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ORIGINAL RESEARCH PAPER

Optimizing cadmium and mercury specificity of CadR-based *E. coli* biosensors by redesign of CadR

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Abstract The metalloprotein, CadR, was redesigned to optimize cadmium and mercury specificity of CadR-based *E. coli* biosensors. By truncating 10 and 21 amino acids from the *C*-terminal extension of CadR, CadR-TC10 and CadR-TC21 were obtained, respectively. The genes *cadR*, *cadR-TC10* and *cadR-TC21* were used as sensing elements to construct green fluorescent protein based *E.coli* biosensors. Induction at 30 °C for 4 h in supplemented M9 medium was the optimized condition for the biosensor. Compared with CadR-based biosensor, there was a clear decline in induction coefficient for CadR-TC21-based biosensor (decreased by 86 % in Zn(II), 44 % in Hg(II), and only 37 % in Cd(II)). While in CadR-TC10-based biosensor, the induction coefficient decreased by 95 % in Zn(II), 70 % in Hg(II), and 67 % in Cd(II). Improved performances of CadR mutants based *E. coli* biosensors indicated that truncating *C*-terminal extension of CadR could improve the specificity.

Keywords Cadmium · CadR · Mercury · Specificity · Whole cell biosensor

Introduction

Cadmium is a non-essential heavy metal element to plants, animals and human beings. It is toxic, and can act as a cumulative poison in low doses (Haouem et al. 2007). Existing chemical and physical methodologies of cadmium monitoring can be accurate and sensitive, but provide data neither on its bioavailability nor its toxic effects on living systems. Therefore, various whole cell biosensors have been developed due to the possibility of monitoring bioavailability and biological effects (Gu et al. 2004; Yagi 2007; van der Meer and Belkin 2010).

Generally, a whole cell biosensor consists of a sensing element and a reporter element for monitoring, which are always genetically fused to construct a promoter-reporter biosensor system (Gu et al. 2004). The cadmium-sensing regulatory protein, CadR which is a transcription factor and regulates the cadmium resistance operon in Gram-negative bacteria, has been

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used as a component of sensing elements (Ivask et al. 2009; Wu et al. 2009). In these papers, the sensing elements were genes of *cadR* that specifically responded to the presence of Cd by encoding CadR that can activate the promoter of reporter genes, such as fluorescent proteins, and encode proteins that are easy to detect. The specificity and sensitivity of a whole cell biosensor mostly depend on the transcription factor and promoter used in its sensing element.

Other Cd(II) whole cell biosensors have been well constructed (Tauriainen et al. 1998; Ivask et al. 2002; Mandon et al. 2005; Park et al. 2007; Joe et al. 2012). However, experiments rarely have tested specificity and sensitivity of whole cell biosensors systematically.

In this study, the cadmium specificity and sensitivity, as well as the performance of optimized CadR-based biosensors, were investigated. CadR-TC10 and CadR-TC21 were redesigned by truncating 10 and 21amino acids from the *C*-terminal extension of CadR, respectively. Genes of *cadR*, *cadR-TC10* and *cadR-TC21* were used to construct the sensing element of the GFP based *E. coli* biosensors. Induction conditions were optimized with CadR-based biosensors. The specificity and sensitivity of the biosensors were also evaluated.

Materials and methods

Chemicals

Stock solutions of $CdCl_2$, $Pb(NO_3)_2$, $ZnSO_4$, $NiCl_2 \cdot 6H_2O$, $MnCl_2 \cdot 4H_2O$, $CuCl_2 \cdot 2H_2O$, $CoCl_2 \cdot 6H_2O$ and $HgCl_2$ were prepared with analytical grade chemicals in Milli-Q water.

Bacterial strains, culture conditions

Escherichia coli TOP10 (Invitrogen) was used as the host. The bacterial strains used are listed in Supplementary Table 1. Strains were cultured in M9 medium (0.6 % Na_2HPO_4, 0.3 % KH_2PO_4, 0.05 % NaCl, 0.1 % NH_4Cl, 0.025 % $MgSO_4 \cdot 7H_2O$, 0.001 % $CaCl_2$) supplemented with glucose at 0.1 % and Casamino acids at 0.5 %. LB medium (1 % Tryptone, 0.5 % yeast extract, 0.5 % NaCl) was also used to optimize induction conditions. All the plates and mediums contained 40 μg kanamycin/ml. Before induction with metal solutions, strains were cultured at 37 °C with shaking (220 rpm).

Plasmid construction

The genes *cadR* (AF333961), *cadR-TC10* (cutting off 30 nucleotides from the 3′-end of *cadR*) and *cadR-TC21* (cutting off 63 nucleotides from the 3′-end of *cadR*) were amplified by PCR from the vector pMD18-TR (synthesized by Sangon Biotech, Shanghai, China), which was constructed by which gene *cadR* inserted pMD18-T vector (Takara, Shiga, Japan), using the primer pairs as follows: CadR-R and CadR-F, TC10-R and CadR-F, TC21-R and CadR-F (Supplementary Table 2), respectively. After purification, PCR products were digested with *Eco*RI and *Hin*dIII and ligated with the *Eco*RI-*Hin*dIII digested larger fragments of pPROBE-NT (Miller et al. 2000) (AF286453) with *gfp* (Fig. 1a) as a reporter gene, yielding pNTCOG, pNTCOG-TC10 and pNTCOG-TC21, respectively. The recombinant plasmid products were transformed into *E. coli* TOP 10 using the $CaCl_2$ competent cell method, to yield COG, COG-TC10 and COG-TC21, respectively.

Induction and measurement of fluorescence intensity

For the test, the cultures in mid-growth phase ($OD_{600} = \sim 0.6$–0.8, see Supplementary Fig. 1) were diluted to $OD_{600} = 0.1$ (dry wt ~0.4 mg/ml) with supplemented M9 medium. Then, serial dilutions of metal solutions were added into a black 96-well ELISA plate (100 μl per well), and an equal volume of the bacterial suspension, prepared as above, was added. After induction, fluorescence intensity was measured with EnVision 2104 Multilabel Reader (Perkin Elmer).

Optimization of induction conditions

To achieve reasonable sensitivity of the biosensors, different conditions were tested using strains COG induced by serially diluted Cd(II) solutions. Cells were grown in LB medium and supplemented M9 medium for 2 h at 30 °C; then they were grown at 25, 30, 37 and 42 °C for 2 h with supplemented M9 medium. In addition, the effect of induction time was investigated

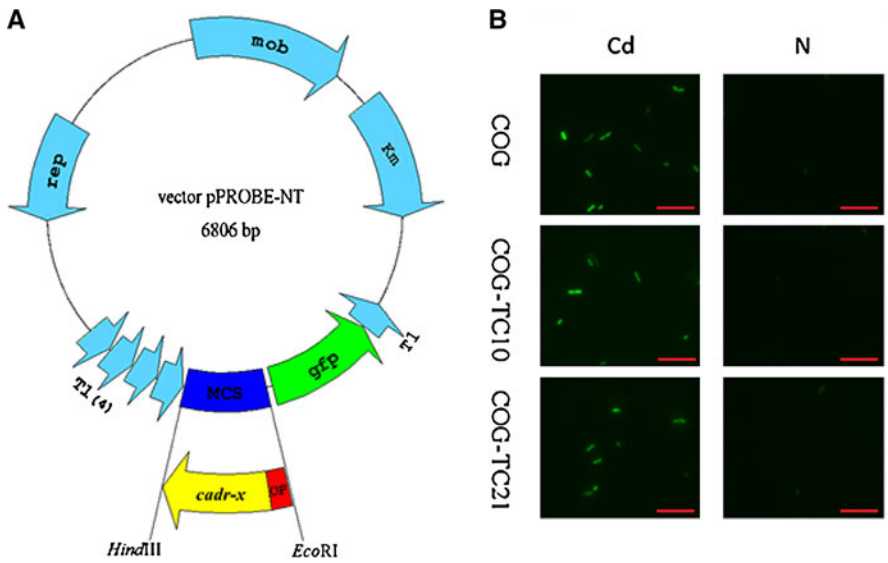

Fig. 1 **a** scheme of the sensor vectors construction. Vector pPROBE-NT was digested with *Eco*RI and *Hin*dIII. Fragments amplified by PCR with the primers described in Section 2,were purified, cut with *Eco*RI and *Hin*dIII, repurified and ligated to the pPROBE-NT digested fragment. Abbreviations used: *cadr-x* stands for *cadR*, *cadR-TC10* or *cadR-TC21*, genes encoding the regulative protein of the *cad* promoter; *OP*, the *cad* operator and promoter; MCS, the multicloning site; *gfp*, gene encoding green fluorescence protein; *T1*, *T1* terminator; *Km*, gene encoding kanamycin resistance; *rep*, genes required for replication; *mob*, genes required for mobilization; *T1*(4), four tandem copies of the *T1* terminator. **b** Green fluorescence image of the biosensor strain. After induction for 4 h at 30 °C in M9 supplemented medium, bacteria in liquid culture visualized microscopically and captured using an Axio Cam MRm camera (Zeiss, Jena, Germany) with a magnification of 400. Images in the first row, labeled Cd, induced by 100 μM Cd(II); in the second row, labeled N, by water. *Bars* = 5 μM

at 0.5, 1, 2, 3, 4 and 5 h at 30 °C with supplemented M9 medium.

Sensitivity and specificity test

The induction of fluorescence intensity was measured after 4 h at 30 °C with $CdCl_2$, $Pb(NO_3)_2$, $ZnSO_4$, $NiCl_2$, $MnCl_2$, $CuCl_2$, $CoCl_2$ and $HgCl_2$, ranging from 0.1 to 10 mM.

Data analysis

The responses of the biosensors were expressed as induction coefficient (*IC*), calculated by the following formula:

$$IC = F_i/F_b \quad (1)$$

where F_i is the fluorescence measured in metal solution, F_b is the mean background fluorescent (non-affected by added metals) value of the sensors, Milli-Q water was added to bacterial suspension instead of metal dilutions (4–6 replicates). Each sample was analyzed in triplicate. Two independent assays were performed for every strain.

The limit of determination in *IC* values (IC_{LOD}) by the biosensors was determined as follows:

$$IC_{LOD} = (F_b + 3SD)/F_b \quad (2)$$

where *SD* is standard deviation. The limit of determination (*LOD*) as μmol of metal per liter was the metal concentration corresponding to IC_{LOD} in the calibration curve (see Eq. 3).

The metal-dependent fluorescence-responses of the biosensors were fitted by a nonlinear regression using the Prism software package of GraphPad Software (Hakkila et al. 2011), calculated according to the following formula:

$$IC = \text{Bottom} + (\text{Top} - \text{Bottom})/(1 + 10^{((\text{LogEC50}-X)\times\text{Hillslope})}) \quad (3)$$

where *X* is the log value of metal concentration; Bottom is minimum effective concentration; Top is the maximum effective concentration; *EC50* is 50 % effective metal concentration for short; Hill slope describes the steepness of the curve.

Results

Constructed biosensor strains

CadR-TC10 was a truncated product of CadR by cutting off 10 amino acids (HSHVGRSHGH) from the *C*-terminal, a special histidine-rich region. For CadR-TC21, a further 11 amino acids were deleted from *C*-terminal, containing two conserved amino acids, G and S, with no identical function hitherto

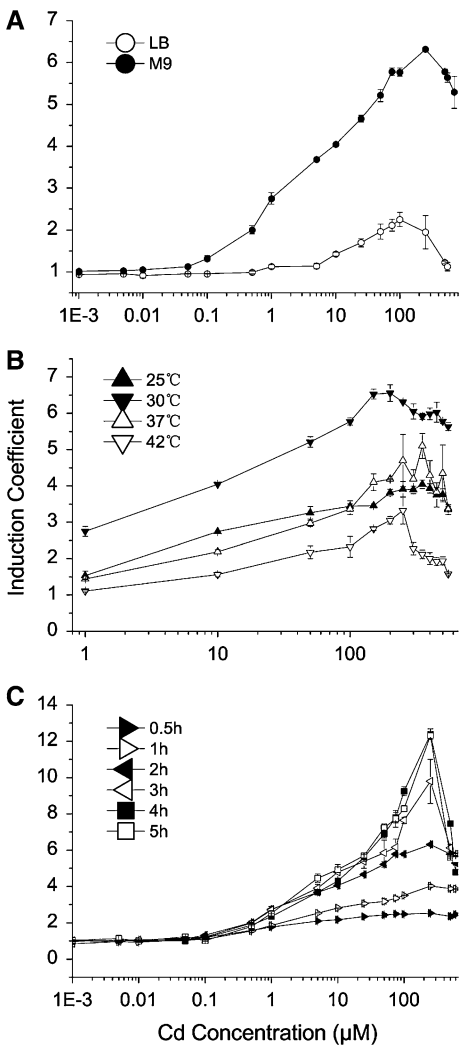

Fig. 2 Conditions optimization of COG inducting with cadmium. **a** The induction coefficients of media LB (open circle) and M9 supplemented with 0.25 % hydrolyzed casein (solid circle) being incubated at 30 °C for 2 h. **b** The induction coefficients under different induction temperatures with supplemented M9 medium for 2 h induction. **c** The effect of induction time, in supplemented M9 medium at 30 °C

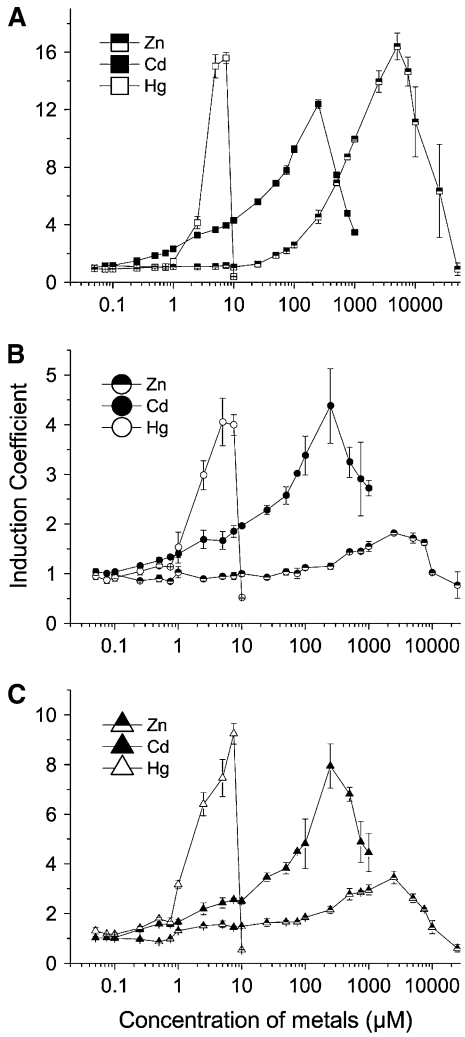

Fig. 3 Response curves of COG, COG-TC10 and COG-TC21 to different concentrations of Zn(II), Cd(II), Hg(II) after 4 h incubation at 30 °C. **a** COG. **b** COG-TC10. **c** COG-TC21

(Supplementary Figs. 2 and 3). The redesign of CadR avoided the introduction of amino acid changes that could reduce the functionality.

The genes *cadR*, *cadR-TC10* and *cadR-TC21* were cloned into the broad-host-range vector pPROBE-NT (Miller 2000) to construct three biosensors plasmids (pNTCOG, pNTCOG-TC10 and pNTCOG-TC21 as shown in Fig. 1a) in which CadR and CadR mutants controlled the expression of GFP via the *cad* promoter.

Recombinant strains expressed GFP in response to Cd(II) resulting in green fluorescence relative to that of strains without stimulant. A representative green fluorescence image of the biosensor strains is shown in Fig. 1b.

Table 1 Characteristic of COG, COG-TC10 and COG-TC21

Measurement and data analysis were done according to Eqs. 1, 2 and 3. EC_{50} the half-maximum response; *Hill slope* indicating the sensitivity of the response. *Span* is *Top* minus *Bottom*, describing the sensitivity and specificity of the response. The quality of fit is expressed as R^2 value

Stain	Metals	EC_{50} (M)	Hill slope	Span	R^2	LOD (μM)
COG	Zn	7.5×10^{-4}	1.2	15.6	0.9944	1.1
	Cd	2.1×10^{-5}	0.8	9.6	0.9175	2.0
	Hg	3.1×10^{-6}	6.3	14.6	0.9994	1.0
COG-TC10	Zn	4.0×10^{-4}	1.3	0.8	0.9578	0.9
	Cd	3.6×10^{-5}	0.7	3.2	0.9365	1.3
	Hg	2.0×10^{-6}	2.4	3.3	0.9958	1.6
COG-TC21	Zn	1.8×10^{-4}	0.7	2.2	0.9031	1.2
	Cd	4.0×10^{-5}	0.7	6.1	0.9024	1.5
	Hg	2.0×10^{-6}	1.9	8.2	0.9876	3.8

Optimization of induction conditions

The results are given in Fig. 2. The sensitivity was distinctly higher in supplemented M9 medium than in LB medium (Fig. 2a). Therefore supplemented M9 was used for culture of sensor strains in subsequent tests. 30 °C was the most suitable induction temperature (Fig. 2b) and was used in the experiments that followed. The optimum induction time was 4 h (Fig. 2c) and was therefore used in further experiments.

Sensitivity and specificity

Cultivation, measurement and data analysis were done according to the information in the Materials and methods section. There was no significant fluorescence of COG, COG-TC10 and COG-TC21 with $Pb(NO_3)_2$, $NiCl_2$, $MnCl_2$, $CuCl_2$ and $CoCl_2$ (data not shown). However, there were successive responses to $CdCl_2$, $ZnSO_4$, and HgC_{l2}. The results are given in Fig. 3 and further details are shown in Table 1 and in Supplementary Table 3 (Equations).

The maximum detection concentrations of Hg(II), Cd(II) and Zn(II) were 7.5, >250, >5000 μM, respectively. The lowest maximum detection concentration with a sharp decrease after maximum detection concentration indicated that Hg(II) had the highest toxicity of bacteria. Cd(II) was second. Zn(II) was the most moderate one.

There was no significant change of *LOD* induced by Zn(II) (Table 1). *LOD* of COG-TC21 responding to Hg(II) was higher than that of COG by 280 %, which indicated a declining sensitivity in the response of COG-TC21 and to Hg(II). Compared with COG, *LOD* of COG-TC10 responded to Cd(II) reduced by 35 %, at 1.3 μM.

CadR displayed an ultrasensitive response that rose more rapidly than the concentration increase that the inducer would predict (Hakkila 2011) to Hg(II) with a Hill slope of 6.3. But the sensitivity of CadR-TC10 and CadR-TC21 were lowered significantly (Table 1), which indicates that this repressor-to-activator transition was less efficient in CadR-TC10 and CadR-TC21 than in CadR. Therefore, COG-TC10 and COG-TC21 were less sensitive to Hg(II) compared with COG.

Spans (see Table 1) of COG were higher than that of COG-TC10 and COG-TC21 no matter which metal was induced (Table 1). While compared with COG's *Span*s, COG-TC21 showed a decline trend in Zn(II) induction by 85.6 %, 43.8 % in Hg(II) induction, and only 36.5 % in Cd(II) induction. Furthermore, response of COG-TC10 to Zn(II) fell to 0.8 in *span* while there was a significant drop of 95 % for COG, Cd(II) induction remained at 33 % of its original *span*, and Hg(II) at 30 % of its original *span*. Thus, we conclude that mutants showed a higher Cd(II) and Hg(II) specificity than COG.

Discussion

In this study, two mutant recombinant fluorescent bacterial strains were constructed by deleting *C*-terminal amino acids which exhibited an high specificity to Cd(II) and Hg(II), for decreases of 85.6 and 95 % in response to Zn(II), respectively. The results show that it was possible to improve the specificity of CadR while maintaining the protein's repressor/activator functions by truncating the *C*-terminus.

Zn, Cd, Hg are in the same group in which the elements have very similar properties. Therefore, CadR-based biosensors can detect them all. In

practice, Cd contaminations and always accompanied Zn pollution. So Cd specific biosensors with less or no response to Zn would be more accurate in Cd(II) detection. COG-TC10 and COG-TC21 displayed this characteristic which should have an application for detecting Cd(II) when no Hg(II) exists. Further research is though needed to construct exclusive biosensors in which only one ionic species is detected.

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