

# Modification of response behavior of zinc sensing HydHG two-component system using a self-activation loop and genomic integration

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Received: 16 July 2012 / Accepted: 2 October 2012 / Published online: 3 November 2012  
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**Abstract** Characterizing the dynamics of HydHG—a two-component transcriptional regulatory network for exogenous zinc in *E. coli*—is essential in understanding the biology of these regulatory and signaling pathways. Here, we used a synthetic biology strategy to modify the dynamic characteristics of the HydHG network in two ways. First, a self-activation loop for HydHG network was created under the control of *zraP* promoter, after which the threshold  $Zn^{2+}$  concentration for the self-activated HydHG network significantly decreased from 200 to 10  $\mu M$ . Second, the self-activation loop was integrated into the *E. coli* genome allowing the threshold  $Zn^{2+}$  concentration to be elevated to 500  $\mu M$ . As the threshold  $Zn^{2+}$  concentration could be modified in both directions, the introduction of a self-activation loop and the entire genomic integration strategy may prove useful for the creation of a two-component bacterial biosensor with varying sensitivities.

**Keywords** *Escherichia coli* · HydHG two-component system · *zraP* promoter · Zinc · Self-activation loop

## Introduction

The signaling of bacterial two-component systems (TCS) changes transcriptional programs in response to a variety of external factors that affect bacterial growth, including osmolality, temperature, and the concentration of other important extracellular molecules. Each TCS is composed of a membrane-located histidine sensor kinase and a cytoplasmic transcriptional regulator. When activated and autophosphorylated by ATP, the sensor transfers a phosphoryl group to the regulator, thereby increasing the binding between the regulator and DNA and ultimately modulating the expression of certain target genes [1, 2]. However, engineering the dynamic behavior of signal transduction networks like TCS is a challenging topic.

In recent years, a number of biological sensing systems have been constructed for a zinc-sensing purpose [3–7]. In *E. coli*, some of proteins involved in zinc homeostasis systems have been identified. Among those proteins, HydH and ZntR were found to respond better to zinc, through involvement in transcriptional regulation of zinc homeostasis genes. The ZntA–ZntR system, which is composed of the zinc efflux protein and zinc binding MerR-like transcriptional activator, respectively, detoxifies zinc but these transcriptional regulators are unable to sense zinc in the environment [8, 9]. HydHG, a two-component membrane associated sensor kinase system in *E. coli*, can sense extracellular zinc [10].

Self-activation loops are regulatory elements found in bacteria, fungi, plants, and archaea that modulate certain variables of gene expression output such as response timing, response level, and genetic noise [11–13]. Accordingly, the introduction of self-activation loops may play a defining role in further understanding of the dynamic behavior of signal transduction networks, a topic that has remained largely unexplored. In order to construct an efficient biosensor, the

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output signal must be inducible at various concentrations of target molecule. Here, we intend to utilize the HydHG TCS integrated with a self-activation loop as a zinc-sensing biosensor. Since the HydHG system responds preferentially to higher concentration of  $Zn^{2+}$  [10], there remains a limit on using this bacterial TCS. For  $Zn^{2+}$  of low concentrations, the HydHG TCS system is not efficient. In this study, we aim to construct a HydHG-based zinc sensor functional at varying sensitivities by introducing a rational regulatory network. First, the dynamic behavior of the *E. coli* HydHG TCS was monitored, after which an inducible positive transcriptional self-activation loop network was introduced to induce HydG activation at low concentrations of  $Zn^{2+}$  (Fig. 1). Second, a self-activation loop was integrated into the *E. coli* genome to decrease the system sensitivity. Via this protocol, three separate types of zinc-sensing bacteria were created with differing threshold  $Zn^{2+}$  concentrations.

## Materials and methods

### Plasmid construction

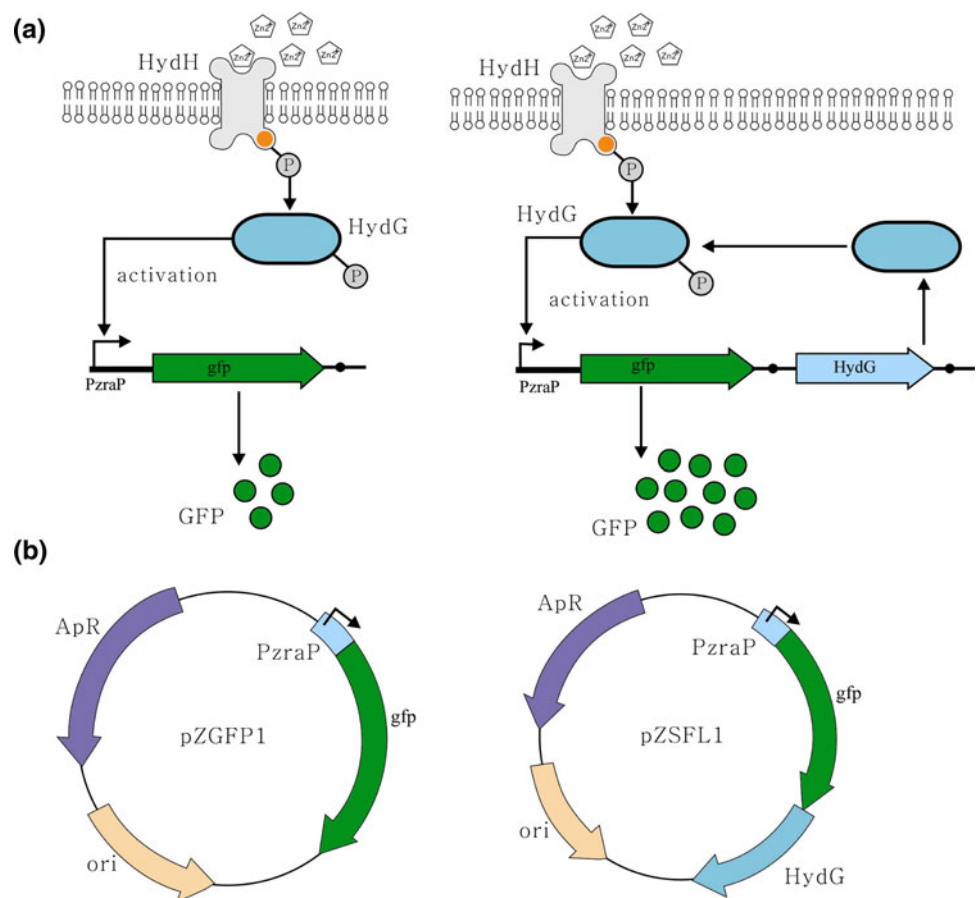
All bacterial strains and plasmids used in this study are listed in Table 1. In all cases, *E. coli* XL1-Blue was used as

the host strain for plasmid construction. To create a self-activation loop plasmid, a 237 bp *zraP* PCR fragment corresponding to the target promoter gene was generated with *zraP*prt\_FSacI and *zraP*prt\_RKpnI primers (Table 2). This particular fragment was then cloned into the pUC19 vector plasmid at the SacI/KpnI site, ultimately resulting in pUZup1 plasmid [14]. The second PCR fragment of the *gfp* gene coding for green fluorescent protein (GFP) was also amplified from the plasmid PROBE-NT' [15] with *GFP*prt\_FKpnI and *GFP*prt\_RBamHI primers (Table 2). This *gfp* gene was then ligated into pUZup1 at the KpnI/BamHI site to create the pZGFP1 plasmid (Table 1). The third PCR fragment of the *hydG* gene was also amplified from *E. coli* genomic DNA using *HydG*\_FBamHI and *HydG*\_RXbaI primers (Table 2), then digested with BamHI/XbaI and ligated into the pZGFP1 vector backbone, ultimately producing the pZSAL1 plasmid (Table 1). Both the pZGFP1 and pZSAL1 plasmids were transferred into the *E. coli* XL1-Blue [16].

### Genomic integration

The chloramphenicol resistance gene was inserted upstream of the *zraP* promoter in the opposite direction to create the pZSAL1-C plasmid (Table 1). A pKD46 plasmid

**Fig. 1** Design of the TCS-mediated expression system. **a** In pZGFP1, the *zraP* promoter consists of a 237 bp sequence containing the operator sequence, located immediately upstream of the green fluorescent protein, and is under the control of the *zraSR* TCS of *E. coli*. In pZSAL1, the *zraP* promoter drives the expression of green fluorescent protein (GFP) and HydG, a regulatory protein. HydG in turns drives the transcription of GFP and HydG by the *zraP* promoter. **b** The plasmid-based transcriptional reporters for pZGFP1 and pZSAL1



**Table 1** Bacterial strains and peptide plasmids

Strain/plasmid	Relevant genotype/property	Reference source
<i>Escherichia coli</i> strains		
XLB 1	SupE44 hsdR17 recA1 endA1 gyrA96 thi relA1 lac F' (proAB <sup>+</sup> lacI <sup>q</sup> lacZΔM15 Tn10 (tet <sup>R</sup> ))	Laboratory stock
<i>E. coli</i> Z-SAL	SupE44 hsdR17 recA1 endA1 gyrA96 thi relA1 lac F' (proAB <sup>+</sup> lacI <sup>q</sup> lacZΔM15 Tn10 (tet <sup>R</sup> ))cm <sup>R</sup> <i>zraP</i> GFP HydG	This work
Plasmids		
pUC19	Ap <sup>r</sup>	New England Biolabs <sup>a</sup>
pKD46	<i>AmpR</i> , <i>beta</i> , <i>exo</i> , <i>gam</i> (lambda red), <i>orits</i>	New England Biolabs <sup>a</sup>
pUCup1	pUC19 (273 bp <i>zraP</i> -HydG intergenic region), containing <i>zraP</i> promoter region, Amp <sup>r</sup>	This work
pZGFP1	pUC19 (273 bp <i>zraP</i> -HydG intergenic region), <i>zraP'</i> - <i>gfp</i> transcriptional fusion vector, containing <i>zraP</i> promoter region, Amp <sup>r</sup>	This work
pZSAL1	pUC19 (273 bp <i>zraP</i> -HydG intergenic region), <i>zraP'</i> - <i>gfp</i> <i>HydG</i> transcriptional fusion vector, containing <i>zraP</i> promoter region, HydG regulatory protein, Amp <sup>r</sup>	This work
pZSAL1-C	pUC19 (273 bp <i>zraP</i> -HydG intergenic region), <i>zraP'</i> - <i>gfp</i> <i>HydG</i> transcriptional fusion vector, containing <i>zraP</i> promoter region, HydG regulatory protein, Amp <sup>r</sup> , Cm <sup>r</sup>	This work

<sup>a</sup> New England Biolabs, Beverly, MA, USA

**Table 2** PCR primers

Oligonucleotide primers	
Name	Sequence
<i>zraP</i> _FSacI	5'-GAGCTCGGTTAATCCTCCAGTGGTTGTC-3'
<i>zraP</i> prt_RKpnI	5'-GGTACCCTTCTTCTTTGCTGCTCATCCC-3'
<i>GFP</i> prt_FKpnI	5'-GGTACC ATGAGTAAAGGAGAAGAAGACTTTTC-3'
<i>GFP</i> prt_RBamHI	5'-GGATCCCCTTAGCTCCTGAAAATCTCG-3'
<i>HydG</i> _FBamHI	5'-GGATCCAAGAAGGAGATATCC ATGACGCACGATAATATCGA-3'
<i>HydG</i> _RXball	5'-TCTAGACTAACGCGACAGTTTTGCCA-3'
<i>CmR</i> _FSacI	5'GAGCTCTACCTGTGACGGAAGATCAC-3'
<i>CmR</i> _REcoRI	5'GAATTCTTACGCCCCGCCCTGCCACT-3'
ZSAL_F	5'TGGCAAACCTCTGATTCTGTGCTGGCAACCCAAC GAGAAAACGTTCTGATTACGCCCCGCCCTGCCACT-3'
ZSAL_R	5'-GCGCTTAACCATTTAGCTAAGGAGTCTTTAGAACG TTGCATAAAACGCATCTAACGCGACAGTTTTGCCA-3'

with the bacteriophage red operon (containing the genes *exo*, *beta*, and *gam*) was used to disrupt a gene in the *zraP* promoter region of the *E. coli* XL1-Blue chromosome [17]. Recombinant *E. coli* XL1-Blue harboring pKD46 was cultured at 30 °C, with recombinases induced via the introduction of L-arabinose (1 mM) when cultures were at an optical density at 600 nm (OD600) of 0.4. Electro-competent cells were also prepared using the standard protocol. A homologous recombination gene was amplified by PCR using ZSAL\_F and ZSAL\_R primers and the pZSAL1-C plasmid as a template (Table 2). After homologous recombination transfer into the *E. coli* XL1-Blue, colonies were selected from LB agar plates using the

appropriate antibiotic. Successful gene replacement with the antibiotic marker was then verified by PCR.

*zraP*-promoter-driven GFP expression analysis

Samples from the culture were obtained after 2 h of induction, washed with phosphate-buffered saline (PBS), and re-suspended in PBS supplemented with 0.3 % of agarose. A reflected fluorescence microscope with a 100× objective (Olympus, Japan) was used for cell fluorescence screening.

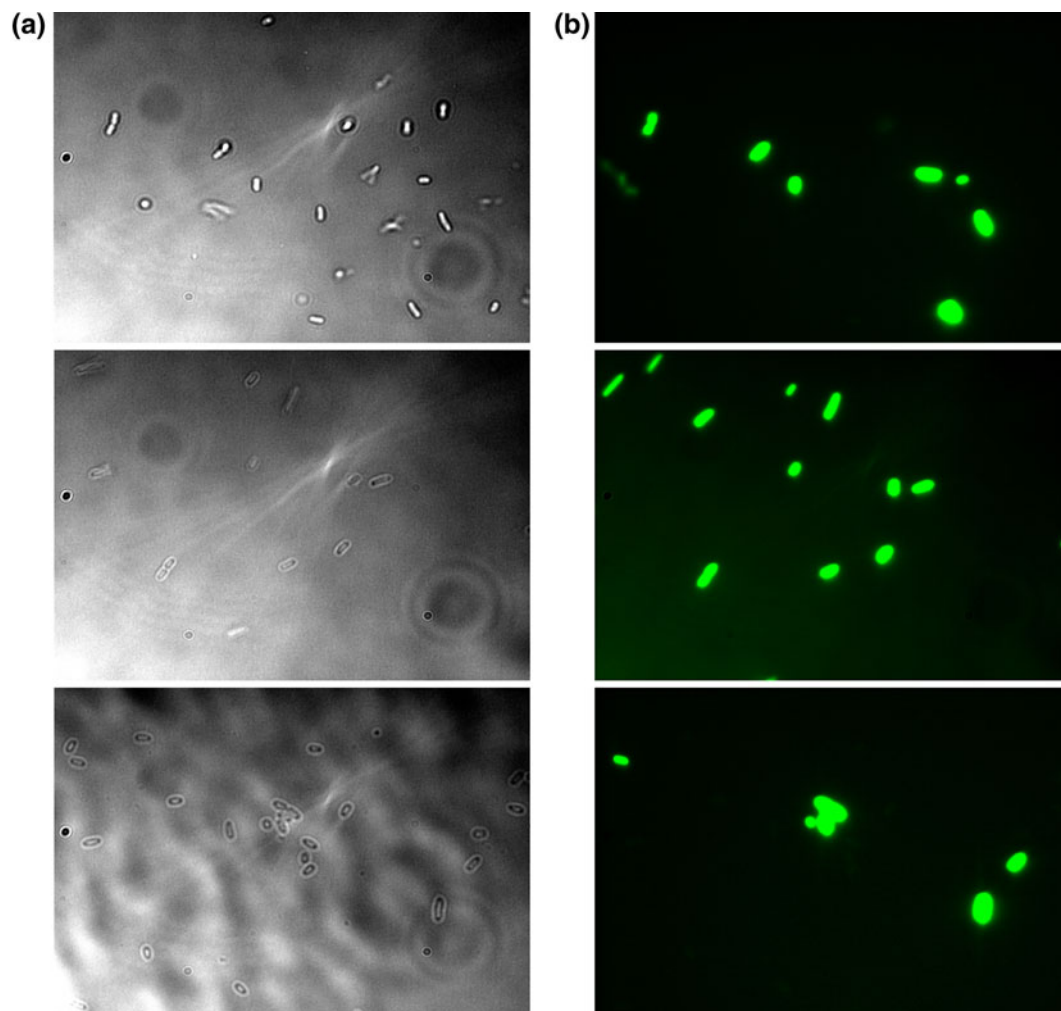
Dynamic behavior characterization of the pZGFP1, pZSAL1, and Z-SAL *E. coli* strains.

Unless otherwise stated, Luria–Bertani (LB) media was used as a liquid culture for transcriptional response studies. All bacterial strains were grown in 200-ml Erlenmeyer flasks containing antibiotics (ampicillin 100 mg/L, chloramphenicol 25 mg/L) at 37 °C under constant rotary shaking (225 rpm). For all induction experiments, GFP levels were quantified using a RF-5301PC spectrofluorimeter (Shimadzu, Japan) with an excitation wavelength of 490/10 nm and an emission wavelength of 510/10 nm. *E. coli* strain harboring the pUC19 plasmid was used as a control to calibrate the instrument prior to all measurements. A UV/vis spectrophotometer (Shimadzu, Japan) was used to measure the optical density (OD) of samples at 600 nm. The specific fluorescence intensity (SFI) was calculated using the raw fluorescence intensity expressed in relative fluorescence units at an optical density of 600 nm. At least three measurements were obtained for each sample.

## Results

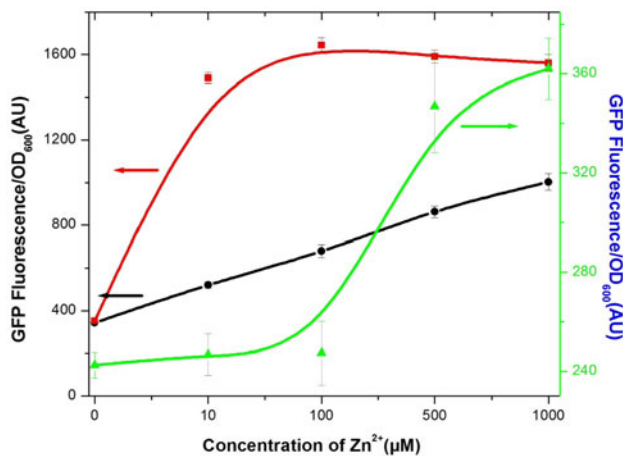
### Engineering bacteria with a self-activation loop

The *E. coli* HydHG TCS was engineered to respond to different threshold  $Zn^{2+}$  concentrations, with the threshold level reduced via the introduction of a self-activation loop and elevated by genomic integration. To create a self-activation loop, the *hydG* gene was cloned downstream of the *gfp* gene and its expression was regulated by a *zraP*-responsive promoter (Fig. 1b, pZSAL). If phosphorylated HydG induced the *zraP* promoter, then HydG was expressed in addition to GFP, thereby further activating the *zraP* promoter. As such, activation of the above network would allow for self-induction, thus further amplify the resulting output signal (Fig. 1a). A feedback activation loop system was then integrated into the *E. coli* genome using homologous



**Fig. 2** Color imaging of cells harboring pZGFP1, pZSAL1 and *E. coli* Z-SAL exposed to 100  $\mu$ M of  $Zn^{2+}$ . 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 before (a) and after exposure to  $Zn^{2+}$  (b)

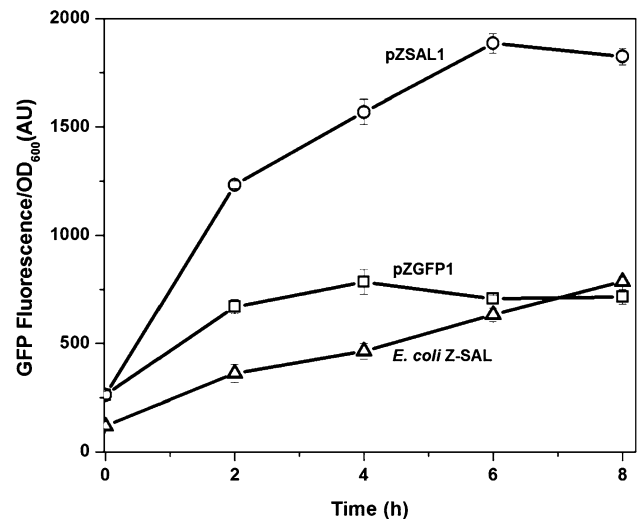


**Fig. 3** The relative dependence of the *zraP* promoter on its cognate response regulator was quantified by measuring its activity at varying concentrations of  $Zn^{2+}$ . The *E. coli* harboring pZGFP1 (black, closed circle), pZSAL1 (red, closed square), and Z-SAL strains (green, closed triangle). The error bars indicate the standard deviation from the mean for the three experiments. The SFI as measured by fluorometer is defined as the culture fluorescence divided by the culture at an optical density of 600 nm (color figure online)

recombination. Using this protocol, three different types of engineered cells were created: cells harboring pZGFP1 (no self-activation network), pZSAL1 (self-activation network) and *E. coli* Z-SAL (genomic integration network). Notably, these recombinant bacterial strains with self-activation loops behave like normal bacteria until they are exposed to  $Zn^{2+}$ . In the presence of  $Zn^{2+}$ , the reporter protein synthesis is upregulated and the strains are consequently transformed into fluorescent cell (Fig. 2).

#### Dynamic behavior monitoring

The two recombinant plasmids and the strain described above were cultured in varying concentrations of  $Zn^{2+}$  (Fig. 3). All the cells were under the control of the *zraP* promoter, and were activated at different sensitivity according to the  $Zn^{2+}$  concentration. The *E. coli* (pZGFP1) fluorescence increased in proportion to the  $Zn^{2+}$  concentration between 10 and 1,000  $\mu M$ . However, the detection sensitivity was not sufficient for concentrations below 10  $\mu M$  of  $Zn^{2+}$ , as only small increases in GFP fluorescence occurred. In comparison to *E. coli* (pZGFP1), the output fluorescence of the *E. coli* (pZSAL1) increased 3-fold when exposed to  $Zn^{2+}$  concentrations as low as 10  $\mu M$ . Accordingly, the bacterial sensitivity to low concentrations of  $Zn^{2+}$  was greatly enhanced by the introduction of a self-activation loop. The fluorescence did not significantly change in response to further increases of  $Zn^{2+}$  concentration ( $>10 \mu M$ ), indicating a fluorescence saturation at 10  $\mu M$  of  $Zn^{2+}$  that



**Fig. 4** The activation kinetics of the fluorescence proteins. Cells were treated at  $t = 0$  with concentrations of  $Zn^{2+}$  in LB media. Shown in this figure are: the *E. coli* (pZGFP1) strain exposed to 10  $\mu M$  of  $Zn^{2+}$  (open square), the *E. coli* (pZSAL1) strain exposed to 10  $\mu M$  of  $Zn^{2+}$  (open circle), and the *E. coli* Z-SAL strain exposed to 500  $\mu M$  of  $Zn^{2+}$  (open triangle). The fluorescence intensity was then followed for up to 8 h. The error bars represent the standard deviation from the mean for the three experiments. Specific fluorescence intensity as measured by fluorometer is defined as the culture fluorescence divided by the culture at an optical density of 600 nm

may have resulted from a maximum expression capacity of this engineered cell. Given these results, we conclude that the incorporation of a self-activation loop was a successful strategy for the construction of a fine zinc sensing apparatus. With regard to *E. coli* (pZSAL1), the threshold concentration for fluorescence was increased to 500  $\mu M$ , indicating a decrease in zinc sensitivity. These data suggest that genomic integration could successfully be employed for zinc-sensing bacteria over a larger range of  $Zn^{2+}$  concentrations.

To quantify the effect of the self-activation loop on the dynamic behavior of *zraP* expression, time-dependent fluorescence profiles of three strains were characterized (Fig. 4). The fluorescence of *E. coli* (pZGFP1) increased after 2 h of cultivation, after which it remained at a similar level for the following 6 h. The *E. coli* (pZSAL1) and *E. coli* Z-SAL fluorescence continued to increase over time, suggesting signal amplification after the introduction of the self-activation loop. Notably, the fluorescence of the *E. coli* Z-SAL strain was roughly half that of the *E. coli* (pZGFP1) strain after 2 h of induction. However, the *E. coli* Z-SAL fluorescence increased continuously, and ultimately surpassed that of *E. coli* (pZGFP1) after 8 h of induction. Such data clearly demonstrate that the signal-amplifying self-activation loop functioned as anticipated in vivo.

## Discussion

The zinc sensing behavior of HydHG TCS was modified via the introduction of a self-activation loop. After the incorporation of this self-activation loop, the signal was fully activated at 10  $\mu\text{M}$  of  $\text{Zn}^{2+}$ . In comparison to this low concentration of  $\text{Zn}^{2+}$ , the previously reported native HydHG TCS network was activated at a higher  $\text{Zn}^{2+}$  concentration of 200  $\mu\text{M}$  [10]. We also could reduce the sensitivity of our zinc sensor using *E. coli* Z-SAL strain, where the sensitivity was reduced by 50-fold since it was activated at 500  $\mu\text{M}$  of  $\text{Zn}^{2+}$  concentration. These results indicate that bacterial TCS sensitivity can be engineered via the introduction of synthetic genetic loops.

The strategy herein may also prove promising for the development of biosensors with varied sensitivities. *E. coli* (pZSAL1) produced a great signal even at 10  $\mu\text{M}$  of  $\text{Zn}^{2+}$  concentration, so possibly replacing the present complicated processes for quantifying  $\text{Zn}^{2+}$  of low concentration. The *E. coli* Z-SAL strain may be used as a simple biosensor recognizing the presence of zinc in severely contaminated sites with relatively high concentration of  $\text{Zn}^{2+}$ . In conclusion, our data demonstrated the feasibility of TCS-based biosensors for the detection of  $\text{Zn}^{2+}$  at various concentrations. The strategy also can be applied to optimize the signal transduction kinetics of other TCSs.

**Acknowledgments** This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2010-0012850).

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