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Iron Chelator-Inducible Expression System for *Escherichia coli*

Lim, Jae-Myung[1], Mi-Ju Hong[1], Seonghun Kim[1†], Doo-Byoung Oh[1], Hyun Ah Kang[2], and Ohsuk Kwon[1]*

[1]*Omics and Integration Research Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon 305-333, Korea*
[2]*Department of Life Science, College of Natural Science, Chung-Ang University, Seoul 156-756, Korea*

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The P_{entC} promoter of the *entCEBA* operon encoding enzymes for enterobactin biosynthesis in *Escherichia coli* is tightly regulated by the availability of iron in the culture medium. In iron-rich conditions, the P_{entC} promoter activity is strongly repressed by the global transcription regulator Fur (ferric uptake regulator), which complexes with ferrous ions and binds to the Fur box 19-bp inverted repeat. In this study, we have constructed the expression vector pOS2 containing the P_{entC} promoter and characterized its repression, induction, and modulation by quantifying the expression of the *lacZ* reporter gene encoding β-galactosidase. β-Galactosidase activities of *E. coli* transformants harboring pOS2-*lacZ* were highly induced in the presence of divalent metal ion chelators such as 2,2'-dipyridyl and EDTA, and were strongly repressed in the presence of excess iron. It was also shown that the basal level β-galactosidase expression by the P_{entC} promoter was drastically decreased by incorporating the *fur* gene into the expression vector. Since the newly developed iron chelator-inducible expression system is efficient and cost-effective, it has wide applications in recombinant protein production.

Keywords: *E. coli*, iron chelator, Fur, expression system

The Gram-negative bacterium *Escherichia coli*, perhaps the best characterized organism for genetic and molecular biology studies, grows rapidly and to high densities on relatively inexpensive substrates. Furthermore, recent developments in genomics and functional genomics have begun to provide the more fundamental knowledge essential for the understanding and engineering of this organism as a cell factory. These advantages, combined with the numerous commercially available expression vectors, make *E. coli* the preferred host for production of heterologous proteins [4, 21]. However, in spite of these many advantages, not every recombinant protein can be expressed efficiently in *E. coli*. The expression system for each recombinant protein must be chosen based on multiple factors including desired expression level, toxicity to host, cellular localization, and biological activity.

One of the most critical features in selecting an expression system is the choice of promoter used to induce target protein expression [14]. In general, the promoter must be strong enough to allow accumulation of the target protein (up to 10–30% of the total cellular protein); should be highly repressible to minimize unwanted background level expression (especially critical in production of recombinant proteins detrimental to the host); and, finally, should be inducible in a simple and cost-effective manner appropriate for industrial large-scale production.

Currently, the widely used promoters in *E. coli* are the *lac* promoter of the lactose utilization operon and its derivatives, such as *tac* [8] and *trc* [7]. The *lac*-derived promoters can be effectively induced by isopropyl-β-D-thiogalactopyranoside (IPTG) [1], making IPTG induction an extremely valuable tool for achieving high levels of recombinant protein expression in laboratory-scale applications [10]. However, the use of IPTG for large-scale production is limited since IPTG is expensive, not digestible, and toxic [12, 21]. Thus, many alternative expression systems have been developed for *E. coli* by employing a variety of inducible promoters including the arabinose-inducible promoter P_{BAD} [13], the tetracycline-inducible promoter P_{tet} [9], the alkaline-inducible promoter P_{alkB} [26], the phosphate-regulated promoter P_{ugp} [27], and the propionate-inducible promoter P_{prpB} [19].

In *E. coli*, the *entCEBA* operon encodes enzymes for the biosynthesis of enterobactin, the cyclic trimester of 2,3-dihydroxybenzoylserine [22]. The P_{entC} promoter of this operon is known to be tightly regulated by iron availability

**Corresponding author*
Phone: 82-42-860-4459; Fax: 82-42-860-4594;
E-mail: oskwon@kribb.re.kr

†Present address: Institute of Microbiology, Department of Biology, Eidgenössische Technische Hochschule (ETH) Zurich, 8093 Zurich, Switzerland.

in the environment [5], such that it is strongly repressed in iron-rich conditions by the Fur (ferric uptake regulator) protein, but fully derepressed in iron-deficient conditions [2, 3]. In general, the Fur protein complexes with ferrous ions and binds with high affinity to the 19-bp inverted repeat consensus sequence known as the Fur box (GATAATGATAATCATTATC) [6, 15]. The Fur box is usually found in the promoter regions of iron regulated genes. Since iron availability in the medium can be readily modulated by addition of iron chelators or excess iron, we were interested in employing the iron-dependent regulatory elements for development of a new, more cost-effective, and inducible expression system.

In the present study, we have used the P_{entC} promoter to construct new expression vectors that can be repressed by excess iron and induced by iron chelators. We report here the induction, basal expression, and modulation of these vectors by using the *lacZ* reporter gene encoding β-galactosidase.

Materials and Methods

Bacterial Strains, Plasmids, and Culture Conditions

E. coli DH5α (*recA1 endA1 gyrA96 thi1 hsdR17 supE44 relA1 lacZ* ΔM15) (Clontech) and JM109 [*recA1 endA1 gyrA96 thi1 hsdR17 mcrA⁻ supE44 relA1* Δ(*lac-proAB*)] (Promega) were used as host strains for cloning and reporter expression. *E. coli* MC4100 [F⁻ *araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR*] [25] was used as the source of genomic DNA to use as a template for PCR cloning of the P_{entC} promoter of the *entCEBA* operon and the *fur* gene. Plasmid pUC18 (Stratagene) was used as the parental plasmid to construct a new expression vector. Plasmid

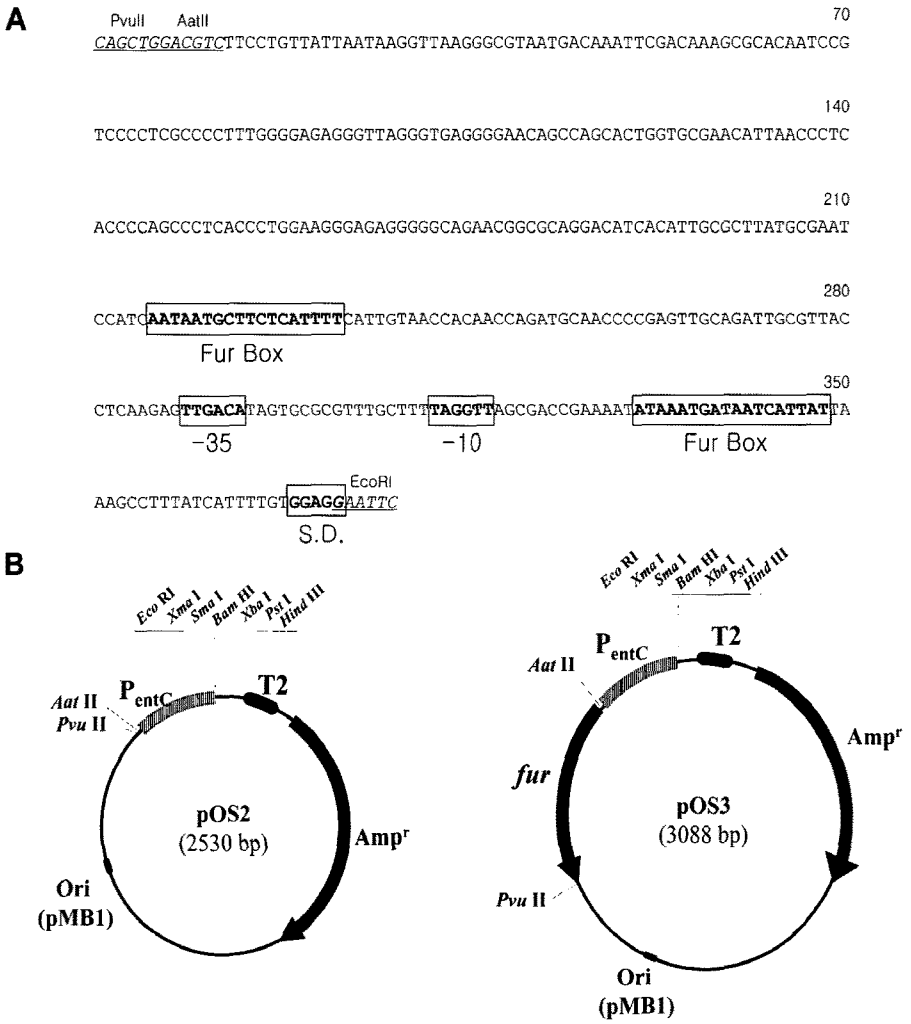

Fig. 1. Construction of expression vectors.
A. Nucleotide sequence of the P_{entC} promoter of the *entCEBA* operon. The boxes indicate the Fur box, promoter sequence (−10 and −35), and ribosome binding sites (S.D.). Restriction enzyme recognition sites were introduced into the primers for PCR amplification. **B.** Schematic diagram of expression vectors. The restriction enzyme sites in the multicloning sites are unique. pOS2 was constructed by joining DNA fragments containing the P_{entC} promoter, the MCS of pUC18 followed by *rrnBT2* terminator (T2), and PvuII-ScaI digested pUC18. The AatII restriction site was introduced during PCR amplification of the P_{entC}. pOS3 contains the *fur* gene cloned between the PvuII and AatII restriction sites of pOS2.

pEXT20 [11] was used as the source of the multicloning site and the *rrnBT2* terminator [6]. Plasmid pRS415 [23] was used as the template for PCR cloning of the *lacZ* reporter gene encoding β-galactosidase.

For routine growth of cultures, Luria-Bertani (LB) broth containing 1% (w/v) bactotryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl, or LB agar [1.5% (w/v)] was used. Cultures were stored in glycerated L broth at −80°C. When required, ampicillin was added into the medium at a concentration of 100 μg/ml. Cultures were grown in 250-ml baffled flasks under aerobic conditions at 37°C with shaking at 180 rpm, and cell growth was monitored spectrophotometrically at 600 nm.

Recombinant DNA Techniques and PCR

Chromosomal and plasmid DNAs were isolated using the AccuPrep Genomic DNA and AccuPrep Plasmid extraction kits, respectively (Bioneer, Daejeon, Korea). DNA fragments were recovered from the agarose gel using the AccuPrep gel purification kit (Bioneer). Oligonucleotides used in this study were synthesized by Bioneer. Polymerase chain reactions (PCR) were carried out using the AccuPower PCR premix and employing the MyGenie 32 Thermal block (Bioneer). PCR products were purified using the AccuPrep PCR purification kit (Bioneer). Sequence verification of PCR-amplified DNA fragments was performed by SolGent Co., Ltd. (Daejeon, Korea). Restriction enzyme digestion, agarose gel electrophoresis, ligation, and transformation were all carried out as described previously [24].

Plasmids Construction

To construct plasmid pOS2, the promoter region of the *entCEBA* operon was amplified by PCR using the primers P_{entC}-up (5'-CCGCAGCTGGACGTCTTCCTGTTATTAATAAGG-3') and P_{entC}-down (5'-CCCGAATTCCTCCACAAAATGATAAAGGC-3') with the chromosomal DNA of *E. coli* MC4100 as template. The resulting 0.37 kb DNA fragment was digested with PvuII and EcoRI (Fig. 1A). To prepare a fragment containing the multicloning site (MCS) and the *rrnBT2* terminator, plasmid pEXT20 [11] was digested with EcoRI and ScaI and the 0.61 kb DNA fragment gel-purified. DNA fragments containing the P_{entC} promoter and the MCS with *rrnBT2* were then ligated with the 1.55 kb DNA fragment prepared by PvuII-ScaI digestion of pUC18.

To construct plasmid pOS3, the *fur* gene was amplified by PCR using the primers Fur-up (5'-CCCGACGTCATGACTGATAACAA-TACC-3') and Fur-down (5'-CCGCAGCTGGCAGGAAATACGCA-GTAA-3') and using the chromosomal DNA of *E. coli* MC4100 as template. The resulting 0.57 kb PCR products were digested with AatII and PvuII and ligated with AatII-PvuII digested pOS2.

To construct reporter expression vectors, the coding region of the β-galactosidase gene was amplified by PCR using the primers lacZ-up (5'-CGGAATTCATGACCATGATTACGGA-3') and lacZ-down (5'-CGGGATCCTTATTTTTGACACCAGAC-3'), using pRS415 [23] as the template. The resulting 3.1 kb fragment was digested with EcoRI and BamHI, and inserted between the EcoRI and BamHI restriction sites of pOS2 and pOS3, yielding pOS2-*lacZ* and pOS3-*lacZ*, respectively.

β-Galactosidase Activity Analysis

For the assay of β-galactosidase, a single colony of *E. coli* JM109 transformants harboring pOS2-*lacZ* or pOS3-*lacZ* was inoculated into 5 ml of LB medium supplemented with 100 μg/ml ampicillin, and incubated at 37°C with shaking. After overnight growth, a 1% inoculum was transferred into 20 ml of fresh medium of the same composition and growth continued at 37°C with shaking. When required, various amounts of 2,2'-dipyridyl, EDTA, or $FeSO_4 \cdot 7H_2O$ were added to the culture medium. β-Galactosidase activity was assayed and the units of activity were calculated as described previously [22].

SDS-Polyacrylamide Gel Electrophoresis

The expression of β-galactosidase was monitored on SDS-12% polyacrylamide gel electrophoresis (PAGE) gels as described by Laemmli [17]. The mini PROTEAN 3 cell apparatus of Bio-Rad Laboratories was used in accordance with the instructions of the manufacturer. The Precision Plus Protein standards of Bio-Rad were used.

RESULTS

Construction and Characteristics of the Expression Vectors

In order to develop a new inducible expression system, we employed the *E. coli* P_{entC} promoter, which is strongly repressed under iron-sufficient conditions, but fully derepressed in iron-deficient conditions [16]. This new expression vector, pOS2, contains the P_{entC} promoter followed by a SD box and a multiple cloning site [MCS] (Fig. 1B). Thus, any gene cloned with translational initiation sequences into the MCS is under the P_{entC} promoter and tightly regulated by iron availability. The iron availability in turn is modulated by the addition of either iron or iron chelators such as 2,2'-dipyridyl and EDTA. Immediately downstream of the MCS, the strong *rrnBT2* transcription terminator derived from the *rrnB* rRNA operon of *E. coli* [6] was inserted to avoid read-through transcription. The plasmid also carries an origin of replication of pMB1 [28] and the selectable marker, ampicillin resistance gene, *bla*.

Since pOS2 is a derivative of a multicopy plasmid, it is possible that the level of Fur proteins expressed from the host chromosomal allele is insufficient for tight regulation of the target gene expression. Thus, we have introduced a *fur* gene into pOS2 upstream of the P_{entC} promoter and in the opposite direction under the divergent promoter P_{fcbB}, as construct pOS3.

Inducible Expression from the P_{entC} Promoter by Iron Chelators

To investigate the changes in expression patterns of the P_{entC} promoter in response to the environmental iron availability, the reporter *lacZ* gene encoding β-galactosidase was cloned into pOS2 and pOS3, yielding pOS2-*lacZ* and pOS3-*lacZ*, respectively. These recombinant vectors were then transformed into *lacZ*-deficient *E. coli* strain JM109 and transformants were cultured in LB medium and

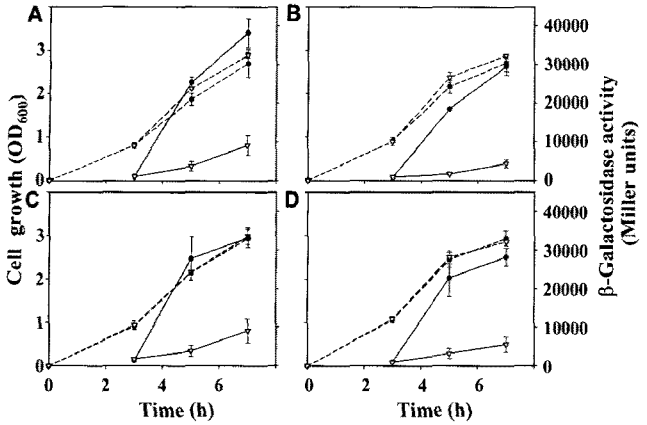

Fig. 2. Iron chelator-induced expression of the P_{entC}-controlled β-galactosidase.
E. coli JM109 transformants harboring pOS2-*lacZ* (A, C) or pOS3-*lacZ* (B, D) were cultured in LB medium for 3 h and supplemented with 200 μM 2,2'-dipyridyl (A, B) or EDTA (C, D). Samples were taken at indicated times from cultures grown in the absence (open symbol) or presence (closed symbol) of the chelator to monitor cell growth (broken line) and β-galactosidase activity (solid line). The error bars represent the standard deviation (SD) for three independent experiments, performed in duplicate.

samples were taken at various intervals to measure the expression of the P_{entC} promoter-derived β-galactosidase (Fig. 2). Interestingly, a slight increase in β-galactosidase activity was observed at the late stages of growth, even in control cultures without addition of a chelator. This result suggests that the available iron in the medium was consumed by the cells, causing a natural iron deficiency. Alternatively, it is possible that another regulatory mechanism(s) is responsible for the induction of the P_{entC} promoter activity, depending on the age of the culture. The basal β-galactosidase activities from pOS3-*lacZ* was less than half of those from pOS2-*lacZ* at all time points, suggesting that the excess Fur proteins produced from the *fur* in pOS3 allowed tighter repression of the P_{entC} promoter activity.

To evaluate the induction of the P_{entC} promoter under iron deficiency, the divalent metal ion chelator 2,2'-dipyridyl was added to a final concentration of 200 μM to exponentially growing (OD_{600}=~1.0) transformants harboring either pOS2-*lacZ* or pOS3-*lacZ*. β-Galactosidase activity was then measured following 2,2'-dipyridyl induction. We observed that the addition of dipyridyl caused a dramatic increase in β-galactosidase expression from the P_{entC} promoter without apparent effect on cell growth. Then, to test the efficiency of EDTA, a cost-effective chelator, 200 μM of EDTA was added into the culture, and β-galactosidase expression was monitored and visualized by PAGE (Fig. 3). Again, we were able to observe the dramatic increase in *lacZ* expression from the P_{entC} promoter (Fig. 2B, Fig. 3). The above results suggest that the addition of chelators to the medium creates an iron-deficient condition, as monitored by β-galactosidase induction.

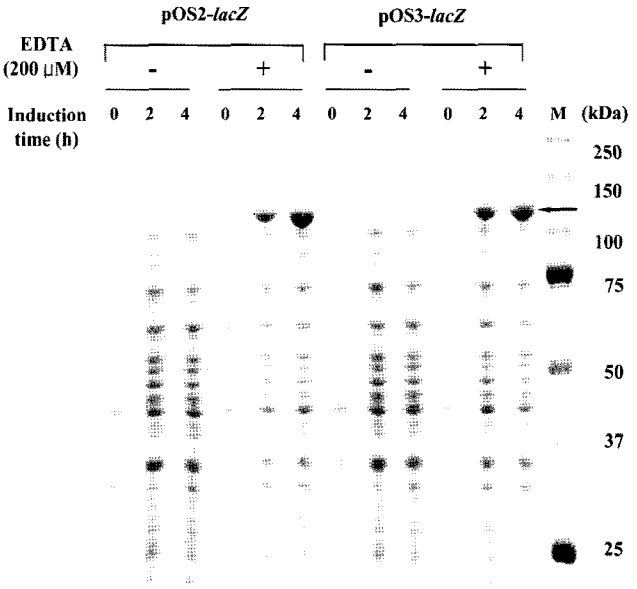

Fig. 3. SDS-PAGE analysis of the P_{entC}-controlled β-galactosidase expression.
For induction of β-galactosidase expression, *E. coli* transformants harboring pOS2-*lacZ* or pOS3-*lacZ* were incubated in LB broth for 3 h and 200 μM of EDTA was added. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12%) was performed with crude extracts of culture samples (5 μl) taken at indicated times from induced and control cultures. Proteins were visualized by Coomassie brilliant blue staining by using the protein molecular mass marker (M). The arrow indicates the band of β-galactosidase.

EDTA Concentration-Dependent Induction of P_{entC}

The ability to modulate the extent of the target gene expression by partial induction of the promoter activity by applying

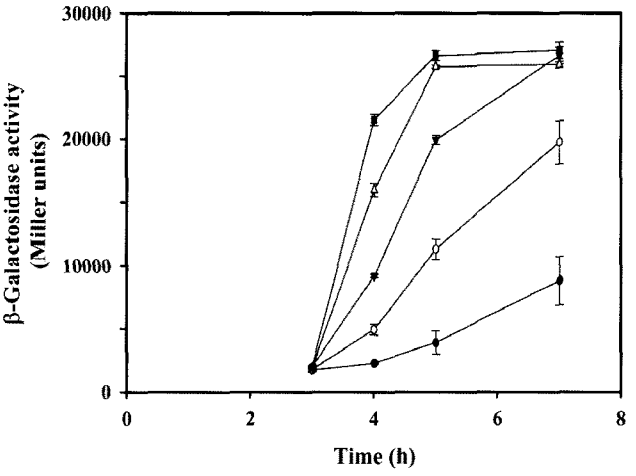

Fig. 4. Chelator concentration-dependent induction of the P_{entC}-controlled β-galactosidase expression.
E. coli JM109 transformants harboring pOS2-*lacZ* were cultured in LB medium for 3 h, and EDTA was added into the culture at final concentrations of 10 (-●-), 50 (-○-), 100 (-▼-), 200 (-△-), and 500 (-■-) mM. Samples were taken at indicated times after the addition of EDTA to measure the β-galactosidase activity. The error bars represent the standard deviation (SD) for three independent experiments, performed in duplicate.

different amounts of inducer is a desirable feature of a controllable expression system. To investigate the possibility to modulate the P_{entC}-derived target gene expression in an inducer concentration-dependent manner, different amounts of EDTA (at final concentrations of 10, 50, 100, 200, and 500 μM) were added into the exponentially growing cultures of transformants harboring pOS2-*lacZ*, and the β-galactosidase activity was measured at different times after EDTA addition (Fig. 4). β-Galactosidase activity was induced along the cell growth in all cultures treated with EDTA. However, the extent of β-galactosidase activity induction in the culture treated with 10 μM EDTA was quite similar to that of cultures without EDTA treatment, indicating that 10 μM EDTA is not enough to create an iron-deficient condition. This may be due to the high concentration of metal ions in the LB medium used in this study. The β-galactosidase activity, however, was drastically induced in cultures treated with EDTA at concentrations of above 50 μM, and the level of induction was increased depending on the EDTA concentration. These results suggest that the variation in β-galactosidase expression from the P_{entC} promoter as a function of EDTA concentration is resulted from partial derepression of the P_{entC} promoter, which was accomplished by different levels of iron chelation by EDTA in the culture medium. At EDTA concentrations up to 200 μM, the cell growth was not affected compared with that of control culture without EDTA addition. However, by treating with 500 μM EDTA, the cell growth was reduced about 30% compared with that of control condition, suggesting that a high concentration of EDTA might cause growth inhibition by chelating many divalent cations in the medium.

Repression of the Basal Level of Expression by Addition of Excess Iron

Tight regulation is an especially important feature of an expression system when recombinant proteins are toxic to the host even at low levels of expression. Under these conditions, it would be desirable to construct a system that limits target gene expression to basal levels until an inducer is applied. The EDTA-inducible expression system presented here is slightly leaky with β-galactosidase activity detected in the absence of chelators. Moreover, the basal expression level increased during cell growth, suggesting that the metal ions are consumed by growing cells. Thus, we tested whether it is possible to minimize the background level of target gene expression from the P_{entC} promoter by providing excess iron to ensure repression of the P_{entC}. Transformants harboring pOS2-*lacZ* or pOS3-*lacZ* were cultured in LB medium supplemented with different amounts of $FeSO_4$, and the β-galactosidase activity was measured after 7 h incubation. β-Galactosidase activity was strongly repressed down to less than 20% in cultures grown in LB supplemented with >5 μM iron compared with that in control cultures grown in LB without addition of iron (data not shown). The decrease in background levels of β-galactosidase activity was more dramatic in cells harboring pOS3-*lacZ* and only 10% of the activity was detected in cultures grown in iron-supplemented LB. However, the extent of repression in both pOS2-*lacZ* and pOS3-*lacZ* transformants was similar when cultured in iron-rich conditions, indicating that the iron-dependent repression was fully saturated.

Controlled Expression of the P_{entC} Promoter

The above results confirm that the P_{entC} promoter-based expression system is sensitive to iron availability in the medium and can be either strongly repressed by the addition of excess iron, or dramatically induced by addition of iron chelators. To evaluate the fine control of target gene expression, we examined the expression of β-galactosidase from transformants cultured in the presence of excess iron and subsequently induced by treatment of EDTA. As shown in Fig. 5, the basal level of β-galactosidase was strongly repressed by cultivating transformants in the presence of 5 μM $FeSO_4$, but the repression was lifted by addition of 200 μM of EDTA. Both pOS2-*lacZ* and pOS3-*lacZ* exhibited similar repression and induction responses with treatment by iron and EDTA. At 2 h after addition, β-galactosidase from pOS2-*lacZ* was induced more than 20-folds compared with the level of expression from cultures treated with 5 μM $FeSO_4$. However, the induction was more drastic for pOS3-*lacZ* and it showed 100 times higher activity than background level. Finally, at four hours post EDTA addition, β-galactosidase activity was

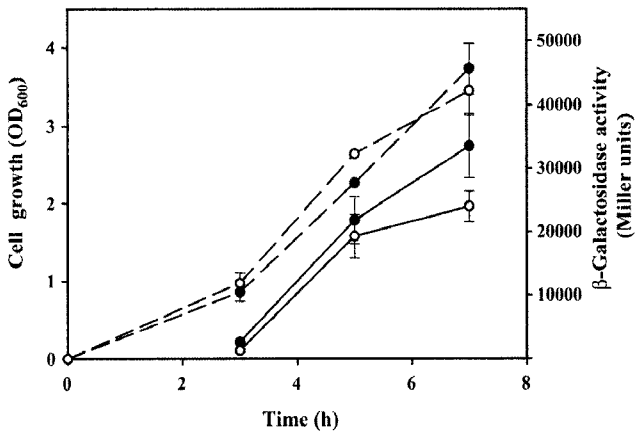

Fig. 5. Fine control of the P_{entC}-derived β-galactosidase expression. *E. coli* JM109 transformants harboring pOS2-*lacZ* (closed circle) or pOS3-*lacZ* (open circle) were cultured for 3 h in LB medium supplemented with 5 μM $FeSO_4$ and then 200 μM EDTA was added. Samples were taken at indicated times to measure cell growth (broken line) and the β-galactosidase activity (solid line). The error bars represent the standard deviation (SD) for three independent experiments, performed in duplicate.

increased to 25-folds and 320-folds of backgrounds for pOS2-*lacZ* and pOS3-*lacZ*, respectively.

Discussion

High-level production of heterologous proteins is very important for both basic research and practical applications, and numerous expression systems are commercially available. However, since most of these systems are not tightly regulated, they cannot be used for expression of those toxic proteins, which even at low levels severely affect the host cell's growth. Moreover, some expression systems are too costly for the large-scale production of recombinant proteins owing to high costs for prerequisite inducers.

In this work, we have characterized a new inducible *E. coli* gene expression system using the P_{entC} promoter of the *E. coli entCEBA* operon [22]. The P_{entC} promoter activity is regulated by the Fur iron responsive transcriptional regulator by iron availability [5, 16]. Even though the *lacZ* reporter gene cloned under the control of the P_{entC} promoter was expressed at low basal level in LB medium, it was easily controlled by the addition of either an iron chelator or iron in the culture medium. The system can be strongly induced in LB medium by the addition of 200 μM 2,2'-dipyridyl or EDTA without growth defects. On the other hand, the system is tightly repressed by the addition of >5 μM of $FeSO_4$. It is possible to finely regulate the system by the sequential repression and induction of cells in a medium initially supplemented with excess iron and subsequently induced by addition of a chelator to allow target protein expression. Furthermore, the efficiency of the iron-availability-dependent expression control was further improved by incorporation of a *fur* gene into the vector. Even though this system may require additional optimization depending on the type of target protein, it will be very useful for controlled expression of proteins that become toxic or insoluble when induced at high level. In addition, this system can be further improved for purification of target proteins by employing an affinity tag or for expression in other bacterial strains by incorporating an origin of replication from a broad-host-range vector.

In conclusion, we have created an iron chelator-inducible expression system that shows tight regulation of gene expression in response to iron availability in the culture medium. This system will have wide applications in expressing recombinant proteins since it employs a simple, cost-effective, and readily available repressor and inducer, iron and EDTA, respectively. The efficiency of the expression system can be further improved by use of a defined medium with less iron content or by optimization of culture conditions [30]. Finally, we note that similar expression systems could be readily developed for other organisms, since most organisms have mechanisms to tightly regulate iron homeostasis [15, 18, 20, 29].

Acknowledgments

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