


**โครงการ: การวิเคราะห์คุณสมบัติหน้าที่ของ** CxxC **motif** ของยีน ***mcsA*** **จาก แบคทีเรีย *Staphylococcus aureus***

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# รายงานวิจัยฉบับสมบูรณ์

## โครงการ: การวิเคราะห์คุณสมบัติหน้าที่ของ CxxC **motif** ของยีน ***McsA*** จากแบคทีเรีย ***Staphylococcus aureus***


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## บทคัดย่อ

ในงานวิจัยครั้งนี้ได้ศึกษาคุณสมบัติของยีน *mcsA* จากเชื้อ *Staphylococcus aureus* โดย McsA ในเชื้อ *S. aureus* เป็นโปรตีนที่มี metal binding domain ที่มี CxxC motif 4 domain อยู่ที่ N-terminal และทำหน้าที่ควบคุมกลไกการตอบสนองต่อ stress ในแบคทีเรีย McsA ทำหน้าที่ควบคุมการทำงานของ repressor โปรตีน *ctsR* ในแบคทีเรีย *S. aureus* พบว่า *ctsR* operon ประกอบด้วยยีน *ctsR, mcsA, mcsB* และ *clpC* และเมื่อศึกษาการแสดงออกของยีนใน *ctsR* operon หลังกระตุ้นเซลด้วยโลหะหนักพบว่ายีน *mcsA* และ *mcsB* จะมีการแสดงออกเพิ่มขึ้นตอบสนองต่อโลหะ copper, cobalt, cadmium และ zinc เมื่อนำยีน *mcsA* มาโคลนและผลิตโปรตีนรีคอมบิแนทท์ของ McsA เพื่อนำมาศึกษาคุณสมบัติการจับกับโลหะหนักพบว่าโปรตีนรีคอมบิแนทท์ของ McsA สามารถจับกับโลหะหนัก $CuCl_2$, $ZnCl_2$, $CdCl_2$ และ $CoCl_2$ การทำ site directed mutagenesis เพื่อเปลี่ยนกรดอะมิโนที่ Cysteine residues ไปเป็น Alanine ที่ตำแหน่ง $Cys^{3, 6, 29, 31,104.107}$ ของ CxxC motif ใน 3 metal binding domain ของ McsA พบว่า การจับของ CXXC motif กับโลหะหนักแต่ละชนิดมีความแตกต่างกันโดยพบว่าการจับของ McsA กับ Copper ต้องการเพียง 1 CXXC motif เมื่อศึกษาการทำปฏิสัมพันธ์ระหว่างโปรตีนโมเลกุลโดยวิธี bacterial two-hybrid system พบว่าโปรตีน McsA สามารถปฏิสัมพันธ์กับโปรตีน McsB และ CtsR โดย CxxC motif ของ McsA มีความสำคัญในการจับ จากข้อมูลในการศึกษาครั้งนี้แสดงให้เห็นว่า Cys residues ใน CxxC motif ของ McsA เกี่ยวข้องกับการจับโลหะหนักและปฏิสัมพันธ์กับโปรตีน และอาจทำหน้าที่สำคัญในรับสัญญานจากโลหะหนักและเกี่ยวข้องกับกลไกการตอบสนองต่อโลหะหนักในเซล



**คำหลัก:** ctsR regulon, modulator of ctsR gene, CXXC motifs, thiol-cysteine residues,metal binding domain, *Staphylococcus aureus*

## ABSTRACT

McsA from *Staphylococcus aureus* was characterized. McsA protein is a modulator of stress response that contained four metal binding domains (CxxC motif) at the N-terminal and functions as a modulator that regulates CtsR repressor. In *S. aureus*, *ctsR* operon codes for a putative *ctsR, mcsA, mcsB* and the downstream *clpC* gene. The expression of *ctsR* operon was determined in response to heavy metals and shown that *mcsA* and *mcsB* are induced in response to some extent by copper, cobalt, cadmium, and zinc. McsA was cloned, expressed and purified and tested for the metal binding activity. It was shown that the protein can bind to Cu (II), Zn (II), Co (II) and Cd (II). Site directed mutagenesis of Cys residue [3, 6, 29, 31,104.107] in the CxxC motif to Ala of three metal binding domains of McsA shown that two conserved cysteine ligands provided by one CxxC motifs is important to bind one copper ion. In addition, bacterial two-hybrid system shown that McsA was able to bind McsB and CtsR of *S. aureus* and CxxC motif is important in the binding. This data indicates that these Cys residues in the CxxC motif might be involved in metal binding and may have an important role in metal induced signaling system and involve in intracellular metal stress response mechanism.



**Keywords:** ctsR regulon, modulator of ctsR gene, CXXC motifs, thiol-cysteine residues,metal binding domain, *Staphylococcus aureus*

## Executive Summary

**1. Rationale (ความสำคัญและที่มาของปัญหา)**

*Staphylococcus aureus* is a Gram-positive bacterium that causes a variety of diseases ranging from minor skin infections to life-threatening diseases (Easmon and Adam, 1983). Most of the *S. aureus* strains develop antibiotic resistance which is a serious problem in their treatment. It poses a major and immediate threat to public health. *S. aureus* is capable of growing in a wide range of adverse environmental stress conditions and a number of genes that involved in environmental stress response (alkali, antibiotic, cold, heavy metal and heat) in *S. aureus* have been identified.

*ctsR* gene is a regulator of stress resistance gene which is a member of heat shock class III (Clement and Foster, 1999). It's important in the virulence and survival of several pathogens and its synthesis is stimulated in response to a variety of stress such as heat shock and acid shock (Derre et al., 1999; Anderson, et al., 2006, Bore et al., 2007). *ctsR* operon in gram positive bacteria consisting of four genes, designated as *ctsR*, *Mctsr*$_A$, *Mctsr*$_B$ and *clpC.*

In the *S. aureus* strains, modulator of ctsR gene (in this study, designed as *Mctsr*$_A$) is located downstream of ctsR gene. MctsR$_A$ contains metal binding motif (CxxC) at the N-terminal and functions as a modulator of the heat shock repressor ctsR. CxxC motif always found in heavy metal chaperone or thiol-disulphide oxidoreductase superfamily and CxxC motif from the N-terminal of metal binding from copper ATPases and metal chaperones have been identified in both eukaryotes and prokaryotes (Harrison *et al*., 2000)

The paired cysteine residues in this metal binding domain play an important role in heavy metal binding and may involve in the binding and protein interaction with other molecules. (Walker *et al*., 2002; Walker *et al*., 2004; Zdanowski *et al.,* 2006; Gaskell *et al.,* 2007; Yabe *et al.,* 2008).

To date, little is known about molecular mechanism of the ctsR and its modulator in *S. aureus*. Additionally, there have been no studies on the function of the cysteine residue in protein interaction in any organisms. Here I propose to investigate the molecular mechanism of Mcts$R_A$ and ctsR in *S. aureus* and characterize the function of CxxC motif from Mcts$R_A$ genes involve in metal binding activity and protein interaction. CxxC motif at the N terminus of the Mcts$R_A$ may responsible for the binding with ctsR and clpC molecule and involved in regulation of heat shock and virulence gene expression. The results from this study will lead to explain the function of CxxC motif and its mechanism in bacterial protein interaction and gain new insight into intracellular stress response mechanism under physiological and pathological condition.

**2. Specific aims (วัตถุประสงค์)**

The specific objectives of this study are:

1) To clone and characterize CxxC motif from Mcts$R_A$ genes of *S. aureus*.

2) To perform site directed mutagenesis to change cysteine to alanine in the CxxC motif of the Mcts$R_A$ and characterize its metal binding activity.

3) To construct bacterial two-hybrid system and determine protein interaction of Mcts$R_A$ or $\Delta$Mcts$R_A$ with ctsR and clpC molecule.

## 3. Research design and methods (ระเบียบวิธีวิจัย)

### Bacterial strains and growth conditions

*S. aureus* strain N315, ATCC12600 and *E. coli* strain JM109, DH5 α, BL21 (DE3) PLysS and MC1061 will be used in this study. *S. aureus* will be grown in tryptic soy broth (TSB) and *E. coli* will be grown in Luria- Bertani broth (LB). When needed, ampicillin (50 µg/ml), carbenicillin (50 µg/ml), erythromycin (20 µg/ml), and tetracycline (10 µg/ml) will be added to the growth medium.

### Methodology

1. Molecular genetic procedures

Plasmid and chromosomal DNA isolation, DNA manipulation, digestion of DNA with restriction enzymes, DNA ligation, polymerase chain reactions will be performed as described by Sambrook et al., (1989).

2. Characterization of metal binding activity from $MctsR_A$ of *S. aureus*

2.1 Cloning and overexpression of $MctsR_A$ in *S. aureus*

To clone the $MctsR_A$ from S. aureus, two oligonucleotide primers (mctsR-F1 and mctsR-F2) will be designed with a *BamHI* site at the 5' end and a *HindIII* site at the 3' end of the DNA fragment. The PCR will be performed using S. aureus genomic DNA as template and the PCR product will be first cloned into PCR2.1 vector (Invitrogen) and subsequently into the *BamHI* and *HindIII* sites of pRSETa (Invitrogen). The resulting plasmid will be transformed in *E. coli* BL21 (DE3) PLysS (Novagen) by electroporation. To overexpress the cloned gene product, the transformants will be grown in LB (1000 ml)

containing ampicillin and chloramphenicol to an $OD_{600}$ of 0.5, the cells will be induced for the expression of protein by the addition of 2.0 mM IPTG for three hrs. The induced culture will be harvested, washed with 20 mM Tris-HCl containing145 mM NaCl and resuspended in 1 ml 40 mM imidazole, 0.4 M NaCl, 160 mM Tris-HCl pH 7.9, 1 mg/ml lysozyme and 200 µM PMSF. Cell suspensions will be sonicated and treated with DNaseI (1 µg/ml) and Triton-X (1%). Cell debris and unbroken cells will be removed by centrifugation at 4ºC. The supernatant will be applied to a nickel-charged-agarose-affinity column and eluted with 200-400 mM imidazole. Eluted fractions will be subjected to a 12.5 % SDS-PAGE analysis. Fractions containing the overexpressed His-tag protein will be pooled and dialyzed in a dialysis cassette against 25 mM Tris, pH 8.0,100 mM sucrose, 50 mM NaCl, and 1 mM DTT. The purified protein will be estimated by Bradford method and stored at -20 $^{O}$C.

2.2 Site directed mutagenesis to change cysteine to alanine in the CxxC motif of $MctsR_A$

Site directed mutagenesis will be performed in order to replace Cys residues by Ala in the CxxC motif using PCR-based method with megaprimer as described by Brøns-Poulsen et al., 1998. The two primers that will be used to introduce Cys [3, 6, 29, 31] to Ala are mctsR-N1a and mctsR-N2 (Table1). PCR will be performed from the two primers using *S. aureus* SH1000 as a template to generate a megaprimer. This fragment will be purified and used as a megaprimer in a second round PCR with mctsR-B1 primer and *S. aureus* SH1000 as a template. The PCR product will be first cloned in frame into PCR2.1 vector (Invitrogen)

and mutation will be confirmed by DNA sequencing. The fragment corresponding to mutated gene will be gel purified and subcloned into the BamHI and HindIII site of pRSETa and overexpressed in *E. coli* BL21(DE3) pLysS as described above.

2.3 Cation binding specificity of the CxxC motif by iminodiacetic acid-agarose chromatography.

Iminodiacetic acid-agarose columns equilibrated with different heavy metals will be used to determine the cation binding specificity as described by Lutsenko et al. (1997). The columns containing 100 μl of iminodiacetic acid-agarose (Sigma) will be washed with 50 mM sodium phosphate buffer (pH 7.5) and then separately equilibrated with 10 volumes of the same buffer containing one of several heavy metal chloride compounds ($CdCl_2$, $CuCl_2$, $CoCl_2$, $MnCl_2$, $ZnCl_2$ and $FeCl_3$) at a final concentration of 1 mM. Excess metal ions will be removed by extensive washing with sodium phosphate buffer and then 100 μg of purified McstR protein or mutated protein (ΔMcstR) will be added to the resin and incubated for 10 min at room temperature. Columns will be centrifuged to remove unbound proteins. Columns will be washed with 500 μl sodium phosphate buffer and bound proteins will be eluted from the column with 50 mM EDTA in sodium phosphate buffer. Both eluted and unbound proteins will be concentrated and analyzed by 12.5% SDS-PAGE.

3. bacterial two-hybrid system to assess protein-protein interaction

3.1 Constrction of the vector

Bacterial two-hybrid system will be constructed using pB2H Δα and pB2 Δω vector as described by Borloo et al., 2007. DNA fragment of the upstream and downstream

regions of ctsR or clpC will be amplified from genomic DNA using primer pairs as shown on Table 1. The PCR product will be first cloned in frame into PCR2.1 vector (Invitrogen) and subsequently into the SphI and BamHI sites of pB2H $\Delta\alpha$ . The $MctsR_A$ will be amplified from genomic DNA using primer pairs using primer mctsR-F2 and mctsR-B2 (Table1). The PCR product will be first cloned in frame into PCR2.1 vector (Invitrogen) and subsequently into the BamHI and NcoI sites of pB2H $\Delta\omega$ . To test the function of the CxxC domain from the $MctsR_A$, site directed mutagenesis will be performed in order to replace Cys residues by Ala at the N terminus of the MctsR (SAV5203) as described above. PCR will be performed from the two primers (mctsR-N1b and mctsR-N2) using *S. aureus* SH1000 as a template to generate a megaprimer. This fragment will be purified and used as a megaprimer in a second round PCR with mctsR-B2 primer and *S. aureus* SH1000 as a template. The PCR product will be first cloned in frame into PCR2.1 vector (Invitrogen) and mutation will be confirmed by DNA sequencing. The fragment corresponding to mutated protein will be gel purified and subcloned into the Sph I and BamHI site of pB2H $\Delta\omega$.

3.2 Coexpression of the fusion proteins

To coexpress the fusion proteins, an *E.coli* MC1061 containing pB2H $\Delta\alpha$-ctsR will be transformed with pB2H $\Delta\omega$-$MctsR_A$ or pB2H $\Delta\omega$-$\Delta MctsR_A$. Coexpression of the fusion protein will be achieved by inoculating the growth medium with proper double transformant and supplemented with IPTG and grown overnight before $\beta$-galactosidase assay.

3.3 β-galactosidase assays

β-galactosidase assay will be performed after preparation of cells as described by Borloo et al., 2007. Assay will be performed at room temperature by following o-nitrophenyl-β,D-galactose (ONPG) hydrolysis and 2-nitrophenol formation at 420 nm in a double beam spectrophotometer. The enzyme activity will be expressed as Miller units.

**4. Outline of study plan (แผนการดำเนินงานวิจัย)**

แผนการดำเนินงานวิจัยแบ่งเป็น 4 ช่วง โดยมีระยะเวลาช่วงละ 6 เดือน รวมระยะเวลาทั้งสิ้น 2 ปี

| แผนงาน | ระยะเวลา |
|---|---|
| Panel 1:<br>-To cloned and overexpressed the $MctsR_A$ of *S. aureus* and clone $MctsR_A$ in overexpression vector<br>- To characterize the metal binding activity of CxxC motif from $MctsR_A$ and of *S. aureus* | เดือนที่ 1-เดือนที่ 6 |
| Panel 2:<br>-To performed site directed mutagenesis of CxxC motif from $MctsR_A$ of *S. aureus* and clone $\Delta$ *mctsR*$_A$ in overexpression vector<br>- To characterize the metal binding activity of CxxC motif from $\Delta MctsR_A$ and of *S. aureus* | เดือนที่ 7-เดือนที่ 12 |
| Panel 3:<br>-To performed site directed mutagenesis of CxxC motif of $MctsR_A$ from S. aureus and clone ctsR, $MctsR_A$ and $\Delta$ $MctsR_A$ in bacterial two-hybrid system | เดือนที่ 13-เดือนที่ 18 |
| Panel 4:<br>-To coexpress the fusion proteins and perform β-galactosidase assays<br>-Data analysis, manuscript preparation | เดือนที่ 19-เดือนที่ 24 |

**5. ผลงาน/หัวข้อเรื่องที่คาดว่าจะตีพิมพ์ในวรสารวิชาการระดับนานาชาติในแต่ละปี**

ปีที่ 2 ชื่อเรื่องที่คาดว่าจะตีพิมพ์: Molecular characterization of the *mctsR* from *Staphylococcus aureus*

ชื่อวรสารที่คาดว่าจะตีพิมพ์: FEMS Microbiology letter (ค่า impact factor 1.48 )

**6.งบประมาณโครงการ**

| | ปีที่ 1 | ปีที่ 2 | รวม (บาท) |
|---|---|---|---|
| 1. หมวดค่าตอบแทน<br>- ค่าตอบแทนหัวหน้าโครงการ | 120,000 | 120,000 | 240,000 |
| 2.หมวดค่าวัสดุ<br>-ค่าสารเคมีและวัสดุวิทยาศาสตร์<br>-เครื่องเขียน กระดาษ หมึกพิมพ์ | <br>114,000<br>5000 | <br>114,000<br>5000 | <br>228,000<br>10000 |
| 3. หมวดค่าใช้สอย<br>-ค่าถ่ายเอกสาร เข้าเล่ม | <br>1000 | <br>1000 | <br>2,000 |
| รวมงบประมาณโครงการ | 240,000 | 240,000 | 480,000 |

## เนื้อหางานวิจัย

### บทนำ (Introduction)

*Staphylococcus aureus* is bacteria that capable of growing in a wide range of adverse environmental stress conditions and a number of genes that involved in environmental stress response in *S. aureus* have been identified. During stress conditions, cellular proteins tend to unfold and aggregate and protein quality control serve to maintain cellular proteins by preventing misfolding and aggregation or degradation of proteins that cannot be refolded (Gottesman et al., 1997). CtsR is a regulator of stress response which is a member of heat shock class III that play an important role in protein quality control (Clement and Foster, 1999; Molière and Turgay, 2009). It's important in the virulence and survival of several pathogens and its synthesis is stimulated in response to a variety of stress such as heat stress, acid stress, oxidative stress and copper stress (Derre et al., 1999; Mostertz et al., 2004, Anderson, et al., 2006, Bore et al., 2007 and Baker et al., 2010). *CtsR* operon has been identified in some microorganisms such as *Bacillus subtilis, Lactobacillus plantarum, Oenococcus oeni* and *Listeria monocytogenes (*Nair et al., 2000; Grandvalet et al*,* 2005; Kirstein et al., 2005; Fiocco *et al*, 2010*)* . In gram positive bacteria*, ctsR operon cons*isting of four genes, designated as *ctsR, mcsA, mcsB* and *clpC.* In the *S. aureus* strains, modulator of *ctsR* gene (in this study, designed as *mcsA*) is located downstream of ctsR gene. McsA functions as a modulator of the heat shock repressor ctsR. The amino acid sequence of McsA contains two Cys2-Cys2 zinc finger motifs (Krüger et al., 2001). Each zinc finger motif contains two CxxC motifs which always found

in heavy metal chaperone or thiol-disulphide oxidoreductase superfamily. The paired cysteine residues in this CxxC motifs play an important role in heavy metal binding (Walker et al., 2002; Walker et al., 2004) and may involve in the binding and protein interaction with other molecules ( Zdanowski et al., 2006; Gaskell et al., 2007; Yabe et al., 2008).

To date, little is known about molecular mechanism of the *ctsR* and its modulator in *S. aureus*. In this study, we investigated the function of CxxC motif from McsA genes involved in metal binding activity and protein interaction. McsA from *S. aureus* was cloned, overexpressed in *E. coli* and the metal binding activity was determined in vitro. The requirement for cysteine residues in the heavy metal binding domains was investigated by site-directed mutagenesis in the McsA protein, in which six cysteine residues in three metal binding domains were replaced by alanine. Additionally, CxxC motif at the N terminus of the McsA may responsible for the binding with other molecules and involved in regulation of heat shock and virulence gene expression. Bacterial two-hybrid system was constructed to determine the protein interaction of McsA with CtsR and mcsB molecule.

## วิธีดำเนินการวิจัย (Material and Method)

### Bacterial strains and growth conditions

*S. aureus* strain SH1000, *E. coli* strain JM109, DH5 α, BL21 (DE3) PLysS and *E. coli* strain MC1061 was used in this study (Table 1). *S. aureus* was grown in tryptic soy broth (TSB) and *E. coli* was grown in Luria- Bertani broth (LB). When needed, ampicillin (50 µg/ml), carbenicillin (50 µg/ml), and chloramphenicol (10 µg/ml) was added to the growth medium.

**TABLE 1. Bacterial strains used in this study**

| Bacterial strains | characteristics | Source or reference |
|---|---|---|
| ***S. aureus* strains** | | |
| SH1000 | NCTC 8325-4 with rsbU mutation repair | Horsburgh *et al.,* 2002 |
| ***E. coli* strains** | | |
| JM109 | rec A1 supE44endA1hsdR17 gyrA96 relA1 thiΔ (lac-proAB) F' (traD36proAB+ laclQ Z Δ M15) | Promega |
| BL21(DE3) | (pLyS) F- ompT hsdSB(rB-m-B) gal dcm | Promega |
| MC1061 | Δ(araA-leu)7697, [araD139]B/r, Δ(codB-lacI)3, galK16, galE15(GalS), &lambda-, e14-, mcrA0, relA1, rpsL150(strR), spoT1, mcrB1, hsdR2 | *E. coli* genetic stock center |

**Cloning and overexpression of *mcsA* in *E. coli* BL21 (DE3)**

PCR of *mcsA* gene was performed using two oligonucleotide primer (McsA-F[a] and McsA-B[a]) (Table 2) and *S. aureus* genomic DNA as a template. The PCR products were cloned into the TA vector (RBC) and subsequently cloned into *BamHI* and *HindIII* sites of pRSETa (Table 3). The resulting plasmid was transformed in *E. coli* BL21 (DE3) PLysS (Novagen) by heat shock. Protein was overproduced by induction with IPTG and purified by nickel-charged agarose affinity column (Novagen).

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

| gene | Primer name | Primer sequence 5'-3' |
|---|---|---|
| ***mcsa*** | Mcsa-F[a] (BamHI) | GCGGATCCGGTGCTTTGTGAAAATTGTCAACTTAA |
| | Mcsa-B[a] (HindIII) | GCAAGCTTTTATGCGTCATCATGTTGCAC |
| **Δ- mcsa** | mcsA-F[b](BamHI) | GCGGATCCGTGCTT**GCG**GAAAAT**GCG**CAACTTAAT |
| | mcsA-B[b] | CAG**C**GCGAGTTTGG**CGC**ACCATTTTTTC |
| | mcsA-DR[b] | TGCATA**CGC**ATTAGC**CGC**CCCAAATTTACC |
| | mcsA-DF[b] | GGTAAATTTGGG**GCG**GCTAAT**GCG**TATGCA |
| | mcsA-R[b](EcoRI) | GCAAGCTTAGGGACACCATCCGTGG |
| **ClpC** | ClpC-F[c](sphI) | GCGCATGCATGTTATTTGGTAGATTAACTGAGCGTG |
| | ClpC-B[c] (BamH) | CGGGATCCTGCTTGCGATGGTGTTTTAGT |
| **mcsa** | Mcsa-F[c](sphI) | GCGCATGC GTGCTTTGTGAAAATTGTCAACTTAA |
| | Mcsa-B[c] (Bam) | GGGTCTAGATGCGTCATCATGTTGCACCTCA |
| **mcsB** | mcsB-F[c](sphI) | GCGCATGCATGACGCATAATATTCATGATAATATCAGC |
| | McsB-B[c] (Bam) | GGGTCTAGACTTTATATGTTCTCTTAGTATATCTGCTCGTTT |
| **ctsR** | ctsR-F [c](sphI) | GCGCATGC ATGCACAATATGTCTGACATCATAGAA |
| | ctsR-B[c](Bam) | CGGGATCCGTAATAATTTATAACTGGTAACAAACGTTTTAAA |

- The restriction sites are indicated by underline
- [a]Primers were used for amplication of protein expression
- [b]Primers were used for amplication for CxxC mutagenesis and the modified bases are indicated by bold red letters.
- [c]Primers were used for bacterial hybrid system and RT-PCR

**TABLE 3. Plasmids and vector used in this study**

| Plasmid | Relevant characteristics | Source or reference |
|---|---|---|
| **Plasmids** | | |
| pCR2.1 | PCR cloning vector, AmpR, , KanR | Invitrogen |
| pRSETa | Overexpression vector, AmpR | Invitrogen |
| pB2HΔα | pACYCDuet-1Ωtac with the *E. coli* β-galactosidase fragment lacking the sequence for amino acids 11-41 (Δα)cloned in the BamHI-NcoI site; CmR,7.5 kb | Borloo et al.,2007 |
| pB2HΔω | pETDuet-1ΔSphI Ωtac with the *E. coli* β-galactosidase fragment lacking the sequence for amino acids 789-1023(Δω) cloned in the BamHI-NcoI site; CbR,8.3kb | Borloo et al.,2007 |
| pB2HΔω*mcsA* | pB2HΔα with the *mcsA* gene cloned in the *BamHI-SphI* site, CbR,8.8 kb | This study |
| pB2HΔωΔ*mcsA* | pB2HΔα with theΔ *mcsA* gene cloned in the *BamHI-SphI* site, CbR,8.8 kb | This study |
| pB2HΔα*ctsR* | pB2HΔα with the *ctsR* gene cloned in the *BamHI-SphI* site, CmR, 8.0 kb | This study |
| pB2HΔα*mcsB* | pB2HΔαwith the *mcsB* gene cloned in the *BamHI-SphI* site, CmR, 7.7 kb | This study |

**Site directed mutagenesis to change cysteines to alanine in the metal binding domain of McsA**

Site directed mutagenesis was performed in order to replace six of the Cys residues with Ala in the metal binding domains of Mcsa using the PCR-based method with megaprimer and PCR base overlapping (Brøns-Poulsen *et al.,* 2002, Kanoksilapatham et al., 2007). The primers (Mcsa-F[b], McsA-B[b], McsA-DR[b], McsA-DF[b] and McsA-B[b]) were used to exchange Cys at positions 3, 6, 29, 31,104, 107 for Ala residues. Polymerase chain reaction-based site-directed mutagenesis was performed using mcsA-F[b] and mcsA-B[b] primers and *S. aureus* SH1000 genomic DNA as template. The fragment was gel purified and used as a megaprimer in the second round of PCR with mcsA-DR[b] primer. The PCR product was cloned in frame in PCR2.1 vector (Invitrogen) to genetate plamid TA-ΔmcsA which was used to replace Cys, 104,107 to Ala using PCR base overlapping method (Kanoksilapatham et al., 2007). Plasmid TA-ΔmcsA and used as a template to generate the first PCR fragment using primer mcsA-F[b] และ mcsA-DR[b] . The overlapping frangment was generate using primers mcsA-DF และ mcsA-R[b]. Overlaping extention was performed as described by Kanoksilapatham *et al.* (2007) and the mutated fragment was cloned into PCR2.1 vector (Invitrogen). Mutations were confirmed by DNA sequencing. The mutated fragment was gel purified and subcloned into the *BamHI* and *HindIII* site of pRSETa and overexpressed in *E. coli* BL21(DE3) as described previously (Sitthisak *et al*., 2007).

**Metal binding properties of McsA**

**1. Cation binding specificity of the CxxC motif by iminodiacetic acid-agarose chromatography.**

Iminodiacetic acid-agarose (IAA) columns equilibrated with different heavy metals were used to determine the cation binding specificity as described by Lutsenko et al. (1997). The columns containin IAA were extensively washed with 50 mM sodium phosphate buffer (pH 7.5) and then separately equilibrated with 10 volumes of the same buffer containing one of several heavy metal compounds ($CdCl_2$, $CuCl_2$, $CoCl_2$, $MnCl_2$, $ZnCl_2$, $MgCl_2$, $Pb(NO_2)_3$ and $FeCl_3$). Excess metal ions were removed by repeated washing with sodium phosphate buffer and then 100 μg of purified McsA or Δ McsA protein was added to the resin. Columns were centrifuged to remove unbound proteins and washed with sodium phosphate buffer. Bound proteins were eluted from the column with 50 mM EDTA in sodium phosphate buffer. Both eluted and unbound proteins were analyzed by 12.5% SDS-PAGE.

**2. Involvement of cysteine residues in the MBD**

Involvement of the cysteine residues was demonstrated by the ability of copper to protect the cysteine residues in the metal-binding domains against labeling with the cysteine-directed fluorescent reagent, 7- diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin (CPM). Briefly, 50 μg of protein was incubated in the presence of different concentrations of heavy metals for 10 min, and then pulse-labeled with a 20 molar excess

of CPM for 1 min in the dark. Proteins were separated on 15% SDS-PAGE. The fluorescent coumarin-labeled proteins were monitored under UV light.

**RT-PCR of *ctsR* and *mcsA***

*S. aureus* strains were grown overnight in TSB at 37$^{O}$C with shaking and then diluted 1: 100 in TSB. Bacteria were grown in TSB until mid-log phase ($OD_{600}$ =0.4). Culture for control RNA was collected and $CuCl_2$, $CdCl_2$, $CoCl_2$ and $ZnCl_2$ were add to a final concentration of 2 mM, 0.125 mM, 2 mM and 1 mM respectively. All cultures were then incubated at 37$^{O}$C with shaking for 15 min. Total RNA was extracted from uninduce culture (control) and various heavy metal stress cultures using RNA easy RED. 1 μg of total RNA from each samples were reverse transcribed into cDNA with Revoscript RT Premix kit (iNtRON biotechnology, inc). 0.5 microliters of the mixture was added to a PCR mixture using the primer pair to amplify *ctsR* gene (*ctsR*-F$^{c}$ and *ctsR*-B$^{c}$), *mcsA* gene (*mcsA*-F$^{c}$ and *mcsA*-B$^{c}$) and clpC (*clpC*-F$^{c}$ and *clpC*-B$^{c}$). Genomic DNA from *S. aureus* was used as a positive control and RNA templates without the reverse transcriptase were used as a negative control. All PCR products were analyzed by 0.8% DNA agarose gel electrophoresis.

**Bacterial two-hybrid system to assess protein-protein interaction**

**Constrction of the vector**

Bacterial two-hybrid system was constructed using pB2H $\Delta\alpha$ and pB2 $\Delta\omega$ vector as described by Borloo *et al*., 2007. DNA fragment of the upstream and downstream regions of *ctsR* and *mcsB* were amplified from genomic DNA using primer pairs as shown on

Table 2. The PCR product was cloned in frame into PCR2.1 vector (Invitrogen) and subsequently cloned into the *SphI* and *BamHI* sites of pB2H $\Delta\alpha$. The *mcsA* was amplified from genomic DNA using primer pairs using primer mcsA-F$^{c}$ and mcsA-B$^{c}$ (Table2). The PCR product was first cloned in frame into PCR2.1 vector (Invitrogen) and subsequently into the *SphI* and *BamHI* sites of pB2H $\Delta\omega$. To test the function of the CxxC domain from the McsA, site directed mutagenesis was performed in order to replace Cys residues by Ala at the N terminus of the McsA as described above. The fragment corresponding to mutated protein will be gel purified and subcloned into the *SphI* and *BamHI* site of pB2H $\Delta\omega$.

**Coexpression of the fusion proteins**

To coexpress the fusion proteins, an *E.coli* MC1061 containing pB2H $\Delta\omega$-mcsA or pB2H $\Delta\omega$-$\Delta$mcsA was transformed with pB2H $\Delta\alpha$-*ctsR* or pB2H $\Delta\alpha$-*mcsB.* Coexpression of the fusion proteins was achieved by inoculating the growth medium with proper double transformant and supplemented with 1 mM IPTG and grown overnight before $\beta$-galactosidase assay.

**$\beta$-galactosidase assays**

$\beta$-galactosidase assay was performed after preparation of cells as described by Borloo et al., 2007. Briefly, Cell culture were collected at 10000g for 10 min and the pellet were resuspended in 1 ml Z buffer and homogenized by sonicator and cell debris was removed by centrifugation at 10,000Xg at 4 ºC. The supernatants contained the soluble protein fraction of the cells were used to determine the enzymatic activity. $\beta$-galactosidase assay was performed at room temperature by following o-nitrophenyl-$\beta$,D-galactose (ONPG)

hydrolysis and 2-nitrophenol formation at 420 nm in a double beam spectrophotometer. Cell lysate protein concentrations were determined by the Bradford assay using the Bio-Rad protein assay solution. The enzyme activity was expressed as nmol/min/mg protein.

$$\text{Activity} = \frac{(OD_{420} - 1.75 \times OD_{550})}{\text{Mg protein} \times T \times \text{vol}} \times \frac{1}{0.0045} \times V$$

- $OD_{420}$ and $OD_{550}$ are read from the reaction mixture.
- T = time of the reaction in minutes.
- vol = ml cells added to the assay tubes
- V = total vol
- 0.0045 OD420/nmol = e420 o-nitrophenol

**Molecular genetic procedures.**

Plasmid and chromosomal DNA isolation, DNA manipulation, digestion of DNA with restriction enzymes, DNA ligation, RNA isolation and polymerase chain reactions were performed as described by Sambrook & Russell (2001).

**ผลการวิจัย (Result)**

**1) Analysis of *mcsA* genes in *S. aureus* genome**

The genome analysis of *S. aureus* strains COL, N315, Mu3, Mu50, MW2, MRSA252 showed the presence of similar *ctsR* operon consisting of 4 genes, designated as *ctsR* (482bp), *mcsA* (567bp), *mcsB* (1008 bp) and *clpC* ( 2457 bp) (Figure 1). Promoter prediction of ctsR show that upstream of the ctsR is a potential -35 (TTGAAA) and -10 (TCATATAAT). The genome database analyses suggested that the genes involved in *mcsA* are conserved in *S. aureus. mcsA* shows almost 100% sequence identity among *S. aureus* strains and about 80% with other staphylococcal species. *mcsA* encodes a protein with 188 amino acid with a predictive molecular mass of 24 KDa. Four CxxC motifs containing $C^{3}XXC^{6}$, $C^{29}XXC^{32}$, $C^{87}XXC^{90}$ and $C^{104}XXC^{107}$ has been identify in mcsA protein.

**2) Cloning and overexpression of *mcsA* and $\Delta$*mcsA* in *E. coli* BL21 (DE3)**

*McsA* and $\Delta$*mcsA* were cloned, overexpressed as described in the Methods. The purified McsA protein produced a band with molecular mass of approximately 24 kDa as shown in Figure 2, whereas the purified $\Delta$McsA protein produced a band with molecular mass of approximately 24 kDa as shown in Figure 3.

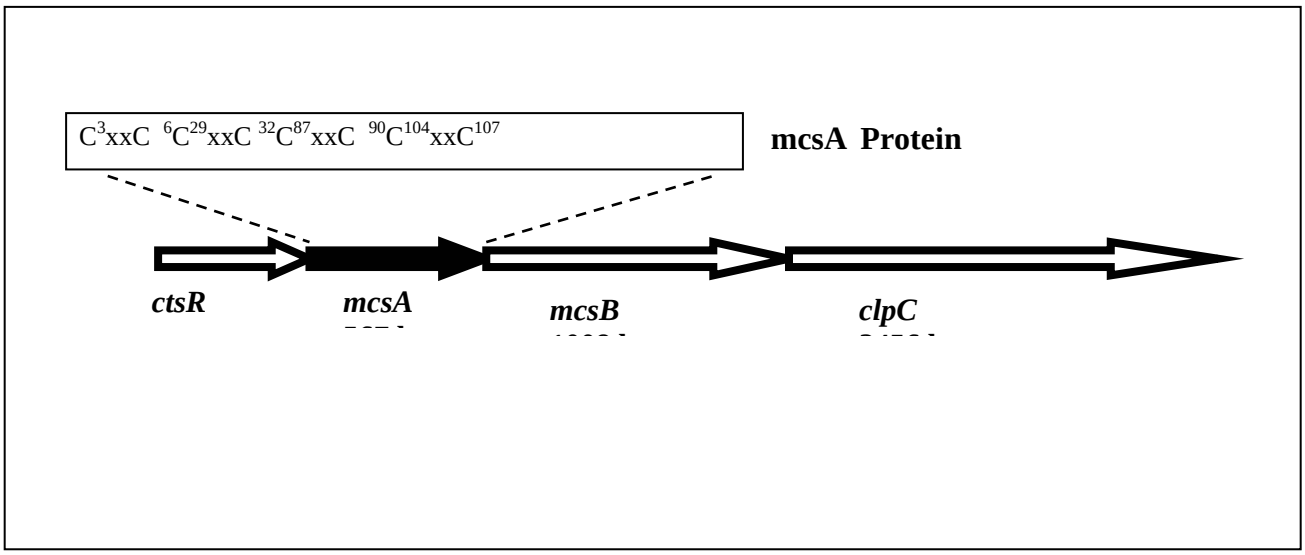


**Figure1.** Schematic representation of the *ctsR* operon in *S. aureus*. The arrow indicates predicted genes and their orientations. A schematic representation of the Mcsa protein with four heavy metal-binding CxxC motifs is shown above the *mcsA* gene.

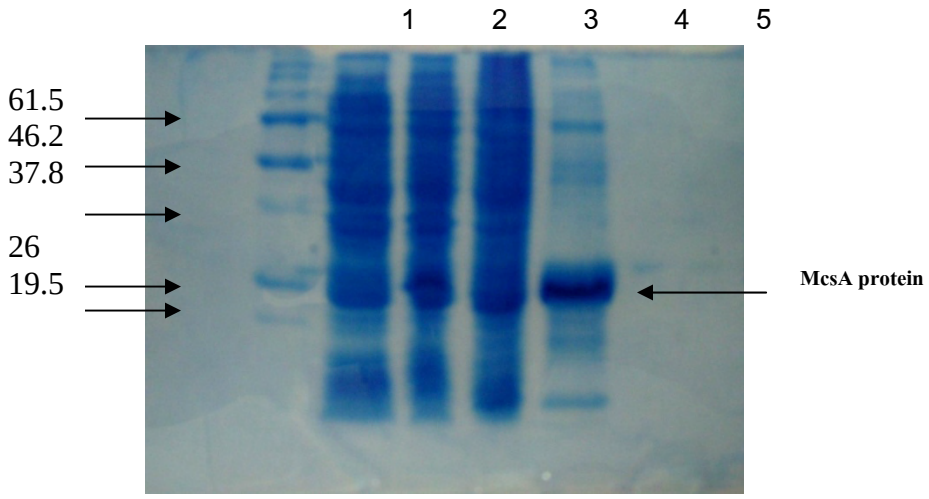


**Fig. 2**. SDS-PAGE analysis of purified McsA. Protein samples were heat denatured prior to electrophoresis and the gel (12.5%) was stained with Coomassie blue. Lane 1, molecular weight marker; lane 2, *E. coli* uninduced extract; lane 3, *E. coli* induced extract; lane 4, *E. coli* crude extract ; lane 5, purified McsA protein. The values on the left are molecular weight masses (kDa).

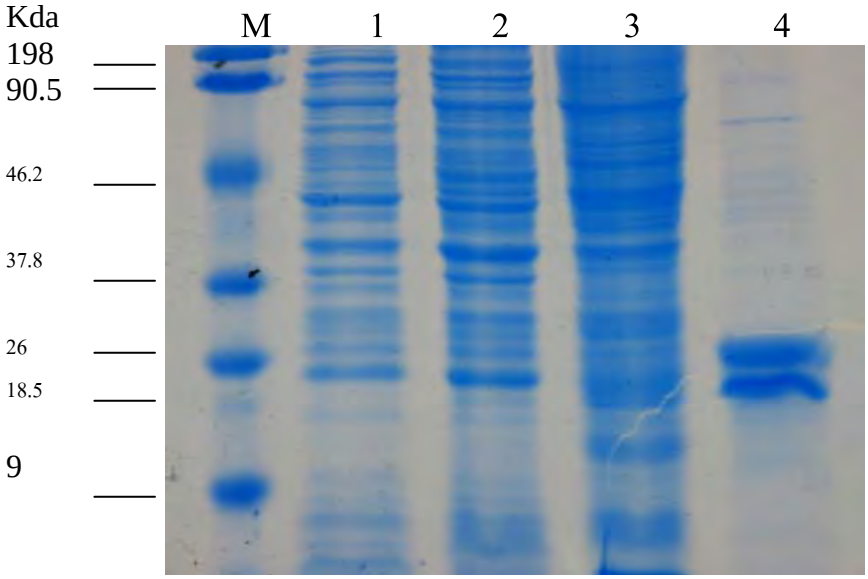


**Fig. 3**. SDS-PAGE analysis of purified ΔMcsA. Protein samples were heat denatured prior to electrophoresis and the gel (12.5%) was stained with Coomassie blue. Lane 1, molecular weight marker; lane 2, *E. coli* uninduced extract; lane 3, *E. coli* induced extract; lane 4, purified Δ McsA protein. The values on the left are molecular weight masses (kDa).

**Metal binding properties of McsA and Δ McsA protein**

In this study, the ability of CxxC motif from McsA protein to bind different heavy metals was investigated using heavy metal-chelating chromatography (IAA resin). As shown in Figure 4a, McsA protein binds specifically to copper, zinc, cadmium, and cobalt. No binding was observed to the columns charged with iron, manganese, magnesium and lead (Figure 4b). No binding with any metals except copper was observed in the Δ McsA protein (Figure 4c and 4d).

To further confirm the role of cysteine residues in the metal-binding domains of McsA protein, a cysteine-directed fluorescent reagent (CPM) was used as described in the materials and methods. As shown in Figure 5a, 200 μmol of copper prevented the labeling of cysteine residues with CPM when the CxxC motif from McsA was incubated with fluorescence dye in the presence of various concentrations of copper ions. In addition, inhibition of fluorescent labelling was also seen when the McsA protein was incubated with zinc and cadmium, and cobalt (Figure 5b and 5c). These results confirm that cysteine residues bind to heavy metals.

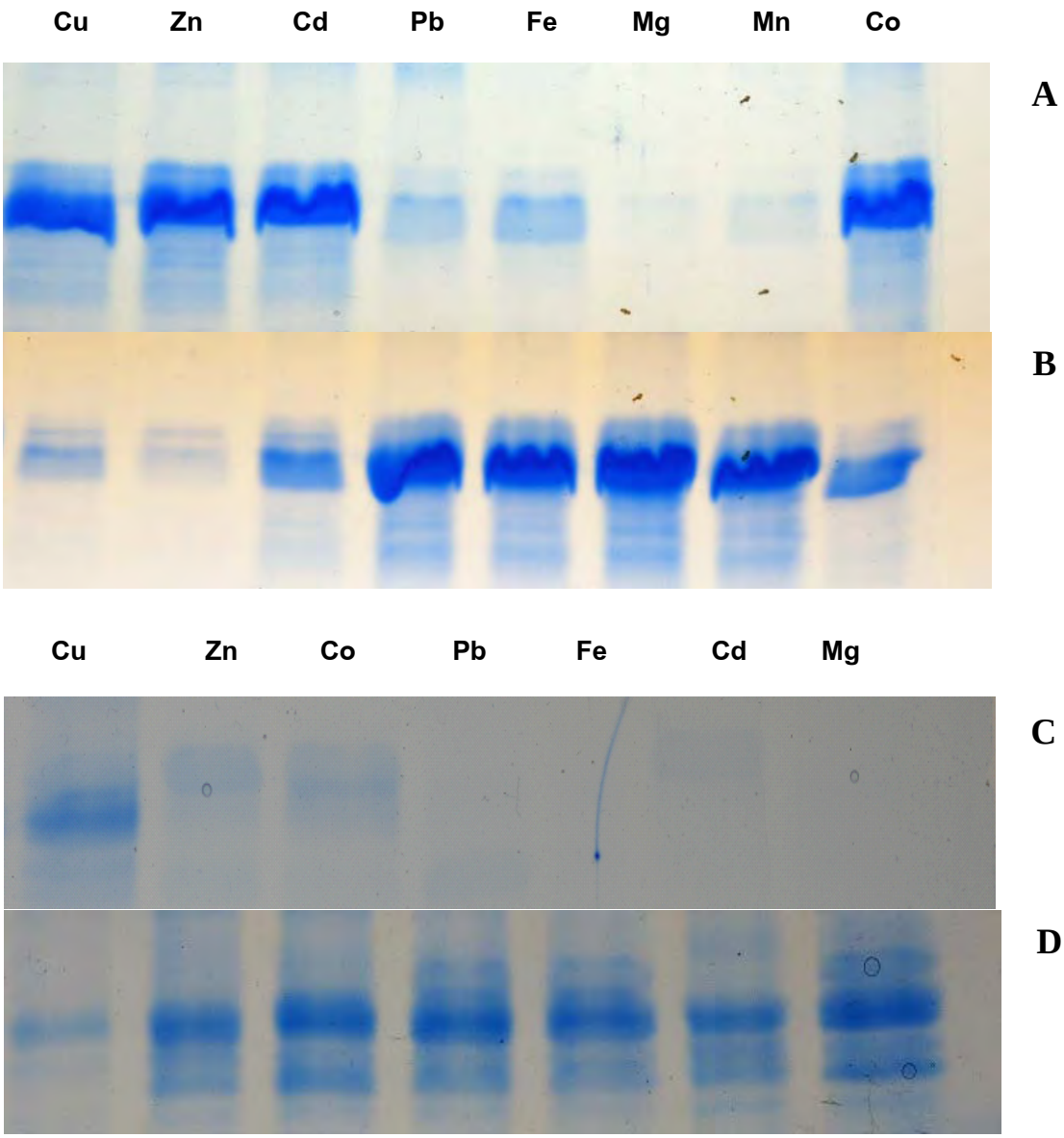


**Figure 4.** Metal binding properties of McsA protein and Cys- mutated McsA protein. IAA-resin was charged with different heavy metal as indicated above the respective lane. Bound and unbound proteins were analyzed on 12.5 % SDS-PAGE. Row A, Bound McsA protein; Row B, Unbound McsA protein; Row C, Bound Cys- mutated McsA protein, Row D, Unbound Cys- mutated McsA protein.

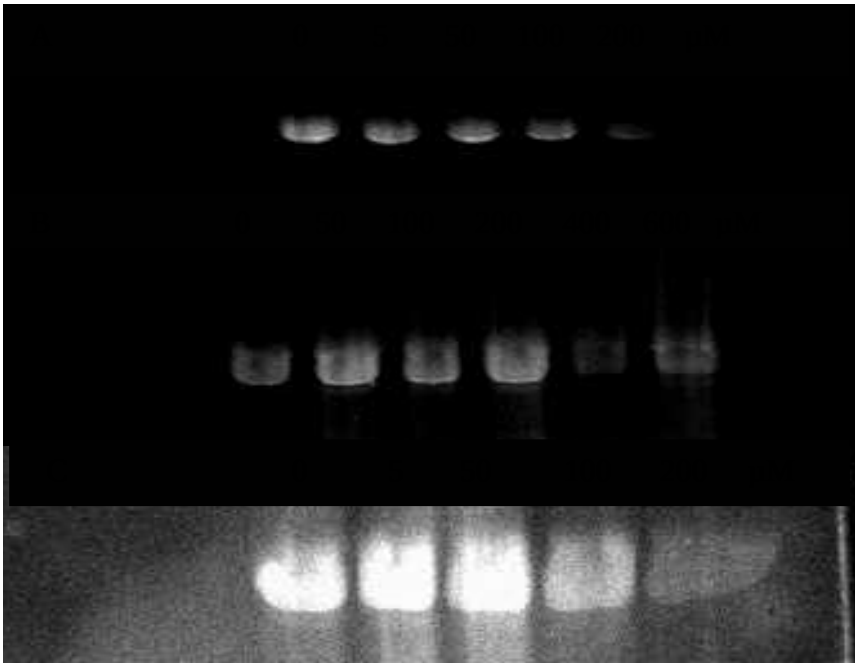

**Figure 5.** Cysteine-directed fluorescent reagent labeling to examine the role of cystein residues in metal binding of McsA protein. McsA protein was incubated the presence of different concentrations of heavy metals for 10 min., and then pulse-labeled with a 20 molar excess of cysteine-directed fluorescent reagent, 7- diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin (CPM) for 1 min in the dark. Proteins were separated on 15% SDS-PAGE. The CPM-labeled proteins were then monitored under UV light. Row A, McsA protein was incubated the presence of $CuCl_2$. Row B. McsA protein was incubated the presence of $ZnCl_2$. Row C. McsA protein was incubated the presence of $CdCl_2$.

**Expression of *ctsR, mcsA, mcsB* and *clpC* is induced by heavy metal stress**

In this study, we would like to analyzed whether the genes in *ctsR* regulon was induced by heavy metals directly. Cu (II), Zn (II), Co (II) and Cd (II) were used in this study base on the metal binding assay. RT-PCR was performed to evaluated the effect of heavy metals on the expression of and *ctsR, mcsA, mcsB* and *clpC* . As shown on figure 6, upon heavy metals stress, transcription of *mcsA* and *mcsB* were increasingly expressed compared with uninduced cell control. The decrease of expression of *ctsR* gene was detected when *S. aureus* was exposed to Cu (II), Zn (II), Co (II) and Cd (II). No PCR product was detected in the negative control using RNA without the reverse transcriptase as PCR templates.

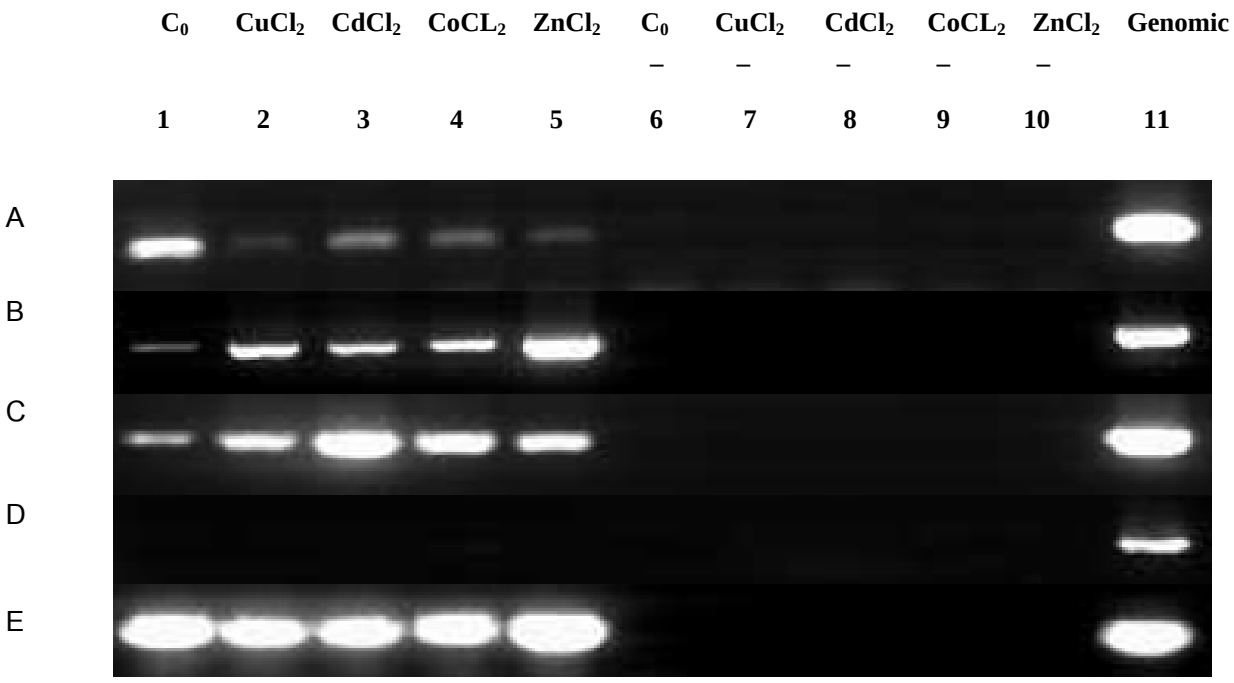


**Figure 6.** RT-PCR analysis of genes in *ctsR* operon in response to various heavy metals. Total RNA from each samples were reverse transcribed into cDNA with *Revoscript RT.* PCR was performed with primers ctsR-F and ctsR-B (row A), primers mcsA-F and mcsA-B (rowB), mcsB-F and mcsB-B (rowC), clpC-F and clpC-B (rowD) and 16S-F and 16S-B (RowE). Total RNA was extract from uninduce culture (control) (lane 1), culture after induce with 2 mM $CuCl_2$ (lane 2), culture after induce with 0.125 mM $CdCl_2$(lane 3), culture after induce with 2 mM $CoCl_2$ (lane 4), culture after induce with 1 mM $ZnCl_2$ (lane 5). Genomic DNA from *S. aureus* was used as a positive control (lane 11) and RNA templates from each samples without the reverse transcriptase were used as a negative control (lane 6-10). Total RNA from each samples were reverse transcribed into cDNA with *Revoscript RT Premix kit* (iNtRON biotechnology, inc). 0.5 microliters of the mixture was added to a PCR mixture using the primer pair to amplify *ctsR*,*mcsA, mcsB, clpC* genes. Genomic DNA from *S. aureus* was used as a positive control and RNA templates without the reverse transcriptase were used as a negative control. All PCR products were analyzed by 0.8% DNA agarose gel electrophoresis

**Bacterial two-hybrid system to assess protein-protein interaction**

In this study, we tested whether there was an interaction between McsA protein with McsB protein or ctsR protein using bacterial two hybrid system (Borloo et al., 2007). pB2HΔω-mcsA and pB2HΔα-mcsB or pB2HΔα-ctsR was cotransformed into *E. coli* MC1061 and tested for the β-galactosidase assays. The result of the β-galactosidase assays was shown in Figure 7. *E.coli* MC1061 with Plasmid pB2HΔω- *mcsa* coexpress pB2HΔαctsR gave a unit of activity equal to 1218.5 nmol/min/mg protein. *E. coli* MC1061 with Plasmid pB2HΔω mcsa coexpress pB2HΔαmcsB gave an activity value of 1088.25 nmol/min/mg protein. β-galactosidase activity was not found in *E.coli* MC1061 co-expressed with pB2HΔα/ pB2HΔω(negative control). Lower β-galactosidase activity was detected when pB2HΔωΔmcsa coexpress pB2HΔαctsR (105 nmol/min/mg protein) and pB2HΔωΔmcsa coexpress pB2HΔαmcsB(70.5 nmol/min/mg protein).

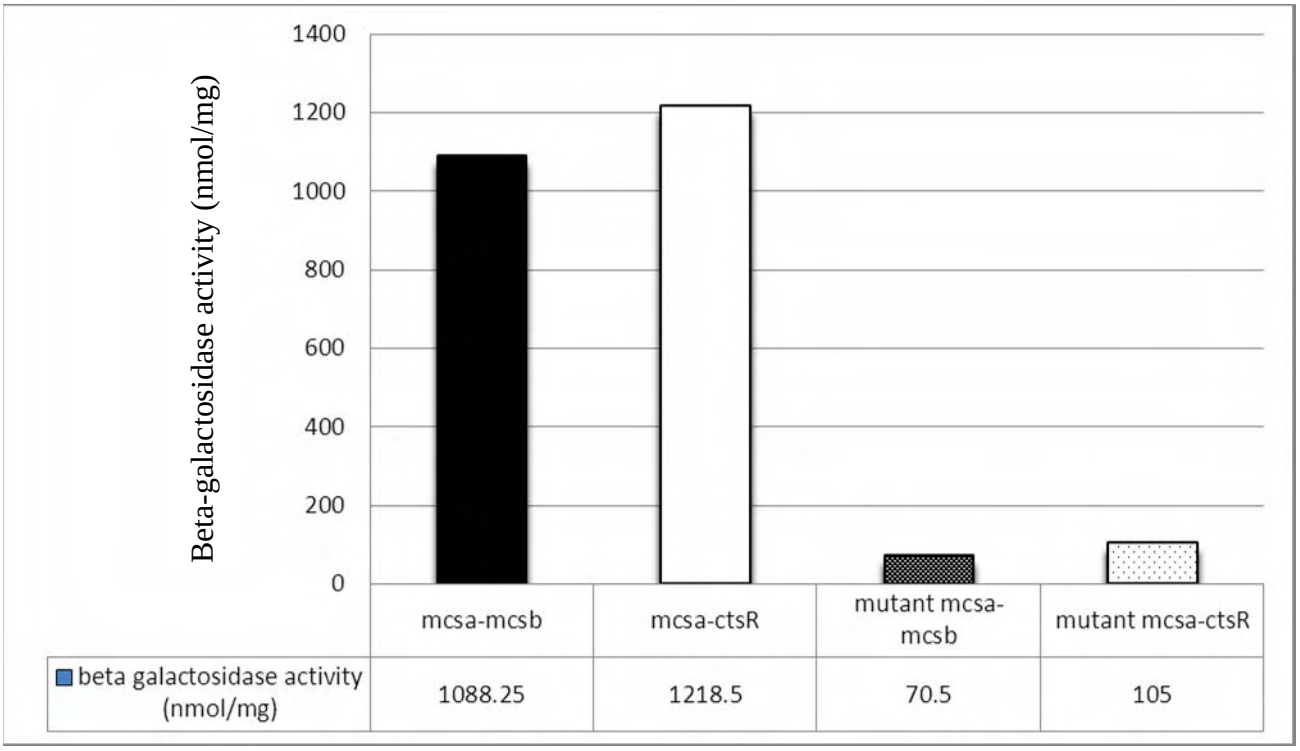


**Figure 7. β-galactosidase activity for protein interaction.**

β-galactosidase expression of *E.coli* MC1061 with Plasmid pB2HΔω*mcsa* coexpress pB2HΔαmcsB (■),pB2HΔωΔmcsa coexpress pB2HΔαctsR (□), Plasmid pB2HΔωmcsa coexpress pB2HΔαmcsB(▩), Plasmid pB2HΔωΔmcsa coexpress pB2HΔαmcsB (▨) Cotransformant *E.coli* MC1061 were induced with 1 mM IPTG and grown at 37 °C overnight. Cells were homogenized and enzyme activity was measured by hydrolysis of o-nitrophenyl galactoside .β-galactosidase activitys was expressed as nmol/min/mg protein, mean± standard deviations of 3 experiments.

## ข้อวิจารณ์ (Discussion)

In this study, the putative McsA from *Staphylococcus aureus* encoded a protein with 189 amino acids which contained four metal binding domains with CxxC motif was characterized. CxxC motif have been identify in many proteins in various organisms. Previous study has shown that the paired cysteine residues in this metal binding domain of various organisms play an important role in heavy metal binding and transporting (Nash et al., 1993; Walker et al., 2002; Walker et al., 2004, Sitthisak et al., 2007; Agarwal et al., 2010). CxxC motif in the metal binding domain from CopA and CopZ of *S. aureus* can bind bind specifically to copper, cobalt and cadmium (Sitthisak et al., 2007). In common with previous work CxxC motif in McsA can bind to copper, cobalt, cadmium and zinc. We changed six of the eight conserved cysteines of the CxxC motifs in McsA protein. In agreement with the previous study, the metal binding activity determined by the mutated CxxC show that the CxxC domain require two conserved cysteine ligands provided by one CxxC motifs to bind one copper ions (Lutsenko, et al 1997; Sitthisak et al., 2007) while four conserved cysteine ligands provided by two CxxC motifs require to bind zinc ion (Allen et al 2006, Zimmermann et al., 2009).

Expression of genes in *ctsR* operon was induced by heat, cold, osmotic pressure and disulfide stress (Derre et al., 1999; Anderson, et al., 2006, Bore et al., 2007; Fiocco D. *et al.,* 2010, Elsholz AK *et al.,* 2011). The *S.aureus* McsA which contains CxxC metal binding motifs may bind, and hence sense, excess heavy metals, thus modulating CtsR activity and therefore inducing heavy metal response. Previous work shows that copper

shock results in the induction of the *mcsA* response (Baker et al., 2010). In this study, RT-PCR study show that copper, cobalt, cadmium and zinc stress induce the expression of genes in *ctsR* operon. During heavy metal stress conditions, cellular proteins tend to unfold and aggregate due to its toxicity. Metal ion is an important componets in several regulatory proteins (Berg, 1990). Metal can bind and oxidized the Cys residues and induce thiol-specific oxidative stress and the Cys-X-X-Cys motif is essential for their catalysis of redox reactions (Chivers et al., 1997, Quan et al, 2007). Previously study have shown that McsB is able to bind to a ctsR:DNA complex (Kruger et al., 2001; Kirstein et al., 2005) and inactivate CtsR during thiol-specific oxidative stress (Elsholz et al,2011). In addition, in *B. subtilis,* the expression of *ctsR* regulons is controlled by redox-active cysteines which brought by disulfide stress (Leichert et al., 2003; Elsholz AK et al., 2011) and HxxxCxxC motif in ZAS protein from *Streptomyces coelicolor* has been identified as a redox-sensing molecule (Zdanowski et al., 2006). Recently study has shown that CtsR is inactivated during oxidative stress by a thiol-dependent regulatory pathway and the regulatory nano switch of mcsA is located within the second zinc finger of mcsA (Elsholz et al., 2010; Elsholz et al., 2011). When the thiols of McsA become oxidized, the strong interaction between McsA and McsB is interrupted and free McsB is no longer inhibited by McsA, resulting in the inactivation of CtsR (Elsholz et al., 2011).Thus, in response to heavy metal stress, metal can bind directly to the Cys residues of the CxxC motif and activate the *ctsR* reguron through this pathway.

CxxC motif involved in the interaction between two molecules through a (2Fe -2S) clusters- containing protein (Gaskell et al., 2007; Yabe et al., 2008). In this study, the bacterial hybrid system show that McsA can interact with CtsR and McsB molecule. This data was agreement with previous studies by Fress et al (2007) that CtsR of *B. subtillis* can bind specifically to McsA. In *B. subtilis*, Mcsa form ternary complex with Mcsb and ClpC. In response to stress, clpC release from the complex result in sequestration of CtsR from its target promoters. Then, CtsR bind to the mcsA and mcsB complex and mediated the target gene expression (Frees et al., 2007). Previous study has shown that changing the XX residues can perturb the reduction potential of the active-site disulfide bond of the CxxX motif in *Escherichia coli* enzymes thioredoxin (Chivers et al., 1997).

## สรุปและข้อเสนอแนะ (Conclusion and Recommendation)

In this study, the putative McsA from *Staphylococcus aureus* contained four metal binding domains with CxxC motif was shown to Cu (II), Zn (II), Co (II) and Cd (II). Function of the CxxC motif was studied by creating mutations in CxxC motif and determining the metal binding activity and protein interactions between McsA with other protein molecules. Two conserved cysteine ligands in one CxxC motif of McsA is important to bind one copper ion. Bacteria two-hybrid system shows that McsA are able to bind McsB and CtsR of *S. aureus* and the Cysteine residues are important in the binding. The data from this study show that the CxxC motif in McsA protein play central roles in binding to heavy metals and mediating interaction between protein molecules. The metal-ion ligand interaction may induce redox-active cysteines and play an important role in metal induced signaling system.

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**กิตติกรรมประกาศ**

ผู้วิจัยขอขอบคุณกองทุนสนับสนุนการวิจัย (สกว) ที่ให้การสนับสนุนโครงการวิจัยนี้จนเสร็จสิ้นสมบูรณ์ ขอขอบคุณ Professor Bart Devreese จาก Department of Biochemistry, Physiology and Microbiology, Laboratory for protein Biochemistry and Protein Engineering. Ghent, Belgium ที่ให้ความเอื้อเฟื้อพลาสมิด ผู้วิจัยขอขอบคุณนักวิจัยที่ปรึกษาได้แก่ ศ. ดร. ศกรณ์ มงคลสุข คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล และ Professor R.K. Jayaswal จาก Department of Biological Sciences, Illinois State University ที่ให้คำปรึกษาจนงานวิจัยสำเร็จลุล่วงและสุดท้ายนี้ผู้วิจัยขอขอบคุณภาควิชา จุลชีววิทยาและปรสิตวิทยา คณะวิทยาศาสตร์การแพทย์ มหาวิทยาลัยนเรศวรที่เอื้อเฟื้อสถานที่และอุปกรณ์ในงานวิจัย

**Output ที่ได้จากโครงการวิจัย**

1. เข้าร่วมนำเสนอผลงาน ในงานประชุมวิชาการนานาชาติ Conference on Cadmium in Food and Human Health (Thailand Reverse Brain Drain) ระหว่างวันที่ 15 ถึง17 มกราคม พ.ศ. 2553 โดยนำเสนอในรูปแบบโปสเตอร์ หัวข้อ Characterization of metal binding properties of CxxC motif from McsA protein in *Staphylococcus aureus*

3. manuscript in preparation คาดว่าจะตีพิมพ์ใน Microbiology-SGM ในหัวข้อเรื่อง CXXC Motif from McsA in *Staphylococcus aureus* play a role in metal binding and protein interaction