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Engineering a chimeric malate two-component system by introducing a positive feedback loop in *Escherichia coli*

Irisappan Ganesh, Murali kannan Maruthamuthu, and Soon Ho Hong†

Department of Chemical Engineering, University of Ulsan, Ulsan 44610, Korea
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Abstract−Previous studies constructed a chimeric MalKZ two-component system to sense environmental malate. In this study, we used a positive feedback loop to accelerate and amplify the output signal indicating malate concentration. The positive feedback loop was constructed by cloning *ompR* gene, which encodes *ompC* and induces OmpR protein; *ompC* promoter was used to control the process. The transcriptional expression profile showed that the expression level of *ompC* gene increased about two-fold after the positive feedback loop was introduced. When GFP was used as a reporter protein, a 71% increase in fluorescence level was observed. The results indicate that the signal transduction kinetics of MalKZ can be engineered by introducing the positive feedback loop.

Keywords: *Escherichia coli*, Malate, Positive Feedback Loop, Two-component System

INTRODUCTION

In the TCA cycle, malate, succinate, and fumarate are intermediate compounds. Malate is potentially an attractive candidate from which biopolymer and biorefinery products can be synthesized, so malate is considered as one of the top ten building block chemicals that can be produced by a biological process [1,3]. Since the demand for malate has been increasing steadily, studies focus on developing a biological process for the efficient synthesis of malate [2]. As the TCA cycle is primarily involved in the metabolism of living organisms, it is difficult to direct carbon flux to malate in this process. Therefore, the metabolism of living organisms must be modified extensively to produce microorganisms that would enable efficient synthesis of malate. With the help of a high-throughput screening technique, we can improve microbial strains associated with malate production. A bacterial biosensor is a promising candidate for the high-throughput screening system.

In a two-component system (TCS), organisms can sense and respond to changes in different environmental conditions via basic stimulus-response coupling mechanisms [4,5]. Each TCS consists of a membrane containing sensor protein-histidine kinase (HK) and a cytoplasmic response regulator (RR). The cytoplasmic RR senses a specific environmental signal that is activated by HK-modulated phosphorylation [6]. Because TCS is strain-specific, some stimulus could not be sensed naturally in a certain bacterial strain. So, chimeric TCS strategy has been employed to construct TCS with a desired stimulus-response mechanism. Chimeric TCS can be constructed by domain swapping. In this process, the catalytic domain from different HKs is combined with the sensory domain. A chimeric malate sensing MalKZ HK was successfully constructed by integrating MalK and EnvZ [7].

†To whom correspondence should be addressed.
E-mail: shhong@ulsan.ac.kr

Although the chimeric MalKZ HK showed good malate sensing ability, MalKZ had to undergo further improvement in order to be used as a malate biosensor. It has been proven that the dynamic behavior of TCS can be engineered by introducing a positive feedback loop, which modulates the gene expression output variables, such as response timing, response level, and genetic noise [8,9]. Previous studies have reported that the dynamic behavior of zinc sensing HydHG TCS can be engineered by introducing a positive feedback loop. When the positive feedback loop was introduced, the output signal was amplified and the system was fully activated with the concentration of Zn^{2+} being as low as 10 μM [10]. Furthermore, researchers have reported that the sensitivity of copper-sens-

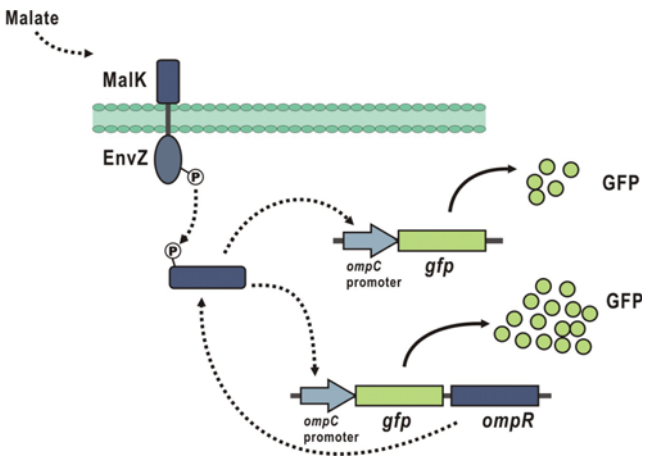

Fig. 1. Design of the TCS-mediated expression system. In pOGFP1, the *ompC* promoter consists of a 250 bp sequence containing the operator sequence; it is located just upstream of the green fluorescent protein, which is under the control of R/TCS of *E. coli*. In pOSAL1, the *ompC* promoter drives the expression of the green fluorescent protein and OmpR, a regulatory protein. OmpR RR drives the transcription of GFP and OmpR from the *ompC* promoter.

ing CusSR TCS can be increased by up to ten-old as the intensity of the output signal is increased by introducing a positive feedback loop [11].

In this study, a positive feedback loop was introduced to engineer the dynamic behavior of the chimeric MalKZ TCS. To construct the positive feedback loop, *ompR* gene was cloned downstream of a *gfp* gene, which can be easily folded in the cytoplasm [12]. Its expression is induced by an *ompC* promoter (Fig. 1). The effect of the positive feedback loop was evaluated by real-time quantitative PCR (qRT-PCR) and green fluorescent protein (GFP).

MATERIALS AND METHODS

1. Bacterial Strains and Media

E. coli XL1-Blue was used as a host strain for recombinant DNA manipulation and malate sensing experiment. The bacterial strains used in this study were XL1- Blue and BL21 (DE3). The plasmids used in the study were pUC19, pOGFP1 [7], pOSAL1, pACYC-Duet-1, pMalKZ1 [7]. All *E. coli* strains were grown in Luria-Bertani (LB) broth (10 g/L bacto-tryptone, 5 g/L bacto-yeast extract and 5 g/L NaCl) and M9 minimal salts medium (Sigma-Aldrich, St. Louis, MO, USA), unless otherwise stated, with glucose (0.4%) as a carbon source, and supplemented with 2 mM $MgSO_4$, 0.1 mM $CaCl_2$ and 1% Thiamine HCl per mL supplemented with antibiotics (ampicillin,100 mg/L or chloramphenicol, 25 μg/mL). The cultures was shaken vigorously (225 rpm) at 37 °C.

2. Construction of Plasmid

To construct a positive feedback loop that would continuously activate *ompC* promoter, we amplified the *ompR* gene from *E. coli* genomic DNA using the following oligonucleotides: OmpR_F_SalI and OmpR_R_PstI. Then, the PCR product was digested with SalI and PstI. Subsequently, it was ligated overnight at 16 °C by using T4 DNA ligase and pOGFP1 vector, which was digested using Sa1I and PstI.

3. Monitoring of *ompC* Gene Expression by Real-time Quantitative PCR

The transcriptional activities of the *ompC* gene in response to the presence of malate were measured by qRT-PCR. The single colony of *E. coli* BL21 (DE3) harboring pMalKZ1 with pOSAL1 was grown overnight at 37 °C in LB medium. The overnight culzwith appropriate antibiotics [100 μg/mL ampicillin and 25 μg/mL chloramphenicol] and incubated aerobically in an orbital shaker at 37 °C at 250 rpm until the optical density at 600 nm (OD_{600}) reached 0.5. To induce the expression of the chimeric HK, 10 μM IPTG was added to the culture and the cells were grown aerobically for an additional 4.5 h at 30 °C in the presence of varying concentrations of malate. After 4.5 h, strains were harvested by centrifugation for total RNA preparation using the RNeasy Mini Kit (Qiagen, CA, USA), followed by DNase treatment. Reverse transcription was performed with a cDNA synthesis kit (Applied Biosystems, MA, USA) using random hexamer primers according to the manufacturer's instructions. Specific primers were designed with OLIGO software (version 5.0; Molecular Biology Insights, Cascade, CO, USA) for quantitative analysis of the expression of *ompC* gene and 16sRNA. Samples for which the RT step was omitted were used as negative controls to ensure that the extracted RNA was not contaminated with DNA. qRT-PCR reactions were performed on the Mini Opticon detection system using the SYBR Green PCR Master Mix as recommended by the manufacturer. Each qRT-PCR experiment was performed in triplicate for biological samples using separate cultures grown under aerobic and identical conditions (n=3) and were calculated automatically by the Mini-Opticon software using 16sRNA as an internal control [13,14].

4. Monitoring of GFP Expression by Spectrofluorometer

A single colony of *E. coli* BL21 (DE3) harboring pMalKZ1 with pOSAL1 was grown overnight at 37 °C in LB medium. The overnight cultures were diluted to 100-fold in fresh M9 medium supplemented with appropriate antibiotics [100 μg/mL ampicillin and 25 μg/mL chloramphenicol] and incubated aerobically in an orbital shaker at 37 °C at 250 rpm until the optical density at 600 nm (OD_{600}) reached 0.5. To induce the expression of the chimeric HK, 10 μM IPTG was added to the culture and grown aerobically for 8 h with various concentrations of malate (pMalKZ1 with pOSAL1) and at 30 °C at 250 rpm. Concurrently, pACYCDuet-1/pUC19 in BL21 (DE3) was used as a control.

Cell density and fluorescence were measured under various concentrations of malate during 8 h of culture. Cell density was monitored by measuring the optical density at 600 nm with a spectrophotometer (Shimadzu, Japan). GFP fluorescence was measured using a RF-5301PC spectrofluorometer (Shimadzu, Japan). The excitation wavelength of the spectrofluorometer was set at 485/10 nm and the emission wavelength was set at 515/10 nm. The specific fluorescence intensity (SFI) was defined as the raw fluorescence intensity expressed in relative fluorescence units divided by the optical density at 600 nm measured at each time point. At a minimum, triplicate measurements were obtained for each sample.

The strain was also screened for fluorescence with a 100x objective on a reflected fluorescence microscope (Olympus, Japan) with a cooled charge-coupled device camera (B&W SenSys, KAF1401). Emission intensity was recorded using MetaMorph image analysis software (Molecular device, Silicon valley, CA, USA) with excitation and emission filters sets optimized for EGFP imaging [15].

RESULTS AND DISCUSSION

1. Construction of pOSAL1

To engineer the dynamic properties of the malate sensing chimera TCS, the *ompR* gene was cloned into the downstream of the *gfp* of the pOGFP1 plasmid. As a result of this feedback loop in pOSAL1 plasmid, the continuous activation of *ompC* promoter transiently elevated the reporter GFP expression level. The bacterial biosensor behaves like normal bacteria until it comes in contact with C_4-dicarboxylates like malate. At the time of detection by the bacterial biosensor, the synthesis of reporter protein is activated. If the *ompC* promoter was induced by phosphorylated OmpR, the GFP was expressed with OmpR, which in turn activated the *ompC* promoter again. Therefore the network was self-induced, which consequently amplifies the resulting output signal (Fig. 1).

2. Monitoring of *ompC* Gene Expression by Real-time Quantitative PCR

We used qRT-PCR technique to monitor the transcriptional expression of *ompC* which was induced by OmpR. Control samples

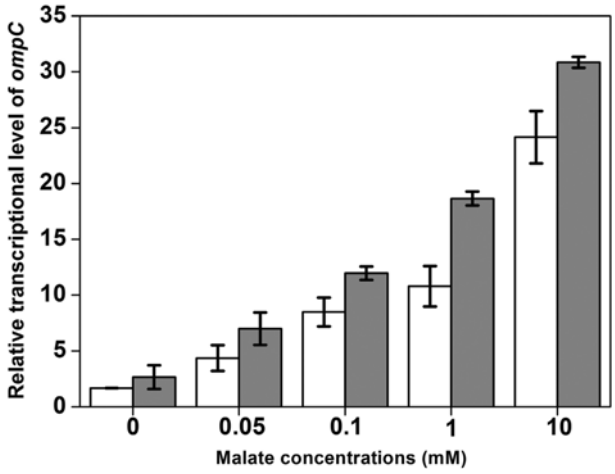

Fig. 2. The relative transcriptional level of *ompC* in strains harboring pOGFP1 (white) and pOSAL1 (gray) at varying malate concentrations. Calculations were performed automatically by the Mini-opticon software using 16sRNA as an internal control. The data are aggregated results from the replicated experiments (n=3).

E. coli (pMalKZ1 and pOGFP1) and *E. coli* harboring pMalKZ1 and pOSAL1 were grown in various concentrations of malate (0, 0.05, 0.1, 1.0 and 10 mM). Generally, strains with the positive feedback loop showed higher relative transcriptional levels than those shown in control strains. In particular, at 1 mM malate, introduction of the positive feedback loop yielded about two-fold increase in *ompC* expression (Fig. 2). Based on this result, it can be deduced that dynamic behavior of chimeric malate TCS was successfully engineered to generate stronger output signals through the introduction of the positive feedback loop.

3. Monitoring of GFP Expression by Spectrofluorometer

To determine whether *E. coli* with a positive feedback loop can be used as a fluorescence-based biosensor system for malate detection, we prepared a culture of *E. coli* BL21 (DE3)—harboring pMalKZ1 and pOSAL1—in various concentrations of malate (0, 0.05, 0.1, 1.0 and 10 mM)—and measured the fluorescence of the culture. A culture of *E. coli* (pMalKZ1 and pOGFP1) was prepared under identical conditions and used as a control. In the presence of 10 mM malate, we used chimeric TCS to induce the synthesis of GFP. Subsequently, the strains showed fluorescence, so they were observed under a reflected fluorescence microscope (Fig. 3(b)).

We compared the relative fluorescence data obtained from *E. coli* (pMalKZ1 and pOSAL1) and *E. coli* (pMalKZ1 and pOGFP1). Thus, we evaluated the effect of introducing the positive feedback loop in the culture. At all malate concentrations, *E. coli* strains with positive feedback loop produced higher fluorescence levels than *E. coli* without the loop (Fig. 4). Furthermore, the output fluorescence signals increased as the malate concentration increased. When 0.05 mM of malate was added to the media, the fluorescence of *E. coli* (pMalKZ1 and pOSAL1) became 71% higher than that of *E. coli* (pMalKZ1 and pOGFP1). At higher concentrations of malate, the differences in transcriptional levels decreased; this could probably be attributed to the saturation of *ompC* expression in *E. coli*. The fluorescence levels in *E. coli* (pMalKZ1 and pOSAL1) containing

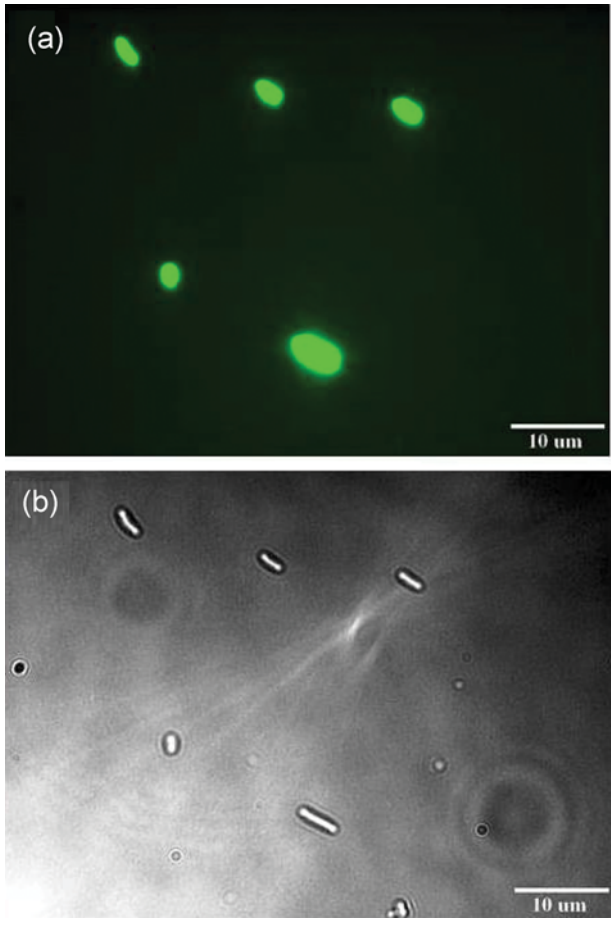

Fig. 3. The image of *E. coli* (MalKZ1 and pOGFP1). (a) Differential interference contrast (DIC) microscopy image of the recombinant cells carrying GFP. (b) The same cells in which reflects the fluorescence in the reflected fluorescence microscope.

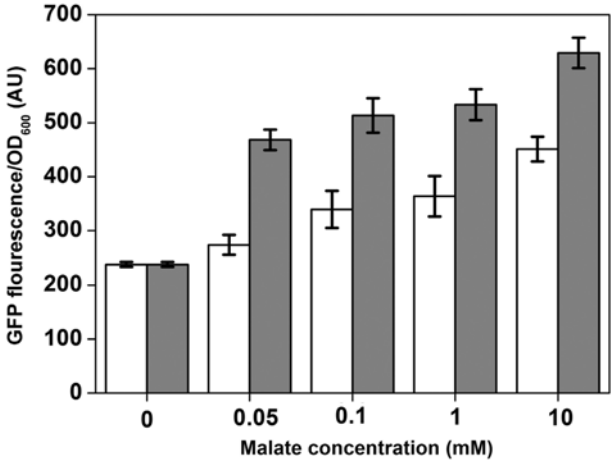

Fig. 4. GFP fluorescence in strains harboring pOGFP1 (white) and pOSAL1 (gray) at various malate concentrations in the 4 h time-period. The data are aggregated results from the replicated experiments (n=3).

0.05 mM malate are higher than those observed in *E. coli* (pMalKZ1 and pOGFP1) containing 10 mM malate (Fig. 4). This indicates

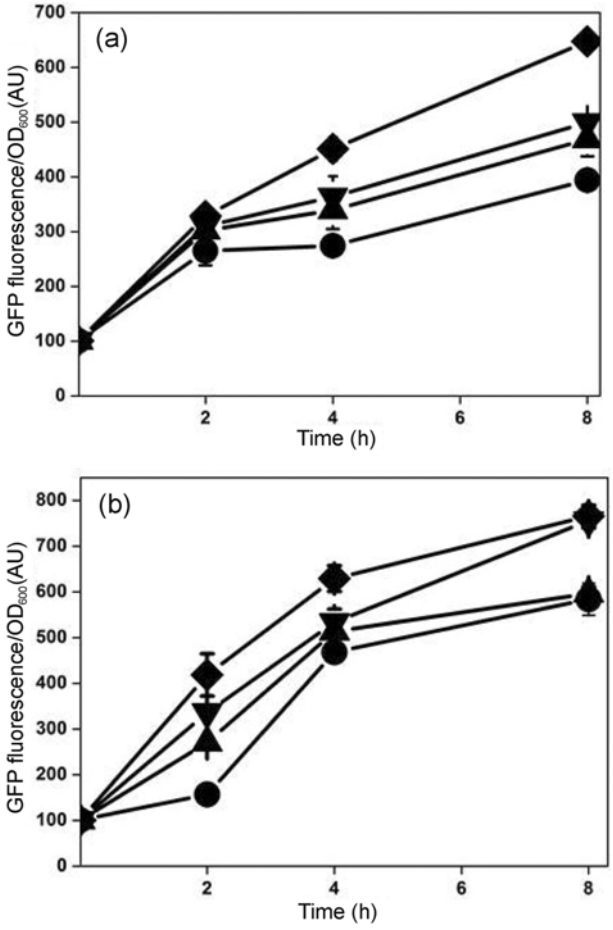

Fig. 5. Time profiles of GFP fluorescence in strains harboring pOGFP1 (a) and pOSAL1 (b) at various malate concentrations: 0.05 mM (●), 0.1 mM (▲), 1.0 mM (▼) and 10 mM (◆). The data are aggregated results from the replicated experiments (n=3).

that the sensitivity towards malate concentration significantly increased in the *E. coli* culture (pMalKZ1 and pOSAL1) after the positive feedback loop was introduced.

To observe the response kinetics of pOGFP1 and pOSAL1 network, we performed time-series experiments: strains harboring pOGFP1 and pOSAL1 network were grown separately. These strains exhibited fluorescence when malate was added to the culture medium. The stability of the fluorescence signal was estimated by measuring the fluorescence at different time-intervals in minimal media (Fig. 5). With pOSAL1, maximum fluorescence was achieved at all concentrations of malate used in this study of 8 h duration (Fig. 5(b)). While comparing pOGFP1 and pOSAL1, we realized that the expression of GFP by *ompC* promoter was augmented within 4 h in culture containing pOSALI. This was observed at all concentrationss of malate. However, maximum fluorescence was exhibited at 8 h in *E coli* culture harboring pOSAL1. As the concentration of malate was increased in the medium, the fluorescence also increased in a linear fashion. The transient GFP expression induced by pOSAL1 was much higher than that induced by pOGFP1. Moreover, the equilibrium between HK and RR was shifted due to the positive regulation of chimeric MalKZ1 TCS by pOSAL1.

CONCLUSION

For sensing environmental malate in a rapid and amplified manner, we employed the strategy of positive feedback loop. As expected, the positive feedback loop network showed higher fluorescence with the increasing concentration of malate in the medium. The transcription factor activated itself in the culture medium containing a positive feedback loop as the malate concentration was increased steadily. In addition, a set of target genes also became activated. These events initiated a specific response in the chimeric MalKZ/R TCS signalling pathway. This indicates that the signal-transduction kinetics of the chimera TCS were engineered to generate an appreciable output signal even at low concentrations of malate. We believe our strategy can be instrumental in optimizing other signal-transduction kinetics of TCS. This study has shown the feasibility of biosensor of the whole cell based on TCS for the detection of malate.

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