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A Glucose-Insensitive T7 Expression System for Fully-Induced Expression of Proteins at a Subsaturating Level of L-Arabinose

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S Supporting Information

ABSTRACT: The L-arabinose (Ara)-controlled T7 expression system was previously constructed by creation of an *Escherichia coli* BL21(BAD) strain. The production of recombinant proteins in this strain was stringently regulated and reached a high level upon induction with Ara. Nevertheless, this system is still associated with inherent problems of interference with glucose and of the all-or-nothing induction profile at a subsaturating level of Ara. In this study, these problems were circumvented by modifying the physiological traits of BL21(BAD) strain. This was followed by deletion of *ptsG* gene and the *araFGH* and *araBAD* operon. The former encodes the glucose transporter while the latter two gene operons produce proteins responsible for Ara uptake and catabolism. In addition, the expression of genomic *araE* (encodes the Ara transporter) was constitutively enhanced. The resulting strain was designated BAD-5. By expression of the faster degrader GFP(LAA) at a subsaturating level of Ara, 80% of BAD-5 strain was found visually bright in the presence or absence of glucose. A further analysis by flow cytometry showed a uniform distribution of GFP expression for BAD-5 strain. In marked contrast, BL21(BAD) strain exhibiting visual brightness was less than 10% of the cell population and remained dark in the presence of glucose. Moreover, a saturated level of luciferase from *Renilla reniformis* (Rluc) could be readily obtained in BAD-5 strain at 20 μM Ara regardless of glucose. Rluc in BL21(BAD) strain was produced in an Ara dose-dependent manner, and the protein production became arrested when glucose was present. Overall, it illustrates the usefulness of the improved system for overproduction of recombinant proteins in an efficient, homogeneous, and glucose-insensitive way.

KEYWORDS: T7 expression system, recombinant protein, *araBAD* promoter, *Renilla* luciferase

INTRODUCTION

Overproduction of recombinant proteins in an isolated form is a fundamental task for both the scientific community and industry. In this context, the application of bacteria as a producer host is relatively simple and efficient. The reason is manifest that bacterial cells can be grown rapidly on cost-effective medium and enabled to largely produce proteins when harboring an expression vector that contains the cloned gene.[1] In particular, the protein production scheme based on bacteria is usually scalable.[2] *Escherichia coli* is well studied and appears to be the most commonly used producer cell. The availability of many expression plasmids and mutant strains makes this prokaryote appealing for various applications of protein production.[3] Generally, most expression plasmids have high copy numbers and are equipped with the high protein-synthesized machinery. Among them, the T7 expression system has received the most attractiveness for protein overproduction. It employs an *E. coli* strain, designated BL21(DE3), carrying a chromosomal copy of the T7 gene 1 (encoding T7 RNA polymerase) under control of the *lacUV5* promoter.[4] To make it work, the gene of interest is fused with the T7 promoter (P_{T7}) carried on a plasmid. After transformation of the recombinant plasmid, strain BL21(DE3) is cultured and induced for protein production by adding isopropyl-β-D-thiogalactopyranoside (IPTG). As a result of IPTG induction, T7 RNA polymerase is synthesized and binds specifically to P_{T7}. The cloned gene regulated by P_{T7} can then be expressed exclusively at a high level because T7 RNA polymerase exhibits superior processivity.[5]

One major disadvantage of the T7 expression system lies in the basal expression of T7 RNA polymerase due to the relaxed regulation of the *lacUV5* promoter.[5] This results in an uninduced expression of cloned genes, allowing the establishment of an environment in favor of selecting variant strains or causing plasmid instability if the produced protein is highly toxic to the producer cell.[6] The situation is especially unfavorable in the time-consuming fermentation process. One way to reduce the basal level of T7 RNA polymerase is to use the producer strain that harbors either pLysS or pLysE vector. These two vectors provide T7 lysozyme that naturally inhibits the function of T7 RNA polymerase.[7] However, T7 lysozyme is also able to degrade the peptidoglycan chain of *E. coli* cell wall. Consequently, it leads to a slow growth of bacteria and reduced production of recombinant proteins.[8] Moreover, IPTG is costly and has potential toxicity, thereby limiting the industrial usefulness of the T7 expression system.[9]

Previously, we have tackled the above-mentioned problem by developing an L-arabinose (Ara)-controllable T7 expression

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Table 1. Bacterial Strains, Plasmids, and Primers Applied in This Study[a]

	relevant characteristic (sequence)	source
Strain		
DH5α	*deoR endA1 gyrA96 hsdR17 supE444 thi* Δ(*lacIZYA-argF169*) *recA1* ϕ*80 lacZM15*	lab collection
BL21(BAD)	*dcm gal ompT hsdS*(r_B^- m_B^-) *lacZ*::P_{BAD}-G1	11
JW1087-2	△*ptsG*::FRT-*kan*-FRT	NIG[b]
JW2809-1	△*araE*::FRT-*kan*-FRT	NIG
BAD-1G	as BL21(BAD) but △*araBAD* and △*ptsG*	this study
BAD-5	as BAD-1G but EM7::*araE*	this study
BAD-5E	as BAD-1G but △*araE*	this study
BAD-L	as BL21(BAD) but $Rluc^+$	this study
BAD-5L	as BAD-5 but $Rluc^+$	this study
Plasmid		
pRK8	carry GFP(LAA)	13
pET-28	carry P_{T7}	Novagen Co.
pET-GFPm	as pET-28 but carry P_{T7}::GFP(LAA)	this study
pTH18Kr	kan^+ pSC101 *ori*	14
pTH-GFPm	as TH18Kr but carry P_{T7}::GFP(LAA)	this study
pBlue-Ruc	carry Rluc	15
pET-20b	carry P_{T7}	16
pET-Rluc	as pET-20b but carry P_{T7}::Rluc	this study
pHK-Km	carry HK022 attachment (*att*) site	17
pHK-lux	as pHK-Km but carry P_{T7}::Rluc	this study
pKD13	carry FRT-*kan*-FRT	20
pKD-EM7	carry FRT-*kan*-FRT-EM7	this study
pKD46	carry P_{BAD}::λ Red	20
pCP20	carry P_{LAM}::FLP	20
pLacYA177C	carry *lacY* A177C	13
pSF1	bla^+ pMB1 *ori*	18
pSF1-Y177	as pSF1 but carry *lacY* A177C	this study
Primer		
RC0512	TGCACCATATGACTTCGAAAGTTTATG	
RC0513	TATGCTCGAGTTATTGTTCATTTTTGAGAACTC	
T7A	ACTATGGATCCCGCGAAATTAATACGAC	
T7B	ACATGCTGCAGTTCCTCCTTTCAGCAAAAAAC	
RC0751	ATTCCGGGGATCCGTCGACC	
RC0752	GTGTAGGCTGGAGCTGCTTC	
RC0753	TTGTCGTATTATACTATGCCGATATACTATGCCGATGATTAATTGTCAAGTGTAGGCTGGAGCTGCTTC	
RC0754	GAGCGATTTTTAGTTCCTCAC	
RC07201	ACCTTCTAGAAGATATACATGCGTAAAGGAGAAGAAC	
RC07202	TTGGAAGCTTATTAAACTGCTGCAGCGTAG	
RC06143 (A2)	GAAGCAGCTCCAGCCTACACGGTTTTCAGTGCCGCTTCC	
RC06144 (B1)	GGTCGACGGATCCCCGGAATGTTCTGGTCCATTCCCACG	
RC06145 (A1)	ATGGCGATTGCAATTGGCCTC	
RC06146 (B2)	TTACTGCCCGTAATATGCCTTC	
RC0725 (A2)	GAAGCAGCTCCAGCCTACACTTCAATGTTTTTTCGCCATC	
RC0726 (B1)	GGTCGACGGATCCCCGGAATTCTCCGCCTGCGTTTTAG	
RC0727 (A1)	ATGCACAAATTTACTAAAGC	
RC0728 (B2)	TCAGACAGTGCGTTTCG	
RC0729 (A1)	CCAATTGCTGCACCGAGC	
RC0730 (A2)	GTGAGGAACTAAAAATCGCTCCTGGCAGGAAAAAATGGTTAC	
RC0731 (B1)	GGTCGACGGATCCCCGGAATATAGTGAAAAAATACGTGAAC	
RC0732 (B2)	CTCCAGCGCTTCAGATTATG	

[a] 5′- or 3′-extensions of primers are underlined. Abbreviations: *ori*, origin of replication; P_{BAD}, L-arabinose BAD promoter; P_{LAM}, λP_R P_L promoter; G1, T7 gene 1; EM7, EM7 promoter; *bla*, ampicillin-resistant gene. [b] NIG, National Institute of Genetics.

system.[10,11] This system is essentially based on the creation of an *E. coli* strain which carries the genomic copy of T7 gene 1 regulated by the *araBAD* promoter. As illustrated, it is marked with high stringency and the cloned gene on a plasmid can be readily expressed upon induction with Ara. However, the nature of stringency and inducibility of this system strongly depends on the composition of culture medium. In the presence of glucose, the expression of cloned gene in this system becomes noninducible by Ara. Like the *lac*-type promoter, the *araBAD* promoter exhibits the kinetics of an "autocatalytic" induction mechanism due to accumulation of the inducer by active transport.[12] This results in all-or-nothing induction of gene expression when a subsaturating level of Ara is employed. Under this condition, there is no way to achieve the maximum production of recombinant proteins with a mixed population consisting of fully induced and uninduced cells.

In this study, the inherent problems associated with the *ara* system as mentioned were circumvented in several steps. By removal of the *araBAD* operon, Ara is not metabolized by bacteria and able to retain its persistent inducibility. The *ptsG* gene responsible for the transport of glucose in *E. coli* was deleted to eliminate the glucose-mediated catabolite repression on the *ara* system. Moreover, the expression of *araE* transporter was constitutively enhanced along with the knockout of the *araFGH* operon that encodes the active transporter for Ara. As a consequence, it leads to a fully induced and homogeneous expression of recombinant proteins at a subsaturating level of Ara irrespective of glucose.

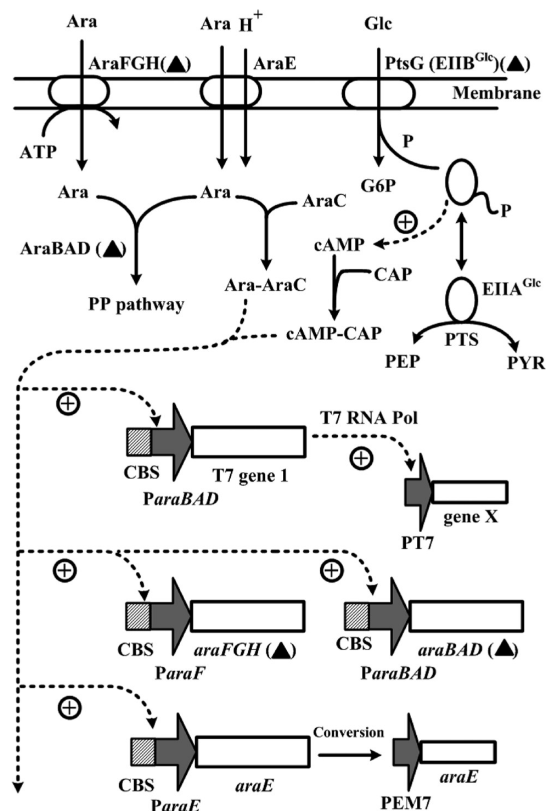

Figure 1. A schematic illustration of strategies applied in this study. Refer to text for details. The phosphoryl group (P) donated from phosphoenolpyruvate (PEP) is translocated to $EIIA^{Glc}$ though the PEP: carbohydrate phosphotransferase system (PTS). Phosphorylated $EIIA^{Glc}$ stimulates (⊕) the synthesis of cyclic AMP (cAMP). cAMP is then complexed with catabolite activator protein (CAP) to form cAMP−CAP. During uptake via PtsG, glucose (Glc) receives the phosphoryl group and converts to glucose 6-phosphate (G6P) in bacterial cytoplasm. Consequently, this makes $EIIA^{Glc}$ dephosporylated and lowers the synthesized level of cAMP. In addition, Ara enters *E. coli* with the aid of AraGFH and AraE transporter. After processing of Ara by AraBAD, the resulting metabolite is further metabolized in the pentose phosphate (PP) pathway. Expression of the Ara metabolism-related genes is fully induced (⊕) by the combined act of Ara-AraC and cAMP−CAP complex protein. Upon induction, expression of T7 gene 1 produces T7 RNA polymerase (Pol), which in turn selectively activates the transcription of the target gene (X) under control of the T7 promoter (PT7). To improve the Ara-controlled T7 expression system, several genes required for glucose and Ara metabolism are deleted as denoted (▲). In addition, the native promoter of *araE* (P*araE*) is replaced by the EM7 promoter (PEM7). Other abbreviations used: PYR, pyruvate; P*araBAD*, *araBAD* promoter; CBS, cAMP−CAP binding site; PTS, phosphoenolpyruvate:carbohydrate phosphotransferase system.

MATERIALS AND METHODS

Plasmid Construction and Genomic Insertion of Genes. *E. coli* strains, plasmids, and primers applied in this study were summarized in Table 1. The work for gene cloning was carried out using *E. coli* strain DH5α. The fast degrader GFP (GFP(LAA)) was amplified from plasmid pRK8[13] by the polymerase chain reaction (PCR) with primer RC07201−07202. After digestion with *Xba*I−*Hin*dIII, the PCR DNA was incorporated into plasmid pET-28 (Novagen Co., USA) to give pET-GFPm. The DNA fragment containing the P_{T7}-driven GFP(LAA) was then recovered from pET-GFPm by *Sma*I−*Sph*I cleavage. Subsequent ligation of this purified DNA into plasmid pTH18Kr[14] produced pTH-GFPm. For protein production, luciferase from *Renilla reniformis* (Rluc) was amplified from pBlue-Ruc[15] using PCR with primer RC0512−RC0513. After digestion by *Nde*I−*Xho*I, the synthesized Rluc gene was spliced into pET-20b[16] to give pET-Rluc. Furthermore, the DNA containing Rluc fused to the P_{T7} was obtained from plasmid pET-Rluc by PCR using primer T7A−T7B. The PCR DNA treated with *Bam*HI−*Pst*I was incorporated into integration plasmid pHK-Km[17]to generate plasmid pHK-lux. Using plasmid pHK-lux, the DNA comprising the P_{T7}-regulated Rluc was integrated into bacterial genome according to the reported protocol.[17] Plasmid pSF1-Y177 carries a mutant lactose transporter gene (*lacY* A177C). It was obtained by incorporation of *lacY* A177C from plasmid pLacYA177C, digested with *Eco*RI−*Hin*dIII, into plasmid pSF1.[18]

In addition, plasmid pKD-EM7 was constructed for replacement of the wild-type promoter of genomic genes with the EM7 promoter.[19] It was derived from plasmid pKD13[20] that contains the kanamycin-resistant gene (*kan*) flanked by the FRT site (FRT-*kan*-FRT). Primer RC0753 was designed to contain the sequence of the EM7 promoter with a 3′-extension complementary to pKD13. On incorporation of the primer sequences (RC0751−RC0753), plasmid pKD13 was modified with the creation of staggered nicks. Treated with *Dpn*I, the PCR DNA was self-ligated to produce plasmid pKD-EM7 that contains FRT-*kan*-FRT-EM7. The incorporated sequence of the EM7 promoter was further confirmed by DNA sequencing.

Strain Construction. Deletion of the *araBAD* operon in BL21-(BAD) strain was carried out essentially following the protocol as outlined (Supplementary Figure S1 in the Supporting Information). In essence, the template DNA containing FRT-*kan*-FRT was amplified from plasmid pKD13 by PCR with primers RC0751 and RC0752. Meanwhile, the N-terminus of *araB* and the C-terminus of *araD*, called megaprimers (∼200 bp), were synthesized with PCR using the targeting primer pair RC06143−RC06145 and RC06144−RC06146, respectively.

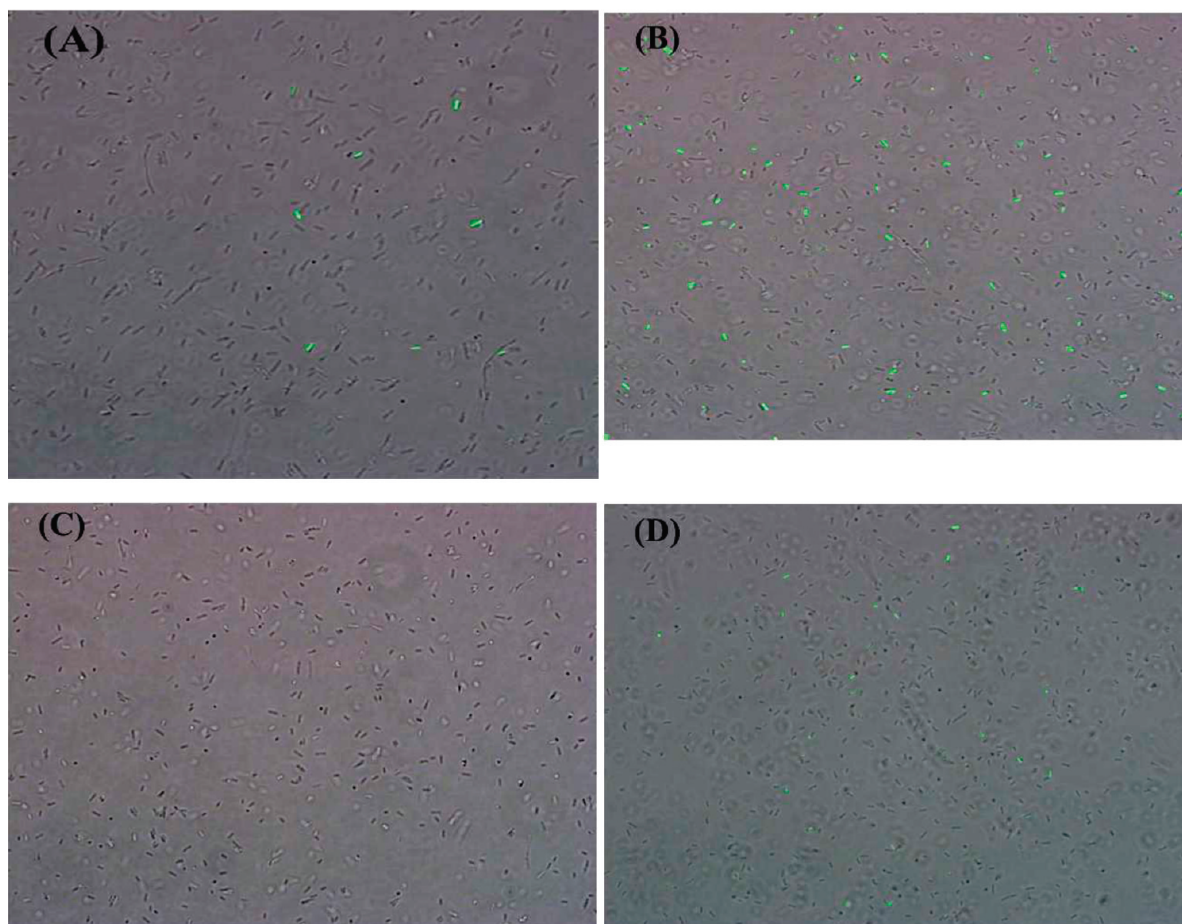

Figure 2. Analysis of GFP(LAA) expression in *E. coli* strain by fluorescence microscopy. BL21(BAD) and BAD-1G strain with plasmid pTH18-GFPm were grown on LB medium with (0.4%) or without glucose and induced with 30 μM Ara upon reaching 0.3 at OD_{550}. After induction for 2 h, bacterial cultures were harvested in 0.1 M phosphate buffer (pH 7.0) and diluted to reach 10^6 cells. Bacterial samples were then mounted on glass slides for observation under fluorescence microscopy. (A) BL21(BAD) strain grown on the medium without glucose. (B) BAD-1G strain grown on the medium without glucose. (C) BL21(BAD) strain grown on the medium with glucose. (D) BAD-1G strain grown on the medium with glucose.

Primers RC06143 (corresponding to A2) and RC06144 (corresponding to B1) contain a 5′-extension that complements with two termini of FRT-*kan*-FRT. With megaprimers, the overlapping PCR was performed using FRT-*kan*-FRT as the DNA template. As a consequence, it produced a targeting DNA cassette that comprises the FRT-*kan*-FRT fragment linked to megaprimers. BL21(BAD) strain harboring helper plasmid pKD46[20] was grown on LB medium[21] and induced with 1 mM Ara for production of λ-Red recombinase at 30 °C. After induction for 3 h, bacterial cultures were made electrocompetent by concentrating 100-fold and washing two times with ice-cold 10% glycerol. Moreover, the targeting DNA cassette (100 ng) was purified and electroporated into competent cells. The electroshocked cells were then incubated in SOC medium (0.5% yeast extract, 2% tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose) at 37 °C for 2 h. By the act of λ-Red recombinase, the *araBAD* operon was crossed over with FRT-*kan*-FRT via homologous recombination. The resulting cells were spread onto agar plates containing LB medium with kanamycin at 37 °C. One integrant which exhibited ampicillin sensitivity (indicating loss of plasmid pKD46) was obtained. To remove the inserted *kan* marker, this integrant cell was transformed with helper plasmid pCP20 expressing Flp as reported previously.[20] Followed by thermal induction at 39 °C, transformants were screened for conferring sensitivity to both kanamycin (loss of the marker gene) and ampicillin (loss of pCP20). In addition, the gene integration and deletion events were verified by *in situ* PCR based on primer RC06145 (corresponding to A1)−RC06146 (corresponding to B2) according to our previous report.[17]

The genomic *ptsG* gene or *araE* gene was further removed by P1 transduction with either JW1087-2 (△*ptsG*::FRT-*kan*-FRT) strain or JW2809-1 (△*araE*::FRT-*kan*-FRT) as the donor cell. P1 transduction was performed following the previous method.[22] Similarly, the kanamycin-resistant gene of one transductant was eliminated with Flp from helper plasmid pCP20.

Likewise, the method for deletion of the *araFGH* operon in *E. coli* strain followed the protocol of *araBAD* knockout. Two megaprimers, consisting of the N-terminus of *araF* and the C-terminus of *araH*, were synthesized by PCR using the targeting primer pair RC0725 (corresponding to A2)−RC0727 and RC0726 (corresponding to B1)−RC0728. Overlapping PCR was then conducted to produce the targeting DNA cassette with the megaprimers that prime FRT-*kan*-FRT. Followed by electroporation of the targeting DNA cassette, the *araFGH* operon of BAD-1G strain was inserted with FRT-*kan*-FRT by the act of λ-Red recombinase. Finally, the inserted antibiotic marker in bacteria was removed by Flp. *In situ* PCR was further carried out to verify the gene integration and deletion events with primer RC0727 (corresponding to A1)−RC0728 (corresponding to B2).

In addition, the promoter of genomic *araE* was replaced with the EM7 promoter. This was achieved in a similar way (refer to Supplementary Figure S1 in the Supporting Information). Two megaprimers

that comprise the upstream region and the N-terminal structural gene of *araE* were generated by PCR with the targeting primer pair RC0731 (corresponding to B1)−RC0732 and RC0729−RC0730 (corresponding to A2), respectively. Both primers RC0730 and RC0731 contain a 5′-extension complementary to two termini of FRT-*kan*-FRT-EM7. From plasmid pKD-EM7, the template DNA (e.g., FRT-*kan*-FRT-EM7) was synthesized by PCR with primer RC0751−RC0754. The targeting DNA consisting of FRT-*kan*-FRT-EM7 flanked with two homologous regions was then synthesized by overlapping PCR. With the help of λ-Red recombinase, genomic *araE* in bacteria was fused with the EM7 promoter. The inserted antibiotic marker in bacteria was finally removed by Flp.

Bacterial Culturing and Analytical Methods. LB medium with or without glucose was used in this study. For growth of bacterial strains harboring plasmids, indicated antibiotics (e.g., 30 μg/mL kanamycin or/and 20 μg/mL ampicillin) were supplemented in culture medium. Bacteria were grown overnight, and cell biomass was measured turbidimetrically at 550 nm (OD_{550}). The overnight culture was seeded into shake flasks containing 30 mL of fresh LB medium supplemented with or without glucose. The initial cell density was kept at 0.08 at OD_{550}, and the culture was then maintained in an orbital shaker set at 37 °C and 200 rpm. Bacterial growth was monitored and sampled for analyses at time intervals.

Protein production in bacterial strains was analyzed by 8% dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) as reported previously.[23]

Analysis by Fluorescence Microscopy and Flow Cytometry. The analysis by fluorescence microscopy and flow cytometry was carried out essentially following our previous report.[24] In brief, bacterial strain was grown and induced by adding Ara. After induction for an indicated time, bacteria were harvested, washed, and resuspended in phosphate buffer (pH 7.4). Bacterial cells were then mounted on glass slides for analysis by fluorescence microscopy (Olympus IX71, Japan). Meanwhile, 10,000 bacteria were employed for measurement of green fluorescence by BD FACSCanto (BD Bioscience, USA). Data were further analyzed by Diva software (De novo Software, USA) and FlowJo (TreeStar, USA).

RESULTS

Strategies for Improving BL21(BAD) Strain. BL21(BAD) strain was previously constructed with genomic insertion of the T7 gene 1 under regulation of the *araBAD* promoter.[10] In this work, the Ara-regulated T7 expression system based on this strain was improved by the strategy outlined in Figure 1. First, BL21(BAD) strain was made incapable of metabolizing Ara. Constant metabolism of Ara in bacterial cells would lower the inducer concentration over time such that the inducibility of Ara could be progressively lost.[13] Therefore, the *araBAD* operon responsible for Ara metabolism was deleted as described. Engineered strains were further examined for their phenotype by growing on MacConkey plus 1% Ara plates. One resulting strain that gave no color change on this selective plate was kept for the remaining experiment.

It is well recognized that the *araBAD* promoter is regulated through catabolite repression.[25] This renders the Ara-based T7 expression system nonfunctional in the presence of glucose. Glucose is transported into *E. coli* by the glucose-specific permease $EIICB^{glc}$ (encoded by *ptsG*). In a *ptsG*-null mutant, the expression of *lacZ* in the *lac* operon, known to be regulated by catabolite repression, is induced regardless of glucose.[26] This result indicates the loss of the glucose-mediated control circuit. To circumvent this control loop, *ptsG* of BL21(BAD) strain

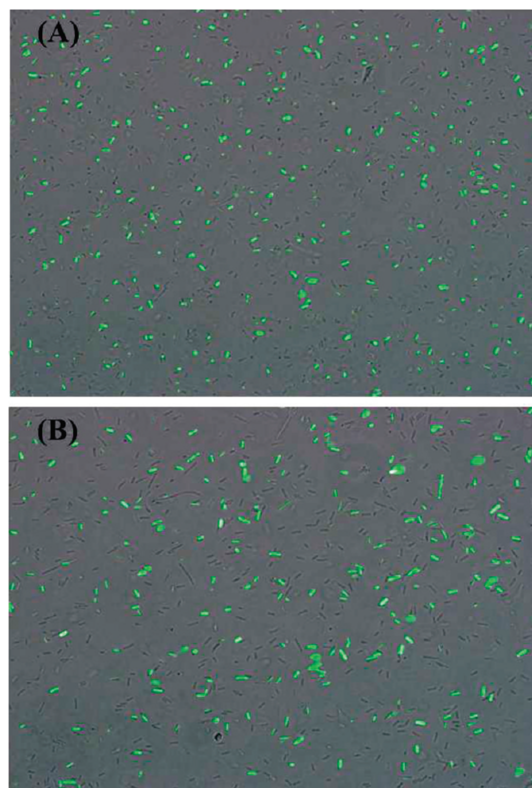

Figure 3. Analysis of GFP(LAA) expression in BAD-5 strain by fluorescence microscopy. In a similar manner, BAD-5 strain with plasmid pTH18-GFPm was cultivated and induced with 30 μM Ara for 2 h. Bacterial cultures were processed and analyzed by fluorescence microscopy. Photomicrographs were taken for bacterial cells grown on the medium without (A) and with glucose (B).

deficient in Ara metabolism was then removed as described. One *ptsG*-lacking strain, designated BAD-1G, exhibited a phenotype of slower growth relative to its parent strain.[27]

The performance of the constructed strain was further investigated for expression of the fast degrader GFP(LAA). Due to its very short half-life (e.g., 23 min), the GFP(LAA) level in the cell closely reflects the expression rate instead of the apparent accumulation rate.[13] Accordingly, plasmid pTH18-GFPm of low copy number was constructed to carry the GFP(LAA) gene under control of the T7 promoter. After transformation with plasmid pTH18-GFPm, both BL21(BAD) and BAD-1G strain were cultivated in shake flasks containing LB with or without glucose. As analyzed with fluorescence microscopy, very little BL21(BAD) strain was visually bright whereas 40% or so of BAD-1G strain remained bright when glucose was absent (Figures 2A and B). In the presence of glucose, brilliant BL21(BAD) strain was not observed (Figure 2C) and the fraction of BAD-1G strain staying bright was greatly decreased (Figures 2D).

Enhanced Expression of Genomic *araE*. As illustrated above, the Ara-regulated T7 expression system in BAD-1G strain is subjected to the glucose-mediated regulation. The result also showed the cell-to-cell variation in gene expression. This phenomenon is known as autocatalytic induction that occurs in the case where the inducer transporter is regulated by the inducer itself.[12] One effective method to abolish the autocatalytic mechanism was reported to decouple the inducer transport-induction loop.[28] Therefore, the *araFGH* operon encoding for

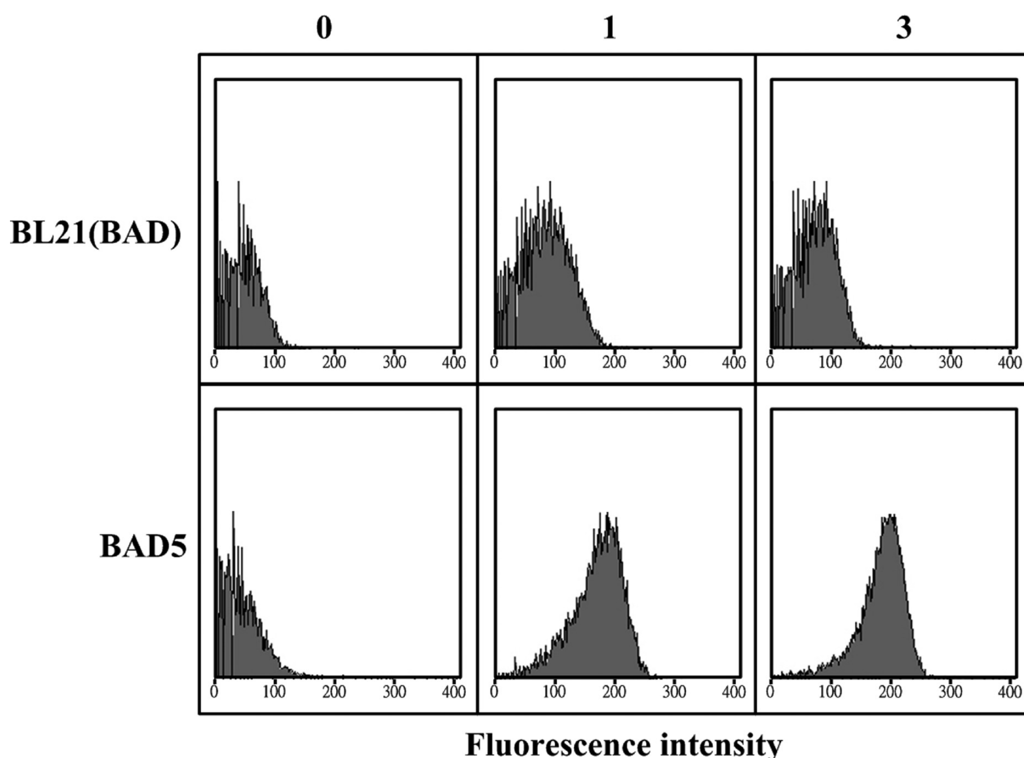

Figure 4. Uniformity of *E. coli* strain expressing GFP(LAA). Plasmid pTH18-GFPm-harboring BL21(BAD) and BAD-5 strain were grown on LB medium with 0.4% glucose and induced with 30 μM Ara. Bacterial cultures were sampled before and after induction and processed for further analyses by flow cytometry. The induction time in hours is denoted above each panel. Time zero indicates the sampling taken immediately before the addition of inducer.

the high-affinity Ara transporter in BAD-1G strain was eliminated as described. In addition, the expression of the low-affinity Ara transporter was constitutively enhanced by *in situ* fusion of the EM7 promoter with genomic *araE* (Figure 1). One resulting strain, designated BAD-5, was obtained and transformed with plasmid pTH18-GFPm for expression of GFP(LAA). The result showed that 80% of BAD-5 strain was visually bright irrespective of glucose (Figure 3A,B).

A previous study illustrated the use of a mutant lactose transporter (*lacY* A177C) to eliminate the autocatalytic regulation.[13] We were also interested to learn the feasibility of this method. BAD-5 strain was then made *araE*-deficient, thus giving BAD-5E strain. Meanwhile, plasmid pSF1-Y177 of high copy number was constructed for constitutive expression of the mutant *lacY* A177C. After transformation with plasmid pSF1-Y177 and pTH18-GFPm, the resulting strain was grown on medium with or without glucose and induced for production of GFP(LAA) in a similar fashion. Consequently, the proportion of BAD-5E strain exhibiting visual brightness was found roughly comparable to that of BAD-5 strain (Supplementary Figure S2 in the Supporting Information).

Furthermore, the analysis by flow cytometry was carried out to examine the homogeneity of induced gene expression in BAD-5 strain grown on glucose-containing medium. As shown in Figure 4, the parent strain, BL21(BAD), expressed very low expression level of GFP as expected. In sharp contrast, BAD-5 strain exhibited a uniform distribution of GFP expression along the time course.

Full Induction of Proteins at a Subsaturating Level of Ara. It is our ultimate goal to overproduce recombinant proteins in an effective way. Therefore, the usefulness of BAD-5 strain was investigated by expression of Rluc. As described, the DNA containing Rluc gene fused with the T7 promoter was inserted into the genome of BL21(BAD) and BAD-5 strain at the phage HK022 attachment locus, consequently producing BAD-L and BAD-5L strain, respectively. Shake-flask cultures of the resulting strains were first carried out in LB medium and induced for Rluc production with various concentrations of Ara upon reaching 0.3 at OD_{550}. As analyzed by SDS−PAGE (Figures 5A and 5B), the protein level was readily saturated for BAD-5L strain induced with 20 μM Ara. In contrast, the production of Rluc in BAD-L strain increased with the Ara concentration. At 100 μM Ara, Rluc produced in BAD-L strain was only half of the saturated level obtained in BAD-5L strain.

Moreover, Rluc production was performed with the two recombinant strains grown on LB medium plus 0.4% glucose. As shown in Figure 5C, the protein production was hardly detected in induced BAD-L strain whereas a saturated level of Rluc could be obtained for BAD-5L strain at 20 μM Ara.

DISCUSSION

The Ara-controlled T7 expression system in BL21(BAD) strain is mainly based on the regulated expression of T7 RNA polymerase by the *araBAD* promoter. This system has several advantages, including stringent regulation, easy modulation, high-level expression, nontoxic inducers, and being scaleable.[10,11] In particular, tight repression of this system could be easily achieved by supplement of glucose. However, the dilemma arises that glucose remains the most favorable carbon source for industrial fermentation in terms of cost and of energy generation

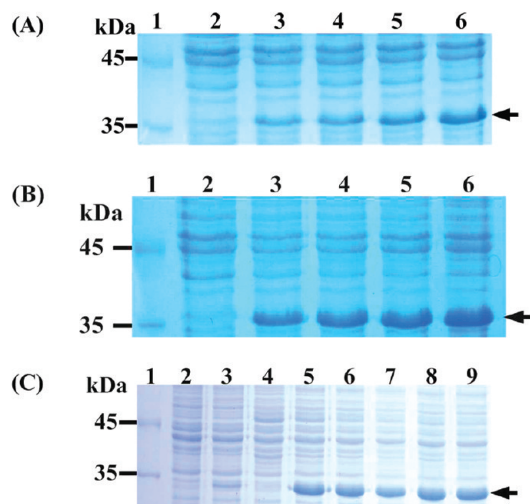

Figure 5. Analysis of Rluc production in *E. coli* strains by SDS−PAGE. Shake-flask cultures of BAD-L and BAD-5L strain were carried out in LB medium with (0.4%) or without glucose. After induction for 6 h, bacterial cultures were harvested and processed for SDS−PAGE analyses. (A) BAD-L strain grown on the glucose-lacking medium. Key: lane 1, protein marker; lane 2, without induction; lane 3, induction with 20 μM Ara; lane 4, induction with 30 μM Ara; lane 5, induction with 50 μM Ara; lane 6, induction with 100 μM Ara. (B) BAD-5L strain grown on the glucose-lacking medium. Key: lane 1, protein marker; lane 2, without induction; lane 3, induction with 20 μM Ara; lane 4, induction with 30 μM Ara; lane 5, induction with 50 μM Ara; lane 6, induction with 100 μM Ara. (C) BAD-L and BAD-5L strain grown on the glucose-containing medium. Key: lane 1, protein marker; lane 2, BAD-L strain without induction; lane 3, BAD-L strain induced with 1 mM Ara; lane 4, BAD-5L strain without induction; lane 5, BAD-5L strain inducted with 20 μM Ara; lane 6, BAD-5L strain inducted with 30 μM Ara; lane 7, BAD-5L strain inducted with 50 μM Ara; lane 8, BAD-5L strain inducted with 70 μM Ara; lane 9, BAD-5L strain inducted with 100 μM Ara. The position of Rluc is indicated by an arrow.

in *E. coli*. To be more practical, this system was modified to improve its effectiveness. This was first approached by deleting *araBAD* and *ptsG* of BL21(BAD) strain to give BAD-1G strain (Table 1). As an illustration, expression of GFP(LAA) in the producer strains was carried out at a subsaturating level of Ara (30 μM). The result showed that much more BAD-1G strain was visually bright than BL21(BAD) strain (Figure 2A,B). Note that GFP(LAA) has a very short half-life and the effect of protein accumulation rate can be minimized. It is expected that Ara catabolism in BL21(BAD) strain depletes the inducer concentration such that the protein degradation rate overrides its synthesis rate. In contrast, the inducer remains intact in BAD-1G strain which lacks Ara metabolism and the protein synthesis prevails. Therefore, the sharp discrepancy in induction level could be attributed to progressive loss of Ara induction in BL21(BAD) strain.

In the presence of glucose, expression of GFP(LAA) in BL21(BAD) strain was repressed (Figure 2C). Unlike BL21-(BAD) strain, BAD-1G strain still enabled to emit fluorescence (Figure 2D). There are two main Ara uptake systems in *E. coli*, including the high affinity AraFGH transporter and the low affinity AraE transporter.[25] The Ara metabolism-related genes encoding the Ara transporters and Ara catabolism (e.g., *araBAD*) are regulated through AraC (Figure 1). Upon binding to Ara, AraC acts like a positive regulator (Ara-AraC). Transcription of these Ara metabolism-related genes is then fully activated by the cooperation of Ara-AraC with the transcriptional activator, the cAMP−CAP complex.[25] Glucose is the most preferable sugar and enters *E. coli* with the aid of $EIICB^{glc}$ in the PTS, recognized as glucose-PTS. When glucose is taken up, $EIIA^{glc}$ in glucose-PTS largely exists in the dephosphorylated form. Induced synthesis of cAMP is negated by dephosphorylated $EIIA^{glc}$, thereby disabling the function of CAP. As a result, the transcription of genes whose expression requires cAMP−CAP is repressed, a regulation mechanism known as catabolite repression.[29] It has been reported that inactivation of $EIICB^{glc}$ or a null *ptsG* allele could lead to the interruption of catabolite repression.[30,31] Therefore, the result showing production of fluorescence in *ptsG*-deficient BAD-1G strain in the presence of glucose is consistent with the previous reports. However, the amount of brilliant BAD-1G strain within the cell population greatly reduced in the presence of glucose (Figure 2D) and the underling mechanism is unclear.

Apparently, the result illustrated by BAD-1G strain suggests the heterogeneous induction nature of this system. This type of carbohydrate-responsive system has long been known to suffer from the all-or-none induction phenomenon.[12] To improve the system, BAD-1G strain was deprived of the high affinity AraFGH transporter. Moreover, expression of genomic *araE* in the strain was enhanced and independently regulated by the EM7 promoter (Figure 1). This strong promoter consists of the consensus sequence at -10 and -35 region and permits high and constitutive expression of the regulated gene.[19] It turned out that the resulting BAD-5 strain displayed a homogeneous induction of GFP(LAA) expression at all times, and the population homogeneity was not influenced by glucose (Figure 4). One previous study reported that AraE could cause the problem of cell-to-cell heterogeneity, which could be solved using LacY A177C mutant transporter.[13] Like AraE, LacY A177C permease is a cation/solute symporter that couples the transport of Ara with that of H^{+}.[32] However, why LacY A177C functions better than AraE is not manifest in that report. In their study, plasmid-borne GFP(LAA) and *araE* were under control of the *araBAD* promoter and *tac* promoter, respectively. According to their report, a detectable level of GFP(LAA) could be monitored only at a high Ara level (e.g., 150 μM or above). Note that induced expression of the *tac* promoter-regulated *araE* requires IPTG. As reported previously, IPTG exhibited a cross-inhibition effect on expression of the *araBAD* promoter.[33] Accordingly, IPTG might interfere with the expression level of the araBAD promoter-driven GFP(LAA). This perhaps leads to the protein degradation rate exceeding its expression rate in their system. In contrast, the present work showed that expression of the T7 promoter-driven GFP(LAA) was measurable at a low Ara level (e.g., 30 μM). The discrepancy in the expression efficiency of promoter systems is likely to explain the disagreement between the previous study and ours. Nevertheless, both AraE and LacY A177C worked equally well in our system.

Finally, the usefulness of improved BAD-5 strain was illustrated for production of recombinant Rluc. Regardless of glucose, the saturated level of soluble Rluc could be readily achieved in BAD-5 strain at a subsaturating concentration of Ara (e.g., 20 μM). Without addition of inducer, Rluc production was barely detectable (Figures 5B and 5C). In marked contrast, Rluc produced in parent BL21(BAD) strain was in an Ara dose-dependent manner and largely negated by glucose. Ara of mM level is usually required to obtain the saturated production

of recombinant proteins in BL21(BAD) strain.[10] One added advantage of BAD-5 strain deficient in *ptsG* is to retard the glucose uptake rate. This merit has been utilized for increasing production of recombinant proteins due to less excreted synthesis of acetate.[34,35] In conclusion, the Ara-based T7 expression system was improved to circumvent the interference of glucose and to achieve homologous and efficient expression of recombinant proteins with Ara at a μM level. The usefulness of this improved system would be acknowledged for overproduction of recombinant proteins in an efficient, tightly regulated, and economic way.

ASSOCIATED CONTENT

Supporting Information. Supplementary Figures S1, illustrating the deletion of gene operons and replacement of genomic gene promoter with EM7 promoter, and S2, depicting the analysis of GFP(LAA) expression in BAD-5E strain by fluorescence microscopy. This material is available free of charge via the Internet at http://pubs.acs.org.

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