGGCAGACA (BamHI, SmaI)	
WZ733	CGCGGATCCGCTTCGGCAATCATCAGTC (BamHI)	*upp* deletion
WZ734	**CCGCTTTTCCGA**CCGCCCAGAAGAAGACC	
WZ735	**TCTTCTGGGCGG**TCGGAAAAGCGGTGGT	*upp* deletion
WZ736	CCGGAATTCTGGGTATTTTGCGTCCTC (EcoRI)	
WZ739	CCCAAGCTTATGGACATCACCATCGTCAACC (HindIII)	*upp* amplification expression
WZ740	CCGGAATTCCCGTAATGCCCTTAGAAACT (EcoRI)	
WZ741	CCCAAGCTTTAATGAGCGACAACAACG (HindIII)	*odhI* amplification expression
WZ742	CCGGAATTCCTGCAAAGAACTTTCCTAG (EcoRI)	
WZ743	CCCAAGCTTATGAGTAAAGGAGAAGAACTT (HindIII)	*gfpmut3a* amplification
WZ746	CCGGAATTCTTATTTGTATAGTTCAT (EcoRI)	

[a] The sites for the restriction enzymes (indicated in parentheses) are underlined. Complementary sequences are bold.

equipped with an argon laser (emission at 488 nm and 15 mW) and a 525-nm band-pass filter. The cells were diluted to an OD_{600} of 0.2 using phosphate-buffered saline (PBS) buffer (pH 7.2) and placed on ice prior to analysis. For each sample, 50,000 events were collected at a rate between 1,000 and 2,000 events per s. Cells cultured in the absence of inducer were used as a control to determine background fluorescence.

Shake flask fermentation. *C. glutamicum* was cultured in 500-ml shake flasks containing 30 ml of CGIII medium for 16 h. Five percent

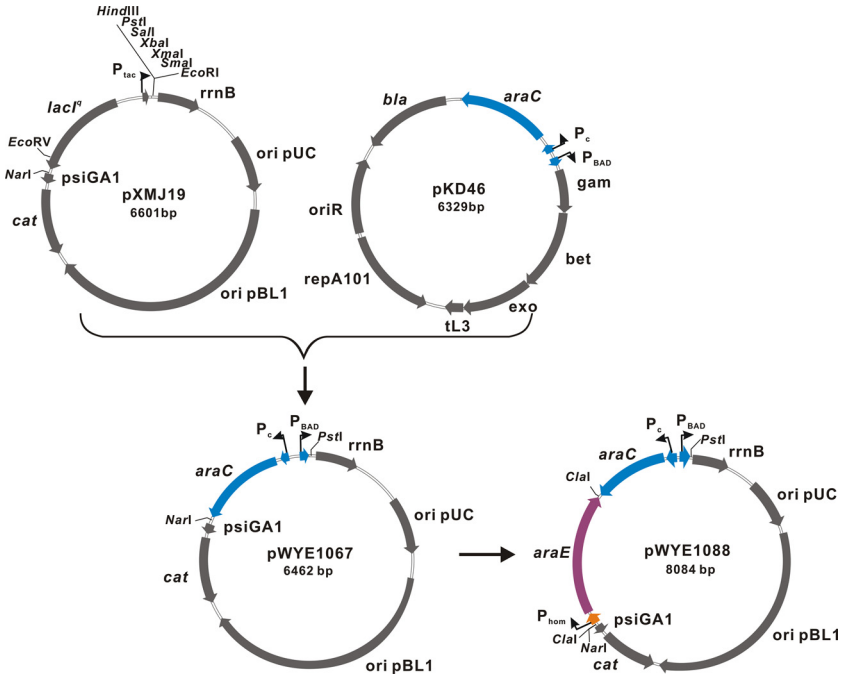

FIG 1 Construction of the arabinose-inducible expression vectors pWYE1067 (*araC*-P_{BAD}) and pWYE1088 (P_{hom}-*araE*, *araC*-P_{BAD}). The *araC*-P_{BAD} fragment was amplified by PCR using the *E. coli* vector pKD46 as the template. The PCR product was digested with NarI and PstI and ligated into the *E. coli*-*C. glutamicum* shuttle vector pXMJ19 to generate the vector pWYE1067 (*araC*-P_{BAD}). P_{hom} and *araE* were amplified by PCR using *C. glutamicum* and *E. coli* chromosomes, respectively, as the templates. The two fragments were fused by overlap extension PCR and ligated into the ClaI-digested vector pWYE1067 to generate the vector pWYE1088 (P_{hom}-*araE*, *araC*-P_{BAD}). rrnB, the transcriptional terminator; *cat*, chloramphenicol acetyltransferase gene; ori, origin of replication.

(vol/vol) inocula were added to shake flasks (500 ml) containing 30 ml of CGX medium (3), and fermentation was performed at 30°C and 200 rpm. After sterilization, glucose and $CaCO_3$ were added to final concentrations of 4 and 2%, respectively. Cell growth was monitored by measuring the absorbance at 600 nm using a UV-visible spectrophotometer.

Analytic methods. The dry cell weight was estimated based on the correlation 1 OD_{600} unit is equal to 0.28 g of dry cell weight/liter (17). The glucose concentration was determined using an SBA-40D biosensor automatic analyzer (Shandong, China). The L-glutamate concentration was measured using a high-performance liquid chromatography system equipped with an Eclipse XDB-C18 column (Agilent Technologies, Wilmington, DE) after derivatization with 2,4-dinitrofluorobenzene.

RESULTS AND DISCUSSION

Construction of the L-arabinose-inducible expression vector pWYE1067. The entire *araC* and the P_{BAD} promoter fragment was amplified from pKD46 and ligated into the *E. coli-C. glutamicum* vector pXMJ19 to create the vector pWYE1067 (Fig. 1). To estimate the inducibility of *araC*-P_{BAD} promoter system in *C. glutamicum*, the *lacZ* gene encoding β-galactosidase from *E. coli* was used as a reporter gene. In the presence of 0.2% L-arabinose, β-galactosidase activity was maintained at a low level, whereas activity noticeably improved after the addition of 1% L-arabinose (Fig. 2A). In contrast, β-galactosidase activity was barely detectable at each time point during cultivation in the absence of L-arabinose, demonstrating that the P_{BAD} promoter was tightly activated by L-arabinose. However, P_{BAD}-based expression was efficiently induced only at high L-arabinose concentrations in *C. glutamicum* compared to *E. coli*, in which 0.03% L-arabinose was sufficient to induce significant P_{BAD}-based expression (8). The sensitivity of the promoter to inducer concentrations depends on the ability of cells to take up the inducer. The inability of some strains to transport IPTG or the deletion of *lacY* gene encoding lactose permease in *E. coli* led to the inefficient induction of P_{lac} promoter (6, 9, 22). The results from previous investigations indicated that L-arabinose might enter *C. glutamicum* through aqueous channels or a low-affinity nonspecific transporter (1, 11); therefore, L-arabinose uptake by *C. glutamicum* might be a major factor influencing P_{BAD}-based expression.

Screening for an appropriate promoter to express the *araE* gene from pWYE1067. To improve the sensitivity of the P_{BAD} response to L-arabinose, we further modified the vector pWYE1067 by introducing the *araE* gene encoding the L-arabinose transporter from *E. coli* under the control of a constitutive promoter from *C. glutamicum*. Five candidate promoters derived from *C. glutamicum* were ligated into the vector pXMJ19-*lacZ* on the upstream of the promoter-less *lacZ* gene to evaluate their activities. As shown in Fig. 2B, P_{45} showed 2-fold-higher *lacZ* expression compared to P_{eno} and P_{fda}, both of which had relatively moderate strength, and P_{hom} exhibited the lowest *lacZ* expression among the promoters tested. Notably, P_{glyA} displayed a high constitutive expression profile with 4-fold-higher *lacZ* expression compared to P_{tac} in the presence of 1 mM IPTG (Fig. 2B). In *E. coli*, the different expression levels of *araE* gene under the control of constitutive promoters slightly influence the degree of P_{BAD} induction (12). However, the excess expression of the plasmid-based *araE* gene and *araBAD* operon did not make the recombinant *C. glutamicum* grow on L-arabinose (34), indicating that the overexpression of membrane protein (AraE) might interfere with the metabolic process and be unfavorable for the growth of *C. glutamicum*. Therefore, to appropriately control the *araE* expression and alleviate the adverse effects on cell growth, the weaker constitutive P_{hom} promoter was used to regulate *araE* expression from vector pWYE1067, generating the resulting vector pWYE1088 (Fig. 1).

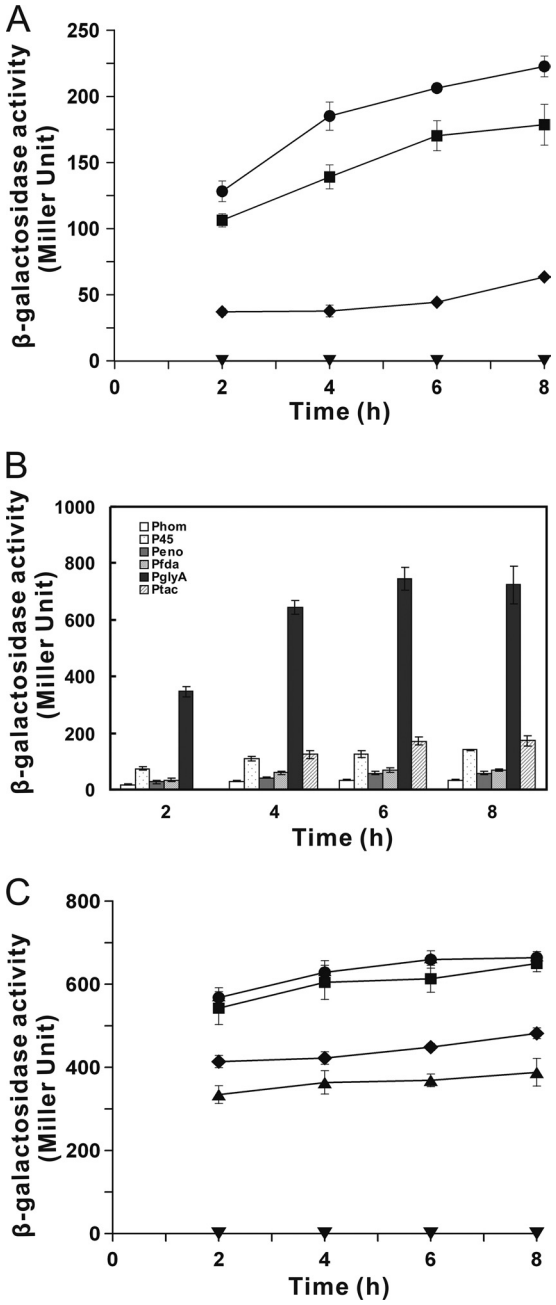

FIG 2 Assessment of different promoter activities in *C. glutamicum* ATCC 13032. (A) P_{BAD} activities in *C. glutamicum* carrying pWYE1067-*lacZ* exposed to different L-arabinose concentrations. The L-arabinose concentrations are represented by different symbols: 0% (▼), 0.2% (◆), 1% (■), and 2% (●). (B) Promoter activities of P_{hom}, P_{45}, P_{glyA}, P_{fda}, and P_{eno} in *C. glutamicum* ATCC 13032. To induce the P_{tac} promoter, IPTG was added at a final concentration of 1 mM after cultivation for 2 h. (C) The P_{BAD} activities in *C. glutamicum* carrying pWYE1088-*lacZ* exposed to different L-arabinose concentrations. The L-arabinose concentrations are represented by different symbols: 0% (▼), 0.02% (▲), 0.2% (◆), 1% (■), and 2% (●).

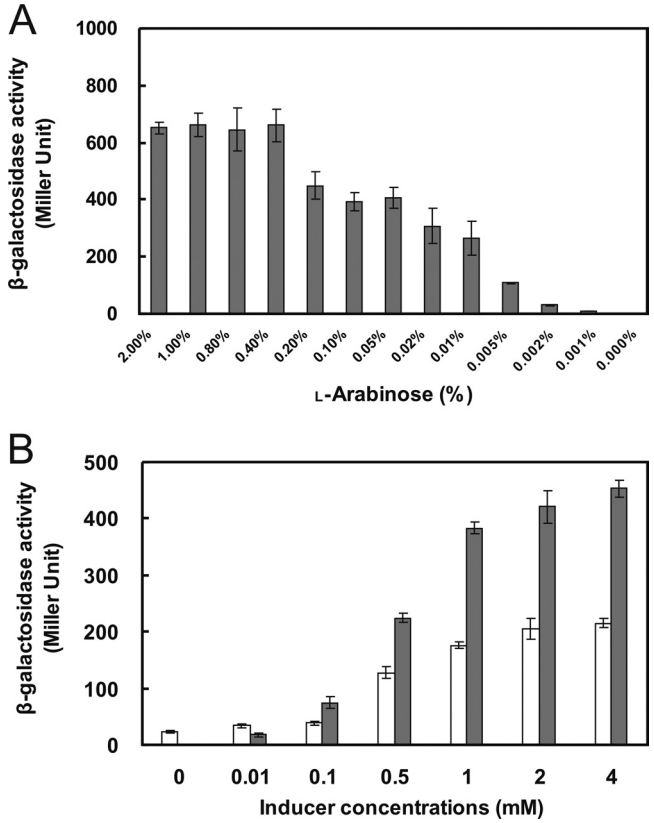

FIG 3 Characterization of the dynamic range of L-arabinose induction. (A) β-Galactosidase activities in *C. glutamicum* ATCC 13032 carrying pWYE1088-*lacZ*. Cells were harvested after 4 h of induction at the indicated L-arabinose concentrations for analysis. (B) Comparison of the strength of the P_{BAD} and P_{tac} promoters in the presence of the same molar concentrations of L-arabinose (■) and IPTG (□). The mean values from at least three independent cultures are shown with the standard deviations.

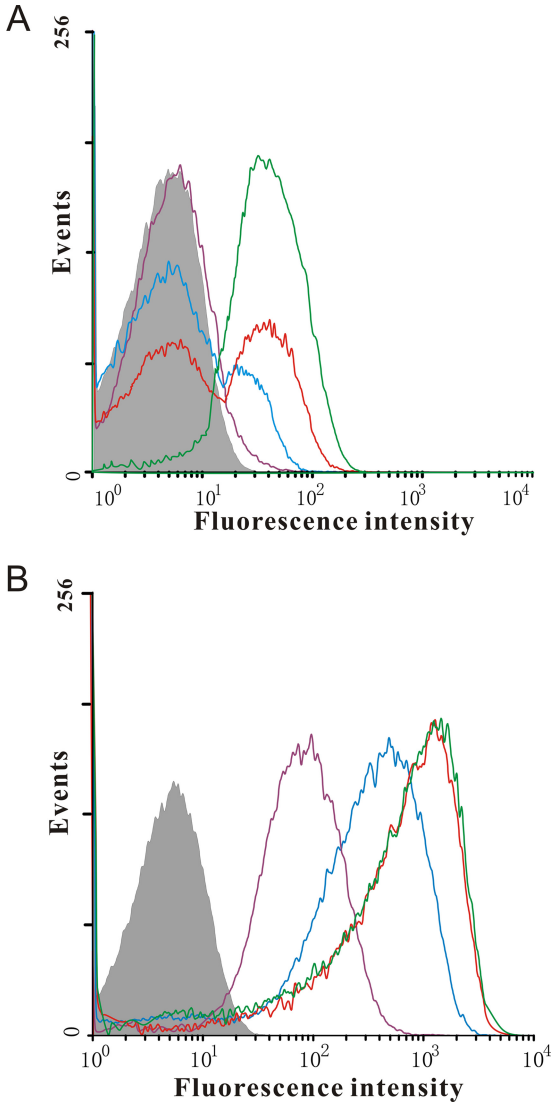

FIG 4 Flow cytometry analysis of GFP expression. Histograms showing the numbers of cells and the fluorescence intensity of cultures of *C. glutamicum* strains harboring the *gfpmut3a* reporter plasmids. (A) All cultures harbored the *gfpmut3a* gene on the vector pWYE1067. (B) All cultures harbored the *gfpmut3a* gene on the vector pWYE1088. The fluorescence intensity of individual cells was measured by flow cytometry 2 h after the addition of L-arabinose at the indicated concentrations (gray-shaded curve, 0%; purple curve, 0.002%; blue curve, 0.02%; red curve, 0.2%; green curve, 2%).

Dose-dependent control of P_{BAD}-based expression by L-arabinose. As expected, when the *araE* gene was expressed in *C. glutamicum*, the L-arabinose concentration to induce P_{BAD}-based expression was significantly decreased, and the level of P_{BAD}-based expression increased by 10-fold in response to 0.2% L-arabinose (Fig. 2C). In addition, *C. glutamicum* P_{BAD}-based expression increased with increasing incubation time and then remained constant. This effect is attributed to a deficiency in the L-arabinose degradation pathway of *C. glutamicum* (11) that makes the intracellular pool of L-arabinose invariable during the induction process. This expression system achieved an effective induction of *lacZ* gene expression in a wider dynamic range from 0.001 to 0.4% L-arabinose (Fig. 3A). In contrast, the P_{tac} promoter regulated the *lacZ* expression over a concentration range from 0.01 to 1 mM IPTG (Fig. 3B). Furthermore, the level of P_{BAD}-based expression was ~2-fold higher than that of P_{tac} in the presence of the same molar concentration of L-arabinose or IPTG (Fig. 3B). Therefore, this expression system could provide the high-level expression in *C. glutamicum* compared to the previously available expression system.

***P_{BAD}*-based expression is tightly regulated by L-arabinose.** The *upp* gene encoding uracil phosphoribosyltransferase, which converts 5-fluorouracile (5-FU) to a toxic product for cell growth (7), was chosen as a reporter gene to assess the stringency of L-arabinose induction in *C. glutamicum*. To inhibit basal levels of *upp* expression, this gene was deleted from the chromosome of *C. glutamicum* by homologous recombination. The resulting *upp*-null mutant was used as the parental strain for the inducible expression of the *upp* gene from pWYE1088. The mutant strain harboring pWYE1088-*upp* exhibited normal growth on CGX medium containing 5-FU in the absence of L-arabinose but was unable to grow in the presence of 0.02% L-arabinose (see Fig. S1 in the supplemental material). It indicated that this system tightly controlled the expression of the *upp* gene by L-arabinose without leaky expression.

Homogeneous expression of the P_{BAD} promoter associated with P_{hom}-*araE*. To assess the homogeneity of P_{BAD}-based expres-

TABLE 3 Effects of a variety of carbon sources on P_{BAD} strength in *C. glutamicum*

Carbon source	Mean β-galactosidase activity ± SD at an L-arabinose concn of[a]:			
	0.1%		0.2%	
	Miller unit	Ratio	Miller unit	Ratio
Glucose	389.7 ± 40.6	100	409.5 ± 6.2	100
Sucrose	403.4 ± 40.1	1.1 ± 0.2	531.4 ± 20.3	1.3 ± 0.2
Fructose	353.8 ± 78.8	0.9 ± 0.3	378.2 ± 26.1	0.9 ± 0.1
Gluconate	640.9 ± 13.2	1.6 ± 0.2	651.2 ± 33.9	1.6 ± 0.1
Ribose	585.4 ± 14.0	1.5 ± 0.1	607.7 ± 30.8	1.5 ± 0.1
Acetate	362.4 ± 17.8	0.9 ± 0.1	400.1 ± 19.4	1.0 ± 0.1

[a] β-Galactosidase activities, expressed in Miller units, represent means from three independent experiments. Ratios are calculated relative to the glucose culture for each carbon source.

sion, *C. glutamicum* harboring either pWYE1067-*gfpmut3a* (pWYE1067-GFP strain) or pWYE1088-*gfpmut3a* (pWYE1088-GFP strain) was cultivated in the presence or absence of L-arabinose and harvested for flow cytometry analysis. As shown in Fig. 4A, cultures of the pWYE1067-GFP strain exhibited little fluorescence in the presence of 0.002% L-arabinose. However, two distinct subpopulations were observed in the presence of 0.02 and 0.2% L-arabinose, indicating that the response to L-arabinose induction is heterogeneous. It might be attributed to the differences between individual cells in L-arabinose transport (13). In contrast, the fluorescence of individual cells was reliably detected in all of the cultures of the pWYE1088-GFP strain that were induced with different concentrations of L-arabinose (Fig. 4B). Nearly all of the pWYE1088-GFP population exhibited a positive homogeneous fluorescence signal at 0.02% L-arabinose compared to the pWYE1067-GFP population, demonstrating that the expression of *araE* under the control of the P_{hom} promoter resulted in a homogeneous population of cells, a finding consistent with a previous report for *E. coli* (12). In addition, the population-averaged fluorescence intensities of the pWYE1088-GFP strain increased with increasing L-arabinose concentration, indicating that variable promoter control occurs in each cell within the population rather than in a fraction of the population.

Effects of various carbon sources on the strength of the P_{BAD} promoter. To investigate the P_{BAD}-based expression in response to different carbon sources, *C. glutamicum* harboring pWYE1088-*lacZ* was cultivated in CGX medium using glucose, sucrose, fructose, ribose, gluconate, and acetate as the sole carbon source. The β-galactosidase activity of cells grown on glucose showed a modest decrease compared to that of cells grown on LB medium (Table 3). The strength of P_{BAD}-based expression in cells grown with ribose and gluconate was slightly increased, with as much as 1.5- and 1.6-fold-higher β-galactosidase activities relative to expression in glucose. Moreover, cells grown with sucrose, fructose and acetate showed similar β-galactosidase activity compared to cells grown with glucose (Table 3). In *E. coli*, the P_{BAD} promoter is subjected to significant catabolic repression in response to glucose (8) because this bacterium preferentially uses glucose and inhibits the uptake rate of secondary carbon sources by phosphotransferase (PTS) systems (33). As for *C. glutamicum*, the various PTS systems are expressed constitutively (47). In addition, the constitutive expression of *araE* resulted in an increase in the intracellular pool of L-arabinose for P_{BAD} induction and did not interfere with the uptake of other carbon sources. Therefore, the different carbon sources did not have a strong effect on the strength of P_{BAD}-based expression.

Application of the L-arabinose-inducible system in glutamate fermentation. In order to evaluate its effect, the current L-arabinose-inducible system was used to regulate the expression of the *odhI* gene, which encodes a regulatory protein that inhibits α-oxoglutarate dehydrogenase activity (14). *C. glutamicum* strains carrying pWYE1088 and pWYE1088-*odhI* were cultivated in 500-ml shake flasks. L-Arabinose was added at a final concentration of 0.02% to induce *odhI* expression. As shown in Fig. 5, cellular growth and glucose consumption of the two strains were identical, whereas glutamate did not accumulate in the strain containing pWYE1088 under biotin nonlimiting conditions. In contrast, the *odhI*-overexpressing strain continuously accumulated glutamate in the late exponential and stationary phases and produced glutamate at levels reaching 13.7 mM after 30 h.

Consequently, the arabinose-inducible expression system generated in the present study provides a novel efficient genetic engineering tool for molecular biology and metabolic engineering in *C. glutamicum*. Furthermore, the strategy of coexpressing a sugar-

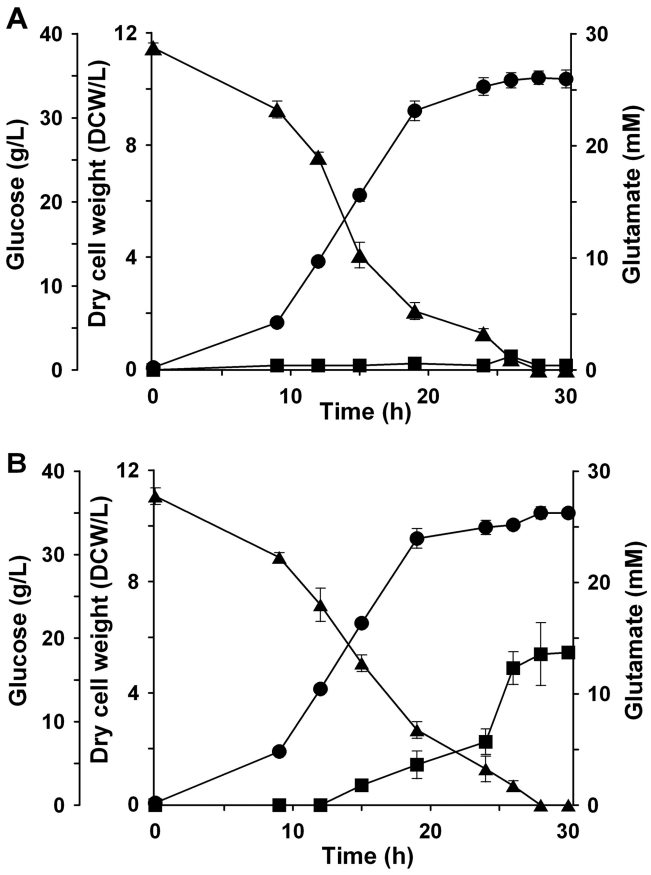

FIG 5 Shake-flask fermentation profiles of *C. glutamicum* ATCC 13032 strain carrying the vector pWYE1088 (A) or the vector pWYE1088-*odhI* (B) under biotin nonlimiting conditions. L-Arabinose (0.02%) was used to induce *odhI* gene expression. The dry cell weight (●), glucose concentration (▲), and glutamate concentration (■) are indicated. Average measurements with the standard deviations from three independent experiments are shown.

regulated promoter and sugar transporter to facilitate the uptake of an inducer provides an effective solution to improve the inducible expression of sugar-responsive promoters in other bacteria that cannot efficiently transport the inducer.

ACKNOWLEDGMENTS

We are grateful to Tong Zhao for the flow cytometry analysis.

This study was supported by the Key Project of the Chinese Academy of Sciences (KSCX2-EW-J-6), the National Natural Science Foundation of China (grant 31100074), the Beijing Natural Science Foundation (grant 5112023), and the Ministry of Science and Technology of China (grant 2010ZX09401-403).

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