Construction of Copper Removing Bacteria Through the Integration of Two-Component System and Cell Surface Display

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Abstract Synthetic biological systems are becoming more and more feasible for commercial and medical purposes through the genetic engineering of several components. The simple assembly of a genetic circuit was shown to stimulate the removal of copper by bacteria through the engineering of a two-component system. The CusSR two-component systems is a regulator of *Escherichia coli* copper homeostatic system. In this system, genetic circuits of CusSR were fused to a cell surface display system for metal adsorption; this system is suitable for the display of a copper binding peptide through outer membrane protein C (OmpC). *E. coli ompC* codes for an outer membrane pore protein (porin) are induced at high osmolarity and temperature, which can also be used as an anchoring motif to accept the passenger proteins. The bacteria that produce the chimeric OmpC containing the copper binding peptide adsorbed maximum concentrations of 92.2 μmol of Cu^{2+}/gram dry weight of bacterial cells. This synthetic bacterial system senses the specific heavy metal and activates a cell surface display system that acts to remove the metal.

Abbreviations
TCS   Two component system

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Introduction

As a result of industrialization, heavy metal pollution has become a serious problem in both developing and developed countries throughout the world. In order to solve this problem, a variety of expression systems promoting the display of chimeric proteins on the surfaces of microbial cells have emerged for the bioremediation of heavy metals [1, 2]. Cell surface display technology has made possible a wide range of applications in the biotechnological and industrial fields, such as the recovery of harmful chemicals and heavy metals, live-vaccine development, and screening of peptide libraries without causing metabolic abnormalities in the host system [3–6]. This technology allow for the expressions of proteins or peptides on the surfaces of the cells using the surface proteins of bacteria, yeast, or even mammalian cells as anchoring motifs [7–9].

Copper can be extremely toxic to microbes and thus necessitates homeostatic mechanisms for cell survival [4, 10, 11]. Copper induces transcription of both *copA* and *cueO*, which encode for the copper transporting ATPase and periplasmic multicopper oxidase, respectively, leading to the detoxification of copper [12, 13]. At higher concentrations of Cu^{2+}, the system described above fails to exogenously efflux and sense the metal. However, the two-component system (TCS) CusSR allows for exogenous copper detection via membrane-associated sensor kinase. The response regulator in CusSR is involved in the transcription of copper and silver efflux systems under anaerobic conditions and is utilized in aerobic conditions under extreme copper stress [10, 14]. In response to high Cu^{2+} concentrations, the CusSR system present in the bacteria specifically activates the expressions of CusCFBA genes. The CusC is an outer membrane protein that undergoes specific acquisition of tolerance under high Cu^{2+} and Ag^{2+} concentrations [15]. Therefore, mechanistic path from copper stimulus to regulation of CusCFBA genes depends on a number of characteristics of the TCS. By manipulating these characteristics, we can create sensor kinases that respond to new signals, promoters that respond to different response regulators or actuators. Currently, the main application of bacterial two-component system is its use as a sensor for chemical and biological components in their surrounding enviornment by altering actuators (reporters), but in the near future, TCS can be exploited for the design of complex synthetic network suitable for commercial or medical application.

A number of outer membrane proteins are now in use for the display on the cell surface and action of enzymes, peptide libraries, antigenic determinants, or single-chain antibodies on the surface of gram negative bacteria [16]. *Esherichia coli* OmpC, one of the most abundant and well characterized. OmpC molecule consists of 16 transmembrane, antiparallel β-strands, which produce a β-barrel structure surrounding a large channel and are connected by seven internal loops and eight external loops [17]. In general, the amino acid sequences of the external loops are less conservative, and thus, these loops may be relatively tolerant to insertion and deletion. Therefore, we decided to use C-terminal deletion–fusion strategy on the external loops as the point of insertion for foreign peptides for cell surface display. In this study, we took the initiative in broadening the application of TCS by investigating the display of a copper binding peptide (CBP) on the surface of *E. coli* using manipulated actuators OmpC as an anchoring motif in response to exogenous Cu^{2+} in the two-component CusSR signal transduction system (Fig. 1). The dynamic characteristic of *cusC* gene expression via exogenous Cu^{2+} was studied, and a simple copper adsorption system was constructed to adsorb exogenous Cu^{2+} on the bacterial surface. These engineered bacteria behave like normal bacteria until it senses copper. If this bacteria senses copper, they activate genetic circuit, display peptide, and remove copper automatically

Fig. 1 Engineered *E. coli* to detect and remove copper. Periplasmic metal sensing receptors detect Cu^{2+}, which phosphorylate the histidine kinase domain and response regulator from the OmpR family of TCS in the receptor. This activates the synthetic genetic circuit of CusSR TCS, resulting in a Cu^{2+}-binding peptide on the *E. coli* surface available for bioadsorption
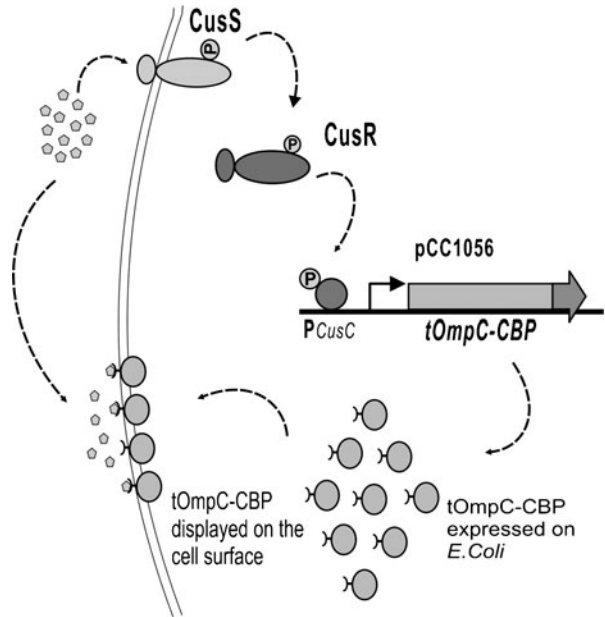
Materials and Methods

Bacterial Strains and Media

E. coli XL1-Blue was used as a host strain for all recombinant DNA manipulation. All recombinant *E. coli* were cultivated in Luria–Bertani (LB) medium (10 g/l bacto-tryptone, 5 g/l bacto-yeast extract, and 5 g/l NaCl) supplemented with 100 μg/ml of ampicillin at 37 °C with vigorous shaking (200 rpm) unless otherwise noted.

Construction of the Plasmid for the Copper Removal System

The genomic region containing the 156-bp *cusC–cusR* intergenic region was amplified from *E. coli* genomic DNA with oligonucleotides cusC_FEcoRI and cusC_RsacI (Table 1). The polymerase chain reaction (PCR) was performed with the MJ Mini personal thermal cycler (BioRad Laboratories, USA) using the Expand High Fidelity PCR System (Roche Molecular Biochemicals, Mannheim, Germany). The PCR products were then digested with *Eco*RI and *Sac*I and were ligated with pUC19 to construct the pUCup1 plasmid [18]. *E. coli ompC* genes were amplified from the genomic DNA of *E. coli* using primers that were designed based on reported genome sequences [19]. The truncated, metal-binding peptide-containing *E. coli* outer membrane protein C (*ompC*$_t$) were integrated using the C-terminal deletion–fusion strategy [17, 20]. For the expression of CBP (SPHHGGW) on the cell surface [21], the *ompC*$_t$ genes encoding the 331 amino acids from the N terminus were amplified using two complimentary pairs of oligonucleotides, as shown in Table 1. The PCR product was cloned into the pUCup1 plasmid using *Sac*I and *Kpn*I to construct pCC1056, in which the chimeric protein was under the control of a *cusC* promoter (Fig. 2). This plasmid was transformed into chemically competent *E. coli* cells for further studies.

**Table 1** Primers used in this study

Oligonucleotide name	Sequence of primers (5′–3′)
cusC_FEcoRI	5′-GAATTCATTTCCTCCGCATGTTGCCCG-3′
cusC_RsacI	5′-GAGCTCAGGCTCATAATTTCTGGTGA-3′
ompCCuprt_FsacI	5′-GAGCTCATGAAAGTTAAAGTACTGTCC-3′
ompcCuprt_RkpnI	5′-GGTACCTTACCAGCCGCCATGATGCGGGGAGCCAGCGTCA CGAGTGAACTG-3′
cusC RT fwd	5′-TTTCGACCGGCCTTAACG-3′
cusC RT Rev	5′-CGGCTTCGCTCATGTTCTTT-3′

Evaluation of Engineered Bacteria in Response to Copper

A single colony of *E. coli* harboring pCC1056 was grown overnight in nutrient-rich LB medium supplemented with 100 μg/ml ampicillin and was incubated at 37 °C in an orbital shaker at 200 rpm until reaching an optical density of 0.5 at 600 nm (OD_{600}). Then the cells were grown for an additional 4 h in the presence of varying concentrations of $CuSO_4$ to evaluate the dynamics of the CusSR TCS.

The transcriptional activities of the *cusC* gene in response to Cu^{2+} in the *E. coli* cells harboring pCC1056 were measured using real-time quantitative PCR (RT-PCR). A metal–bacteria mixture was obtained using the protocol described above. After 4 h, the cells were then harvested via centrifugation for total RNA preparation using the RNeasy Mini kit according to the manufacturer's instruction (Qiagen), which was then followed by DNase treatment. Reverse transcription was performed with a complementary DNA (cDNA) synthesis kit (Applied Biosystems) using a random hexamer primer mix according to the manufacturer's instructions. Specific primers were designed with OLIGO software (version

Fig. 2 Plasmids containing pCC1056 constructed for display of CBP on the *E. coli* cell surface
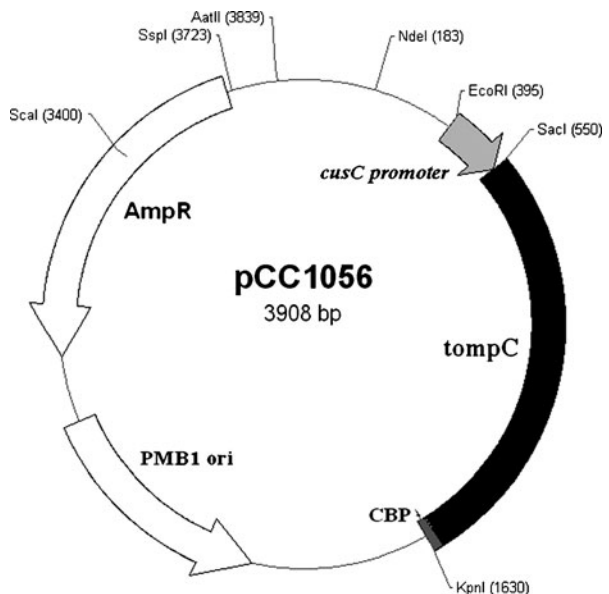
5.0; Molecular Biology Insights, Cascade, CO, USA) for the quantitative expression of the *cusC* gene (Table 1). Samples for which the RT step was omitted were used as negative controls to confirm that the extracted RNA was not contaminated with DNA. RT-PCR reactions were performed on the Mini-opticon detection system with the SYBR Green PCR Master mix, which was used as recommended by the manufacturer. Each quantitative RT-PCR experiment was performed on three replicates of biological samples consisting of separate cultures grown under identical conditions ($n=3$), and the results were calculated automatically using Mini-opticon software with 16s RNA as an internal control [22].

After 4 h in the presence of 0.5 mM concentrations of $CuSO_4$, the cells were then harvested. Protein samples prepared and were analyzed by 12% (w/v) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli [23]. Fractionated protein samples were stained with Coomassie brilliant blue R-250 (Bio-Rad Laboratories, Hercules, CA, USA).

Evaluation of Metal Bioadsorption by the Engineered Bacteria

E. coli strains harboring pCC1056 were grown separately overnight at 37 °C. The overnight culture was diluted 100-fold in fresh LB medium supplemented with 100 μg/ml ampicillin and incubated at 37 °C in an orbital shaker at 250 rpm. When the OD_{600} of the cultures reached 0.5, the cells were induced with varying concentration of 0.5 and 1.0 mM $CuSO_4$ for 4 h. Prior to determination of metal content, bacterial cells were cultivated to the stationary growth phase in LB medium and were washed twice with 0.85% (w/v) NaCl. They were then treated with 5 mM EDTA on ice for 30 min to remove the cell surface-bound Cu^{2+}, and the resulting concentration of Cu^{2+} in the supernatant was measured directly using inductively coupled plasma–mass spectrometry (HP 4500, Yokogawa, Tokyo, Japan). The same procedure was used to determine bioadsorption of Zn^{2+} with *E. coli* harboring pCC1056.

Results and Discussion

Evaluation of Dynamic Characteristics of *cusC*

The bacterial copper adsorption system constructed through integration of chimeric OmpC and *cusC* promoter (P_{cusC}) to respond to and adsorb exogenous copper. To determine whether the response of Cu^{2+} concentration on engineered bacteria activated expression of the *cusC* gene by the CusSR TCS, the dynamics of CusSR TCS was studied using quantitative RT-PCR. In the presence of an exogenous copper concentration, CusSR TCS activates the expression of the *cusC* gene [10]. The RNA samples from *E. coli* cultures grown for 4 h in LB media supplemented with varying concentrations of Cu^{2+} were used, and the results are shown in Fig. 3A. Our findings suggested that the transcript level of the *cusC* gene is significantly increased in cells grown at elevated Cu^{2+} concentration in LB medium. At a 0.1 mM induction, the increases in the transcriptional levels of *cusC* were not significant ($P<0.05$, $n=3$) compared to those of the control; however, with an induction of 1 mM of copper, a significant increase of approximately sixfold ($P<0.05$, $n=3$) was observed in the LB media. The results revealed that the presence of copper ions in the nutrient-rich medium induced the transcriptional level of *cusC* gene to higher concentrations compared to those of the control.
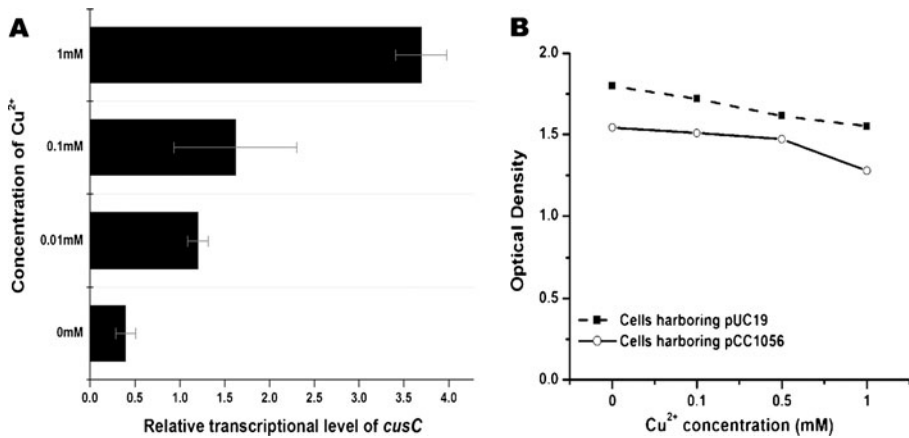
Fig. 3 **A** Transcriptional levels of the *cusC* gene in LB medium after 4 h of Cu^{2+} exposure. The C_t value was normalized to the mean C_t value of the 16s RNA as an internal control. **B** Effect of cells harboring pUC19 and pCC1056 on copper tolerance. Growth curves with varying concentration of $CuSO_4$ are shown. Overnight cultures were diluted 100-fold in fresh LB medium supplemented with 100 μg/ml ampicillin, and after 2 h incubatation at 37 °C, indicated concentration of $CuSO_4$ were added. Cell growth was monitored as the optical density at 600 nm after 4 h of incubation at 37 °C

Evaluation of Copper Bioadsorption

The results presented above demonstrate that the CusSR TCS of the bacteria shows a dynamic response upon exposure to exogenous Cu^{2+} concentrations. As a result, a cell surface bacterial system was constructed to assist in the selective adsorption of exogenous Cu^{2+}. Here, the OmpC coding region was integrated to heterologous peptides (CBPs), which have already been shown to have a high affinity for Cu^{2+} ions. A synthetic DNA fragment encoding this chimeric gene was inserted into the pUCup1 to construct pCC1056, in which the chimeric protein was under the control of a *cusC* promoter (Fig. 2). Localization of target polypepetides (OmpC-CBP) from the harvested recombinant *E. coli* XL1-Blue harboring pCC1056 was determined by SDS-PAGE, indicating that ompC-CBP was expressed under the control of *cusC* promoter in the presence of exogenous copper. Figure 4B shows the SDS-PAGE of OmpC-CBP fusion protein from recombinant *E. coli* harboring pCC1056.

The ability of these engineered bacteria to absorb heavy metals was examined to determine the effect of displaying the CBP. Under these experimental conditions, *E. coli* cells that produced the chimeric *ompC_t*-CBP proteins were cultured, and the wild-type *E. coli* cells with no chimeric protein and only ompC without promoter and metal binding protein served as a control. The cells harboring only OmpC showed identical values with the wild-type *E. coli* cells harboring pUC19 (data not shown). When cultures reached an OD_{600} of 0.5, the cells were induced when exposed to 0.5 and 1.0 mM $CuSO_4$. We also found slow growth rate in cells harboring pCC1056 compared to cells harboring pUC19 due to copper ion induction and cell surface display system (Fig. 3B). As shown in Fig. 4A, cells harboring pCC1056 could adsorb 92.2 and 78.7 μmol of Cu^{2+}/gram DCW when induced with 0.5 and 1 mM $CuSO_4$, respectively, while the uninduced cell strain harboring pCC1056 adsorbed 18.8 μmol of Cu^{2+}/gram DCW.

A slight increase in the Cu^{2+} adsorption by uninduced cells was observed due to leaking expression of *ompC_t*-CBP [24]. Therefore, cells displaying *ompC_t*-CBP at 0.5 and 1 mM
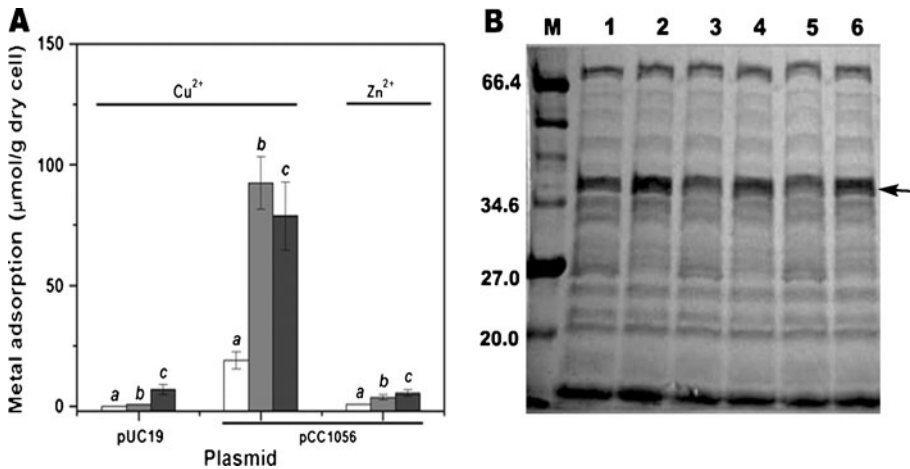
Fig. 4 **A** Bioadsorption of Cu^{2+} by *E. coli* (pCC1056). The metal contents of these samples were determined using inductively coupled plasma–mass spectrometry. The data are aggregate results from replicate experiments ($n=3$). *a* Uninduced cells, *b* 0.5 mM $CuSO_4$ induced, *c* 1.0 mM $CuSO_4$ induced. **B** Localization of the OmpC-CBP fusion in protein extracts and culture medium of pCC1056 transformed XL1-Blue cells (*2*, *4*, and *6*) and non-induced recombinant plasmid as controls (*1*, *3*, and *5*). Overnight cultures were diluted 1/10 in fresh medium (20 ml) containing 100 mg/ml ampicillin, induced after 2 h with 0.5 mM $CuSO_4$, and grown for a further 4 h. The culture medium was subsequently recovered for protein samples; proteins were detected in Coomassie Blue-stained SDS-PAGE gels. The location of the OmpC-CBP fusion protein is indicated by *arrows*

$CuSO_4$ induction adsorbed Cu^{2+} at ~5 and ~4 times higher levels, respectively, than did the cells not displaying *ompC$_t$*-CBP (control plasmid). The 0.5 mM $CuSO_4$ induced cell showed higher Cu^{2+} adsorption than 1.0 mM induced cell due to the toxicity of copper. Induction with $CuSO_4$ concentrations >1 mM was found to decrease cell growth by more than 50%. This is due to the saturation of copper homeostasis systems, which renders them unable to detoxify excess Cu^{2+} in the environment. When low concentrations of $CuSO_4$ (0.01 or 0.1 mM) were used for induction, Cu^{2+} adsorbed <10% of the maximum Cu^{2+} adsorption (data not shown). The CusSR system was not activated by low concentration of $CuSO_4$, and only the *Cue* system was activated to detoxify Cu^{2+} [25, 26]. Another possible mechanism in the decrease in copper adsorption by the cells harboring pCC1056 was copper accumulated into cell by OmpC-CBP fusion protein that fails in translocation into cell membrane.

Regarding the adsorption of other metals, namely, Zn^{2+}, by induced cells, the amount recovered from the cell surface was found to be lower than that of Cu^{2+} due to the selective metal adsorption peptide on the cell surface (Fig. 4A).

Conclusion

In the present study, *E. coli* strain was engineered to sense and remove copper through integration of the copper sensing TCS and cell surface display of OmpC$_t$-CBP chimera protein. The engineered bacteria selectively adsorbed Cu^{2+} in rich LB medium, and this result indicated that the engineered *E. coli* system may be used to sense and remove copper contamination in many locations such as soil, groundwater, or industrial surfaces. The strategy employed in this study can be applied for the development of other heavy metal and contaminant removing system.

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