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CsoR Metalloregulatory Protein: Function, Mechanism and Relevance

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Abstract – Transition metals are required constituent in bacterial metabolism to assist in some enzymatic reactions. However, intracellular accumulations of these metal ions are harmful to the bacteria as it can trigger unnecessary redox reactions. To overcome this condition, metalloregulatory proteins assist organisms to adapt to sudden elevated and deprived metal ion concentration in the environment via metal homeostasis. CsoR protein is a copper(I) [Cu(I)] sensing operon repressor that is found to be present in all major classes of eubacteria. This metalloregulatory protein binds to the operator region in its apo state under Cu(I) limiting condition and detaches off from the regulatory region when it binds to the excess cytosolic Cu(I) ion, thus derepressing the expression of genes involved in Cu(I) homeostasis. CsoR proteins exist in dimeric and tetrameric states and form certain coordination geometries upon attachment with Cu(I). Certain CsoR proteins have also been found to possess the ability to bind to other types of metals with various binding affinities in some Gram positive bacteria. The role of this metalloregulatory protein in host pathogen interaction and its relation to bacterial virulence are also discussed.

Keywords: Copper-sensing operon repressor, CsoR, metalloregulatory protein, dimer, Cu(I), tetramer.

Metalloregulatory protein

Transition metals such as copper, iron and zinc have unique redox reaction and are essential cofactors for some enzymatic reactions in bacterial metabolism. For instance, the presence of trace amount of copper is prerequisite as it acts as a cofactor for the enzymes that are responsible for the growth and functioning of microorganisms besides involving in electron transport in cells (Trevors & Cotter, 1990). Although metals are required element for healthy bacterial growth, it is crucial to ensure that these metals are in correct proportion in the cell. This is because, undesirable redox reaction may occur and enzymes can lose their specificity due to inappropriate binding to metal binding sides. Due to this condition, microorganisms have evolved to sustain metal homeostasis with the aid of metalloregulatory proteins (Chillappagari *et al*., 2009).

Metalloregulatory proteins are members of metal-sensing transcriptional regulators which are commonly known as metal sensor proteins. They are responsible for metal homeostasis in organisms as they control gene expression related to metal homeostasis and allow the organisms to adapt to sudden elevation or deprivation of metal ions in the environment. These metal-sensing transcriptional regulators work by establishing specific metal ion coordination complexes with metal affinities to one or more metal ions. These complexes then either repress or activate expression of metal-regulatory

genes by respectively binding to or release of the operator region of the genes they control (Tan *et al*., 2014) .

To date, there are seven structural metalloregulatory families (Table 1) that have been identified; (i) Arsenic/Antimony (As/Sb) sensor from *E. coli R774* (ArsR), (ii) Zinc (Zn) sensor from *Synechococcus* (Smtb), (iii) Cu sensor *M. tuberculosis* (CsoR), (iv) Cu sensor from *E. hirae* (CopY), (v) Ferum (Fe) sensor from *E. coli* (Fur), (vi) Fe sensor from (*C. diphtheriea*) (DtxR) and (vii) Nickel (Ni) sensor from *E. coli* (NikR) (Giedroc & Arunkumar, 2007).

Table 1: Metalloregulatory Family and its Regulatory Proteins (Caballero *et al*., 2011)

Family	Regulators	Metal affinities	Source
Fur	Fur	Zn(II) / Co(II) / Fe(II) / Mn(II)	*Escherichia coli*
	Zur	Zn(II)	*Escherichia coli*
ArsR	CzrA	Zn(II) / Co(II)	*Staphylococcus aureus*
	SmtB	Zn(II) / Co(II)	*Synechococcus*
	AztR	Zn(II)	*Anabaena PCC7120*
	BxmR	Zn(II) / Cu(I)	*Osciliatoria brevis*
	NmtR	Zn(II) / Ni(II) / Co(II)	*Mycobacterium tuberculosis*
CsoR	RcnR	Ni(II) / Co(II)	*Escherichia coli*
	$CsoR_{BS}$	Zn(II) / Cu(I) / Ni(II) / Co(II)	*Bacillus subtilis*
	$CsoR_{MT}$	Cu(I)	*Mycobacterium tuberculosis*
	$CsoR_{SA}$	Cu(I)	*Staphylococcus aureus*
MarR	AdcR	Zn(II) / Co(II) / Mn(II)	*Streptococcus pneumoniae*
MerR	ZntR	Zn(II)	*Escherichia coli*
	CueR	Cu(I) / Au(I)	*Escherichia coli*
	CupR	Au(I) / Cu(I)	*Ralstonia metallidurans*
NikR	$NikR_{EC}$	Zn(II) / Cu(II) / Ni(II) / Co(II) / Cd(II)	*Escherichia coli*
	$NikR_{HP}$	Ni(II)	*Helicobacter pylori*
DtxR	MntR	Zn(II) / Ni(II) / Co(II) / Mn(II) / Cd(II)	*Bacillus subtilis*

Copper-responsive gene regulation in bacteria

To date, there are 9 classes of copper-sensing regulators that respond either by activation or repression of the metal homeostasis genes which they regulate (Table 2).

Table 2: 9 Classes of copper-sensing regulators and types of copper-responsive gene regulation in bacteria (Rademacher & Masepohl, 2012)

Class	Name of regulator	Source	Accession number	Number of amino acids	Regulator Family	Type of regulation
1	CueR	*Escherichia coli*	EG13256	135	MerR	Activation
2	CuRS	*Escherichia coli*	EG13851	227	TCS	Activation
3	ComR	*Escherichia coli*	EG13435	210	TetR	Repression
4	CopL	*Xanthomonas axonopodis*	AAR85972	122	None	Activation
5	CorE	*Myxococcus xanthus*	MXAN_3426	211	ECF	Activation
6	BxmR	*Osciliatoria brevis*	BAD11074	136	SmtB /ArsR	Repression
7	CopY	*Enterococcus hirae*	CAA86835	145	HTH	Repression

8	CsoR	*Mycobacterium tuberculosis*	Rv0967	119	DUF156	Repression
9	YcnK	*Bacillus subtilis*	BSU03960	190	DeoR	Repression

CsoR Protein

CsoR protein is responsible for copper efflux in microorganisms. In general, CsoR is found to be widespread in prokaryotes, mainly in all major classes of eubacteria (Liu *et al*., 2007). To date, CsoR-like repressors have been found and characterised in *B. subtilis, Corneybacterium glutamicum (C. glutamicum), Geobacillus thermodenitrificans (G. thermodenitrificans), S. aureus, Streptomyces lividans (S. lividans), Listeria monocytogenes (L. monocytogenes),* and *Thermos thermophilus HB8 (T. thermophiles HB8)*, covering five major classes of eubacteria (Higgins & Giedroc, 2014). Apart from this, CsoR type regulators have also been predicted to be present in a large number of proteobacteria, cyanobacteria and deinococci.

CsoR works by repressing their target genes related to copper homeostasis (Table 3) under copper-limiting condition and derepresses their expression when copper concentrations are high.

Table 3: Experimentally verified copper-responsive regulators, phylogenetic group and their target gene (Rademacher & Masepohl, 2012)

Name of Regulator	Source	Phylogenetic group	Target genes
CsoR	*B. subtilis*	Gram-positive	*copZA, ycnJ*
CsoR	*C. glutamicum*	Gram-positive	*ctpV-csoR*
CsoR	*L. monocytogenes*	Gram-positive	*csoR- copZA*
CsoR	*M. tuberculosis*	Gram-positive	*csoR-rv0968-ctpV*
RicR	*M. tuberculosis*	Gram-positive	*mymT; lpqS; rv2963*
CsoR	*S. aureus*	Gram-positive	*copA*
CsoR	*S. lividans*	Gram-positive	*csoR-copZA*
CsoR	*T. thermophilus*	Deinococci	*copZ-csoR-copA*
YcnK	*B. subtilis*	Gram-positive	*ycnJ*

CsoR from M. tuberculosis (Mtb CsoR) and ItsMechanism of Regulation

The existence of CsoR protein was initially discovered in *M. tuberculosis* (Higgins & Giedroc, 2014). It regulates the expression of *copZA* operon in which *copZ* codes for copper chaperone and *copA* codes for copper ATPase. The *csoR* gene is located upstream of *copZA* operon. *Mtb* CsoR in its apo-state represses transcription of *copZA* operon by attaching to the palindromic sequence of the operator region of the operon (Figure 1) under copper-deprived condition. Meanwhile, in the presence of high concentration of copper, CsoR repressor dissociates from the operator region, allowing transcription of the genes to take place. Dissociation of CsoR from the operator region is mediated by the binding of Cu(I) ion to the protein which causes conformational changes to the protein. This subsequently causes the protein to lose its binding affinity towards the DNA (operator), allowing RNA polymerase to transcribe *copZA* operon (Liu *et al*., 2007; Rademacher & Masepohl, 2012). Expression of the genes will subsequently facilitate in the efflux of excessive Cu(I) from the bacterial cell. This is achieved via: (1) CopZ protein transports the excess Cu(I) to the N-terminal domain of CopA protein and (2) CopA protein effluxes of excess Cu(I) ion across the cytoplasmic membrane of the cell coupled with ATP hydrolysis (Hirooka *et al*., 2012).

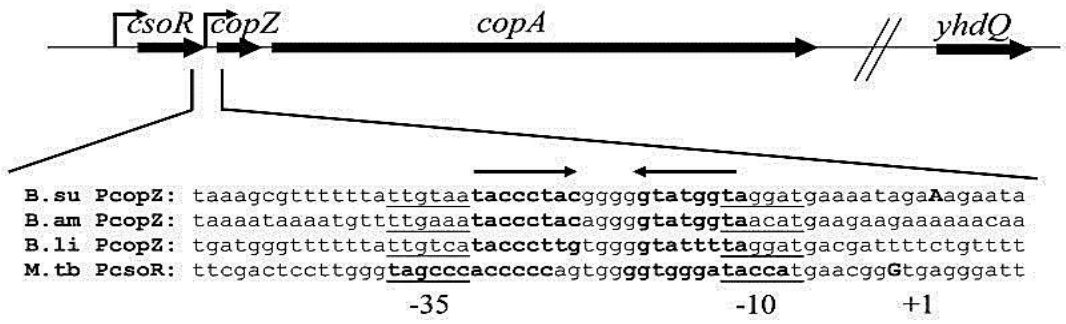

Figure 1: Schematic representation of the *csoR* - *copZA* locus. Palindromic sequence consisting of (GTAGCCCACCCC-N4-GGGGTGGGATAC) overlapping the -35 / -10 region (Gaballa & Helmann, 2003)

Mtb CsoR protein is a dimer that has α-helical architecture. The crystal structure of Cu(I)-bound *Mtb* CsoR revealed that it forms homodimeric antiparallel four helix bundle architecture that binds to four equivalents of Cu(I) ion in trigonal planar coordination geometry and the protomers are composed of three helices (Liu *et al*., 2007).

CsoR from B. subtilis and Its Mechanism of Regulation

CsoR from *B. subtilis* is a homolog of the CsoR protein from *M. tuberculosis*. In *B. subtilis*, CsoR protein forms a tetramer and two molecules of CsoR tetramer bind to the regulatory region of *copZA* operon. When Cu(I) concentration is in excess in the cytoplasm, CsoR protein binds to Cu(I) and detaches from the regulatory region, and thus derepressing the expression of the genes (Figure 2). Similar to the mechanism discussed for Mtb *copZA* copper homeostasis system, CopZ protein functions to transport the excess Cu(I) to the N-terminal domain of CopA protein. CopA protein is responsible for the efflux of excess Cu(I) ion across the cytoplasmic membrane of the cell coupled with ATP hydrolysis. Figure 2 shows the illustration on the regulatory system assisted by CsoR protein in *B. subtilis* (Hirooka *et al.*, 2012), as described above.

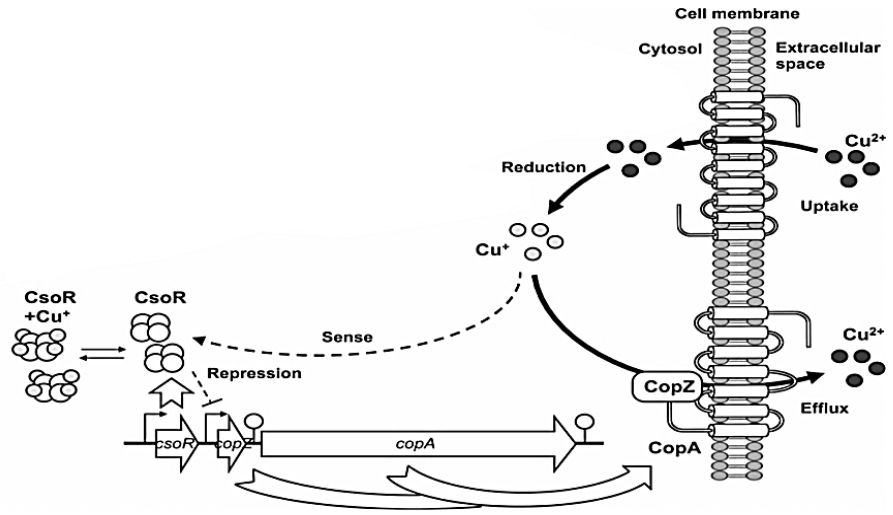

Figure 2: Copper homeostasis by CsoR protein via *copZA* operon in *B. subtilis* (Adapted and modified from (Hirooka *et al.*, 2012)

CsoR from T. thermophiles and Its Mechanism of Regulation

CsoR from *T. thermophilus* forms a homotetramer and acts similarly to CsoRs of *M. tuberculosis* and *B. subtilis*. Uniquely however, CsoR of *T. thermophilus* is able to sense other metal ions apart from Cu(I); such as argentum [Ag(I)], cadmium [Cd(II)], cobalt [Co(II)], Ni(II) and Zn(II). This may be due to its unique binding motif of C-H-H, as compared to the C-H-C motif mostly found in other CsoR proteins. In addition, the X-ray crystal structure of this protein revealed that a histidine residue located at the N-terminal domain is also involved in metal-ion binding. Therefore, the metal-binding motif of CsoR from *T. thermophilus* can possibly be H-C-H-H (Sakamoto *et al.*, 2010), similar to the binding motif of RcnR protein found in *E. coli* (Iwig *et al*., 2008) . CsoR represses the transcription of *copZA* genes of the *copZ-csoR-copA* operon in ways similar to those discussed above for CsoRs from *M. tuberculosis* and *B. subtilis*.

Metal coordination in CsoR proteins

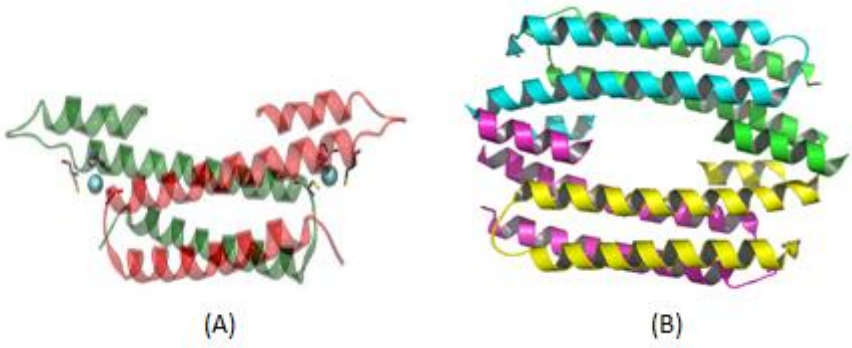

Figure 3: (A) Crystal structure of *Mtb* CsoR homodimer. Cu(I) is bound between two protein subunits (Liu *et al*., 2007) (B) CsoR protein tetramer in *B. subtilis* (Ma *et al*., 2009)

CsoR protein family is known to contain a functional signature motif which is x-C-H-C, whereby x is represented by any amino acid residue (Higgins & Giedroc, 2014). Crystallographic analysis revealed that the *Mtb* CsoR forms homodimer [Figure 3(A)] with antiparallel four alpha helix bundle structural design (α1-α2-α1'-α2') with double symmetrical-like subunit joining Cu(I) binding sites. Binding of CsoR to Cu(I) results in the formation of trigonal S_2N complex coordinated by Cys 36, Cys 35' and His 61' (Liu *et al*., 2007), as seen in Figure 4. Based on the X-ray absorption fine structure (EXAFS), double Cu-S and single Cu-(N/O) interaction are formed upon binding of *Mtb* CsoR with Cu(I) (Ma, *et al*., 2009).

Cu(I) adopts S_2N coordination geometry upon binding with *Bsu* CsoR, which is similar to the pattern with Cu(I)-saturated *Mtb* CsoR. Binding of Cu(I) produces double Cu-S interaction and a single Cu-N/O coordinated by Cys45', His70 and Cys74 within a dimer, which is comparable with the Cu(I) ligands in *Mtb* CsoR dimer [Figure 3(B)]. The tetramers are non-separable for both the apo and Cu(I) bound *Bsu* CsoR. Remarkably, this protein was also found to be able to bind to other divalent metals, forming different coordination geometries with various affinities. Metal ions including Co(II), Zn(II) and Ni(II) tend to bind to *Bsu* CsoR, forming nontrigonal coordination geometry which weakly regulate DNA-binding at the operator region *in vitro* (Ma *et al*., 2009).

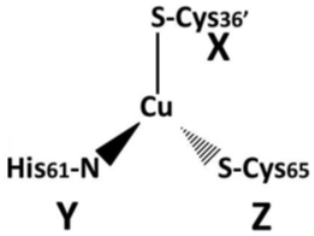

Figure 4: Cu(I)-bound *Mtb* CsoR. Each Cu(I) is coordinated by Cys 36, Cys 35' and His 61'at the subunits bridging site of the dimeric protein (Higgins & Giedroc, 2014)

Metalloregulatory protein in host-pathogen interaction

Bacterial pathogen is defined as any type of bacteria that has the potential to cause pathogenicity in host organism. Pathogenic bacteria carries virulence factor which causes diseases in host organism. Virulence factors are usually in the form of hydrolytic enzymes, bacterial toxins, cell surface proteins that either protect the bacterium or conduct the bacterial attachment (Hodgkinson & Petris, 2012); (Wu *et al*., 2008).

Similar to other organisms, bacteria has evolved metal homeostasis system to regulate Cu, Zn, Fe, Co and manganese (Mn) which is aided by metalloregulatory proteins. Metal homeostasis is crucial in maintaining a balanced intracellular environment, particularly in mitigating the harmful effect caused by the excess amount of these metal ions (Giedroc & Arunkumar, 2007).

Copper is known to possess antimicrobial activity and it has been widely used to treat medical ailments until the emergence of modern era antibiotics. Nevertheless, the use of copper as anti-microbial components is still practiced. For example, copper is added in hospital drinking water as an electrolytic ionizer to battle against *Legionella* (Hodgkinson & Petris, 2012). Besides that, dry copper surfaces have been documented to be able to kill various types of bacteria within few minutes upon contact (Espírito Santo *et al*., 2011). Currently, innovative studies are focussing on the role of copper-binding properties to develop anti-cancer and anti-HIV drugs and this can be applied in the development of novel antibiotics (Pontel *et al*., 2015).

Recent findings have shown that copper has been used by host organisms as a weapon to combat bacterial infections. Controlled accretion of the copper directed to pathogenic bacteria is the defence strategy used by the host organism against invading pathogens. However, over time, pathogenic bacteria have evolved to tackle copper toxicity with well-established mechanisms (Pontel *et al*., 2015). Copper toxicity in microorganisms is tackled by copper homeostasis system via (1) Cu(I) oxidation, (2) copper efflux across the plasma membrane and (3) copper seizure by copper-binding proteins (Hodgkinson & Petris, 2012).

For mechanism (1), copper oxidation in bacteria is conducted by copper-dependent enzymes such as copper/zinc-containing superoxide dismutases (SodC proteins) and multi-copper oxidases like CueO. CueO helps bacteria to combat copper toxicity via oxidation of enterobactin, a catecholate iron siderophore. Enterobactin, when in combination with copper, can cause toxicity to its bacterial host (Grass *et al*., 2004). Hence, with the oxidation of enterobactin, toxicity caused by the non-oxidized siderophore in combination with copper can be circumvented. CueO is also known to be a responsible element of virulence in *Salmonella typhimurium* (Achard *et al*., 2010). For mechanism (2), regulatory protein such as CsoR, and copper chaperones like CopA and CopZ proteins are involved in the efflux of excess copper out of bacterial cells. Regulatory proteins sense the presence of excess copper and allow transcription of copper chaperones while copper chaperone transports the copper ions to the plasma membrane and efflux the metal in the presence of ATP (Smaldone & Helmann, 2007). As for mechanism (3), copper metal ion is seized within the cytoplasm or periplasm by copper-binding proteins. Metallothionein-like proteins such as MymT is highly expressed by *M. tuberculosis* under

high copper concentrations and this helps facilitate copper tolerance of the pathogen the protein has the ability to bind to more than six copper atoms by forming copper-thiolate bonds, protecting the pathogen from copper toxicity (Gold *et al.*, 2008).

Conclusion

As much as copper metalloregulatory proteins help in maintaining copper concentrations intracellularly, their direct link to bacterial pathogenicity has recently been established. In light of this, it cannot be denied that they may serve to be attractive drug targets to combat bacterial infections in the near future.

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