Appl Microbiol Biotechnol (2013) 97:5423–5431
DOI 10.1007/s00253-012-4633-8

Induction of gene expression in bacteria at optimal growth temperatures

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Received: 30 August 2012 / Revised: 29 November 2012 / Accepted: 1 December 2012 / Published online: 28 December 2012

Abstract Traditional temperature-sensitive systems use either heat shock (40–42 °C) or cold shock (15–23 °C) to induce gene expression at temperatures that are not the optimal temperature for host cell growth (37 °C). This impacts the overall productivity and yield by disturbing cell growth and cellular metabolism. Here, we have developed a new system which controls gene expression in *Escherichia coli* at more permissive temperatures. The temperature-sensitive *c*I857-P_L system and the classic lacI-P_{lacO} system were connected in series to control the gene of interest. When the culture temperature was lowered, the thermolabile *c*I857 repressor was activated and blocked the expression of lacI from P_L. Subsequently, the decrease of LacI derepressed the expression of gene of interest from P_{lacO}. Using a green fluorescent protein marker, we demonstrated that (1) gene expression was tightly regulated at 42 °C and strongly induced by lowering temperature to 25–37 °C; (2) different levels of gene expression can be induced by varying culture temperature; and (3) gene expression after induction was sustained until the end of the log phase. We then applied this system in the biosynthesis of acetoin and demonstrated that high yield and production could be achieved using temperature induction. The ability to express proteins at optimal growth temperatures without chemical inducers is advantageous for large-scale and industrial fermentations.

Keywords Expression system · Temperature induction · *c*I857 · lacI · Acetoin

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Introduction

Engineered microorganisms can be used to produce a variety of recombinant proteins including antibiotics and enzymes by cloning and overexpressing the coding genes. These systems can also produce metabolites including but not limited to biofuels and medications by cloning and expressing the biosynthetic pathways. In both cases, the expression of heterologous genes or pathways would consume excess resources inside the cells and result in a decreased growth rate. So, although some successful biosyntheses have been reported utilizing constitutive expression systems (Lee et al. 2007), in most cases, inducible expression strategy is used to divide the fermentation into two phases: in the preinduction phase, foreign gene expression is repressed; the yield of the desired product is low; and cell growth is favored; in the next phase, when enough cells have been accumulated, foreign gene expression is induced to generate the goal product at a high productivity. To a successful biosynthesis, the ability to induce gene expression at the correct time and at the appropriate level is thought to be very important (Gadkar et al. 2005; Solomon and Prather 2011; Anesiadis et al. 2008).

A variety of inducible systems exist, each triggered by different means including chemical inducers, temperature changes, pH changes, or metabolic signals related to cell density (Terpe 2006; Jana and Deb 2005; Tsao et al. 2010). Among them, the lacI-*lacO* system is most common and is widely used in both labs and industry. The *lac* operon is a DNA unit responsible for the metabolism of lactose in *Escherichia coli* and is only activated in the presence of lactose. In this system, the repressor protein lacI can bind tightly to the operator DNA *lacO* and block gene transcription. Conversely, inducer allolactose (a lactose metabolite) and its molecular analog, isopropyl β-D-1-thiogalactopyranoside (IPTG), bind to lacI protein, cause conformational change, release the lacI-*lacO* bound, and start the gene expression (Simons et al. 1987). The lacI-*lacO* couple is recruited in many popular expression vectors, like P_{tac}, P_{trc}, and P_{T7lacO} vectors. High levels of induced recombinant protein production and relatively low "leak expression" can be achieved. However, IPTG is costly, making it prohibitive in large-scale fermentation. Furthermore, IPTG is toxic to humans, making it unsuitable for producing therapeutic proteins and products (Lee and Keasling 2008).

The lambda *c*I857-P_L (or P_R) system is inducible by heat shock and is widely utilized in recombinant protein production. In this system, the transcription from P_L or P_R is controlled by temperature-sensitive repressor *c*I857 which binds the P_L promoter at low temperatures (28–30 °C), repressing transcription. At higher temperatures (40–42 °C), *c*I857 is inactivated, allowing transcription (George et al. 1987). This thermal inducibility avoids expensive and toxic chemical inducers, is easy to operate, and scales well for large fermentation (George et al. 1987; Villaverde et al. 1993; Andreeßen and Steinbüchel 2012). However, the heat shock system is flawed as well. First, the proteins synthesized at the heat shock temperature tend to fold incorrectly, leading to formation of inclusion bodies, proteolytic digestion, resulting in protein inactivation and decreased net yield (Schein 1991). Also, the correctly produced enzymes commonly have a shorter half-life time at higher temperature, further decreasing net yields. This is why most biosynthetic fermentations using engineered microorganisms are performed at a lower temperature (28 or 30 °C), although the preinduction cell growths are performed under 37 °C (Bokinsky et al. 2011; Steen et al. 2010). Second, heat shock is known to trigger a variety of stress responses in host cells, and the overproduction of recombinant protein itself also triggers the heat shock and SOS responses. All these stresses overlap in time and compromise the product yield and quality. This dual-stress problem is discussed in details in a recent review (Valdez-Cruz et al. 2010).

Culturing at low temperatures is usually beneficial for protein solubility and activity by improving the folding process (Schein 1991), and IPTG induction can be performed at low temperatures (for example, at 30 °C) (Vasina and Baneyx 1997). Cold shock-inducible systems based on *cspA* promoter which can be trigged by temperature downshift were also developed (Chao et al. 2002; Qing et al. 2004). CspA is a protein found in *E. coli* which can help the cell to adapt to low temperatures. Its expression can be induced by rapid downshift of temperature through a mechanism involving mRNA stability (Fang et al. 1997). Cold shock-inducible systems are preferred when the foreign protein is unstable or toxic at high temperatures (Qing et al. 2004). However, the inducing temperature of the cold shock is very low (15–23 °C) which led to more than 4.5-fold decrease in growth rate (Mujacic et al. 1999). This is acceptable for the production of high value-added therapeutic proteins, but problematic when the productivity is a concern (Stephanopoulos 2007).

Overall, temperature-controlled expression systems have many advantages including avoiding chemical inducers and ease of use. But, traditional heat and cold shock systems use inducing temperatures too high or too low for optimal cell growth. In this study, we described the construction and application of a new system that allowed inducible gene expression near optimum growth temperatures.

Materials and methods

Bacterial strains and growth conditions

E. coli DH5α (Invitrogen) was used for routine DNA manipulation; BL21(DE3) (Invitrogen) was used as the host for green fluorescent protein (GFP) expression and acetoin biosynthesis. W3110 was used for gene knockout. Antibiotics were used if necessary as follows: ampicillin (50°mg/mL), kanamycin (50°mg/mL), and chloramphenicol (30°mg/mL). IPTG was used to induce gene expression at final concentration of 0.5°mM.

DNA manipulation

Primer oligonucleotides were purchased from Generay Biotech Company (China) and were listed in Table 1. DNA sequencing was performed by BGI Company (China). Pyrobest™ DNA polymerase from Takara (Japan) was used for PCR.

To construct the LC cassette, the *c*I857-P_L sequence was amplified from plasmid pCP20 (Datsenko and Wanner 2000) using primers pair *c*I857s/*c*I857a; the *lacI* sequence was amplified from pET28a (Novagen) using primers pair p2s/p2a. Those two PCR products were then joined together by overlap extension PCR to generate the LC cassette using primers pair pa/ps. The *lite* sequence cording for *ssrA* tag (AANDENYALAA) was added at the 3′ end of *lacI* by primer pa. The LC cassette was TA cloned into pMD19-T vector (Takara, Japan). For constructing vector plc, the

Table 1 Primers used in this study

Primer name	Sequence (5′–3′)
p2s	agcagcagtcgcttcacg
p2a	tggttgcatgtactaaggaggttgtgtgaaaccagtaacgttatacgatg
*c*I857s	aggagtcgcataagggagagcgtcgagattcttgctcaattgttatca
*c*I857a	catcgtataacgttactggtttcacacaacctccttagtacatgcaac
ps	agattcttgctcaattgttatcag
pa	gcgctgcag*tcaagctgctaaagcgtagttttcgtcgtttgcagcc*ctgcccgctttccagtcggg
LCs1	tcgcataagggagagcgtcgagattcttgctcaattgtta
LCa1	attgcgttgcgcctgcagtcaagctgctaaagcgtagttt
LCs2	tcagctatgcgccgaccaga
LCa2	tcgtcgtttgcagcctgccc
pETs1	aaactacgctttagcagcttgactgcaggcgcaacgcaattaatgtaagt
pETa1	taacaattgagcaagaatctcgacgctctcccttatgcga
pETs2	taatgtaagttagctcactc
pETa2	ctcctgcattaggaagcagc
KO_I_S	ccggaagagagtcaattcagggtggtgaatgtgaaaccagagcgattgtgtaggctggag
KO_I_A	attaattgcgttgcgctcactgcccgctttccagtcgggattaacggctgacatgggaat
KO_I_VS	acagggacaccaggattt
KO_I_VA	ttgaggggacgacgaca
GFP_A	gcgccatgg(*Nco* I)atatggtgagcaagggcgaggag
GFP_S	gcggtcgac(*Sal* I)ttacttgtacagctcgtccatg
alsSD_A	cgcggatcc(*BamH* I)tattcagggcttccttcag
alsSD_S	catgccatgg(*Nco* I)gtatttggcattccaggt

Restriction enzyme cutting site is underlined and followed by the enzyme name in brackets; *lite* sequence is italicized

pMD19-T-LC was amplified with primers pair LCs1/LCa1 and primers pair LCs2/LCa2. pET28a was amplified with primers pairs pETs1/pETa1 and pETs2/pETa2. The four PCR products were mixed and assembled into vector plc by a ligation-free method, SHA (Jiang et al. 2012). The constructed vector was verified by DNA sequencing.

Gene *gfp* was amplified from pDR195-*gfp* (Zhang et al. 2011) using primers pair GFP_S/GFP_A and cloned into the multiple cloning sites (MCSs) of plc and pET28a to create plc-*gfp* and pET28a-*gfp*. The *alsSD* operon (coding for the acetoin biosynthetic pathway) was amplified by PCR from *Bacillus subtilis* 168 genomic DNA template using primers pair alsSD_S/alsSD_A and cloned into the MCSs of plc and pET28a to create plc-*alsSD* and pET28a-*alsSD*.

Deletion of chromosomal *lacI* in *E. coli* W3110 and its verification were performed according to a previously reported method (Datsenko and Wanner 2000). pKD3 was amplified with primers pair KO_I_S/KO_I_A. The PCR product was transformed into W3110/pKD46 to disrupt the *lacI* gene. Cm^r transformants were verified using primers pair KO_I_VS/KO_I_VA.

GFP expression

Individual colonies of *E. coli* BL21(DE3)/plc-*gfp* were grown in 10-mL culture tubes at 42 °C at 180 rpm overnight in 2 mL Lysogeny broth (LB) containing antibiotic. Overnight cultures were subcultured (1:100) into 20 mL fresh media in 100-mL flasks and incubated at 42 °C with shaking at 180 rpm. When the OD_{600} reached about 0.60, gene expression was induced by transferring to the indicated temperatures. Strains of *E. coli* BL21(DE3)/pET28a-*gfp* were cultured similarly, except that 0.5 mM IPTG (final concentration) was added along with the temperature shifting. All cultivations were carried out in triplicate.

GFP expression was determined by measuring the relative fluorescence using a Hitachi F-4600 fluorescence spectrophotometer (Japan) under excitation and emission of 488 and 507 nm, respectively. This fluorescent reading was normalized by simply dividing by the OD_{600} reading.

Acetoin biosynthesis

All experiments were carried out in triplicate. Seed cultures prepared in 50 mL LB medium were subcultured (1:100) into 3 L M9 media plus 0.1 % tryptone in a 5-L Biostat B Plus (Sartorius Stedim, Germany) bioreactor. M9 medium contained per liter the following: 9.8 g $K_2HPO_4{\cdot}3H_2O$, 2.1 g citric acid, 0.3 g ammonium ferric citrate, 0.4 mL H_2SO_4. The pH was adjusted to 7.0 by ammonium hydroxide. Two percent glucose and 0.1 % tryptone were autoclaved individually prior to mixing. The pH was kept at 7.0;

temperature, at 42 °C; air supplied, at 2 L/min; and pO_2, at 20 %. Stirring was controlled in cascade.

When the OD_{600} reached about 0.60, the temperature was shifted to 30 °C to express the pathway genes. One hour after the induction, air supply was shut down to create an oxygen-limited environment. Strains harboring pET28a-*alsSD* were cultured similarly, except that 0.5 mM IPTG (final concentration) was added before the temperature shifting.

Glucose concentration was measured by an enzyme-based biosensor appliance (SBA-40D biosensing analyzer, China). Acetoin concentration was measured by GC. Fermentation samples were centrifuged (13,000×*g*, 5 min), and the supernatants were saturated with sodium chloride and extracted with an equal volume of ethyl acetate; 0.2 % *n*-butanol was added as internal standard. GC analyses were performed with a SP-6890 gas chromatography system equipped with a CP-Wax58 column (25 m length, 0.25 mm ID, 0.2 μm film thickness, Varian). Experimental chromatographic conditions were as follows: injector set at 200 °C; flame ionization detector set at 270 °C; N_2 carrier gas at 0.05 MPa; oven temperature program, 1-min isotherm at 50 °C followed by a linear temperature increase of 3 °C min^{-1} up to 90 °C and then 40 °C min^{-1} to 200 °C held for 5 min. For total protein assays, cells from cultures were pelleted, lysed, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and stained with Coomassie blue as described previously (Yang et al. 2012).

Results

System design and construction

Previously, different regulation systems have been placed in series to create new functions, an example being a genetic toggle switch (Kobayashi et al. 2004) and oscillator (Stricker et al. 2008). The new function was established by putting the expression of the repressor from one system under the control of the operator from another system. Here, we followed a similar strategy to build a gene expression control system with new induction capabilities. The basic design of this system is summarized in Fig. 1. Briefly, the lacI-P_{lacO} system was inserted between the *c*I857-P_L system and the gene of interest. As a result, the regulation behavior of the *c*I857-P_L system was reversed. Gene expression was turned off at higher temperatures while turned on at lower temperatures. The system was also inducible by addition of IPTG regardless of the temperature.

A *lite* sequence coding for the *ssrA* destruction tag was added at the 3′ terminal of *lacI* to reduce the lifetime of the lacI repressor. Such modification has been shown to be able

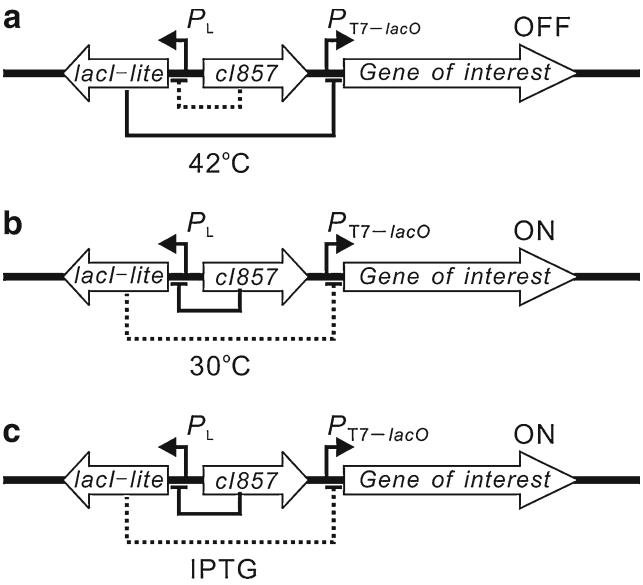

Fig. 1 Network diagram of the temperature-controlled expression system. **a** At 42 °C, *c*I857 is inactivated. lacI is expressed from P_L and blocks expression of the gene of interest. **b** At 30 °C, *c*I857 is activated and represses expression of lacI. With no lacI binding to operator *lacO*, expression of the gene of interest is derepressed. **c** In the presence of IPTG, lacI is inactivated. The gene of interest is expressed regardless of the temperature

to make the network react quicker (Stricker et al. 2008; Elowitz and Leibler 1999).

To facilitate future applications, the *c*I857 gene, P_L, and *lacI-lite* were firstly incorporated together as a DNA cassette (LC cassette), so that many existing vectors that use *lacO* hybrid promoters can be modified into temperature-inducible ones simply by inserting the LC cassette into them. For example, in this study, we created vector plc simply by replacing the *lacI* fragment of vector pET28a (Novagen) with the LC cassette (Fig. 2).

GFP expression induced by temperature downshifting

Following the custom in the literature, we cloned green fluorescent protein into vector plc as an indicator of gene expression (Lee and Keasling 2006; Choi et al. 2010; Lee and Keasling 2008). GFP was used as a quantitative reporter of promoter activity in *E. coli* (Lissemore et al. 2000), and its maturation time was about 6.5 min in *E. coli* (Megerle et al. 2008; Adiciptaningrum et al. 2009). The resulting plc-*gfp* vector was transformed into BL21(DE3). Cell growth and GFP production were determined at various times following induction. As shown in Fig. 3, GFP expression was strongly induced by temperature downshifting from 42 to 35 °C, and the GFP expression lasted for a long time until the end of the log phase.

Next, we investigated the relationship between the gene expression and the inducing temperature. Popular *E. coli*-

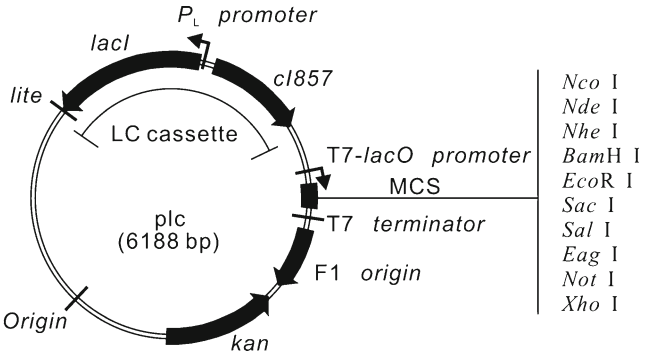

Fig. 2 Physical map of the plc expression vector. plc was modified from pET28a by replacing *lacI* with the LC cassette. plc and pET28a share the same plasmid back bone and T7 expression region

culturing temperatures like 40, 37, and 30 °C were examined as well as several other temperature points in the range from 25 to 42 °C (Fig. 4). Protein expression increased as the inducing temperature decreased from 40 to 30 °C. Within this temperature range, predefined submaximal levels of gene expression can be obtained. At temperatures below 30 °C, gene expression reached the maximum, which is consistent with an earlier report that the *c*I857 was fully activated below 30 °C (Villaverde et al. 1993). This result demonstrates that our system has a different inducible temperature range from that of traditional heat and cold shock systems. Importantly, this range covered the optimum growth temperatures of *E. coli*.

Our system requires preinduction culturing at 42 °C rather than the standard 37 °C. To evaluate the impact of this difference, *E. coli* host was cultured constantly at 37 or 42 °C, or cultured first at 42 then at 37 °C. The cell growth curves were compared (Fig. 5). In LB medium, the lag phase

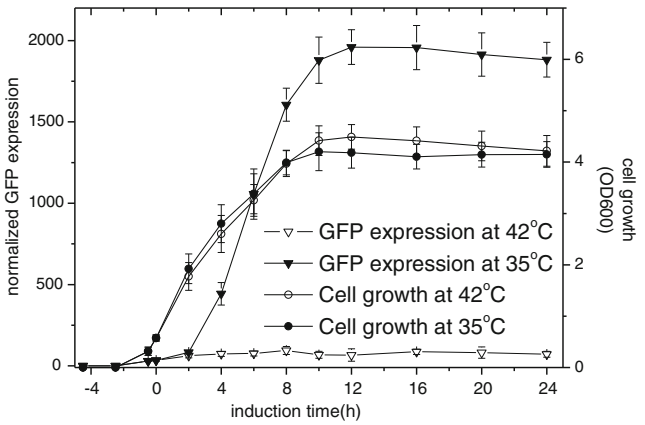

Fig. 3 Time courses of cell growth and GFP gene expression after temperature downshifting. Strain BL21(DE3)/plc-*gfp* was cultured in LB medium at 42 °C and induced by lowering the temperature to 35 °C at time=0 h. Note that GFP expression was normalized by dividing the fluorescent reading by the OD_{600} reading, so it reflected the level of GFP production per cell. Data are the average and standard deviation of three independent experiments

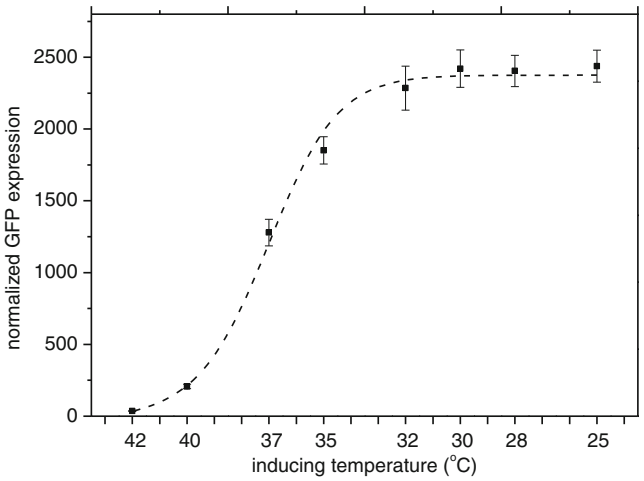

Fig. 4 GFP expression levels at different induction temperatures. Strain BL21(DE3)/plc-*gfp* was cultured in LB medium at 42 °C and induced by shifting to the indicated temperatures. Data are the average and standard deviation of three independent experiments

is not obvious for both temperatures, and the strain reached a little higher final OD_{600} at 37 °C than at 42 °C. In M9 medium, the lag phase at 42 °C was slightly longer (about 1 h) than that at 37 °C, but interestingly, the strain reached higher final OD_{600} at 42 °C than at 37 °C. The growth curve of 42 °C then 37 °C shared the same first part (before temperature shifting) with curve of 42 °C, and its upper limit fell between the 42 and 37 °C curves in both mediums. Nevertheless, these differences are acceptable, especially considering our success in applying this system in GFP expression and acetoin biosynthesis.

Comparison of GFP expression between plc-*gfp* and pET28a-*gfp*

Induced and uninduced GFP expression levels were compared between plc-*gfp* and pET28a-*gfp* (Table 2). The degree of uninduced or "leaky" expression from plc is no higher than uninduced pET28a. The GFP expression of plc-*gfp* induced by temperature shifting was about 22 % of that of pET28a-*gfp* induced by IPTG. plc and pET28a shared the same plasmid backbone and T7-*lacO* promoter, so they were supposed to have the same copy number (about 40 per cell, according to Novagen manual) and promoter strength. The only difference should be the level of lacI activity. In the BL21(DE3)/plc-*gfp* cells, besides the 40 copies of *lacI* on the plc-*gfp* plasmid, there were also two *lacI* copies (one native copy and one copy introduced by DE3) on the chromosome, which were not controlled by the *c*I857-P_L. So at inducible temperature, there would still be low level of lacI expressed from chromosomal *lacI*, and the T7-*lacO* promoter was thus partly repressed. With IPTG added, plc-*gfp* reached similarly high level of GFP expression as pET28a-*gfp*.

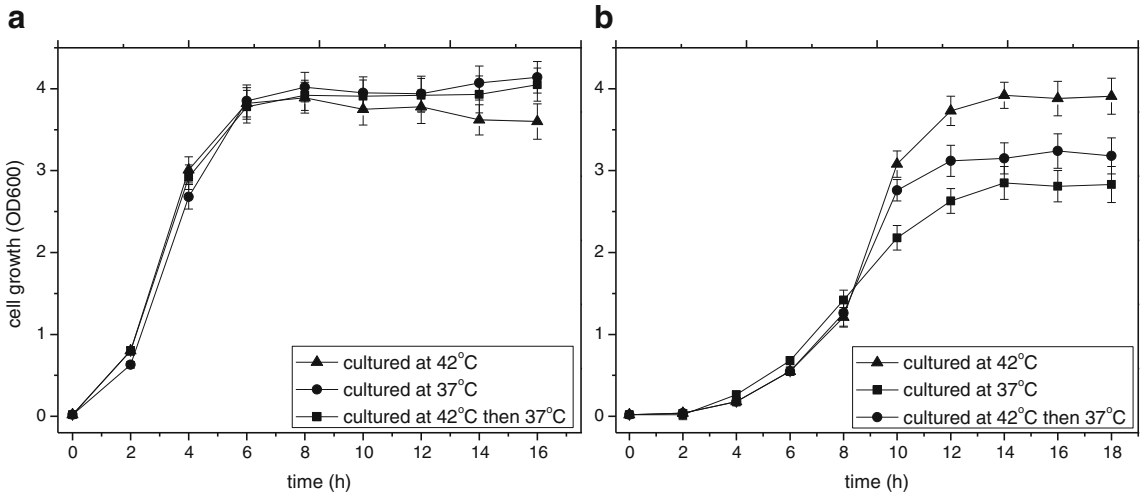

Fig. 5 Growth curves of *E. coli* BL21(DE) at different temperatures in LB (**a**) or M9 medium (**b**). Overnight LB culture was subcultured (1:100) into fresh LB or M9 media and cultured at 42, 37, or 42 °C then 37 °C. The 42 to 37 °C shift was carried out at 2 h for LB culture and 6 h for M9 culture, when the OD_{600} reached about 0.6. Data are the average and standard deviation of three independent experiments

To further increase the expression ability of plc, we knocked out the chromosomal *lacI* of *E. coli* strain W3110 which has only one *lacI* copy and planned to introduce the T7 DNA polymerase gene by a helper plasmid or by gene knock-in method. However, the resulted W3110Δ*lacI* strain grew slowly at 37 °C and was not viable at 42 °C (data not shown).

Application of plc in acetoin biosynthesis

Following successful demonstration of the temperature inducibility of the plc construct, we utilized the plc vector to express the acetoin biosynthesis pathway in *E. coli*. Acetoin (3-hydroxy-2-butanone) is a compound with a pleasant odor, widely used in foods, cosmetic, flavor, and chemical synthesis (Xiao and Xu 2007). It is also a precursor to important industrial chemicals 2,3-butanediol, butanone, and 2-butanol (Nielsen et al. 2010; Siemerink et al. 2011). The acetoin biosynthesis pathway is composed of the acetolactate synthase (coded by *alsS*) and the acetolactate decarboxylase (coded by *alsD*). Through glycolysis and this pathway, one molecule of acetoin can be generated from one molecule of glucose at a stoichiometry of 1:1.

Previously acetoin has been produced by engineered *E. coli* or *Clostridium acetobutylicum* using IPTG-inducible expression systems (Siemerink et al. 2011; Yan et al. 2009; Nielsen et al. 2010; Ui et al. 1997). Here, we compared BL21(DE3)/pET28a-*alsSD* and BL21(DE3)/plc-*alsSD*. Two strains were cultured in parallel conditions, in a 5-L bioreactor, with a working volume of 3 L. Temperatures for both strains were downshifted from 42 to 30 °C when the OD_{600} reached about 0.6; 0.5 mM IPTG (final concentration) was added for BL21(DE3)/pET28a-*alsSD*, but not for BL21(DE3)/plc-*alsSD*. Both strains produced acetoin efficiently after induced by IPTG and temperature shifting, respectively (Fig. 6). The maximum titers and acetoin/glucose yields were summarized in Table 3. Unlike the production of GFP, high titer and yield of acetoin were achieved by both the plc system and the pET28a system. Protein expressions were further examined by

Table 2 Induced and uninduced GFP expressions from plc-*gfp* and pET28a-*gfp*

Strain	Induction	Normalized GFP expression
BL21(DE3)/pET28a-*gfp*	Uninduced, cultured at 42 °C	$(3.7\pm0.5)\times10$
	Induced by 0.5 mM IPTG at 30 °C	$(9.6\pm0.2)\times10^3$
BL21(DE3)/plc-*gfp*	Uninduced, cultured at 42 °C	$(3.0\pm0.1)\times10$
	Induced by downshifting the temperature from 42 to 30 °C	$(2.4\pm0.1)\times10^3$
	Induced by downshifting the temperature from 42 to 30 °C and the addition of 0.5 mM IPTG	$(9.0\pm0.2)\times10^3$

Preinduction culturing was performed at 42 °C. Data are the average and standard deviation of three independent experiments

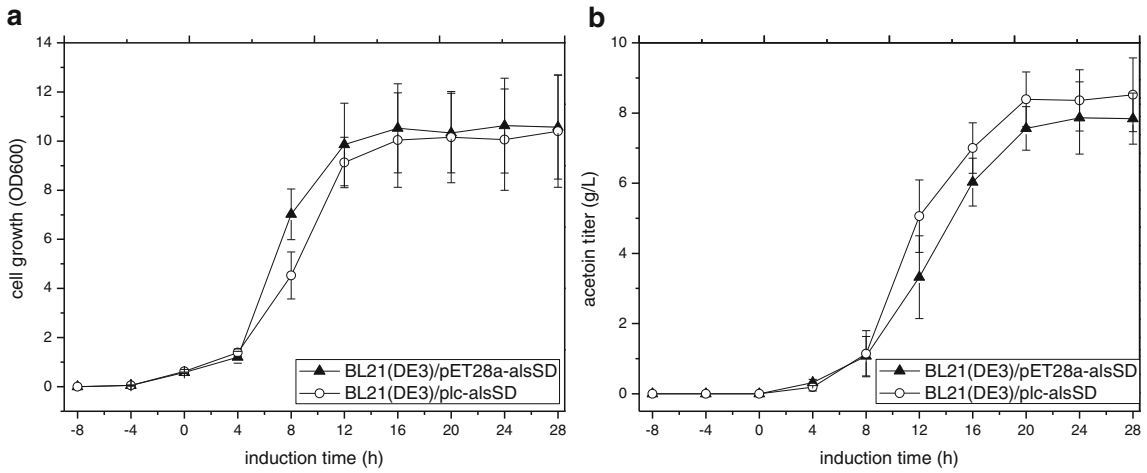

Fig. 6 Growth curves (**a**) and acetoin production curves (**b**) of BL21 (DE3)/pET28a-*alsSD* and BL21(DE3)/plc-*alsSD*. Strains were cultured in 3-L M9 media plus 0.1 % tryptone in a 5-L bioreactor. At 0 h time, the culture temperature was downshifted from 42 to 30 °C. IPTG was added into BL21(DE3)/pET28a-*alsSD* culture, but not into BL21(DE3)/plc-*alsSD* culture. At 1 h time, air supply was shut down to create an oxygen-limited environment. Data are the average and standard deviation of three independent experiments

SDS-PAGE. As shown in Fig. 7, less AlsS and AlsD were produced from plc than from pET28a. Unlike protein production, the genes encoding biosynthetic pathways do not require high expression levels for metabolite production. In this case, substrate concentrations act as the limiting factors in the product biosynthesis not enzyme concentrations. Thus, increasing enzyme induction does not necessarily increase production nor does low enzyme induction prevent high yields. Additionally, protein synthesis itself will compete with the production of desired product for available cellular metabolites (Lee and Keasling 2006, 2008; Solomon and Prather 2011). Our results suggested that the AlsS and AlsD from *B. subtilis* demonstrated high enzymatic activities in recombinant *E. coli* and can be expressed on a relatively low, but sufficient level, for high efficiency acetoin production.

Discussion

Gene expression induction by temperature shifting is a superior method as it can be applied in a simple and cost-effective manner. However, culture temperature is a basic fermentation parameter which directly impacts the cell growth and cellular metabolism and thus should be optimized (Laluce et al. 2009). In traditional heat shock (40–42 °C) and cold shock (15–23 °C) methods, the ability to optimize the culture temperature is limited due to narrow induction temperature spectrums which are very different from optimum growth temperatures (37 °C) (Villaverde et al. 1993; Qing et al. 2004). In this study, we developed a novel system which induces gene expression by reducing temperature from 42 °C to a range of 25–37 °C. It filled the temperature gap left between the traditional heat and cold shock systems. More importantly, it covered the optimal growth temperature.

This system was built by combining the *c*I857-P_L and lacI-*lacO* systems which are both well characterized. Previously, the two systems had been used in conjunction to construct a genetic toggle switch (Kobayashi et al. 2004) and oscillator (Stricker et al. 2008). Their studies demonstrated that signals can be precisely and stably transmitted along the regulation pathway and not interrupted by the cell-division cycle. Consistent with previous findings, in our study, the gene expression correlated with temperature. As shown in Figs. 3 and 4, gene expression can be sustained through the end of the log phase, and expression level can be modulated by varying induction temperature. This is particularly useful in metabolite biosynthesis in which the expressed enzymes are thermolabile, or long production periods are required (Clomburg and Gonzalez 2010).

Table 3 Acetoin production from BL21(DE3)/plc-*alsSD* and BL21(DE3)/pET28a-*alsSD*

Strain	OD_{600}	Maximum titer (g/L)	Molar yield (acetoin/glucose) (%)
BL21(DE3)/pET28a-alsSD	10.6±2.1	7.84±0.73	80.2±6.4
BL21(DE3)/plc-alsSD	10.4±2.3	8.52±1.05	87.1±8.0

Data are the average and standard deviation of three independent experiments

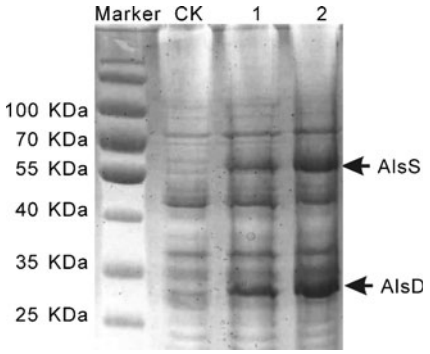

Fig. 7 Comparison of enzyme expression levels from plc and pET28a during acetoin fermentations. Cell samples were collected from the bioreactor 8 h after induction. Proteins were separated by SDS-PAGE and stained with Coomassie blue. Lanes *CK*, BL21(DE3)/plc; *1*, BL21 (DE3)/plc-*alsSD*; *2*, BL21(DE3)/pET28a-*alsSD*. Expected sizes of AlsS and AlsD were 60.5 and 28.2 kDa calculated from their gene sequences. A representative result for three independent experiments was shown

We have not fulfilled the expression potential of plc because of the chromosomal lacI. Further effort is needed to improve this.

This study also demonstrated that new regulation paradigms can be created from existing regulation systems. Through this combinatorial approach, the relationship between gene expression and induction signal can be reversed, and the induction signal spectrum can be changed. This type of complex induction system has been rarely reported for protein or metabolite production. However, in nature, cascade regulation pathways containing multiple steps are common in intracellular signaling networks (Müller and Hausmann 2011). As the result of evolution, they were able to perform more complicated and accurate regulation behaviors to make the organisms to response to internal and external changes. In biosynthetic studies, complex regulation systems may offer potential to achieve better yield and productivity.

Acknowledgments This work is supported by the National Natural Science Foundation of China (no. 21106170), the "Twelfth Five-Year Plan" in the National Science and Technology for the Rural Development in China (no. 2012BAD32B06), the National Defense Innovation Foundation of Chinese Academy of Sciences (no. CXJJ-11-M56), and the project of Academy-Locality Cooperation Chinese Academy of Sciences.

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