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Development of a pigment-based whole-cell biosensor for the analysis of environmental copper†

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Using engineered microorganisms to detect heavy metals in the environment has proven to be highly effective and robust. This paper reports on the development of a novel microbial sensor for the detection of copper ions. To develop this microbial sensor, we screened and characterized various biological parts, including promoters, output signals, and hosts. In addition, we used the plant pigment betaxanthin to output fluorescent signals in order to reduce the detection time. The resulting whole-cell biosensor presented a good sensitivity when detecting copper ions in environmental samples including freshwater pond and tap water.

Metal ions including iron, copper, zinc, and manganese play important roles as cofactors for enzymes involved in the catalysis of metabolic processes, and the maintenance of cell integrity, which is achieved through regulation by osmotic pressure.[1,2] These ions are ubiquitous in all organisms; however, heavy metals can be toxic at high intracellular concentrations.[3,4] For example, an excess of copper ions can cause protein dysfunction by interacting strongly with thiol groups and interfere with assembled iron–sulfur cofactors.[5] This has led many microorganisms to develop the ability to sense small variations in metal concentrations as well as the means to control the influx and efflux of metal ions.[1,6] Many molecular-based biosensors have been developed with high specificity, selectivity, and rapid reaction times for the detection of copper ions. However, these systems do not provide bioavailability of analytes.[7–9] Compared with molecular-based biosensors, whole-cell based biosensors have disadvantages such as slower response and lower sensitivity; however, their ability to measure activities of analytes in a physiologically relevant manner to directly determine the bioavailability of the analytes. *Cupriavidus metallidurans* CH34 is a bacterium which has developed resistance to high concentrations of many heavy metals in the environment in which it is found.[10,11] Therefore, *C. metallidurans* CH34 has recently been used in bioremediation processes and biosensors.[12–15]

In Gram-negative bacteria, there are three classes of copper-induced defence mechanisms including: (i) CueR-like activators,[16,17] (ii) CusRS-like two-component systems,[18–20] and (iii) TetR-like regulators.[21] *C. metallidurans* utilizes the CusRS-like two-component regulatory system, which includes the sensor kinase CopS and the response regulator CopR. The CopS autophosphorylates at a histidine residue and subsequently relays the phosphoryl group to the response regulator, CopR. CopR contains two functional domains: an N-terminal CheY-like receiver domain and a C-terminal DNA-binding helix-turn-helix output domain.[22] The phosphorylation of N-terminal domains induces conformational changes that affect the binding affinity between CopR and its recognition sites on chromosomal DNA.[19,23] Therefore, CopR activates the transcription of *cop* gene clusters in the presence of Cu(I)/(II) ions.

In *C. metallidurans*, the megaplasmid and the pMOL30 plasmids encode a variety of genes which confer resistance to the toxic effects of heavy metals such as Cd(II), Zn(II), Pb(II), Ag(I), Au(III), and Cu(II).[24–26] Two *cop* gene clusters (*i.e.* the *cop* clusters of pMOL30 and the megaplasmid) related to resistance mechanisms which protect against high copper concentrations. Under increasing concentrations of Cu(II), the second *cop* cluster on the pMOL30 regulated by CopSR, containing 19 ORFs (*copVTMKNSRABC-DIJGFLQHE*) organized in nine operons (Fig. S1A, ESI†), showed much greater upregulation by quantitative-PCR and microarray data compared with the gene clusters on the megaplasmid.[27] We therefore examined the regulations between CopSR and its regulators of pMOL30.

There were cop-based biosensors reported by Ng *et al.* and Leth *et al.*[28,29] However, the CopSR regulatory system from *C. metallidurans* was not characterized. Previously, we designed a MerR-type CupR regulatory circuit in *C. metallidurans* to generate a fluorescence-based microbial sensor for the selective detection of gold ions.[12] In current study, we employed a biosensor with a two-component regulatory system to detect bioavailable Cu(II). To optimize the performance of the Cu biosensor, we also examined the promoters of *cop* gene clusters and two reporter signals (red fluorescent proteins (RFP), and yellow fluorescent pigments, betaxanthin by expressing DOPA 4,5-dioxygenase from *Mirabilis*

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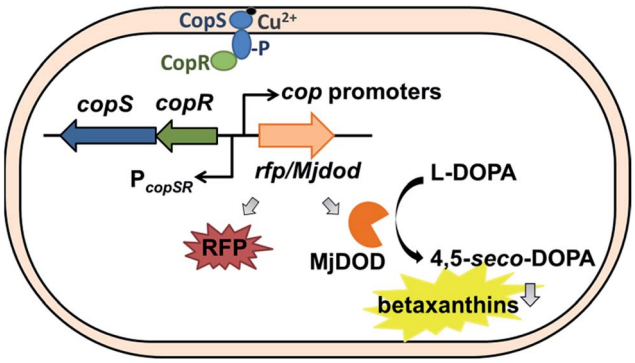

Fig. 1 Schematic diagram of the copper-induced *copSR*-based whole-cell biosensors in this study. CopSR regulatory system was expressed from it native promoter. Arrows indicate the direction of transcription and putative position of promoters. Upon detecting the Cu ions, the CopSR activates the cop promoters and the expression of *rfp* or *Mjdod* providing signal outputs.

jalapa plant (Mjdod)) (Fig. 1). The biosensor plasmid was subsequently transformed into two hosts: the original host and a *Ralstonia eutropha* strain, which does not have copSR homolog and is widely used in industry for biotechnology applications.[30,31]

We began by comparing the CopR regulated promoters at the transcription level with the aim of achieving the better sensitivity for the analysis of Cu(II) (Fig. S1A and B†). Eight promoters of cop gene cluster of *C. metallidurans* (P*copA*, P*copH*, P*copT*, P*copM*, P*copF*, P*copL*, P*copQ*, and P*copE*) were constructed in order to drive the expression of RFP, which allowed the protein expression of individual promoters to be examined quantitatively. Previous reports indicate that the CopR homolog in *E. coli*, PcoR, was able to bind to a copper box containing the 25 bp motif AgxTtACaxaAxTGTaATxaxxxxG.[32] We therefore searched for this motif in locations upstream from the transcription start codon of the cop gene cluster in *C. metallidurans*. However, the conservation of relatively weak consensus sequences in *C. metallidurans* suggests that the sequence recognition is species-dependent (Fig. S1C†).

Details as to the construction of the plasmids are provided as ESI.† CopSR sequences were introduced on the same plasmid (driven by its native promoter) to increase the number of copies of regulatory proteins that were produced (Fig. S1B†). Cells were then transformed and cultivated to exponential phase in lysogeny broth (LB) followed by a 24 h incubation with Cu(II) ions (ESI†). Following this, we quantified and compared the fluorescence of two hosts including *C. metallidurans* and *R. eutropha* cells in the presence of several copper concentrations (0, 10, 100, and 1000 μM) (Fig. S2†). We found that the fluorescence intensity of P*copA*, P*copH*, P*copT*, P*copM*, and P*copQ* promoters increased in conjunction with an increase in the concentration of Cu(II). Interestingly, we observed a similar pattern of induction for both hosts. Nonetheless, no significant induction was observed for p*copF*, P*copL*, and P*copE* promoters in the presence of Cu(II).

We then examined P*copA*, P*copH*, P*copT*. P*copM*, and P*copQ* promoters to determine their fluorescence induction dynamic range of *C. metallidurans* in the presence of Cu(II). As shown in Fig. 2A, for all five promoters, fluorescence increased as the concentration of Cu(II) was increased in the media. Specifically, we observed a linear relationship between fluorescence response and the concentration of Cu(II) within various linear ranges (Fig. 2B). Among the five promoters, the calculated detection limit of PcopT was the lowest (*i.e.*, presented the highest sensitivity) and PcopM, P*cop*A, P*cop*Q, and PcopH showed the second to the fifth lowest detection limits, respectively. Their limit of detection (LOD) was 24.3, 24.5, 99.4, 103.7 and 259.5 μM, respectively. Fig. 2B lists the details of linear regression, calculated LOD values, and the correlation coefficient (R^2) for each promoter. Table S4† showed a comparison between linear ranges obtained with other whole-cell based biosensors.

To distinguish among the various types of interference caused by different heavy metal salts, we monitored the fluorescence intensity of each promoter in the presence of Fe, Co, Ni, Zn, or Pb at 1 mM. Surprisingly, Zn(II) ions significantly induced RFP intensity for PcopH, PcopT, and PcopM at high concentration (Fig. 3). Unlike the other promoters, *copQ* promoter exhibited a Cu(II)-specific induction pattern. Therefore, we used PcopQ for all subsequent tests in this study.

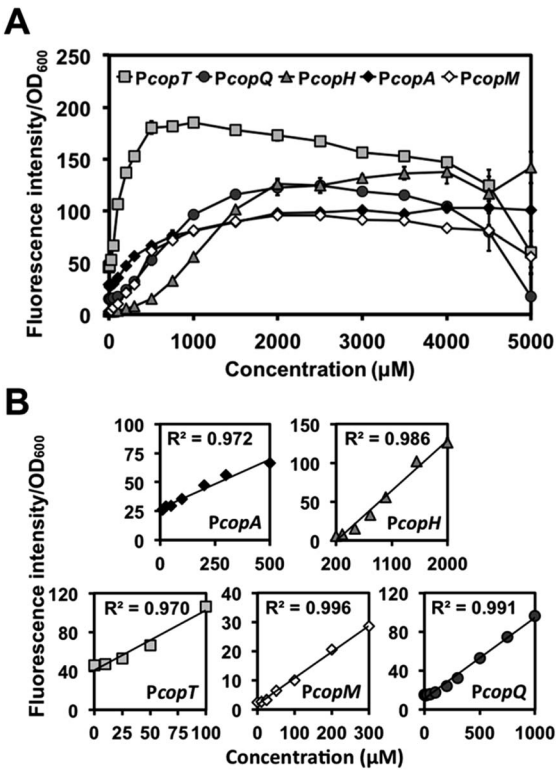

Fig. 2 Expression of RFP from copper-induced *C. metallidurans* cells harboring *copS-copR-PcopSR-cop promoter-rfp*. (A) Dose–response curves and (B) linear calibration curves for cells carrying PcopT, PcopQ, PcopH, PcopA and PcopM. The linear relationships of PcopA, PcopH, PcopT, PcopM and PcopQ were in the concentration range of 10–500 μM ($y = 0.085x + 27.158$, $R^2 = 0.972$), 200–2000 μM ($y = 0.072x - 15.000$, $R^2 = 0.986$), 0–100 μM ($y = 0.621x + 40.443$, $R^2 = 0.970$), 0–300 μM ($y = 0.090x + 1.737$, $R^2 = 0.996$), and 0–1000 μM ($y = 0.083x + 11.104$, $R^2 = 0.991$), respectively. Error bars which are smaller than data symbols represent the standard deviation from triplicate measurements.

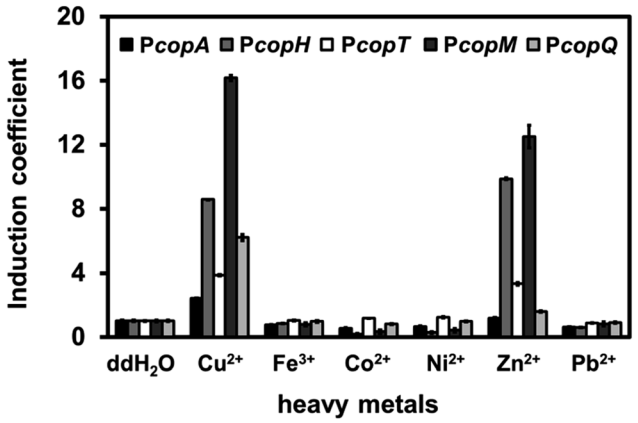

Fig. 3 Induction coefficient of *C. metallidurans* cells harboring *copS-copR-PcopSR-cop promoter-rfp* in response to various metal ions. All of the metal ions were used at the final concentration of 1 mM in LB medium. Error bars represent the standard deviation from triplicate measurements.

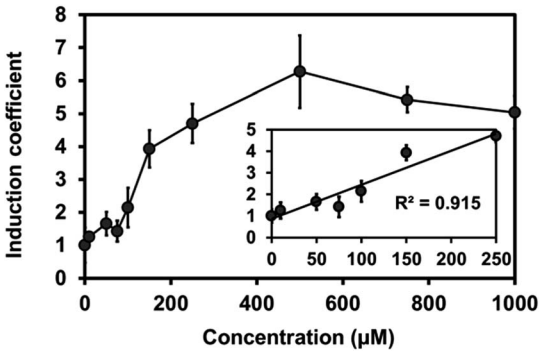

Fig. 4 Induction coefficient of copper-induced *C. metallidurans* cells harboring *copS-copR-PcopSR-PcopQ-Mjdod*. The linear relationship was in the concentration range of 0–250 μM ($y = 0.013x + 1.182$, $R^2 = 0.917$). Error bars represent the standard deviation from triplicate measurements.

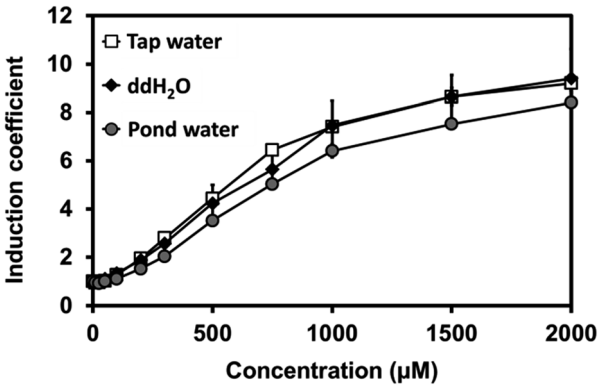

Fig. 5 Induction coefficient of copper-induced *C. metallidurans* cells harboring *copS-copR-PcopSR-PcopQ-rfp* in LB mixing with different water samples. Autoclaved sterilized double-distilled water (ddH_2O) was measured as a control. Error bars represent the standard deviation from triplicate measurements.

Building a regulatory platform requires a mechanism that can output measurable signals in the presence of an input stimulus. Most of whole-cell biosensors involve the use of fluorescent proteins; therefore adopting a pigment-based reporter can be beneficial. Fluorescent proteins allow the easy detection of analytes; however, the production and maturation of these proteins can affect the time required for color to develop. Previously, the DOPA 4,5-dioxygenase, Mjdod, was shown to be involved in the synthesis of betaxanthin, a yellow betalain pigment that contains the chromophore betalamic acid, which is in-turn synthesized from l-dihydroxyphenylalanine (L-DOPA).[33,34]

To test the expression of plant Mjdod in *C. metallidurans*, we constructed a plasmid containing *pBAD-Mjdod*. The production of yellow pigment was visible to the naked eye by inducing the exponential-phase of cells with a 0.2% arabinose and 1 mM L-DOPA for 2 h (Fig. S3†). However, the absence of either L-DOPA or arabinose failed to cause any change in color, indicating that the formation of betaxanthin depends on the presence of Mjdod as well as its own substrate, L-DOPA. We then investigated whether it would be possible to use betaxanthins as output signals for the proposed biosensor. For this, we replaced *rfp* with *Mjdod* and used dose–response curves to evaluate the response to Cu(II) at various concentrations. Specifically, cells were incubated with Cu(II) for 4 h and treated with L-DOPA for 2 h. In performing this investigation, we observed a similar pattern of induction and LOD (87.3 μM) with reduced detection time compared to RFP assays (Fig. 4).

Our novel biosensor demonstrated great performance in the detection of Cu(II) in water under laboratory conditions. We also examined the efficacy of the biosensor using environmental samples, including pond and tap water. For this, sensor cells were grown at a 1 : 1 ratio of 2X LB to tap water, pond water, or pure water in the presence of Cu(II). Three samples presented a nearly identical increase in fluorescence signals, suggesting that the buffer matrix did not significantly affect the efficacy of the biosensors (Fig. 5).

The main advantages that whole-cell microbial biosensors have over conventional analytical techniques are inexpensive, portability, and environmental compatibility. In this study, we developed a whole-cell biosensor for the detection of environmental Cu(II). We examined multiple promoters and identified their Cu(II) response concentrations. We found that betaxanthin was able to report both fluorescent and colorimetric signals within 6 h. These findings suggest that synthetic biology can be applied to improve the sensitivity and detection time of whole-cell biosensors for on-site environmental monitoring.

Acknowledgements

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