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Modification of CusSR bacterial two-component systems by the introduction of an inducible positive feedback loop

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Abstract The CusSR two-component system (TCS) is a copper-sensing apparatus of *E. coli* that is responsible for regulating the copper-related homeostatic system. The dynamic characteristics of the CusSR network were modified by the introduction of a positive feedback loop. To construct the feedback loop, the CusR, which is activated by the *cusC* promoter, was cloned downstream of the *cusC* promoter and reporter protein. The feedback loop system, once activated by environmental copper, triggers the activation of the *cusC* promoter, which results in the amplification of a reporter protein and CusR expression. The threshold copper concentration for the activation of the modified CusSR TCS network was lowered from 2,476.5 μg/l to 247.7 μg/l, which indicates a tenfold increase in sensitivity. The intensity of the output signal was increased twofold, and was maintained for 16 h. The strategy proposed in this study can also be applied to modify the dynamic characteristics of other TCSs.

Keywords *Escherichia coli* · CusSR two-component system · *cusC* promoter · Copper · Positive feedback

Introduction

Two-component systems (TCS) in bacteria are strain-specific and serve as basic stimulus–response coupling mechanisms that allow organisms to sense and respond to changes in different environmental conditions [13, 19]. Each TCS consists of a sensor protein-histidine kinase (HK) that senses a specific environmental stimulus and a corresponding response regulator (RR), which is activated by HK-modulated phosphorylation [8]. Modulation of the phosphatase and kinase activities of HK may therefore induce system responses by shifting the dynamic equilibrium between the phosphorylated and unphosphorylated forms of the RR, which frequently induces a transcriptional program [9]. Among the various TCSs characterized to date, PhoPQ, CpxRA, CusSR, and many other systems have exhibited autoregulatory characteristics [4, 6, 7, 15, 21].

Monitoring of environmental inorganic ions, especially heavy metal ions, through genetically engineered bacteria is one of the most interesting areas of research in bioengineering. *Escherichia coli* senses environmental copper through the CusSR TCS, and it regulates the expression of multiple operons, including the CusCFBA operon, which is involved in the transcription regulation of the genes for copper homeostasis [12]. The cusCFBA operon is organized on the *E. coli* genome next to the genes for CusSR two-component system, which regulates cus-CFBA transcription in copper-dependent manner. The cusSR genes are, however, transcribed in opposite direction from cus-CFBA. The CusSR TCS is activated only at higher

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concentration of copper (>0.5 mM) under aerobic conditions [21]. Considering the relatively high activation concentration, further engineering of CusSR is required to employ the CusSR TCS as a copper-sensing biosensor.

To make native biosystems more sensitive and efficient is a challenging task. Therefore, contemporary research employs a synthetic biology strategy to design efficient synthetic biosystems through the construction of a rational regulatory network. In this work, the dynamic behavior of the CusSR TCS of *E. coli* was monitored, and an inducible positive transcriptional feedback loop network was introduced to allow for the efficient activation of CusSR at lower copper concentrations (Fig. 1). To construct the positive feedback loop, the *cusR* gene was cloned downstream of a *gfp* gene, whose expression is regulated by a *cusC* promoter. If the *cusC* promoter was induced by phosphorylated CusR, the CusR was expressed as well as GFP, which in turn activated the *cusC* promoter again. Therefore, once the network is activated, it will become self-induced and consequently amplify the resulting output signal. The dynamical characteristics of the positive feedback loop in response to the input signal were monitored by quantitative measurements of fluorescence intensity with time. Simple linear dynamic mathematical modeling was conducted to understand the signal transduction kinetics of the inducible positive feedback loop.

Materials and methods

Bacterial strains and media

E. coli XL1-Blue was used as a host strain for recombinant DNA manipulation and construction of a reporter plasmid to respond to copper (Cu^{2+}). The bacterial strains, plasmids, and primers used in this study are listed in Table 1. 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), 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), at 37°C with vigorous shaking (225 rpm).

Construction of plasmids

The genomic region containing the 156-bp *cusC-cusR* intergenic region was amplified from *E. coli* strain genomic DNA with the oligonucleotides *cusC*_FEcoRI and *cusC*_RSacI (Table 1). Polymerase chain reactions (PCR) were performed with the MJ mini Personal Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) using the Expand High-Fidelity PCR System (Roche Molecular Biochemicals, Mannheim, Germany). The PCR product was then digested with EcoRI and SacI and ligated overnight at 16°C with T4 DNA ligase with the similarly digested pUC19 vector backbone, resulting in the creation of pUCup1. The *gfp* gene was amplified from the plasmid PROBE-NT' [10], and was ligated into pUCup1 using the SacI and KpnI restriction enzymes to construct pCGFP1. The pCGFP1 plasmid, in which the expression of *gfp* is under the control of a *cusC* promoter, was introduced into XL1-Blue. To construct a positive feedback loop for continuous activation of the *cusC* promoter, the cusR gene was amplified from *E. coli* genomic DNA with the oligonucleotides *cusR*_FKpnI and *cusR*_RBamHI. The PCR product was then digested with BamHI and KpnI and ligated overnight at 16°C with T4 DNA ligase with the similarly digested pCGFP1 vector backbone, yielding pCSAL1 (Table 1).

Cultivation of bacteria

A single colony of two strains, namely *E. coli* harboring the pCGFP1 or pCSAL1 plasmid, were grown separately overnight at 37°C. The overnight culture was diluted to 100-fold in fresh nutrient-rich (LB) medium and minimal (M9) medium supplemented with 100 μg/ml of ampicillin and incubated at 37°C in an orbital shaker at 225 rpm until the optical density at 600 nm (OD_{600}) reached 0.5. The cells were then grown an additional hour in the presence of varying concentrations of $CuSO_4$ to evaluate the dynamics of the CusSR TCS. These cells were used for further studies.

Microscopic detection of pCGFP1 reporter plasmid

The $CuSO_4$-induced cells were harvested after 4 h of $CuSO_4$ induction by centrifugation at 3,500×*g* for 5 min at 4°C for analysis of the *cusC*-promoter-driven expression of GFP in M9 medium, washed with phosphate-buffered saline (PBS), and re-suspended in PBS supplemented with 0.3% of agarose. The cells were screened for fluorescence with a 100× objective on a reflected fluorescence microscope (Olympus, Japan) with a cooled charge-coupled device camera (B&W SenSys, KAF1401). Emission intensity was recorded by using MetaMorph image analysis software (Molecular Devices Inc., Sunnyvale, CA, USA) with excitation and emission filter sets optimized for EGFP imaging.

Investigation of the dynamic behavior of the pCGFP1 and pCSAL1 plasmids

The transcriptional activity of the two bacterial strains harboring pCGFP1 and pCSAL1 were estimated by the

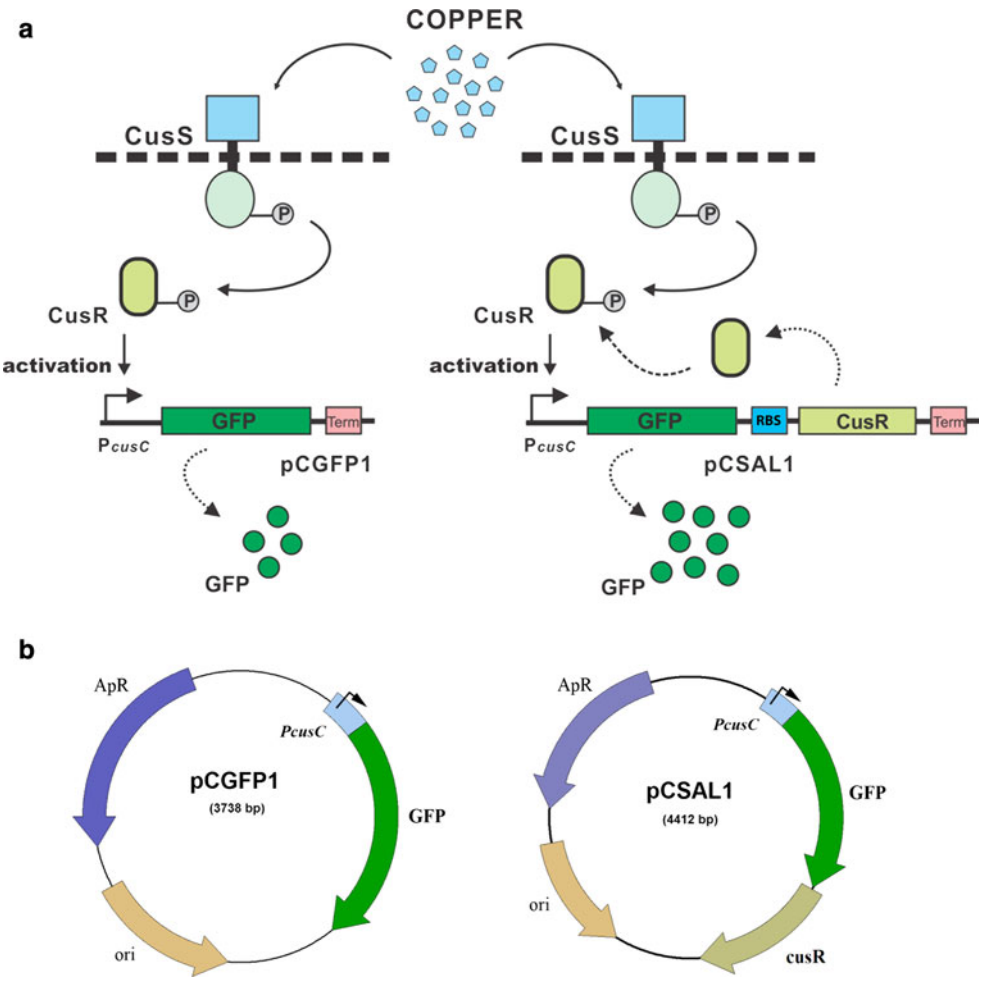

Fig. 1 Design of the TCS-mediated expression system. **a** In pCGFP1, the *cusC* promoter consists of a 156-bp sequence containing the operator sequence and is located just upstream of the green fluorescent protein, which is under the control of the CusSR TCS of *E. coli*. In pCSAL1, the *cusC* promoter drives the expression of the green fluorescent protein and CusR, a regulatory protein. CusR RR in turn drives the transcription of the GFP and CusR from the *cusC* promoter. **b** Plasmid-based transcriptional reporters for pCGFP1 and pCSAL1

Table 1 List of bacterial strains, peptides plasmids, and primers used in this study

Strain, plasmid, or primer	Relevant genotype and/or property	Source of reference
Escherichia coli strains		
XL1-Blue	SupE44 hsdR17 recA1 endA1 gyrA96 thi relA1 lac F'(proAB$^+$ lacI^q lacZΔM15 Tn10 (tet^R))	Laboratory stock
Plasmids		
pUC19	Ap^R	New England Biolabs[a]
pUCup1	pUC19 (156 bp *cusC*-cusR intergenic region), containing *CusC* promoter region, Amp^r	This work
pCGFP1	pUC19 (156 bp *cusC*-cusR intergenic region), *CusC'-gfp* transcriptional fusion vector, containing *CusC* promoter region, Amp^r	This work
pCSAL1	pUC19 (156 bp *cusC*-cusR intergenic region), *CusC'-gfp cusR* transcriptional fusion vector, containing *CusC* promoter region, CusR regulatory protein, Amp^r	This work
Oligonucleotide primers		
*cusC*_FEcoRI	5′-GAATTCATTTCCTCCGCATGTTGCCCG–3′	
*cusC*_RsacI	5′-GAGCTCAGGCTCATAATTTCTGGTGA-3′	
*GFP*prt_FsacI	5′-GAGCTCATGAGTAAAGGAGAAGAACTTTTC-3′	
*GFP*prt_RKpnI	5′-GGTACCCCTTAGCTCCTGAAAATCTCG-3′	
*cusR*_FKpnI	5′-GGTACCAAGAAGGAGATATACCATGAAACTGTTGATTGTCGA-3′	
*cusR*_RBamHI	5′-GGATCCTTATTACTGACCATCCGGCA-3′	

[a] New England Biolabs, Beverly, MA, USA

measurement of GFP fluorescence of cells grown in M9 medium containing various ranges of $CuSO_4$ at different time intervals. Cell growth was monitored by the measurement of the optical density at 600 nm with a spectrophotometer (Shimadzu, Japan). The fluorescence of the GFP-producing cells that were grown in culture was measured using a RF-5301PC spectrofluorometer (Shimadzu, Japan). The excitation wavelength of the spectrofluorometer was set at 490/10 nm, and the emission wavelength was set at 510/10 nm. *E. coli* carrying pCGFP1 without the promoter and the operator of the *cusC* gene was used as the baseline sample to zero the instrument. The specific fluorescence intensity (SFI) is used as a raw fluorescence intensity expressed in relative fluorescence units divided by the optical density at 600 nm measured at each time point. A minimum of three measurements were obtained for each sample.

CuSO4-induced cells were prepared using the protocol described above. The mixture was incubated at 4 h in an orbital shaker at 225 rpm at 37°C. Optical density and fluorescence were measured at varying copper concentrations (1–1,000 μM). At least three measurements were obtained for each sample.

Model-based computational simulation

Numerical simulations were run using Matlab 2010b (MathWorks, Inc., Natick, MA, USA). A simple linear dynamic model was used to predict the promoter activity as a function of the copper concentration. The model was slightly modified to include the basal expression level observed in the absence of copper. Let G represent the average number of GFP proteins per cell. The rate of change of GFP (dG/dt) can be represented as:

$$dG(t)/_{dt} = [k * C(t) - (\Delta) * G(1)] + 28$$

where k is the translation rate constant, C is the concentration of copper used in the study (0–1 mM), delta is the dilution rate of GFP calculated for cells grown on glucose-containing minimal medium, and t represents the time of measurement. The translational rate constant with and without the loop was extracted from the rate of change of the GFP values that were measured experimentally.

Results

Construction of pCGFP1 and pCSAL1 plasmids

The promoter of *cusC* gene was cloned upstream of the *gfp* gene to express GFP in order to respond to environmental copper (Fig. 1b). The *cusC* promoter is tightly governed by environmental signals that are transmitted through the CusSR TCS. The positive feedback loop was constructed by cloning the *cusR* gene downstream of the *gfp* gene of the pCGFP1 plasmid (Fig. 1b). The recombinant bacterial strains behave like normal bacteria until they come in contact with copper. At the time of detection of copper by the bacterial strains harboring pCGFP1 and pCSAL1, the synthesis of the reporter protein is activated, and the strains are consequently transformed into fluorescent bacteria. Unlike *E. coli* (pCGFP1), continuous and amplified fluorescent protein is expected in recombinant bacteria with the positive feedback loop (Fig. 1a).

The responses of the pCGFP1-harboring strains to copper were examined by fluorescence microscopy in minimal medium. Copper-exposed strains appeared green, indicating expression of GFP under the control of the CusSR system, whereas cells that are not exposed to copper ions failed to emit fluorescence (Fig. 2). This indicates that pCGFP1 plasmid was under the control of the *cusC* promoter and was activated in the presence of copper.

Expression profile study

As shown in Fig. 3, we performed calibration response curves of the cells harboring pCGFP1 and pCSAL1 strain in a varying concentration of Cu^{2+}. Fluorescence increased with metal concentration up to 1 mM, the resulting increase in the fluorescence was not linear, which may be due to the toxicity of copper on the bacterial cells. A calibration curve for the pCGFP1strain (1–1,000 μM, $R^2 = 0.974$) and pCSAL1 strain (1–100 μM, $R^2 = 0.997$) was obtained based on a linear relationship between the fluorescence intensity and the amount of metal added to the culture media (Fig. 3b, c). The limits of Cu^{2+} detection by the engineered bacteria harboring pCGFP1 and pCSAL1 strain, i.e., the lowest Cu^{2+} concentration that produce a detectable increase in fluorescence compared to the background was determined using the mean blank values plus three times standard deviation were 38.9 μM for pCGFP1 and 3.9 μM for pCSAL1 strain. The limit of quantification also determined using the mean blank values plus ten times standard deviation were 118 μM for pCGFP1 and 12 μM for pCSAL1 strain.

A detailed analysis of the responses of the pCGFP1- and pCSAL1-harboring strains to various concentrations of copper was conducted (Fig. 3a). The expression profiles exhibited two interesting features. First, the sensitivity of pCSAL1-harboring strains for copper increases to 7.5-fold when compared to the pCGFP1 strain. Second, the quantification limit of the pCSAL1-engineered strain was ten-fold higher than that of pCGFP1. These results suggested that the weak signal induced by 3.35 μM of copper was amplified through the positive feedback loop to express a

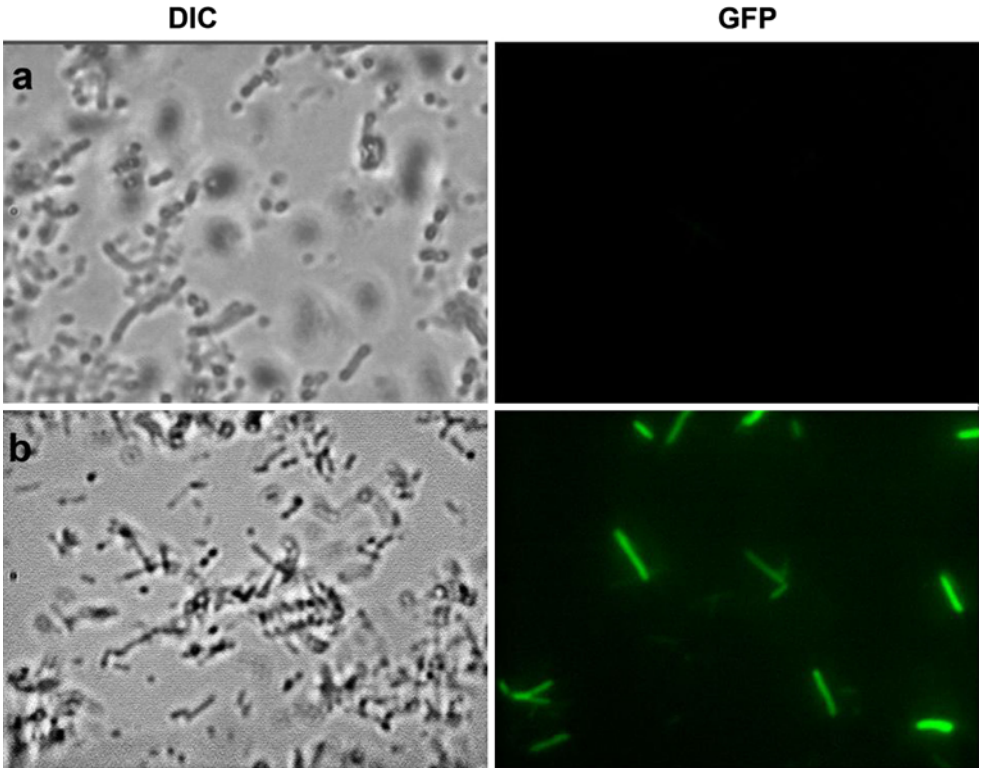

Fig. 2 Color imaging of cells harboring pCGFP1. pCGFP1 exposed to 500 μM of Cu^{2+} and its induction expressing GFP, as imaged by an Olympus reflected fluorescence microscope equipped with a Peltier-cooled CCD camera (Cool Snap, Roper Scientific, Tucson, AZ, USA). Shown are the cells unexposed to Cu^{2+} (**a**) and the cells exposed to Cu^{2+} (**b**)

greater significant amount of GFP and this strain showed higher copper sensitivity.

To understand the kinetic response characteristics, the time-dependent GFP expression profiles of the recombinant strains harboring the pCGFP1 and pCSAL1 plasmids were monitored (Figs. 4 and 5). In the recombinant *E. coli* strain harboring pCGFP1, the maximum fluorescence was observed after 4 h of incubation at higher copper concentrations (500–1,000 μM). At 1,000 μM of copper concentration, fluorescence was decreased, whereas at 500 μM, fluorescence remains saturated with additional time. In case of cells exposed to lower copper concentration (50 and 100 μM) continuously increased their fluorescence until 8 h. In *E. coli* harboring pCSAL1, fluorescence continuously increased for 8 h until it reached a maximum value even at a higher concentration of copper induction, which is approximately twofold higher than that obtained with *E. coli* (pCGFP1). A maximum SFI of 340 was obtained with *E. coli* (pCGFP1), while a SFI of 732 was obtained with the recombinant *E. coli* (pCSAL1) at a 1,000 μM copper concentration. This result indicated that a higher fluorescence output signal can be achieved by employing a positive feedback loop (introduction of CusR regulatory gene within the signal transduction network). Here we need to quantify the cellular CusR concentration of both cells harboring pCGFP1 and pCSAL1 by quantitative real-time PCR in order to compare the efficiency of pCSAL1 in producing intensifying output signal. The result suggested that the transcript level of the CusR gene significantly increased in cells grown at low and high Cu^{2+} concentrations in pCSAL1 strain. In comparison to fluorescence data, a similar pattern of *CusR* gene expression was observed in real-time PCR analysis at different time intervals of copper induction (Fig. S1).

In addition, the maximum obtained fluorescence values were maintained in *E. coli* (pCSAL1), whereas this did not occur in *E. coli* (pCGFP1) (Fig. 5). This result suggested that the *cusC* promoter-inducing signal was prolonged even after the disappearance of the environmental signal by the function of the positive feedback loop as the inducer was produced continuously once the positive feedback loop was activated.

Mathematical analysis: model and dynamics

Mathematical model analysis was carried out to understand the dynamic behavior of the CusSR-mediated *cusC* promoter with and without the positive feedback loop. A simple linear dynamic model was employed to simulate the dynamic behavior of *cusC* promoter in response to

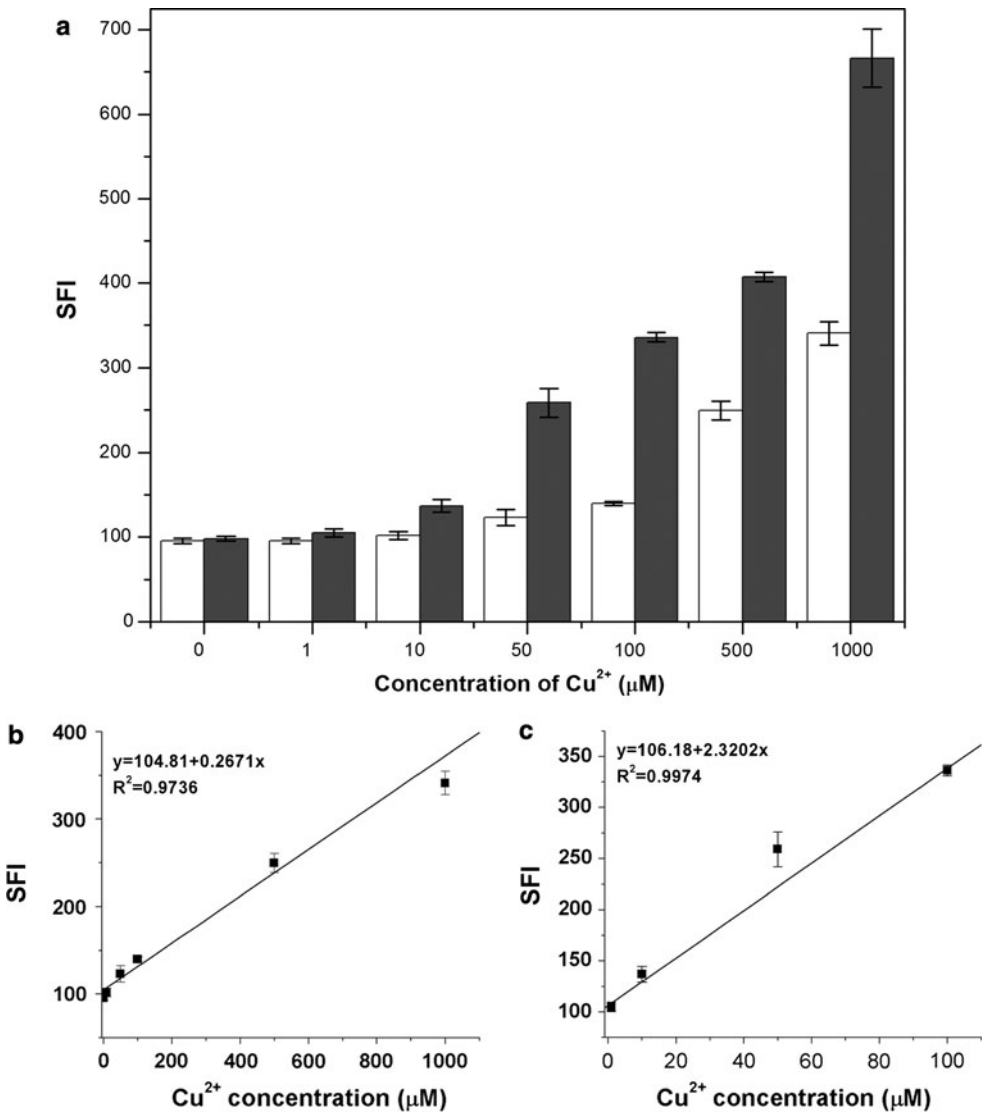

Fig. 3 **a** The dependence of the *cusC* promoter on its cognate response regulator was determined by measuring its activity while varying the concentration of Cu^{2+} in a cell harboring the pCGFP1 (*square*) and pCSAL1 (*filled square*) strains after 4 h. Cell harboring the pCGFP1 (**b**) and pCSAL1 (**c**) strains dose–response to Cu^{2+} under optimum condition and its linear calibration plot. Fluorescence response versus metal concentration. *Error bars* are the standard deviation from the mean of three experiments. Specific fluorescence intensity [SFI] measured with a fluorometer is defined as the culture fluorescence divided by the optical density (600 nm) of the culture (OD_{600} = 0.8–0.9)

environmental copper [1]. In the simple linear dynamic model, variation in copper concentration does not affect the dynamic behavior of the model but affects only the steady-state values, which indicates the level to which the network is activated. Therefore, the model of the pCGFP1 network predicts that, for any concentration of copper, the network will activate CusSR TCS with the same dynamics, possibly reaching different steady-state levels.

The best-fit random parameterization strategy was applied to estimate the translation rates (k) of the *cusC* promoter, based on the fluorescence data of *E. coli* (pCGFP1) and *E. coli* (pCSAL1) (Fig. 6). The fluorescence data obtained with three different copper concentrations were used: no stimulus (0 μM), low (10 μM), and high stimulus (500 μM). All other parameter values had nominal values chosen as described earlier. In the case without the positive feedback loop, the translation rate (k) of 45 showed the best fit with the experimental data obtained with *E. coli* (pCGFP1). The best-fitted translation rate (k) of the *cusC* promoter with the positive feedback loop was increased up to 150. As expected, the predicted translation rate (k) was found to be threefold increased by the addition of the positive feedback loop. The expression profiles of GFP with the estimated translation rates (k) are presented in Fig. 6.

Discussion

Bacterial two-component sensory systems respond to multiple environmental stimuli by sensing molecules of quorum-sensing, ions, drugs, and physicochemical stresses on membrane proteins [2, 14]. Bacterial TCSs have been considered promising candidates for use as bacterial biosensor systems [16]. To be applied as a biosensor system, the sensitivity and response dynamics of the TCS need to

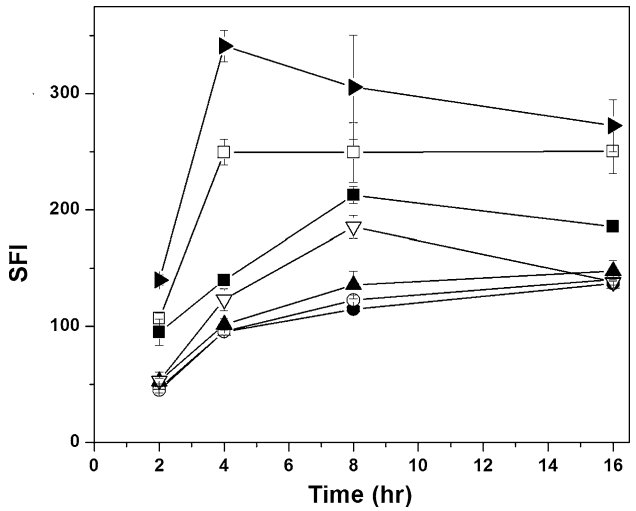

Fig. 4 Activation kinetics of fluorescence protein by pCGFP1. Cells harboring pCGFP1 were treated at $t = 0$ with different concentrations of copper in M9 minimal media: 0 μM (*closed circle*), 1 μM (*open circle*), 10 μM (*closed triangle*), 50 μM (*inverted open triangle*), 100 μM (*closed square*), 500 μM (*open square*), and 1,000 μM (*right-pointing closed triangle*). The fluorescence intensity was followed for up to 16 h. The fluorescence intensity in untreated cells did not yield any significant activation. The data are the aggregate results of copper treatments in replicate experiments ($n = 3$)

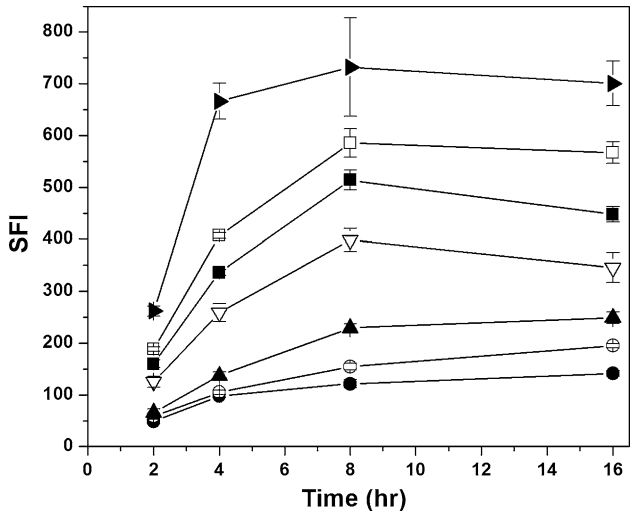

Fig. 5 Activation kinetics of fluorescence protein by pCSAL1. Cells harboring pCSAL1 were treated at $t = 0$ with different concentrations of copper in M9 minimal media: 0 μM (*closed circle*), 1 μM (*open circle*), 10 μM (*closed triangle*), 50 μM (*inverted open triangle*), 100 μM (*closed square*), 500 μM (*open square*), and 1,000 μM (*right-pointing closed triangle*). Fluorescence intensity was followed for up to 16 h. The fluorescence intensity in untreated cells did not yield any significant activation. The data are the aggregate results from copper treatments in replicate experiments ($n = 3$)

be improved. In this study, we have demonstrated the effect of adding a transcriptional positive feedback loop that can amplify the output signal on the dynamic characteristics of the CusSR TCS of *E. coli* [18, 20]. In spite of the

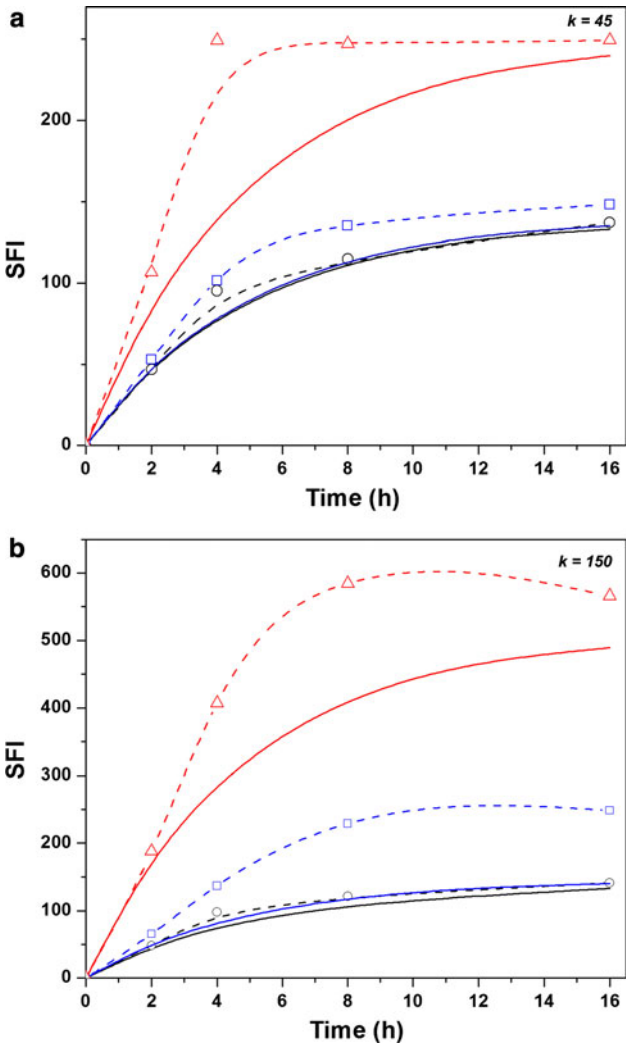

Fig. 6 Activation of *cusC* promoter for varying Cu^{2+} concentrations in a cell harboring pCGFP1 and pCSAL1 from experimental data and model predictions. The model predictions for the activation of fluorescence protein are shown (*solid line*). Experimental quantification of the activation of fluorescence protein is shown (*dashed line*). *Black*, *blue*, and *red lines* correspond to no stimulus, low, and high stimulus, respectively

tremendous theoretical research, there were only a few examples of the experimental demonstration of positive feedback loop using two-component systems [5].

The incorporation of a positive feedback loop resulted in three noteworthy modifications to the dynamic behavior of the *cusC* promoter. First, the CusSR TCS responds to lower concentrations of environmental copper [3]. At standard assay condition in the laboratory, the response of modified CusSR TCS network to Cu^{2+} ions increases with metal concentration (up to 1 mM) with a threshold for detection estimated in ~4 μM or 247.7 μg/l, while the native CusSR TCS network is activated ~39 μM or 2,476.5 μg/l of copper. Therefore, the sensitivity of the CusSR TCS network was increased tenfold by the function of the positive

feedback loop. Second, the intensity of the output signal increased [11]. For an efficient bacterial biosensor, a strong and precise output signal is required but, higher concentration of copper usually results in bacterial toxicity. By introducing the positive feedback loop, modified CusSR system can trigger strong fluorescence even at low concentrations of copper. In our study, the output signal was amplified by twofold compared to that obtained with the native CusSR TCS network. The correlation coefficient between the copper concentration and fluorescence was almost the same as that obtained with the native CusSR TCS network. Third, the duration of the fluorescence response was extended at least for higher concentration of copper in pCSAL1 strain [17]. Fluorescence increased until 8 h of incubation, and the maximum fluorescence value was maintained for the next 8 h.

To the best of our knowledge, this study provides the first example of introducing a positive feedback loop in CusSR bacterial two-component system. We believe that our strategy can be influential in optimizing the behavior of other signal transduction kinetics of TCSs. These studies have shown the feasibility of TCS-based biosensors for the detection of copper metals. However, like other biosensors, there remain challenges to deploying this class of sensors in the environment, and our results also serve as a good model of synthetic biology strategy applications in the field of bioengineering.

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