# Heavy Metal Binding and Accumulation of Genetically Engineered *E. coli* Harboring the CXXC Motif and Histidine Rich Motif Fusion Proteins

**Kamala Boonyodying[1], Thawatchai Kitti[2], Duangkamol Kunthalert[1] and Sutthirat Sitthisak[1,3]**

[1]Department of Microbiology and Parasitology, Faculty of Medical Science, Naresuan University, Phitsanulok, Thailand.
[2]Faculty of Oriental Medicine, Chiang Rai College, Chiang Rai, Thailand.
[3]Centre of Excellence in Medical Biotechnology, Faculty of Medical Science, Naresuan University, Phitsanulok, Thailand.

DOI: http://dx.doi.org/10.13005/bbra/1630

(Received: 20 January 2015; accepted: 06 March 2015)

**Bioremediation is a biological method for removing heavy metals from the environment. Heavy metal binding proteins have been identified in various organisms and have been used to study bioremediation. CXXC and histidine peptide rich motifs are metal binding domains found in proteins of all living organisms. Genes encoding the metal binding domain CXXC motif from the *mcsA* gene and histidine peptide rich motifs from the *copA* gene of *S. aureus* were used to construct 3 recombinant proteins containing various metal binding domains, C4, C8 and C4His recombinant proteins. C4 and C8 recombinant proteins contain four and eight domains of CXXC motifs, respectively. C4His recombinant protein contains four domains of the CXXC motif fused with a histidine-rich metal binding domain. Recombinant proteins were tested for metal binding using IAA chromatography. C4 and C8 recombinant proteins bound $Cu^{2+}$, $Zn^{2+}$, $Cd^{2+}$and $Co^{2+}$ , whereas, the C4His recombinant protein did not bind to any heavy metal tested. *E. coli* expressed with C4 or C8 recombinant proteins showed increased resistance to $Cu^{2+}$ and $Cd^{2+}$. *E. coli* expressed with C4His recombinant proteins showed increased resistance to $Cd^{2+}$. *E. coli* expressed with C4, C8 or C4His recombinant proteins were tested for intracellular bioaccumulation under various heavy metal conditions. The results showed *E. coli*expressed C8 recombinant protein had the highest $Cu^{2+}$ and $Cd^{2+}$ intracellular bioaccumulation above control. This study shows that metal binding domain recombinant proteins can effectively bind and accumulate various types of heavy metals and are good potential tool for studying bioremediation.**

**Key words:** Heavy metal binding proteins, Bioremediation, CXXC motif.

Heavy metals released in the environment pose a risk to humans. Bioremediation is the treatment of hazardous heavy metal contamination in the environment with biological agents, such as bacteria or plants[3]. The expression of heavy metal binding proteins or peptides in microorganisms to enhance heavy metal accumulation has great potential for bioremediation[17]. Heavy metal binding proteins have the ability to bind and absorb various types of heavy metals and have been utilized in the treatment of heavy metal contamination[14,29]. Genetically engineered bacteria expressing heavy metal binding proteins exhibit a successful ability to absorb and accumulate toxic heavy metals[15,22]. The recombinant strain of *Caulobacter crescentus* expressing 6 His fusion protein has an ability to remove cadmium from water[18]. Recombinant spores

* To whom all correspondence should be addressed.
Tel: 66-55-964626; Fax: 66-55-964770
E-mail: sutthirats@nu.ac.th

of *Bacillus subtilis* expressing CotB comprising poly-histidine residues show increased ability to absorb and accumulate $Ni^{2+}$ [12]. Genetically engineered *E. coli* expressing a cadmium transport system and metallothionein can tolerate and accumulate higher concentrations of $Cd^{2+}$ and other heavy metals compared to control[7,21,22]. The CXXC motif is a metal binding domain that contains four Cys-X-X-Cys motif where X is any amino acid. It has been identified at the N-terminal of copper-ATPases and metal chaperones of eukaryotes and prokaryotes[1,10]. Histidine rich peptide is a metal binding domain capable of binding multiple atoms of $Zn^{2+}$, $Ni^{2+}$, $Co^{2+}$, $Cu^{2+}$, $Cd^{2+}$ and $Hg^{2+}$ [9]. Cys-X-X-Cys or histidine rich metal binding motif is present in the soft metal ATPase and chaperone protein of prokaryotes. *S. aureus* contains a heavy metal binding domain at the N-terminal of several proteins, including those encoding by *ctsR* or *cop* operon[23,24,25]. CXXC motifs were found in the N-terminal of copper transporters CopA and McsA in all *S. aureus* strains, whereas histidine rich peptide was found in the N-terminal of copper efflux transporters of some *S. aureus* strains. These heavy metal binding proteins can bind various heavy metals[2], thus, it is possible to take advantage of these proteins in the study of bioremediation. In the present work, we constructed genetically engineered *E. coli* harboring a *mcsA* gene with 4 heavy metal binding domains (CXXC motif) (C4) and 8 heavy metal binding domains (C8). Genetically engineered *E. coli* expressing a combination of 4 heavy metal binding domains and a His rich metal binding domain (C4His) was also constructed. The objective of this study was to compare heavy metal binding and accumulation in these three genetically engineered *E. coli* harboring various heavy metal-binding domains.

## MATERIALS AND METHODS

**Strains, plasmids, media and culture conditions.**

The *S. aureus, E. coli* and plasmids that were used in this study are described in Table 1. PlasmidpRSETa was used to express the C4, C4His and C8 recombinant proteins in *E. coli*, *S. aureus* was grown in TSB and *E. coli* was grown in LB broth at 37°C. When needed, 50 µg/ml ampicillin and 10 µg/ml chloramphenicol were added to the growth medium.

**Cloning and Overexpression of C4, C 8 and C4His recombinant protein**.

To construct C4 recombinant protein, PCR of *mcsA* gene was performed using two oligonucleotide primers (mcsA-F1 and mcsA-R3, Table 2) and *S. aureus* genomic DNA strain SH1000 as a template. The PCR products were cloned into the TA vector (RBC Bioscience, Taiwan) and subsequently cloned into *Bam*HI and sites *Hind*III of pRSETa vector containing an N-terminal 6xHis-tag. C8 recombinant protein was constructed by the PCR of the *mcsA* gene using oligonucleotide primer (mcsA-F1/mcsA-R1 and mcsA-F2/mcsA-R2, Table 2) and the *S. aureus* genomic DNA strain SH1000 as a template. Both PCR products were digested with *Hind*III and ligation, the resulting fragments were cloned into a pBluescript vector (Fermentas) and subsequently cloned into *Bam*HI and *Eco*RI sites of a pRSETa vector. C4His recombinant protein was constructed by the PCR of the *mcsA* gene and the *copA* gene using oligonucleotide primers (mcsA-F1/mcsA-R1 and His-F/His-R) and the *S. aureus* genomic DNA strain SH1000 and ATCC12600 as a template, respectively. Both PCR products were digested with *Hind*III, ligation and the resulting fragments were cloned into a pBluescript vector (Fermentas) and subsequently cloned into *Bam*HI and *Eco*RI sites of the pRSETa vector. The *E. coli* BL21 (DE3) pLysS (Novagen) was transformed by recombinant plasmid by the heat shock method. To overexpress the recombinant protein, the transformants were grown in LB containing 50 µg/ml ampicillin and 10 µg/ml chloramphenicol at 37ºC until the optical density at 600 nm reached 0.4. Then, the cells were induced to express their proteins by adding 1.0 mM of IPTG for 4 hrs. The induced cells were harvested, washed, and re-suspended in a lysis buffer. Pellets were homogenized and the cell debris was removed by centrifugation at 12,000 rpm at 4ºC. Supernatants were applied to nickel-charged agarose affinity columns (Novagen) and eluted with 200-400 mM imidazole. Fractions containing the overexpressed His-tag protein were pooled and dialyzed against dialysis buffer (25 mMTris, 100 mM sucrose, 50 mMNaCl, 1 mM of DTT, pH 8.0).

**Characterization of the metal binding activity of C4, C 8 and C4His recombinant protein by IAA chromatography.**

The metal binding activity of the

recombinant protein was determined by using iminodiacetic acid-agarose (IAA) chromatography as described by Sitthisak, *et al* (2012). Columns containing IAA resin equilibrated with buffer containing one of various heavy metals ($Cd^{2+}$ , $Cu^{2+}{}_2$, $Co^{2+}$, $Zn^{2+}$, $Pb^{3+}$, $Mn^{2+}$, $Mg^{2+}$, $Fe^{3+}$) at a final concentration of 1 mM were used. 50 ¼g of purified recombinant protein were added to the resin. Columns were centrifuged to remove unbound proteins and washed with a sodium phosphate buffer. Bound proteins were eluted from the column with 50 mM EDTA in a sodium phosphate buffer. Both eluted and unbound proteins were analyzed by 15% SDS-PAGE.

**Analysis of growth kinetics of the *E. coli* expressing C4, C 8 and C4His recombinant protein in the presence of heavy metals**

To study the effects of heavy metals in the *E. coli* expressing recombinant proteins, the growth characteristics of *E. coli* strains were determined under various heavy metal conditions and compared to *E. coli* harboring plasmid pRSETa. Bacterial cultures harboring various overexpressed plasmid were grown in an LB broth containing 50 ¼g/ml ampicillin and 10 ¼g/ml chloramphenicol, heavy metals ($Cu^{2+}$, $Cd^{2+}$, $Co^{2+}$ or $Zn^{2+}$) were added to various final concentrations. Bacterial cultures were induced by the addition of 0.1 mM IPTG. The culture tubes were incubated at 37°C with shaking (200 rpm). Growth was measured by optical density determination at 600 nm with a spectronic-20 spectrophotometer.

**Analysis of intracellular heavy metal accumulation.**

Overnight cultures with over-expressed recombinant proteins or control strains were diluted 1:100 in fresh LB media containing 50 ¼g/ml ampicillin and 10 ¼g/ml chloramphenicol. Cultures were grown at 37°C until the optical density at 600 nm of the cultures reached 0.4-0.6. The cultures were subsequently induced with 1 mM IPTG and various heavy metals were added.The cultures were incubated for 4 hrs. Subsequently, cells were harvested by centrifugation for 10 min at room temperature and washed three times with 10 mMTris/HCl (pH 7.4) containing 1 mM EDTA and once with analysis grade $dH_2O$. Cells were dried overnight at 80°C then dissolved in concentrated nitric acid. Disrupted cells were then diluted with analysis grade $dH_2O$ and centrifuged for 10 min at room temperature. The concentration of heavy metals in the soluble fraction was measured by atomic absorption spectrophotometer.

## RESULTS

**Construction and purification of C4, C8 and C4His rich recombinant proteins**

Gene encoding heavy metal binding motifs from *S. aureus*. *copA* and *mcsA* were used to generate the fusion proteins. C4 recombinant protein was cloned from 567 bp of *mcsA* gene from *S. aureus* (Fig 1 (A)). C8 recombinant protein was cloned from fusion gene of two of 360 bp N-terminal of the *mcsA* gene resulting in 720 bp of fusion gene (Fig 1 (B)). C4 His rich was cloned from the hybrid gene of 360 bp N-terminal of *mcsA* and 249 bp N-terminal of *copA S. aureus* resulting in 609 bp of fusion gene (Fig 1 (C)). Genetically engineered *E. coli* harboring C4, C8 and C4His recombinant proteins were constructed. Recombinant proteins were expressed and purified and all recombinant proteins were expressed in soluble form. As shown in Figure 2., the C4, C8 and C4His recombinant proteins were expressed as molecular weights of 25 kDa, 35 kDa and 32 kDa, respectively.

**Analysis of metal binding activity of the C4, C8 and C4His rich recombinant proteins**

The metal binding activity of the three recombinant proteins is shown on Figure 3. In accordance with previous findings, C4 recombinant protein bound specifically to $Cu^{2+}$, $Zn^{2+}$, $Cd^{2+}$, and $Co^{2+}$ [2,5] (Fig 3 (A)). C8 and C4His fusion proteins were constructed and also tested for the binding activity. C8 recombinant protein also bound to $Cu^{2+}$, $Zn^{2+}$, $Cd^{2+}$, and $Co^{2+}$ (Fig 3 (B)), whereas, C4His recombinant protein did not bind to any heavy metal tested since the protein was present in the unbound fraction (Data not shown).

**Growth kinetics of the *E. coli* expressing heavy metal recombinant protein in the presence of heavy metals**

Three genetically engineered *E. coli* harboring heavy metal-binding domains, C4, C8 and C4His rich, were compared for their heavy metals sensitivity using *E. coli* harboring only pRSET plasmid as control. Expression of C4 and C8 metal binding motif conferred increased $Cu^{2+}$ and $Cd^{2+}$ resistance to the engineered *E. coli* strain compared to the control ($p<0.05$). As shown in

Figure 4A and 4B, *E. coli* harboring C4 metal binding motif significantly increased resistance to $Cu^{2+}$ in the presence of 1-1.5 mM to $Cu^{2+}$ and significantly increased resistance to $Cd^{2+}$ at concentrations of 0.125-0.5 mM. In addition, *E. coli* harboring C8 metal binding motif significantly increased resistance to $Cu^{2+}$ in the presence of 1mM to $Cu^{2+}$ and significantly increased resistance to $Cd^{2+}$ at concentrations of 0.125-0.25 mM compared to the control. However, there were no significant differences in the growth of the all *E. coli* strains in the presence of $Zn^{2+}$ and $Co^{2+}$ (data not shown)

**Intracellular accumulation of *E. coli* expressing heavy metal recombinant protein in the presence of heavy metals**

The intracellular accumulations of $Cu^{2+}$ and $Cd^{2+}$ were measured to confirm that $Cd^{2+}$ and $Cu^{2+}$ accumulates in *E. coli* expressing C4, C4His and C8. The *E. coli* strains were grown in LB broth containing 1 mM $Cu^{2+}$ or 0.5 mM $Cd^{2+}$. After 4 hrs incubation, the amount of $Cd^{2+}$ in the cells expressing C4, C4His and C8 was significantly higher than that of control. The level of intracellular $Cd^{2+}$ accumulation was 1.4, 2.5 and 2.5

**Table 1.** Strains and plasmids used in this study

| Strains and plasmid | Relevant characteristics | Reference |
|---|---|---|
| **S. aureus strains** | | |
| SH1000 | NCTC 8325-4 with rsbU mutation repair | Horsburgh, et al., 2002 |
| ATCC12600 | NCTC 8532 [IAM 12544, R. Hugh 2605] | American Type Culture Collection (ATCC) |
| **E. coli strains** | | |
| DH5? | A recombination-deficient amber suppressing strain used for plating and growth of plasmids and hsdR17recA1endA1 gyrA96 thi-1 relA1cosmids. hsdR17recA1endA1 gyrA96 thi-1 relA1cosmids. The O 80 LacZ M15 mutation permit-? complementation with the amino terminus of ? galactosidase encoded in pUC vector | Hanahan, 1983 |
| BL21 (DE3) pLysS | F- omp T hsdSB (rB-m-B) gal dcm(DE3) pLysS (CamR) | Promega |
| **Plasmids** | | |
| TA vector | PCR cloning vector, Ampr, Kanr | RBC Bioscience Corp |
| pRSETa | Overexpression vector, Ampr | Invitrogen, USA |
| pBluescript | DNA cloning and sequencing vector, Ampr | Fermentas, USA |

**Table 2.** Primers used in this study

| Primers (5' 3') | Relevant characteristics | References |
|---|---|---|
| mcsA-F1 | GC*GGATCC*GTGCTTTGTGAAAATTGTCAACTTAA | This study |
| mcsA-R1 | GGG*AAGCTT*GACGATATCAATGATGTCATCTTTAAATG | This study |
| mcsA-F2 | CG*AAGCTT*GTGCTTTGTGAAAATTGTCAACTTAA | This study |
| mcsA-R2 | GGG*GAATTC*GACGATATCAATGATGTCATCTTTAAATG | This study |
| mcsA-R3 | CG*AAGCTT*TTATGCGTCATCATGTTGCACCTCACTCTCAGC | This study |
| His-R | GC*GAATTC*AGCCCCTGATAAAAATGGCTT | This study |
| His-F | G*AAGCTT*ATGGAGCATCATAGTCATCAAGAAC | This study |

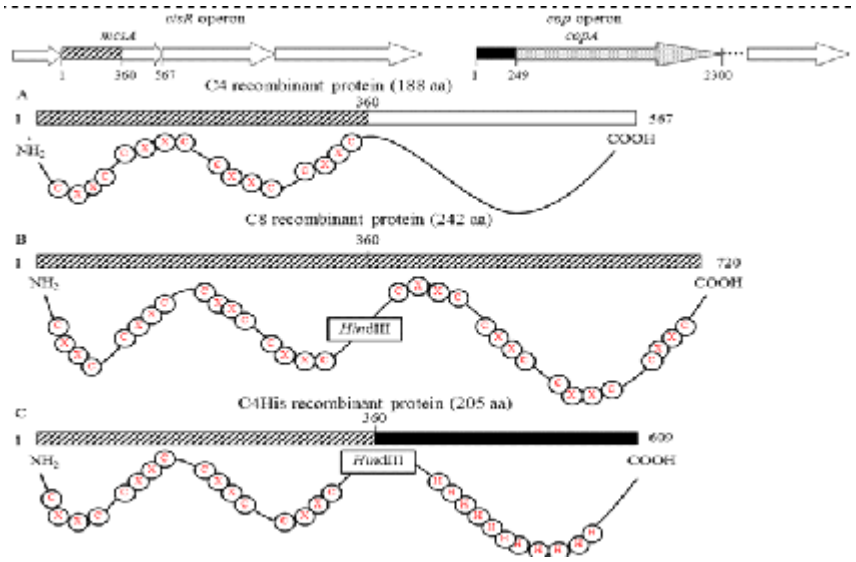


**Fig. 1.**Schematic representation of C4, C8 and C4His rich recombinant fusion protein. The arrow indicates the *mcsA* gene and *copA* gene orientation. The schematic of amino acid representation of C4 with four heavy metal binding CXXC motif (A), C8 with eight heavy metal binding CXXC motif (B) and C4His rich with four heavy metal binding CXXC motif and His rich motif are indicated below (C)

fold higher than the control. *E. coli* expression of C4 and C8 fusion protein was more effective in bioaccumulating $Cu^{2+}$ than that of the control. The level of intracellular $Cu^{2+}$ accumulation was 1.8 and 2.7 fold higher than the control. There was no difference in intracellular copper accumulation of *E. coli* expression C4His fusion protein.

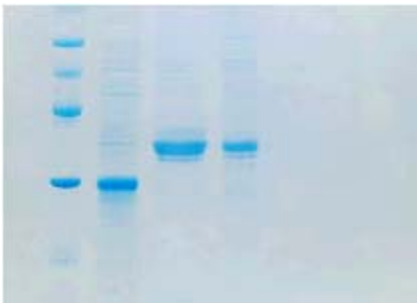

**Fig. 2.**C4, C4His and C8 recombinant fusion protein analysed on 15 % SDS- polyacrylamide gel. The C4, C4His and C8 recombinant protein have a molecular weight of 25, 32 and 35 kDa, respectively. Lane M: Molecular weight marker.

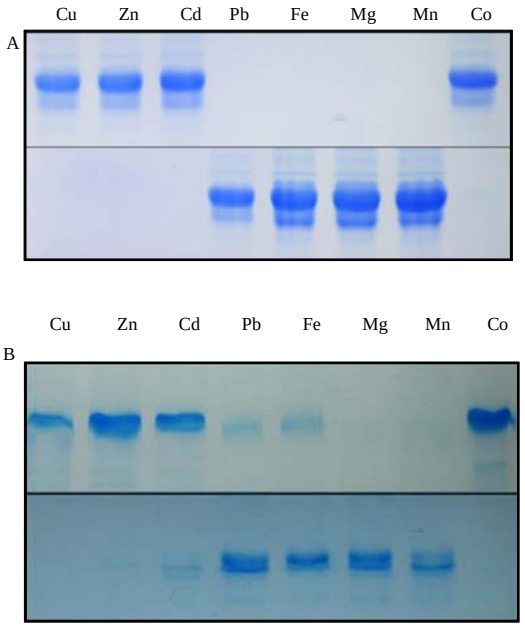


**Fig. 3.** Binding of C4 (A), C8 (B) recombinantprotein to IAA resin chromatography. The IAA-resin was charged with different heavy metals as indicated above the respective lane. Bound and unbound proteins were analyzed by 15% SDS-PAGE. Upper row: Bound protein; Lower row: Unbound protein.

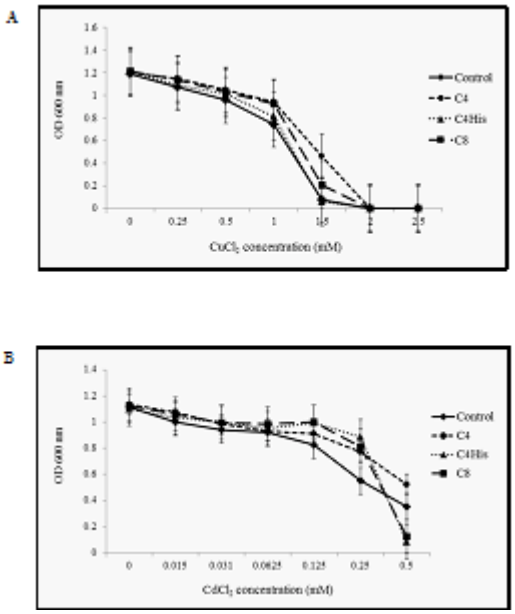


**Fig. 4.** Effects of heavy metals on growth of *E. coli* BL21(DE3) expressing heavy metal binding protein. Overnightcultures were diluted to OD600 in LB with 0.1 mM of IPTG and different concentrations of $CuCl_2$ (A), $CdCl_2$ (B), $CoCl_2$. Cultures were incubated at 37°C with shaking. Cell growth was monitored by measuring the optical density at 600 nm for 18 h. *E. coli* harboring overexpression plasmid pRSETa was used as the control. Each point represents the mean value of three experiments (± standard error of mean) ($P<0.05$).

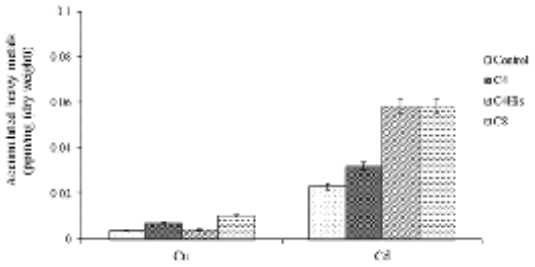


**Fig. 5.** Intracellular bioaccumulation of $Cu^{2+}$ and $Cd^{2+}$ by cells expressing C4, C4His or C8 recombinant protein.*E. coli*BL21 (DE3) harboring overexpression plasmid pRSETa was used as the control.The data shown are the mean value of three experiments ( ± standard deviation ) ($P<0.05$).

## DISCUSSION

Heavy metal binding domains have the capacity to bind and accumulate various types of heavy metal. *E. coli* expressing heavy metal recombinant protein bound heavy metal and accumulated in the cells and increased the heavy metal sensitivity. The heavy metal binding of C4, C8 and C4His recombinant proteins by IAA chromatography showed that C4 and C8 recombinant protein has the ability to capture $Cu^{2+,}$ $Zn^{2+,}$ $Cd^{2+}$ and $Co^{2+}$ , but not $Pb^{3+}$, $Fe^{3+}$, $Mg^{2+}$, $Mn^{2+}$, and $Ni^{2+}$. Previous report on metallothionein protein with cysteine-rich motif showed that it was found to have the ability to capture heavy metals such as $Cd^{2+}$, $Hg^{2+}$, $Cu^{2+}$ and $Zn^{2+}$ [2,17,25]. C4 and C8 recombinant proteins were derived from fusion of gene encode McsA. This protein can bind to $Cu^{2+}$, $Cd^{2+}$, $Co^{2+}$ and $Zn^{2+}$ , but could not bind to the heavy metals $Pb^{3+}$, $Fe^{3+}$, $Mg^{2+}$ and $Mn^{2+}$ [23,25]. Heavy metal binding of proteins is dependentupon the number of electrons outside of the heavy metal and on the number and types of amino acid side chain of the recombinant proteins. Previous studies using NMR spectroscopy analysis of the CXXC motif in copper ATPase showed that it is bound to $Cu^{2+}$ using two cysteine residues[20]. Previous studies in McsA protein showed that when six of the eight conserved cysteines in the CXXC motifs of McsA protein were changed into alanine, the mutated McsA still bound to $Cu^{2+}$with the two cysteine [25]. However, to capture $Cd^{2+,}$ the CXXC motif was found to use 3 or 4 cysteine residues [8]. C4His recombinant protein was found not to bind to any heavy metal tested. This may be explained by the folding of C4His recombinant protein having characteristics different from C4 recombinant protein. C4His recombinant protein is a fusion of N-terminal of *mcsA* and *copA* genes to create the genetically modified protein. This protein has amino acid sequence differences from native protein. The three-dimensional structure and function of a protein is determined by the amino acid sequence. Changing in amino acid sequence of the protein make the three-dimensional structure change and may affect the binding function of the protein [4]. The growth sensitivity of bacteria, *E. coli* BL21 (DE3) pLysS expressed C4, C8 recombinant proteins in media containing heavy metals showed increased $Cu^{2+}$ and $Cd^{2+}$ sensitivity. This study was consistent with the metal binding studies show C4 and C8 recombinant proteins have the ability to capture heavy metals $Cu^{2+}$ and $Cd^{2+.}$ Thus, the increased resistance of $Cu^{2+}$ and $Cd^{2+}$ may be due to their ability to capture heavy metals

thereby reducing the heavy metal toxicity. Bacteria *E. coli* expressing C4 and C8 recombinant protein had intracellular heavy metal accumulation $Cd^{2+}$ and $Cu^{2+}$ higher than the control. This result was consistent with a previous study that showed *E. coli* expressing metallothionein from various species have the ability to accumulate $Cu^{2+}$ and $Cd^{2+}$ over the control[11,16,21]. A previous study showed increased $Cu^{2+}$ sensitivity in *E. coli* expressing the copper transport gene in the cell wall[19]. *E. coli* with the expression of C4His which is a cysteine-rich motif fusion with copA showed no significant differences in the growth and intracellular accumulation of $Cu^{2+}$ compared to the control. No binding was found for this recombinant protein with any heavy metals. C4His recombinant protein cannot bind to $Cd^{2+}$ but *E. coli* that expressed C4His recombinant protein showed increased $Cd^{2+}$ sensitivity. This may be explained by the differences in the status of heavy metal ions that float freely in broth for the sensitivity test or the heavy metal ions that are immobilized on the surface of the resin used for metal binding assay[27, 28]

In conclusion, this study showed that the metal binding domain of C8 recombinant protein bind heavy metals efficiently. Genetically engineered *E. coli* expressing C8 recombinant protein showed highest $Cu^{2+}$ and $Cd^{2+}$ intracellular bioaccumulation and can be used for bioremediation.

## ACKNOWLEDGEMENTS

We are grateful to Prof. Gavin Reynolds and staff from Naresuan University language centerfor help with editing of this manuscript. This work was supported by the grant from TRF-master research grant 2553 (MRG-WII535S021).

## REFERENCES

1. Agarwal, S., Hong, D., Desai, N.K., Sazinsky, M.H., Argüello, J.M., Rosenzweig, A.C. Structure and interactions of the C-terminal metal binding domain of *Archaeoglobus fulgidus* CopA. *Proteins*., 2010; **78**:2450-2458.
2. Boonyodying, K., Watcharasupat, T., Yotpanya, W., Kitti, T., Kawang, W., Kunthalert, D., and et al. Factor involving of heavy metals binding to recombinant heavy metal binding domain protein. *Envi Asia*., 2012; **5**:70-75.
3. Bonaventura, C., Johnson, F.M. Healthy environments for healthy people: bioremediation today and tomorrow. *Environ Health Perspect*., 1997; **105**:5-20.
4. Bystroff, C., Simons, K.C., Han, K.F., Baker, D. Local sequence-structure correlations in proteins. *Curr Opin Biotechnol*., 1996; **7**:417-21.
5. Chen, W., Georgiou, G. Cell-Surface display of heterologous proteins: From high-throughput screening to environmental applications. *Biotechnol. Bioeng*., 2002; **79**:496-503.
6. Das, N., Vimala, R., Karthika, P. Biosorption of heavy metals-An overview. *IJBT*., 2008; **7**:159-169.
7. Deng, X., Yi, X.E., Liu, G. Cadmium removal from aqueous solution by gene-modified *Escherichia coli* JM109. *J Hazard Mater*., 2007; **139**:340-4.
8. DeSilva, T.M., Veglia, G., Porcelli, F., Prantner, A.M., Opella, S.J. Selectivity in heavy metal- binding to peptides and proteins. *Biopolymers*., 2002; **64**:189-97.
9. Gupta, R.K., Dobritsa, S.V., Stiles, C.A., Essington, M.E., Liu, Z., Chen, C.H., and et al. Metallohistins: a new class of plant metal-binding proteins. *J Protein Chem*., 2002; **21**:529-36.
10. Harrison, M.D., Jones, C.E., Solioz, M., Dameron, C.T. Intracellular copper routing: The role of copper chaperones. *Trends Biochem Sci*., 2000; **25**:29-32.
11. He, Y., Ma,W., Li, Y., Liu, J., Jing, W., Wang , L. Expression of metallothionein of fresh water crab (*Sinopotamon henanense*) in *Escherichia coli* enhances tolerance and accumulation of zinc, copper and cadmium. *Ecotoxicol*., 2014; **23**:56-64.
12. Hinc, K., Ghandili, S., Karbalaee, G., Shali, A., Noghabi, K.A., Ricca, E., Ahmadian, G. Efficient binding of nickel ions to recombinant *Bacillus subtilis* spores. *Res Microbiol*., 2010; **161**: 757-764.
13. Horsburgh, M.J., Aish, J.L., White, I.J., Shaw, L., Lithgow, J.K., Lithgow, S.J. SigmaB modulates virulence determinant expression and stress resistance: characterization of a functional *rsbU* strain derived from *Staphylococcus aureus* 8325-4. *J Bacteriol*., 2002; **184**:5457-67.
14. Kao, W.C., Huang, C.C., Chang, J.S. Biosorption of nickel, chromium and zinc by MerP-expressing recombinant *Escherichia coli*. *J Hazard Mater*., 2008; **158**:100-106.
15. Kuroda, K., Ueda, M. Engineering of microorganisms towards recovery of rare metal ions. *Appl Microbiol Biotechnol*., 2010; **87**:53-

60.

16. Ma, Y., Lin, J., Zhang, C., Ren, Y., Lin, J. Cd(II) and As(III) bioaccumulation by recombinant *Escherichia coli* expressing oligomeric human metallothioneins. *J Hazard Mater*., 2011; **185**:1605-1608.
17. Mejáre, M., Bülow, L. Metal-binding proteins and peptides in bioremediation and phytoremediation of heavy metals. *Trends Biotechnol*., 2001; **19**:67-73.
18. Patel, J., Zhang, Q., McKay, R.M.L., Vincent, R., Xu, Z. Genetic engineering of *Caulobacter crescentus* for removal of cadmium from water. *Appl Biochem Biotechnol*., 2010; **160**:232-43.
19. Ravikumar, S., Yoo, I.K., Lee, S.Y., Hong, S.H. Construction of copper removing bacteria through the integration of two-component system and cell surface display. *Appl Biochem Biotechnol*., 2011; **165**:1674-81.
20. Rubino, J.T., Franz, K.J. Coordination chemistry of copper proteins: how nature handles a toxic cargo for essential function. *J Inorg Biochem*., 2012; **107**:129-43.
21. Sauge-Merle, S., Lecomte-Pradines, C., Carrier, P., Cuiné, S., Dubow, M. Heavy metal accumulation by recombinant mammalian metallothionein within *Escherichia coli* protects against elevated metal exposure. *Chemosphere*., 2012; **88**:918-24.
22. Singh, J. S., Abhilash, P.C., Singh, H.B., Singh, R.P., Singh, D.P. Genetically engineered bacteria: An emerging tool for environmental remediation and future research perspectives. *Gene*., 2011; **480**:1-9.
23. Sitthisak, S., Kitti, T., Boonyonying, K., Wozniak, D., Mongkolsuk, S., Jayaswal, R.K. McsA and the roles of metal-binding motif in *Staphylococcus aureus. FEMS Microbiol Lett*., 2012; **327**:126-133.
24. Sitthisak, S., Knutsson, L., Webb, J.W., Jayaswal , R.K. Molecular characterization of the copper transport system in *Staphylococcus aureus. Microbiol*., 2007; **153:**4274-4283.
25. Sitthisak, S., Howieson, K., Amezola, C., Jayaswal, R.K. Characterization of a multicopper oxidase gene from *Staphylococcus aureus. Appl Environ Microbiol*., 2005; **71**:5650-5653.
26. Su, Y.J., Lin, J.Q., Lin, J.Q., Hao, D.H. Bioaccumulation of Arsenic in recombinant *Escherichia coli* expressing human metallothionein. *Biotechnol bioproc eng*., 2009; **14**:565-570.
27. Volz, J., Bosch,F.U., Wunderlin, M., Schuhmacher, M., Melchers, K., Bensch ,K., and et al. Molecular characterization of metal-binding polypeptide domains by electrospray ionization mass spectrometry and metal chelate affinity chromatography. *J Chromatogr A*., 1998; **800**:29-37.
28. Xu, Z., Lee, S.Y. Display of polyhistidine peptides on the *Escherichia coli* cell surface by using outer membrane protein C as an anchoring motif. *Appl Environ Microbiol*.,1999; **65**:5142-7.
29. Zheng, B., Zhang, Q., Gao, J., Han, H., Li, M., Zhang, J., and et al. Insight into the interaction of metal ions with TroA from *Streptococcus suis. PLoS One*., 2011; **6**:19510.