ACCELERATED PUBLICATION

A Tightly Regulated Inducible Expression System Utilizing the *fim* Inversion Recombination Switch

Timothy S. Ham,[1] Sung Kuk Lee,[2] Jay D. Keasling,[1,2,3] Adam P. Arkin[1,3]

[1]*Department of Bioengineering, University of California, Berkeley, California 94720*
[2]*Department of Chemical Engineering, University of California, Berkeley, California, 94720; telephone 510-495-2620; fax: 510-495-2630; e-mail: keasling@berkeley.edu*
[3]*Physical Biosciences Division, Lawrence Berkeley National Laboratory, Berkeley, California 94720*

Received 21 December 2005; accepted 2 March 2006

Published online 13 March 2006 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/bit.20916

Abstract: The *fim* inversion system of *Escherichia coli* (*E. coli*) can behave as a unidirectional switch in an efficient manner. We have developed a new expression system for *E. coli*, comprising the arabinose-inducible *fimE* gene and the *fim* invertible DNA segment containing a constitutively active promoter. In this system, the target gene is cloned with the promoter in the OFF orientation, resulting in no transcribed product. When induced by arabinose, the active promoter is switched to the ON orientation via FimE-catalyzed DNA inversion, and the gene is expressed. Our expression system exhibited very tightly controlled basal expression and high induced expression, with simple induction by inexpensive arabinose. These characteristics make our system suitable for large-scale expression or for production of toxic proteins.

Keywords: *araBAD* promoter; *E. coli*; expression vector; *fim* inversion; genetic switch; GFP expression

INTRODUCTION

Tightly regulated, inducible, gene expression systems are important tools for molecular biology, since controlling gene expression in cells is essential for pathway investigation and manipulation. Thus, numerous inducible expression systems have been developed to express a desired gene in a switch-like fashion, such as the IPTG-induced *trc* promoter (P*trc*) (Amann et al., 1988) and the arabinose-induced *araBAD* promoter (P_{BAD}) systems (Guzman et al., 1995). However, their non-induced basal expression can be fairly significant, limiting their use in complementation studies or in propagation of toxic genes. Leaky expression can be mitigated by altering the ribosome binding site of the target gene (Guzman et al., 1995) but this often results in a reduced induced expression level as well.

Correspondence to: J.D. Keasling
Contract grant sponsor: National Institute of Health
Contract grant number: 5R01GM063525-03

Podhajska et al. (1985) developed an inversion-based expression system using the phage λ Int and the *att*P/*att*B recombination. By decoupling the induction mechanism from the expression promoter, the Int/*att* system was able to overcome leaky expression, showing both tight control in the un-induced state and high induction level when induced (Sektas et al., 2001). However, the Int/*att* system requires not only a specialized host containing an inducible *int*, but also a heat-shock-based induction method, which could make this system undesirable for certain applications.

The invertible promoter system responsible for the phase variation of type 1 fimbriae in *Escherichia coli* (*E. coli*) enables a simpler method of constructing a tightly controlled, decoupled expression system. This phase variation is due to the inversion of a 314-base pair (bp) DNA segment containing the promoter of *fimA* by two invertases, FimB and FimE (Klemm, 1986). FimB is able to invert the DNA segment in both directions, but FimE inverts overwhelmingly from the phase "on" to the phase "off" orientation (Blomfield et al., 1991; review in Blomfield, 2001). By utilizing this unidirectional switching by FimE, we have constructed an expression system that is strictly regulated and strongly induced, without the need for specialized hosts or complex induction methods.

MATERIALS AND METHODS

Bacteria Strains and Culture Medium

The *fim* operon from *E. coli* MG1655 [F^- λ^- *ilvG*$^-$ *rfb*$^-$50 *rph*$^-$] was used in plasmid constructions. *E. coli* DH10B [F^- *mcrA* Δ(*mrr*-hsdRMS mcrBC) Φ80d*lacZ*ΔM15 Δ*lacX74 deoR recA1 ara* Δ*139* Δ(*ara leu*)*7697 galU galK* λ^- *rpsL endA1 nupG* Str^r] (Life Technologies, Rockville, MD) was used as the host for all plasmid constructions. *E. coli* BLR(DE3) [F^- *ompT* $hsdS_B(r_B^- m_B^-)$ *gal dcm* Δ(*srl*-recA)*306*::Tn*10*(DE3)] (Novagen, Madison, WI) was used

in expression assays. Cells were grown in Luria-Bertani (LB) broth or on LB agar plates at 37°C. The following antibiotics were used at the concentrations indicated: ampicillin, 100 μg/mL; chloramphenicol, 30 μg/mL; kanamycin, 50 μg/mL. The LB medium was supplemented with 0.5% (w/v) glucose in order to repress sporadic expression of P_{BAD} during construction of the strains.

Construction of the Expression Plasmid

All DNA manipulations were performed using established protocols (Sambrook et al., 1989). The *fimB* and *fimE* were PCR-amplified and cloned into the *Kpn*I/*Xba*I-digested pBAD30 vector, creating pTSH30 and pTSH32, respectively. The *fim* invertible region in its native phase ''off'' (IRL) orientation was PCR-amplified and cloned into the *Bam*HI/*Eco*RI-digested pPROBE-NT (Miller et al., 2000), resulting in pTSH14. The invertible region in the non-native (IRR) orientation was made by expressing FimB in the same host, and this version of invertible region was used in the construction of the expression vector. The −10 and −35 regions of the promoter within the invertible region were replaced with those of the *trc* promoter without the *lac* repressor binding site, creating pTSH29. Then the invertible region and the upstream T1 terminators of pPROBE were PCR-amplified and cloned into pBAD18 containing *fimE* to yield pFIP (Fim Inversion Promoter) (Fig. 1A). pTrc99A-*gfp* (Lee and Keasling, 2005) was used as a control plasmid.

Determination of In Vivo Promoter Activities

Cells grown overnight in LB medium containing ampicillin (100 μg/mL) and 0.5% (w/v) glucose were subcultured (1:100) into fresh LB medium (5 mL in culture tube and 20 mL in shake flask) containing ampicillin (100 μg/mL) and grown until OD_{600} of around 0.4. Once the desired OD_{600} was reached, the cells were induced with arabinose at 37°C in a Tecan SpectraFluor Plus plate reader (Tecan-US, Durham, NC) or in a shaking incubator. Further kinetic measurements were performed in the Tecan plate reader. A pulse induction experiment was performed by removing the inducer 1 h, 2 h, and 4 h after induction. Cells growing in shaking flasks were harvested by centrifugation and re-suspended in medium lacking arabinose.

RESULTS AND DISCUSSION

Fim Inversion Promoter (FIP) Expression System

The Fim invertases bind to the two non-identical inverted repeat regions surrounding the invertible DNA segment. For the sake of brevity and clarity, we will use the following acronyms: when the promoter within the invertible region is oriented towards inverted repeat left (IRL), we will call this orientation ''Promoter Facing inverted repeat Left'' (PFL), and the opposite orientation ''Promoter Facing inverted repeat Right'' (PFR).

In the native *fim* system, the PFL orientation is the ''off'' (non-expressing) position for the promoter of *fimA*. However, since FimE only performs the inversion from the PFR to PFL orientation, but not vice versa, the FIP system was constructed to have the promoter in the PFR orientation as the ''off'' position. The target gene to be expressed was cloned downstream of IRL (Fig. 1B). Because the constitutive promoter is transcribing in the opposite direction of the cloned gene, no transcript of the gene can result from the promoter. When FimE was expressed by the addition of arabinose, the promoter was inverted in a unidirectional manner to the ''on'' (PFL) orientation, beginning transcription of the target gene (in this case, *gfp*). It was found that the native ribosome binding site (RBS) for *fimE* was susceptible to sporadic, uninduced FimE expression by the leaky P_{BAD}, even in the

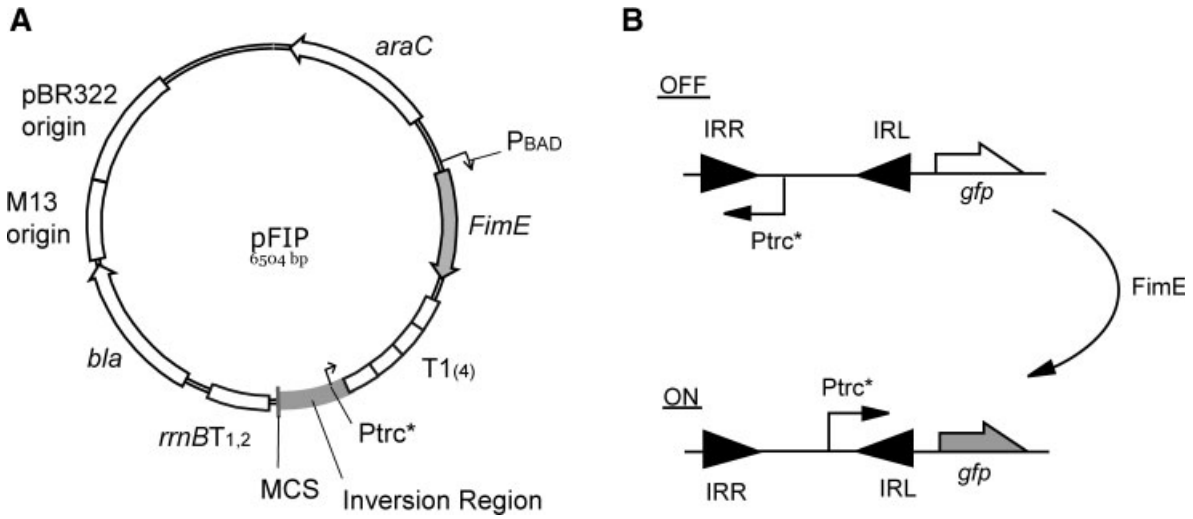

Figure 1. **A**: Map of pFIP vector. **B**: Diagram of pFIP inversion region with *gfp* cloned in the MCS. FimE expression is induced by arabinose, and inverts the promoter switch only in one direction, from facing IRR to IRL. The *trc* promoter used in FIP lacks the *lac* repressor binding site, making it constitutive.

DOI 10.1002/bit

presence of glucose. Because only a low level of FimE is necessary for the rapid inversion reaction, its expression level was optimized by using a weak ribosome binding site (AGGGACAGGAT instead of AGGGAAAAACG) as suggested by Barrick et al. (1994). The weaker RBS was able to eliminate the sporadic expression, but had no effect on inversion when induced (data not shown).

The induction of the FIP system is initiated by expression of FimE via P_{BAD}. Since FimE is required only for initial inversion, but not for subsequent gene expression, the target gene may be expressed without the persistent presence of arabinose. Use of a pulse of inducer could be cost effective in protein production in continuous cultures. Also, we were able to use the pFIP vector in common laboratory strains (*E. coli* DH10B, BL21, and BLR(DE3); data not shown) even though they were not specified as *fim*$^-$ in their genotype. We suspect that they lack functional FimB and FimE.

Expression Characteristics

The green fluorescent protein (GFP) has been utilized previously to analyze the promoter strength in a quantitative fashion (Albano et al., 1998). In this study, the FIP expression system was compared with the well-characterized *trc* promoter by measuring the relative GFP expression levels from pFIP-*gfp* and pTrc99A-*gfp*. Maximal levels of GFP expression were observed upon addition of 60–100 μM of IPTG for pTrc99A-*gfp* and 0.1–5 mM arabinose for pFIP-*gfp* in a variety of strains (data not shown).

Under maximal induction, GFP expression from pFIP-*gfp* was comparable to that of pTrc99A-*gfp* (Fig. 2). Since the FIP system must express FimE before GFP can be expressed, some delay in GFP production was observed. The lower maximal induction level of the FIP system, compared to P*trc*, is due possibly to the presence of additional DNA sequences (IRL) between the constitutive promoter and *gfp*. Previous studies have shown that the sequences between the promoter and the RBS could form mRNA secondary structures that could adversely affect expression (de Smit and van Duin, 1994; Pfleger et al., 2005). If either a weaker or a stronger expression is desired, the expression level could be optimized by cloning the target gene with different RBS's (Barrick et al., 1994).

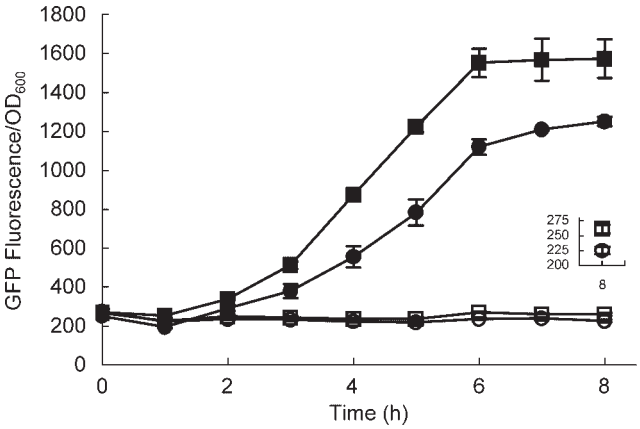

Figure 2. Comparison of maximal and un-induced expression levels between the pFIP-*gfp* and pTrc99A-*gfp* in *E. coli* strain BLR (DE3) in triplicate shake-flask cultures. pTrc99A-*gfp* was induced with 100 μM IPTG. pFIP-*gfp* was induced with 5 mM arabinose. pTrc99A-*gfp* induced (■), un-induced (□); pFIP-*gfp* induced (●), un-induced (○). The cultures were induced at time zero.

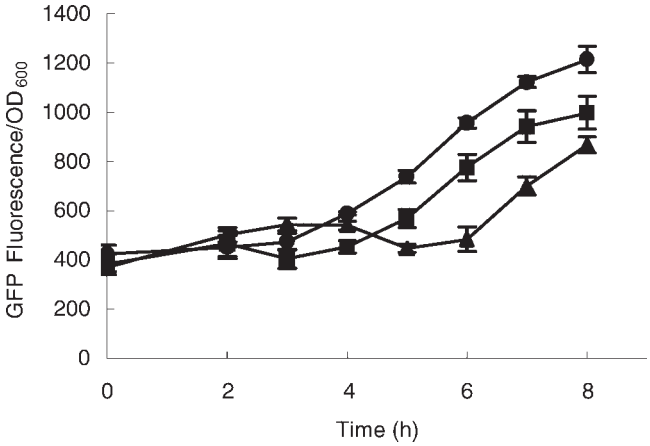

Figure 3. Pulse induction of *E. coli* strain BLR (DE3) containing pFIP-*gfp* in shake-flask cultures. The cultures were induced with 5 mM arabinose at time zero when the OD_{600} reached about 0.4. The inducing medium was removed by centrifugation, and the cells were re-suspended in fresh medium lacking arabinose after induction of 1 h (●), 2 h (■), and 4 h (▲). Error bars show the standard deviation of experiments performed in triplicate.

A pulse induction of the FIP system showed that the duration of induction did not affect expression (Fig. 3). After adaptation to fresh LB medium and a recovery period (approximately 2 h), all cultures expressed normally regardless of the induction interval.

In order to determine the rate of un-induced inversion from the FIP system, the plasmids of cultures grown overnight in LB medium without glucose were isolated and re-transformed. Because the plasmids can only invert unidirectionally, any inverted plasmid would have resulted in a fluorescent colony. No fluorescent colonies were observed among approximately 3,000 colonies (data not shown). Also, no un-induced expression was observed in BLR(DE3) containing the pFIP-*gfp*, unlike BLR(DE3) containing the pTrc99A-*gfp*, which showed leaky GFP expression even in un-induced conditions (Fig. 2 and inset).

In summary, we have developed a new inversion-based expression system that is tightly regulated, and strongly expressed. Additionally, the FIP system can be expressed by a brief exposure to arabinose, after which no further inducer is necessary. The minimal host requirements and the use of a simple-to-use, inexpensive inducer could make the FIP system attractive for complementation studies, or for large-scale protein production.

References

Albano CR, Randers-Eichhorn L, Bentley WE, Rao G. 1998. Green fluorescent protein as a real time quantitative reporter of heterologous protein production. Biotechnol Prog 14:351–354.

Amann E, Ochs B, Abel KJ. 1988. Tightly regulated tac promoter vectors useful for the expression of unfused and fused proteins in *Escherichia coli*. Gene 69:301–315.

Barrick D, Villanueba K, Childs J, Kalil R, Schneider TD, Lawrence CE, Gold L, Stormo D. 1994. Quantitative analysis of ribosome binding sites in *E. coli*. Nucleic Acids Res 22:1287–1295.

Blomfield IC. 2001. The regulation of pap and type 1 fimbriation in *Escherichia coli*. Adv Microb Physiol 45:1–49.

Blomfield IC, McClain MS, Princ JA, Calie PJ, Eisenstein BI. 1991. Type 1 fimbriation and *fimE* mutants of *Escherichia coli* K-12. J Bacteriol 173: 5298–5307.

de Smit MH, van Duin J. 1994. Translational initiation on structured messengers. Another role for the Shine-Dalgarno interaction. J Mol Biol 235:173–184.

Guzman LM, Belin D, Carson MJ, Beckwith J. 1995. Tight regulation, modulation, and high-level expression by vectors containing the arabinose P_{BAD} promoter. J Bacteriol 177:4121–4130.

Klemm P. 1986. Two regulatory *fim* genes, *fimB* and *fimE*, control the phase variation of type 1 fimbriae in *Escherichia coli*. EMBO J 5:1389–1393.

Lee SK, Keasling JD. 2005. A propionate-inducible expression system for enteric bacteria. Appl Environ Microbiol 71:6856–6862.

Miller WG, Leveau JH, Lindow SE. 2000. Improved *gfp* and *inaZ* broad-host-range promoter-probe vectors. Mol Plant Microbe Interact 13: 1243–1250.

Pfleger BF, Fawzi NJ, Keasling JD. 2005. Optimization of DsRed production in *Escherichia coli*: Effect of ribosome binding site sequestration on translation efficiency. Biotechnol Bioeng 92:553–558.

Podhajska AJ, Hasan N, Szybalski W. 1985. Control of cloned gene expression by promoter inversion *in vivo*: Construction of the heat-pulse-activated *att*-nutL-p-att-N module. Gene 40:163–168.

Sambrook J, Fritsch EF, Maniatis T. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory.

Sektas M, Hasan N, Szybalski W. 2001. Expression plasmid with a very tight two-step control: Int/*att*-mediated gene inversion with respect to the stationary promoter. Gene 267:213–220.

DOI 10.1002/bit