Characterization of copper mediated transcriptional responses in *Bacillus subtilis.*

and

Identification of copper and iron connection in *Bacillus subtilis.*

Dissertation

zur Erlangung des Doktorgrades

der Naturwissenschaften

(Dr. rer. nat.)

dem

Fachbereich Biologie

der Philipps-Universität Marburg

vorgelegt von

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aus Indien

Marburg/Lahn, 2009

Vom Fachbereich Chemie

der Philipps-Universität Marburg als Dissertation

am _____ angenommen.

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Tag der Disputation_____

Summary

Copper is an essential cofactor for many enzymes and over a threshold, it is toxic for all organisms. The redox ability of copper to cycle between two oxidation states, Cu(I) and Cu(II) makes it crucial for the structure and function numerous enzymes. Uncontrolled accumulation of copper inside the cell leads to the generation of reactive oxygen species (ROS) through fenton reaction leading to oxidative stress and finally damages DNA and proteins. In this work, the gram positive bacterium *Bacillus subtilis* was used to understand the mechanisms underlying copper homeostasis. Especially, the recently identified copper efflux transcriptional regulator CsoR was mainly in focus. Deletion mutant of Δ*csoR* shows a growth defective phenotype in copper excess conditions and exhibited a slight better growth phenotype under copper limiting conditions. Microarray studies initiated with Δ*csoR* under copper limiting conditions revealed the *ycnJ* gene to encode a protein that plays an important role in copper metabolism, as it shows a significant 8-fold upregulation under copper limiting conditions, and its disruption exhibits a growth defective phenotype under copper limiting as well as copper excess conditions. Native gel shift experiments with the recombinant N-terminal cytosolic domain of the YcnJ membrane protein (135 residues) disclose its strong affinity to Cu(II) atoms *in vitro*. Inspection of the upstream sequence of *ycnJ* revealed the *ycnK* gene to encode a putative transcriptional regulator, whose deletion showed a constitutive expression of *ycnJ*. Further studies supported a predominant role for the YcnJ protein not only as a copper importer under copper limiting conditions, but also possibly as a copper resistance determinant under copper excess conditions. Interestingly, the correlation between copper dependent iron aquisition was also elucidiated. Microarray approach to identify the copper responsive genes using Δ*csoR* mutant exhibited an alternate regulation of iron responsive genes in the presence or absence of copper. Significant downregulation of the following iron responsive genes was observed: 1. *dhbABCEF* which is involved in bacillibactin production, 2. *feuABC* which are responsible for bacillibactin uptake and 3. *besA* and *btr,* which are

involved in sensing iron bound bacilibactin, hydrolysis, cleavage and subsequent release of iron from bacillibactin. Transcription profiling data, quantification of bacillibactin amounts and estimation of total cellular iron and copper concentrations in wildtype and the Δ*csoR* mutant in response to different copper amounts demonstrated an indirect relation between copper and iron. Further, Δ*csoR* mutant exhibited differential regulation of oxygen sensing, cytochromes and anaerobic genes in response to excess copper in the growth media suggesting a probable role for CsoR and its significance in energy generation, cellular respiration, oxygen transport, and iron acquisition under different oxygen availability conditions imposed by the presence of copper.

Zussamenfassung

Kupfer ist ein essentieller Kofaktor für viele Enzyme und über einem Schwellenwert toxisch für alle Organismen. Der Redoxstatus von Kupfer, der zwischen Cu(I) und Cu(II) changiert, ist bedeutungsvoll für die Funktionalität kupferabhängiger Enzyme. Unkontrollierte Akkumulation von Kupfer in der Zelle kann zur Generierung von reaktiven Sauerstoffspezies und dadurch zu oxidativem Stress führen, der DNA und Proteine schädigt. In dieser Arbeit wurde das Gram-positive Bakterium Bacillus subtilis verwendet, um die Mechanismen zu verstehen, die der Kupferhomöostase zugrunde liegen. Insbesondere wurde der kürzlich identifizierte Transkriptions-Regulator CsoR für den Kupferefflux untersucht. Deletionsmutanten von csoR zeigen einen defektiven Wachstumsphänotyp unter Kupferstress und einen etwas besseres Wachstum unter kupferlimitierenden Bedingungen. Globalexpressionsanalysen von ΔcsoR mittels DNA Microarrays unter Kupferlimitation zeigten eine achtfache Überexpression des ycnJ Gens, das eine wichtige Rolle im Kupfermetabolismus zu spielen schien. Die Deletion von ycnJ zeigte einen Wachstumseffekt unter Kupferlimitation und Kupferstress. Native Gelschift-Experimente mit der rekombinanten N-terminalen Domäne von YcnJ zeigten seine starke Affinität zu Cu(II) Ionen in vitro. Das stromaufwärts von ycnJ gelegene Gen ycnK codiert für einen putativen Transkriptionsfaktor, dessen Deletion eine konstitutive Exprerssion von ycnJ zeigte. Weitere Studien unterstützten die prinzipielle Rolle von YcnJ nicht nur als Kupferimporter unter Kupferlimitationsbedingungen, aber auch als möglicher Kupferresistenzfaktor unter Kupferstress. Weiterhin wurde ein Zusammenhang zwischen Kupfer- und Eisenhomöostase aufgedeckt. Microarray-gestützte globale Expressionsanalysen mit der csoR Mutante zeigten eine jeweils andere Expression von Eisenhomöostasegenen in Anwesenheit oder Abwesenheit von Kupfer. Unterexpression der folgenden Gene wurde beobachtet: 1.) dhbABCEF, die an der Bacillibactin-Produktion beteiligt sind, 2.) feuABC, die für die Aufnahme von eisenbeladenem Bacillibactin verantwortlich sind, 3.) btr und besA, die für die intrazelluläre

Perzeption von Bacillibactin zur Regulation der Aufnahme sowie zur Bacillibactinhydrolyse und Eisenfreisetzung notwendig sind. Die Transkriptionsprofile, Quantifizierungen der Bacillibactinmengen und Bestimmungen der Gesamteisen- und Gesamtkupfergehalte im Wildtyp und der csoR Mutante hinsichtlich unterschiedlich angebotener Kupfermengen zeigten einen indirekten Zusammenhang zwischen zellulärem Eisen- und Kupferhaushalt. Zusätzlich zeigte die csoR Mutante eine differenzierte Regulation der Sauerstoffperzeptions-, der Fermentations- und der Cytochromgene unter Kupferstress, was weiterhin auf eine mögliche Rolle von CsoR im Energiehaushalt der Zelle unter unterschiedlichen Sauerstoff-, Eisen- und Kupferkonzentrationen hinweist.

The work presented here has been published or has been submitted for publication:

Shashi Chillappagari, Marcus Miethke, Hein Trip, Oscar P. Kuipers, and Mohamed A. Marahiel(2009) Copper Acquisition Is Mediated by YcnJ and Regulated by YcnK and CsoR in *Bacillus subtilis*. **Journal of Bacteriology**, Apr. 2009, p. 2362–2370.

Shashi Chillappagari, Marcus Miethke,Oscar P. Kuipers, and Mohamed A. Marahiel. Copper Responsive Transcription Factor CsoR Regulates Iron and Copper Homeostasis in *Bacillus Subtilis*.(*manuscript in preparation*)

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1. Abbreviations

A	Ampere
aa	amino acid
Amp	ampicillin
AMP	adenosine-5'-monophosphate
ADP	adenosine-5'-diphosphate
ATCC	American type culture collection
ATP	adenosine-5'-triphosphate
APS	ammonium persulfate
BB	bacillibactin
bp	base pairs
BCIP	5-bromo-4-chloro-3-indolylphosphate
BMM	Belitsky minimal medium
BCS	bathocuprione disulfonate
BSA	bovine serum albumin
CoA	coenzyme A
Cy	Cyanine
Da	Dalton
DHB	2,3-dihydroxybenzoate
DMSO	dimethyl sulfoxide
DMF	*N*,*N*-dimethylformamide
dNTP	2'-de-oxynucleosid-5'-triphosphate
EDTA	ethylene-diamino-tetraacetic acid
Ent	enterobactin
eq.	equivalent
FMN	flavin mononucleotide
FPLC	fast performance liquid chromatography
HEPES	2-*N*'-[*N*-(2-hydroxylethyl)-piperazinyl]-ethansulfonic acid
HPLC	high performance liquid chromatography
ICL	isochorismate lyase
ICP-MS	inductively coupled plasma mass spectrometry
IPTG	isopropyl-β-*D*-thiogalactoside
Kan	kanamycin
kb	kilo bases

Abbreviations

kDa	kilo Dalton
LB medium	Luria-Bertani medium
LC/MS	liquid chromatography/mass spectrometry
MCS	multiple cloning site
Min	minutes
mL	milli liters
MOPS	3-(*N*-Morpholino)-propanesulfonic acid
MS	mass spectrometry
NRPS	nonribosomal peptide synthetases
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
rpm	rotations per minute
RT	room temperature
SBP	substrate-binding protein
SDS	sodium dodecylsulfate
TBS	Tris-buffered saline
TE-domain	thioesterase domain
TEMED	*N*, *N*, *N*′, *N*′-tetramethylethylendiamine
TFA	trifluoroacetic acid
Tris	tris-(hydroxymethyl)-aminomethane
UV	ultra voilet
V	volts
v/v	volume per volume
WT	wild type
w/v	weight per volume

2. Introduction:

2.1 General properties of copper:

Before the advent of oxygen on earth, iron and copper had less importance. The emeregence of oxygen on earth eventually led to oxidation of iron and copper which further make the route for the origin of reactive oxygen species. It is believed that cyanobacteria is responsible for the cause of dioxygen production and the oxidation of Fe(II) to Fe(III) and Cu(I) to Cu(II) changed the life on earth. The biological significance of copper was not known before the appearance of oxygen on earth where the water insoluble Cu (I) exists as highly insoluble sulphides (2). Copper, a transitional metal, is an essential cofactor for most of the enzymes involved in electron transfer utilizing di-oxygen as a prosthetic group (1). The convenient redox potential of copper (200-800 mV) and the transition between two oxidation states, Cu(I) and Cu(II) when bound to protein, makes it biologically important cellular constituent (2,3). Cu (II)-N bonds are often inert and more labile bonds are formed between Copper and oxygen. Cu(I) is considered as a soft metal and shows high affinity to bond with sulfhydryl ligands. Redox cycling and the transfer of electrons between these two oxidation states are made use by numerous metallo-enzymes like cytochrome-c oxidase, lysyl oxidase and superoxide dismutase, enabling them to participate in many biological redox reactions which include cellular biochemical pathways and regulatory functions (e.g. bacterial CueR and CsoR). Copper proteins are involved in many biological events as respiration, oxidative stress, copper dependent iron acquisition pathways in yeast and mammals (Fet3p, ceruloplasmin, hephastein). Although copper is an essential transition metal, excess of cellular copper accumulation is potentially toxic because of its redox properties. Cu(I) and Cu(II) both have a high affinity to bind numerous cellular targets and exhibit favoritism to those rich in thiols. Inappropriate binding to cellular targets due to over accumulation of copper or by defective copper efflux systems might lead to severe implications, since excess copper inside the cell is a potential target for generating highly reactive oxygen radicals (ROS). These reactive oxygen

radicals subsequently attack DNA and RNA and cleave them further, leading to the oxidation of several proteins and lipids making them inactive. Therefore, tight regulation of the copper levels inside the cell is very important for its survival. Defects in copper homeostatsis are directly responsible for human diseases. For example, mutations in ATP7A gene leads to an X-linked lethal disorder of copper hyperaccumulation or mutations in ATP7B gene leads to an autosomal recessive disease characterized by striking hepatic and neuronal copper over load, hepatotoxicity. These genes encode for P-type copper efflux ATPase pumps, the defects of which cause Menkes and Wilson's diseases respectively.

2.2 Copper transporters:

In order to elucidiate the potential transporters and carrier systems involved in transport of copper across the membrane, yeast CTR1 gene sequence information was used to find the analogous genes in other systems. The human CTR1 (hCTR1) cloned and expressed is highly homologous to yeast CTR1 and was shown to complement as well, thus supporting the uptake of copper in yeast. The copper entering the cells is then chaperoned by copper chaperones HAH1/ATOX1 (which are highly homologous to yeast ATX1), which carry the Cu(I) to their respective destinations. The delivery of copper in enterocytes involves ATP7A or MNK while in hepatocytes, it is ATP7B or WND. Ccp is another copper chaperon involved in delivery of copper to Cu/Zn superoxide dismutase (SOD) in cytoplasm which protects the cells against the generation of superoxide radicals. Copper requirement by the terminal cytochrome-c oxidase which is involved in cellular respiration in mitochondria is facilitated by COX-17. Homologues of yeast cytochrome-c oxidase reveal two other proteins Sco1 and Sco2 necessary for the copper incorporation.

In strains of *S. cerevisiae*, cellular copper acquisition requires *CTR1*. Copper-deficient conditions lead to elevated expression of two membrane associates high affininty copper uptake transporters encoded by *CTR1*, *CTR3*, and a cell surface Cu(II)/Fe(III) reductase

FRE1, which reduces Cu(II) to Cu(I) prior to uptake by *CTR1* (12). These transporters were found to be repressed by copper and induced by limitation. With the addition of $CuSo_4$, mRNA level of CTR3 was reduced to 8 fold. Although CTR1 and CTR3 exhibits slight homology at amino acid level, they are functional duplicates. The distinct features of CTR1 and CTR3 lies in the transmembrane domains, where CTR1 consists of putative MX_2MXM metal binding motif which is repeated 8times in the amino terminal domain. In hCTR1 and mCTR1 it is found only twice. In contrast, *Saccharomyces pombe* CTR4 contains 5 repeats. However, CTR3 (lacking this conserved MX_2MXM metal binding motif) was proposed to perform copper uptake through cysteine residues which are abundantly (11 Cys) present throughout the protein sequence, since it was well known that both methionine and cysteine are capable to bind copper (16,17). Ctr1 undergoes copper-induced endocytosis at low copper concentrations (0.1-1μM). Although it was initially speculated that CTR1 is not degraded when exposed to copper, deletion mutants of end3, end4 and chcl-ts (clathrin heavy chain gene- temperature sensitive) which are involved in endocytosis function and pep4 involved in vacuolar degradation were shown to exhibit degradation of CTR1, when exposed to copper concentrations greater than 10μM (18). Degradation of CTR1 in response to copper excess conditions suggested a probable role for homeostatic mechanisms for copper uptake.

Copper homeostasis is well-studied in gram negative bacteria such as *Enterococcus hirae* and *Escherichia coli.* In *E. hirae*, the process occurs at the plasma membrane and includes four genes, i.e. *copY, copZ, copA* and *copB*. The CopA and CopB are two integral membrane P-type ATPases, that are necessary for the transport of copper into the cells under copper limiting conditions and efflux under copper excess conditions respectively. CopA, which serves to import copper, interacts with CopZ that acts as a copper chaperon. CopZ then chaperones the metal atom to the transcriptional repressor CopY, thereby releasing the repression of copper homeostasis genes (19). CopA of E.hirae exhibits 43% sequence identity with human menkes and Wilson copper ATPases. In contast, orthologous proteins of CopA

from other bacteria perform copper efflux. Deletion mutant of *copA* exhibits a growth defective phenotype under copper limiting conditions created by the addition of copper specific chelators in the growth media. Since Ag(I) is able to enter the cell via CopA, the deletion mutants exhibited strong resistance to increasing amounts of Ag(I), which indirectly support *copA* as a copper uptake determinant. Deletion of *copB* resulted in copper hypersensitive phenotype suggesting a probable role in copper efflux (20,21). Similar to most other heavy metal ATPases, CopA possesses a conserved metal binding CxxC motif. Mutational studies were performed with CxxC motif to ensure its role in recognition of CopZ. Mutation of CxxC motif to SxxS motif did not abolish the CopZ and CopA interaction in the absence of copper. Further addition of copper did not result in significant interaction of CopZ with CopA. Increased mRNA levels of CopZ were observed with increase in copper concentrations upto 0.5mM and decreased with further increase in copper amounts. CopZ overexpression in strains resulted in copper hypersensitivity at 0.1mM, in contrast to wildtype *E.hirae* strain which is not sensitive to copper at that concentration. CopZ overexpression in *E.hirae* was shown to be more sensitive to oxidative stress inducing agents such as H_20_2 or paraquat. Similar to copper induced degradation of CTR1 in *S.cerevisiae*, CopZ of *E.hirae* was also degraded by proteolysis with an exception that Cu-CopZ is more sensitive to proteolysis in comparision to apo-CopZ. Over expression of CopZ leads to Cu-CopZ accumulation inside the cell, where binding of copper to the apo-CopZ exposes the solvent channel and can thus actively participate in Fenton-type reaction, leading to cell death.

Copper homeostasis in *E.coli* is mediated by ORF f 834, which encodes an 834 residue P-type ATPase, with 36% identity to copA from *E.hirae*. Copper efflux is carried out mainly by heavy metal exporters which primarily belong to the integral membrane protein family of P-type ATPases. These P-type ATPases are functional in translocating Cu(I) across the cytoplasmic membrane (23), whose expression is mainly controlled at the level of transcription. CopA is believed to be the core element of copper homeostasis under both

aerobic and anaerobic conditions. CopA is an integral inner membrane protein with eight hydropathic transmembrane *α*-helices and shares common features with other P-type ATPases (29). *copA* represents a central component in *E. coli* copper efflux, while *cueO* encodes a multicopper oxidase (24,28). The Δ*copA* deletion strain was shown to be less resistant to copper than the wild-type; complementation with the same gene on a plasmid reverts copper tolerance (2) and it was also shown that CopA is able to transport silver (3). In *E. coli,* there are several sets of genes which are responsible for copper homeostatic functions. The *cusRS* genes for example, form a sensor-regulatory pair which senses copper and activates the *cusCFBA* genes (24, 27). CusA encodes an innermembrane protein acting as a secondary transporter, activated by the proton-substrate antiport required for the substrate specificity and CusC is an outermembrane factor. CusB acts as an adaptor linking both CusA and CusB. CusF is a periplasmic copper binding protein, while *cusCBA* gene products are homologous to a family of proton / cation antiporter complexes homologous to multidrug resistance systems representing TolC-AraAB system. (25). CusF binds Cu(I) *invitro* where the proposed methionine-rich region in CusF binds Cu(I) further interacting with the aromatic ring of tryptophan forming a cation π interaction (26). In addition, the *cutA*, *cutB*, *cutC*, *cutD*, *cutE*, and *cutF* genes are also believed to be involved in copper uptake, storage, delivery and efflux. The *cutA* locus and the *cutE* gene were found to be located at 94 and 15 min on the *E. coli* chromosome, respectively (31). The *cutA* locus consists of two operons, (CutA1) one containing a single open reading frame (ORF) encoding a cytoplasmic protein of 13 kDa and the other consisting of two genes encoding 50-kDa (CutA2) and 24-kDa (CutA3) inner membrane proteins. CutA is a 12 kDa protein which is conserved in bacteria, plants, and animals, including humans. CutA1 was suggested to be possibly involved in atleast two unrelated processes in bacteria and mammals, thus fulfilling different functions. (i) as a "divalent cat-ion tolerant protein" (ii) as a signal transducer. The determined crystal structure and the arrangement of both the proteins in *E.coli* (CutA) and Rat (CutA1) shows a trimeric

assembly, similar to signal transduction proteins PII, like nitrogen regulatory response in bacterial cells and eukaryotic chloroplasts (30). Sequence analysis suggests that *cutC* gene is a cytoplasmic copper-binding protein. *cutC* and *cutD* mutants were shown to result in increased copper reserves and thus are involved in energy-dependent copper export, while *cutE* and CutF are thought to deliver copper to various proteins whose function is dependent on copper as a cofactor, ultimately leading to activate the export system (29). *cutF* gene is allelic to the *nlpE*, an outer membrane lipoprotein which is involved in surface sensing, activating Cpx in response to adhesion in an NlpE-dependent manner. NlpE has also been characterized as a copper homeostasis protein, CutF; *cutF* deletion mutant renders *E.coli* copper sensitive and temperature sensitive phenotype (31, 32). Apart from the above mentioned copper homeostasis mechanisms involved, there are additional components which also effectively participate in copper homeostasis which are plasmid encoded. This plasmid-encoded system, *pcoABCD* constitute a copper-resistance system which is induced under copper stress conditions. These Pco proteins are structurally similar to the predicted Cop proteins from *P. syringae*. Sequence analysis and subsequent complementation studies show that the *pco* group is an operon of four genes which exhibit significant similarities with the *copABCD* operon of *P. syringae* (33,34). Collectively, the copper resistance in *E. coli* is thus considered as a complex system involving chromosomally encoded genes responsible for the copper uptake, storage and distribution which are in turn closely coupled with plasmid-mediated enhanced copper efflux. Thus one could attribute to a specific system for the tight handling of copper is essential for the above mentioned copper management for bacteria which are exposed to high levels of copper in the environment.

P. syringae pv. Tomato strain PT23.2 consists of the plasmid-borne copper resistance operon which consists of four genes *copABCD*. The protein products CopA, CopB, and CopC of CopD proteins were found to mediate sequestration of copper outside the cytoplasm providing copper-resistance mechanism. The functions of the plasmid encoded copper resistance system

were discovered mainly from 1). *Pseudomonas syringae* pathovar (pv.) *tomato,* isolated from tomato fields in Southern California and 2). *Xanthomonas campestris* vesicatoria (pv.) which were isolated from pepper and tomato plants in fields that had been sprayed with copper sulfate for disease control (34). The copper resistance of these bacteria was conferred by the *copABCDRS* operon on plasmid (pPT23D). Similar plasmid encoded copper resistance system *pcoABCDRS* was identified in *E.coli* strain (pRJ1004) isolated from the gut flora of pigs which were fed with copper-enriched diet to promote growth (34). CopA, a 72 kDa periplasmic protein was shown to bind several copper atoms approximately 11 per a polypeptide chain which accounts for the copper sequestration (33). Multiple repeated sequence of histidine residues found in CopA (Met-Xaa-Xaa-Met-Xaa-His-Xaa-Xaa-Met) along with other histidine ligands, could probably assist in copper binding by CopA. The outer membrane protein CopB consists of quiet similar sequence, Asp-His-Xaa-Xaa-Met-Xaa-Xaa-Met octapeptide sequence which evokes interest in thinking that this could also bind copper. But, the stringent purification conditions used to purify CopB might be responsible for the no copper content identified in this protein. CopC is a blue copper protein which is localized in periplasm of gram negative bacteria and found capable of binding Cu(I) and Cu(II) at two different sites, occupied either one at a time or both simultaneously (36). Binding of Cu (II) position is consistent with the coordination arrangement involving His-1, Glu-27, Asp-89, and His-91, where as copper(I) is located in Met-rich region which is constituted by His-48 and three of the four Met residues (40, 43, 46, 51). The switching in the redox state from Cu (I) to Cu (II) results in translocation of copper from one site to another. The redox shift of copper is further facilitated by the CopA, a multicopper oxidase, localized in periplasm of gram negative bacteria. The cooperative role of CopC in partnership with CopA results in metal transfer function or detoxification by oxidizing Cu (I) bound to CopC to the less toxic Cu (II) (10). CopD is a 33-kDa and inner membrane protein containing several predicted transmembrane regions and some conserved His residues, but no Met-rich

motif. Expression of CopC together with CopD make the strain highly sensitive to copper which is not found when either of them was expressed alone which concludes that CopC together with CopD performs copper uptake function. CopC and CopD are not essential to resistance but are required for maximum resistance, and are proposed to maintain uptake of small amounts copper essential to cell growth when the copper-sequestering CopB and CopA proteins are fully induced (37).

Copper homeostasis in *B.subtilis* is induced by copper inducible copZA operon which encodes for a copper chaperone and a CPx-type copper efflux ATPase. Copper efflux is carried out mainly by heavy metal exporters which primarily belong to the integral membrane protein family of P-type ATPases (46), whose expression is mainly controlled at the level of transcription. These P-type ATPases are functional in translocating Cu(I) across the cytoplasmic membrane. CopZ binds the Cu(I) molecule and acts a copper chaperon transferring the Cu(I) molecules to the copper specific P-type ATPase CopA which effluxes the copper out of the cell. However, at low levels of intracellular copper, these P-type ATPases might function in the reverse direction and could able to compensate the minimal copper requirements. A very few copies of these P-type ATPases could complement the copper needs of the cell. The detailed mechanism of copper import into bacterial cells is still not very well documented, although in some bacteria these P-type ATPases have evolved to import minute amounts of copper into the cell. A reversible copper pump would explain the current lack of data about bonafide copper import systems in bacteria (47). Here, we explore the role of *B. subtilis* YcnJ, which is a homologue to *P. syringae* CopCD, in copper homeostasis. The *ycnJ* gene from *B. subtilis* is highly induced under copper limiting conditions and Δ*ycnJ* mutant shows reduced growth under copper limiting conditions. Since uptake components for copper have not been reported in *B. subtilis* so far, we demonstrate that YcnJ is a candidate for such a function (48).

2.3 Transcriptional regulation of copper homeostasis:

Regulation of these transcription events is a major step in maintaining the appropriate metal concentrations inside the cell. Several transcriptional regulators were identified in wide range of organisms from yeast, bacteria and mammals. Regulation of the high-affinity copper uptake system is mediated at transcriptional level by Mac1 which is believed to be a direct Cu(I) sensor (38). The binding of Cu(I) to the Cys rich motifs induces an intramolecular affinity with the N-terminal DNA binding domain. The role of Mac1 in the expression of CTR1 and FRE1 (Fe-reductase) were studied in mutants generated within the *MAC1* locus. Mutation of His to Gln substitution at residue 279 resulted in a semidominant gain in *MAC1* mutation which is designated as $MAC1^{up1}$ (39). The resulting gain in mutation does not seem to inhibit the expression of CTR1 and FRE1 in response to copper. The same mutation exhibits a copper hypersensitive phenotype because of deregulated uptake of copper or gain in the function. A frame shift mutant designated as mac1-1 resulted in complete loss of copper uptake functions in addition to the loss of Cu(II) and Fe(II) reduction. Mac1 regulated genes posses atleast two repeats of the responsive sequence elements TTTGC (T/G)C(A/G) towards its 5'sequences. DNA foot printing studies have shown a loss of protection of this repeats by Mac1 upon addition of copper salts. DNA binding studies have shown that Mac1 not only binds selectively to TTTGCTCA element as a monomer, but also sequentially binds to DNA containing two repeats of the same depending on the selection of the metal binding sites in the Mac1. Studies using the myc-tag fusions to Mac1 revealed that the turnover of Mac1 was decreased in copper depleted cells within 90min in contrast to copper replete cells where the Mac1 levels are significantly stable for 3 hrs.

Mac1 consits of a N-terminal DNA binding domain (DBD 1-159aa residues) and a C-terminal activation domain (AD 240-417aa residues). Two hybrid assay system and coexpression of the two fusion proteins DBD domain-Gal4 and VP16-AD domain resulted in a *GAL1-lacZ* reporter protein in a copper induced manner and cease under copper depletion resulting in

copper dependent regulation of Mac1 as a transcriptional activator in copper deficient cells. Another copper dependent transcriptional regulator Ace1 was identified. In contrast to Mac1, copper dependent activation of gene expression was exhibited by Ace1 while inactive Ace1 was found in copper deficient cells (38). Activation of Ace1 was achieved by formation of tetra copper-thiolate copper complex within the regulatory domain of Ace1; this copper dependent activation of Ace1 induces two copper detoxification genes, *cup1* and *CRS5* (metallothioneins) of which *cup1* confers copper resistance to the yeast under copper excess conditions. A third gene activated by Ace1 is *SOD1* (Super oxide dismuatse) which is also important in controlling cellular copper levels. Upregulation of *CTR1* was shown in cells where there is an over expression of *cup1* and *CRS5* resulted due to the reduced intracellular cytoplasmic copper inside the cells. The copper dependent inactivation of Mac1 and activation of Ace1 results in perfect balance in copper homeostasis in *S.cerevesiae* (38, 39, and 40).In *Enterococcus hirae*, the four copper specific proteins are organized in polycistronic *cop* operon, *copYZAB,* where CopY apparently regulates the expression of the complete operon and acts as a copper-inducible repressor. Deletion of the *cop*Y gene resulted in overexpression of CopA and CopB ATPase. Primer extention and DNase I foot printing studies revealed the transcriptional start site of *cop* operon. CopY exists as a Zn (II) containing homodimer in solution, and the purified CopY was shown to protect the region between the nucleotides -71 to -11, relative to the translational start site and contains hyphenated inverted repeats (41). The consensus binding motif of the CopY like repressors was identified as TACAxxTGTA, also called as 'cop box' much similar to the other motifs recognized by the transcriptional regulators such as BlaI from *Bacillus licheniformis* and MecI from *Staphylococcus aureus.* NMR analysis, sequence similarity and prediction of the secondary structures reveal that CopY-type repressors belong to the family of winged helical β-lactamase repressor proteins. CopZ acts as a copper chaperone which delivers Cu(I) to CopY thereby replacing the Zn(II) and release the repression of CopY from the DNA. This

binding of copper ions to a repressor either allosterically inhibits DNA binding or leading to de-repression of regulated genes (41, 42, and 43).

Copper homeostasis is governed by various transcriptional regulators in *E.coli*, which is a diversified process regulating differently at different stress conditions.Three independent copper transcriptional regulatory systems have been identified in *E.coli*. (i) DNA binding CueR (ii) Chromosomal (cusRS) and (iii) plasmid encoded (PcoRS). Crystallographic studies of *E. coli* CueR reveals that the metals CuI, AgI and AuI, form isostructural coordination complexes and *E. coli* CueR is capable of sensing CuI and AuI (43). CueR is a copper specific transcriptional regulator which is a homologue of MerR, which is broadly distributed in bacteria and has been shown to regulate two genes, *copA* and *cueO*. Inspection of the upstream sequences of *copA* and *cueO* promoter revealed signature elements of promoters which are controlled by metalloregulatory proteins in the MerR family (45). It was initially thought that copA expression was induced by *cusRS* but the primer extension analysis revealed a *cusRS* independent copper induction which indicates that there exists an alternative copper-responsive signal transduction system regulated by a MerR family member. On comparision of the transcriptional regulators CueR, ZntR, and MerR, it is more likely that CueR detects excess copper inside the cell and activates transcription of copper detoxification genes. The C-terminal metal binding region was found to be conserved in CueR, ZntR and MerR. However the differences in the position of the Cys residues in comparision to the ZntR and MerR sequence and also the spacing between the two conserved Cys residues in CueR resulted in selective discrimination between the metals. The fig.1 summarises a short overview of the cysteine residue spacing of metal responsive transcriptional regulators from different sources.

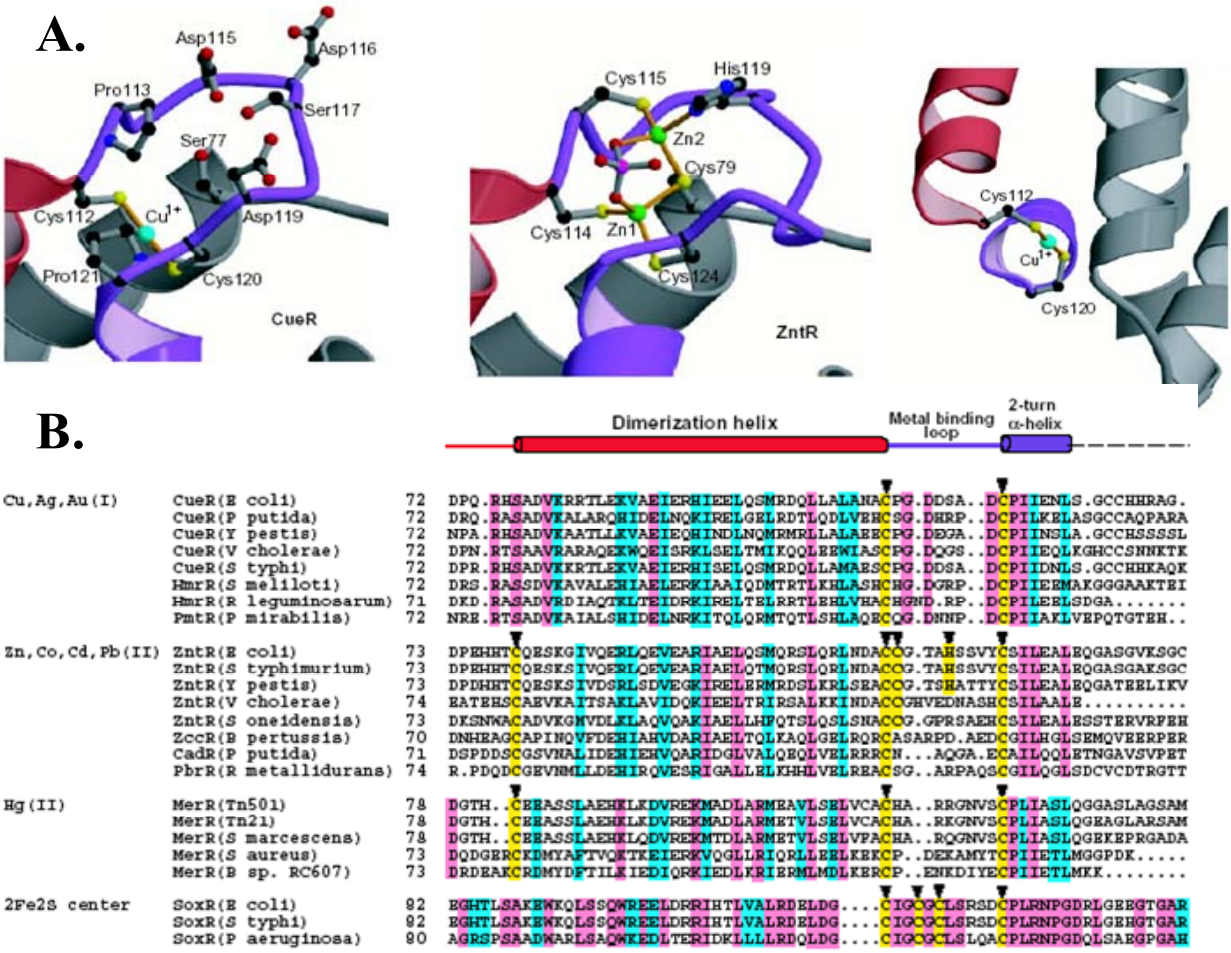


Fig.1: A) A close-up view of the metal-binding region of ZntR is presented in a similar orientation to that of CueR. The domains are shown in the same color scheme used for CueR. B). Sequence alignment of CueR and selected MerR homologues. The positions of Cys residues that are thought to serve as metal ligands in *E. coli* in comparison with other MerR homologues are indicated by (▼).

A deletion mutant of CueR was shown to abolish the copper dependent regulation of CopA. Further, to test if the copper responsiveness is mediated by the MerR-like region or by other upstream elements, mutations in the *copA* promoter were designed and the entire upstream region, including the putative CpxR binding site, was deleted from the 5'-end of the *copA-lacZ* promoter fusion. Copper responsive activation of *copA* was observed despite the removal of the upstream region. However -19 and -31 positions abolished copper responsiveness to the *copA* promoter, suggesting an important role for MerR like transcription regulators. The *cusRS,* a two-component signal transduction system genes form a sensor-regulatory pair which senses copper and activates the *cusCFBA* genes. *cusRS* sense the copper

levels in periplasm, CusS is a histidine kinase which is located at the cytoplasmic membrane and probably senses periplasmic copper stress. CusR here acts as a response regulator and induce the *cusCFBA*. A deletion mutant of *cusCFBA* was not copper sensitive in comparision to wildtype and *ΔcopA* mutant. A double mutant of Δ*cusCFBA* and *ΔcopA* was not more sensitive than *ΔcopA* alone which signify that *cusCFBA* is not an alternative to *copA*. In contrast, the strain where the deletion of both *ΔcueO* and Δ*cusCFBA* exhibits high copper sensitivity suggesting a probable role in copper detoxification at periplasm, quiet homologous to CzcCBA cation diffusion system. The third copper responsive transcriptional regulators *pcoRS* are quite homologous to *CusRS,* CusR and PcoR is 61% and CusS and PcoS exhibits 38% similarity. Despite the high homology these regulatory pairs both function independently. PcoS consists of two transmembrane segments extended into the periplasm and PcoR is a response regulator activated by phosphorylation which reveals similarities with activator and sensor components of the large family of bacterial two component regulatory systems. Regulation of plasmid determined that resistance genes are coupled with regulation of chromosomal genes. Deletion mutants of *pcoRS* exhibits copper sensitive phenotype and suggest a probable role in high level copper resistance determinants. Mutations in the *pcoR* gene might be complemented with *cutR* which is quiet analogus in their function in regulating chromosomal genes and *cutS,* as a corresponding sensor.

The recently discovered copper-specific repressor CsoR in *Mycobacterium tuberculosis* belongs to an entirely new set of copper-responsive repressors, whose homologs are widely spread in all major classes of eubacteria. CsoR from *B. subtilis* which is located upstream of the *copZA* operon is 37% homologous to *M. tuberculosis* CsoR, and elevated copper levels in *B. subtilis* are sensed by CsoR which leads to derepression of the *copZA* copper efflux operon. Copper in excess inside the cells is primarily sensed by copper sensing transcriptional regulator CsoR which efflux the excess copper out by the P-type ATPases copper efflux machinery. CsoR under normal conditions binds specifically to the P-type ATPases copper

efflux machinery, acting as a repressor. Increased intracellular copper levels is sensed by CsoR and binds to Cu(I) molecule and activate the efflux machinery by *copZA* operon. Δ*csoR* mutant was shown to constitutively express *copZA* copper efflux (48, 49). Intrestingly, the *ycnK* gene located upstream from *ycnJ* is investigated and was shown to encode a transcriptional regulator which acts, in addition to the investigated regulator CsoR, as a copper-specific repressor for *ycnJ* (48).

2.4 Copper chaperones.

Copper being both essential and toxic, tight regulation of copper is a prerequisite. Efficient mechanisms have been developed by the organism to efflux the excess copper out. To efflux copper out of the cells, metallochaperones, a newly discovered and an important class of enzymes that transport metal ions into specific export partners (P-type ATPases) are required. There are three major families of copper chaperones. (1) Atx1(or CopZ) family: In yeast, Atx1was first identified as a antioxidant (Atx1), but further investigation led to conclude its primary role as shuttling Cu(I) to copper efflux transporter(Ccc2). Atx1 binding to the cytosolic Cu(I) involves thiol ligands of the two cystein residues and a third aminoacid residue was supposed to stabilize this interaction. Atx1-Cu(1) complex then moves across the transgolgi network and recognizes the receptor site (MxCxxC where x is any aminoacid) in the downstream of CTR1 or upstream of Ccc2 and other related ATPases for proper interaction and efficient copper transport. Apart from these interactions, a positively charged interface on chaperone and a negative charge on interacting copper efflux ATPase is required for efficient docking. This interaction drives transfer of the Cu (I) from Atx1 to the corresponding transporter Ccc2 coupled with the ATP hydrolysis. Homologues of Atx1 in humans and mammals, Hah1 and ATOX1 can complement the functions for Atx1. Apart from the copper efflux, Atx1 functions in a high affinity iron uptake pathway, and is essential for loading of the multi-copper oxidase, Fet3. In *E.hirae,* a 69 aa copper binding protein was

identified and based on its role and function, it was considered as a copper specific chaperone CopZ (EhCopZ). EhCopZ was proposed to interact with a copper responsive repressor copY and two copper transporting ATPases CopA and CopB. The open face sandwich structure of CopZ resolved by NMR consists of a *βαββαβ* global structure where the two *α*-helices lying on a 4-stranded, antiparallel *β*-sheet.

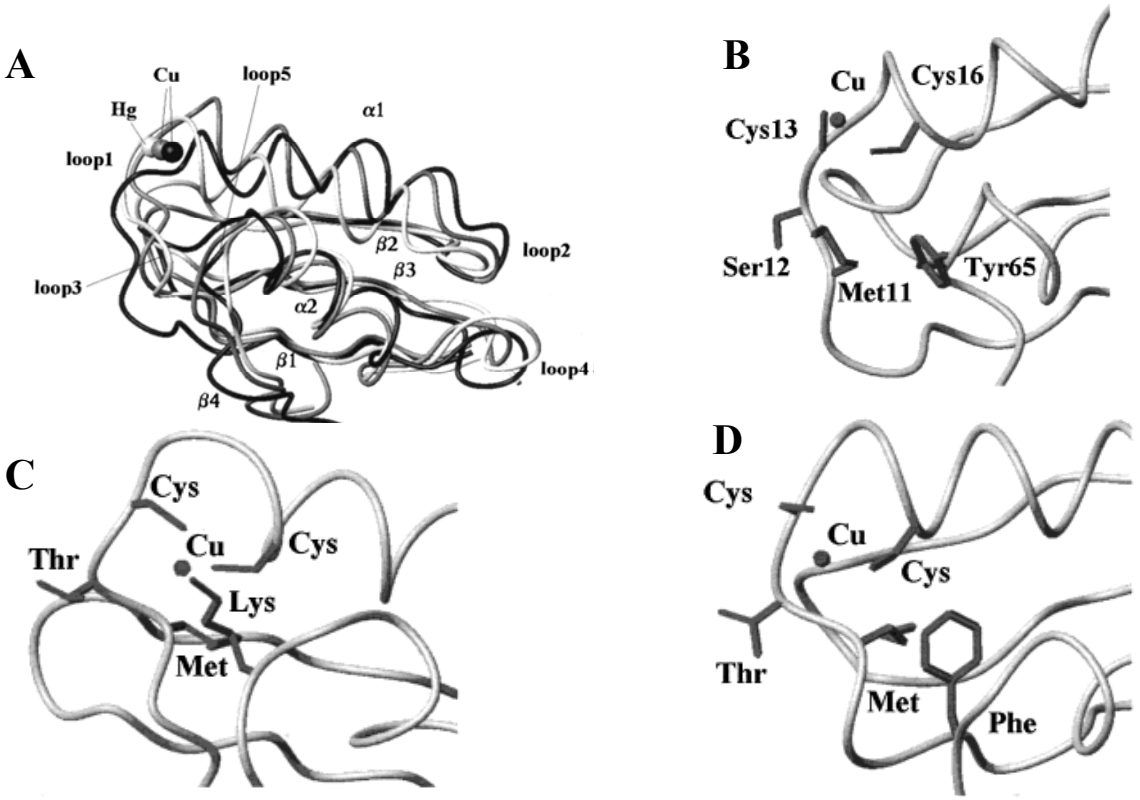


Fig.2: A. Comparision of the structure of BsCopZ-Cu(I) (black) with that of the soluble domain of the Cu-ATPase Ccc2-Cu(I) (gray;15) and with MerP-Hg(II) (light gray; 38). B. Comparison of the active site of BsCopZ-Cu(I) with that of C. yAtx1-Cu and with that D. of Ccc2-Cu(I). *Banci et.al., Biochemistry* 2001, *40,* 15660-15668

The recognition motif (C-x-x-C) as found in other eukaryotes and mammal copper chaperones, Atx1 from yeast, and Atox1 is located between the firs *β*-sheet and the first *α*-helix facilitating the binding of Cu (I) in a novel solvent exposed binding site. Inspite of having same fold and structure, the differences in their arrangement of the critical surface residues guide the variying modes of interactions with several targets for recognition and copper delivery. Mutating the C-x-x-C copper binding motif in the N-terminus of CopA to S-x-x-S abolished the Cu (I) induced decrease in the dissociation rate, without affecting the

association rate suggesting the significance of the cysteine residues in copper recognition and transport (63). There are 1-6 repeating CopZ-like structural elements in the N-termini of numerous metalloenzymes. One or two CopZ like elements are also found in bacterial cadmium and copper ATPases, including CopA of *E. hirae* in mercuric reductases, and in the multi-domain copper chaperones for superoxide dismutase. Around six copies in the N-termini of the human copper ATPases, ATP7A and ATP7B are defective in Menkes and Wilson disease, respectively (63). MelC1, a copper chaperone in *streptomyces* delivers copper to a binuclear Cu_A and Cu_B in tyrosinase, required for the melanin biosynthesis. The apo form of tyrosinase is maintained by MelC1 to facilitate copper incorporation and secretion (2).

The second family of copper chaperones includes the CCS, which supplies copper to Cu-Zn superoxide dismutase (SOD). An enzyme which catalyzes the dismutation of superoxide radicals ($O_2^{\bullet -} + O_2^{\bullet -} + 2H^+ \rightarrow O_2 + H_2O_2$) (65). Oxidative stress inside the cells elicited by hydrogenperoxide or redox metals like iron and copper results in the up-regulation of many antioxidant enzymes. Of many upregulated antioxidant enzymes, one of the primary enzymes includes Cu, Zn superoxide dismutase (SOD1). The copper chaperone for SOD1 (CCS) is involved in physiological SOD1 activation, which mainly acquires its catalytic copper co-factor by direct copper transfer from its copper chaperone protein CCS. Oxidative stress mediated upregulation of SOD1 is considered via transcriptional mechanisms, however, post-translational modification is critical for the activation of SOD1 e.g. oxidation of an intra-subunit disulfide bond by CCS in an oxygen dependent manner. CCS independent activation of SOD1 is achieved by copper insertion. Hence, oxidation of disulfide and copper acquisition are critical for enzyme activity and play an important role in structural stability of SOD1 (65). Expressed periplasmic Cu, ZnSOD from *S.cerevesiae* in *E.coli* exhibited enhanced protection against oxidative stress induced by paraquat or H_20_2 (56).

The third family involves putative copper chaperons called COX17, which deliver the copper to the mitochondrial enzyme cytochrome oxidase and is also responsible for the cytochorome *c* oxidase assembly. Cytochrome *c* oxidase (CcO) 1 is the terminal oxidase of the respiratory chain within the mitochondrial inner membrane (66). This gene was discovered in the search of yeast genes involved in cytochrome *c* oxidase assembly, hence named as COX17. The CcO enzyme is constituted by 13 subunits, 3 of which (Cox1-Cox3) are encoded by the mitochondrial genome, while the remaining 10 subunits are encoded by the nuclear genome. Cox1 contains a monocopper site called Cu_B, Cox2 contains a binuclear center called Cu_A requiring two copper ions. Cox1-3 being encoded by mitochondrial genome, the need for the copper atoms required for the assembly of these subunits have to be imported from the cytoplasm. To fulfill this requirement, several accessory proteins of which include COX11, COX17, COX19 and SCO1 and SCO2 are essential. SCOI transfer the copper to Cu_A site whereas Cox11 loads Cu_B site where both the accessory proteins receive copper directly from COX17. It appears that COX17 delivers copper to SCO1 and SCO2 which inturn transfer the copper atoms to cytochromes; hence these proteins are termed as co-chaperones (67).

The overall consideration of these copper proteins suggest that this extraordinary copper transfer mechanism between their physiological partners and their carefull selection of the interacting partners allows the directional transfer of copper by copper chaperones, protecting the cell from unspecific interactions and damage by copper.

2.5 Multicopper oxidases:

Multicopper oxidases employ the clever electron chemistry of bound copper ions, the enzymes that can be defined by their spectroscopy, sequence homology, and reactivity. Spectroscopic features originating due to the binding of the copper ions to multicopper oxidases they are broadly divided into three different classes which reflect the geometric and electronic structure of the active site. Type 1 (T1) or blue copper, type 2 (T2) or normal

copper, and type 3 (T3) or coupled binuclear copper centers. This division has further included the trinuclear copper clusters which are comprised of a type 2 and a type 3 center, the mixed-valent binuclear Cu_A site, the Cu_B-hemeA center of cytochrome *c*-oxidase and the binuclear Cu_Z center of nitrous oxide reductase (58). The redox properties of multicopper oxidases include outer-sphere electron transfer e.g blue copper proteins, CuA site of cytochrome oxidase and nitrous oxide reductase. Whereas the activation, binding and reduction of dioxygen, nitrite and nitrous oxide involves inner-sphere electron transfer where the substrate oxidation is coupled with four-electrons leading to the reduction of dioxygen to water (58). Transfer of electrons takes place from the substrate to the copper cluster *via* the copper and subsequently to the oxygen molecule bound at the site. Intra molecular electron transfer, substrates and the precise mechanisms vary from protein to protein, whereas the aminoacid sequences for recognition of copper binding ligands are highly conserved (57). The interactions of multicopper oxidases with substrates can be broadly divided into two categories: enzymes with low substrate specificity where plant and fungal laccases which utilize the outer sphere electrons for oxidizing diphenols, aryl diamines, and amino phenols with a K_m in the range of 1-10mM where there is no binding pocket for the substrate. The other multicopper oxidases possess a significant degree of substrate specificity, where the K_m < 1 mM, implying a possible substrate binding pocket. These multicopper oxidases are widely spread; in plants they are identified as laccases which are used in formation of lignin. Fungal laccases are important in pigment formation, detoxifictaion and lignin degradation in yeast (Fet3p) and mammals (hCp, hephaestin), function together with the iron permease, Ftr1p, to support high-affinity Fe uptake in yeast. Fet3p is a ferroxidase that, like ceruloplasmin and hephaestin, couples the oxidation of 4 equiv of Fe^{II} to the reduction of O_2 to $2H_2O$ (59, 60). In bacteria, CueO and MnxG perform copper homeostasis and manganese oxidation respectively. The physiological substrates of these different multicopper oxidases varying from organic compounds to different metals make them more intresting. The copper mediated

toxicity in *E.coli* was overcome by the expression of CueO and was postulated that CueO oxidize the cuprous copper to less toxic cupric copper. Multicopper oxidase (CueO), a laccase like enzyme from *E.coli* transported to periplasm via TAT pathway is able to protect the cells from copper mediated toxicity. It was shown that CueO oxidise cuprous Cu^{+} to Cu^{2+} and helps in maintaining the cuprous – cupric redox balance in aerobic environments similar to other MCO (Fet3) from S.cerevisiae and ceruloplasmin (humans). A disruption of *cueO* renders cells more copper sensitive (52). The broad specificity of CueO to oxidise wide variety of substrates interestingly includes ferrous iron, and catecholate siderophores suggesting a link between copper and iron. Further it was shown that a *fur* mutant over producing enterobactin subsequently facilitates Cu^{+} uptake by reduction of Cu^{2+}. Conversely, the reactive oxygen species generated by copper stress could further oxidize Fur-bound Fe^{2+}, releasing Fe from Fur and relieving Fur-mediated repression. This could be a possible reason for an increase in the *fur* transcript during oxidative stress conditions. Multicopper oxidase (MCO)/Fet3P in *Saccharomyces cerevisiae* plays a major role in re-oxidation of the Fe^{2+} to Fe^{3+} on the membrane. In *S. cerevisiae* and mammals, copper chaperones like ATX1 and ATOX1 are highly homologus to CopZ of *B.subtilis* and deliver copper to copper transporting ATPases, Ccc2P or Wilson (WND)/Menkes (MNK) proteins. This transported copper once gets over into the lumen or secretory pathway vesicles is incorporated into several proteins like apo-Fet3P, apoceruloplsmin in liver. This secreted multicopper-ferroxidase ceruloplasmin catalyse the oxidation of Fe^{2+} to Fe^{3+} in serum facilitating iron loading onto transferrins and subsequently systemic iron distribution. Fe^{3+} can be readily accepted by the iron permeases (Ftr1p) due to the fact that Fe^{3+} may provide increase substrate specificity for the high affinity iron uptake into the cell under iron limiting conditions. Further it might be possible that ferric iron bound to chelators (siderophores) is reduced by cell surface metallo reductases (Fre) and subsequent re-oxidation to Fe^{3+} by MCO favours high affinity iron uptake. Therefore, in *S.cerevisiae* and in mammals, copper deficiency results in iron

deficiency, whereas the unicellular green algae *Chlamydomonas reinhardtii* which served as a valuble model for studying metal metabolism in photosynthetic background led to the identification of several components in iron assimilation pathway which are copper dependent. These include multicopper ferroxidase (Fox1), copper chaperone (Atx1), an iron permease (Ftr1), a copper transporting ATPase and an iron storage encoding *Fer1*. Further in *C. Reinhardtii,* copper limitation did not result in secondary iron deficiency which is likely to be observed in yeast and mammals extending the possibility of copper-independent iron assimilation pathway. In gram negative bacterium *E.coli*, iron acquisition is mediated by catecholic siderophores (enterobactin) and its glucosyl derivatives (salmochelins) which are produced by enteric bacteria *Salmonella* spp. Enterobactin is produced by non ribosomal peptide synthesis (NRPS) and is negatively regulated by Ferric Uptake Regulator (FUR), when iron concentrations falls below 1μM. Copper in combination with catecholate siderophores was found to be highly toxic to the cells than copper alone, since these catecholates can acts as Cu(II) reductants.

2.6 Oxidative stress

The exploration of oxygen on earth has led to the oxidation of iron and copper which were normally present in their reduced forms and has thus resulted in the formation of reactive oxygen species (ROS). Haber and Weiss proposed that the interaction between super oxide ($O2^{\cdot-}$) and hydrogen peroxide (H_2O_2) results in generation of highly reactive hydroxyl radical ($HO^{\cdot}$) through Fenton reaction. The mechanism by which copper poisons the cell has been not very well understood. A current hypothesis is that copper reacts with cellular H_2O_2 to generate hydroxyl radicals in a process analogous to the Fenton reaction, the analogous reaction is driven by iron in vivo (50, 51).

$$Cu^{+} + H_2O_2 \rightarrow Cu^{2+} + OH^{-} + HO^{\cdot}$$

Oxidative stress leads to mutagenesis, aging and also several diseases. Respiration results in generation of ROS (reactive oxygen species) by the incomplete reduction of oxygen to water. Other factors which might induce ROS are exposure to radiation, light, metals or oxidation-reduction and redox active drugs such as paraquat. It is widely considered that copper exerts its effects at the cellular level at least in part through induction of oxidative stress. In general, all organisms which are grown aerobically are prone to oxidative stress and the defence mechanism include ROS-scavenging molecules e.g. superoxide dismutase and catalases. Copper induced oxidative damage of lipid membranes was shown in yeast. Other major targets of ROS include the oxidation of amino acid side chains to hydroxy or carbonyl derivatives or by breaking the peptide bonds, block branched-chain biosynthesis because they directly damage the iron-sulfur clusters of 2 dehydratases. The addition of branched-chain amino acids to the copper excess containing *E.coli* strains restored growth indicates that copper blocks their biosynthesis. Individual proteins might display different susceptibilities to copper induced oxidative stress. Recent investigations in *E.coli* under copper toxicity conditions revealed the oxygen independent inactivation of isopropylmalate dehydratase, an iron-sulfur cluster pathway enzyme. The toxic Cu (I) concentrations inside the cell damages iron-sulphur proteins by clustering to the sulphur ligands which results in the displacement of iron atoms from the solvent exposed cluster. The copper chelation by glutathione and detoxification of copper by dedicated copper efflux mechanisms and the cluster repair by assembly resulted in resistance against copper (51). The oxidative stress response regulator *soxRS* in *E.coli* were found to be strongly upregulated when exposed to increased $CuSo_4$ concentrations by oxidizing the 2Fe-2S clusters in the protein through an unknown mechanism or by blocking the steps in Fe-S pathway assembly (51, 53, 54). Hydrogen peroxide activates the transcription factor OxyR through the oxidation of two cysteines and formation of an intramolecular disulfide bond (54). Activation of OxyR subsequently induces

transcription of a set of antioxidant genes, including *katG* (hydroperoxidase I), *ahpCF* (alkylhydroperoxidase), *dps* (a nonspecific DNA binding protein), *gorA* (glutathione reductase), *grxA* (glutaredoxin I), and *oxyS* (a regulatory RNA) (54). Oxidized SoxR then induces the expression of the second transcription factor SoxS, which directly activates the second set of the following transcription genes involved in protection of cells against oxidative stress *sodA*(manganese superoxide dismutase), *fpr* (ferredoxin/flavodoxin- NADP1 reductase), *zwf* (glucose 6-phosphate dehydrogenase), *fumC* (fumarase C), *nfo* (endonuclease IV), *acnA* (aconitase A), and *micF* (a regulatory RNA). Iron and copper due to their redox cycling properties, are potent elements in the formation of hydroxyl radicals. Iron and copper in most of the metabolic enzymes are regarded as the cofactors for their activity and simultaneously when in excess, promote the formation of hydroxyl radicals through Fenton reaction. In prokaryotic cells, Fur (ferric uptake regulation) which is considered as an iron dependent repressor plays an important role in defence against oxidative stress. Derepression of iron regulon in a Δ*fur* mutant produces an oxidative stress leading to cellular damage (55). Superoxide dismutase (SOD) plays a key role in the defense against oxygen toxicity, is also Fur regulated (47, 55). SOD reduces superoxide levels as the first line of cellular defense against oxidative damage mediated by superoxide anion radicals. Expressed periplasmic Cu, ZnSOD from *S.cerevesiae* in *E.coli* exhibited enhanced protection against oxidative stress induced by paraquat or H_20_2 (56). Copper induced oxidative protein damage results in increased cellular protein carbonyl levels in yeast *Saccharomyces cerevisae* (60). In yeast, methionine sulphoxide reductase protects Fe-S clusters from oxidative inactivation. A double deletion mutant which lacks 2 methionine sulphoxide reductases in yeast are sensitive to many prooxidants but exhibited and increased copper resistance. Microarray analysis in this mutant shows and increases upregulation of Fet3P regulated by transcriptional factor *Aft1*. *Aft1* senses low levels of FeS cluster in mitochondria. Fe^{55}- labelling studies revealed an increased turnover of the FeS clusters in *mxr* deletion mutant which is consistent with the

increase in the oxidative levels inside the cells. Exposure to hydrogen peroxide plus copper in permeabilized yeast cells readily inactived the AMP deaminase, enhanced inactivation of AMP deaminase was observed by the addition of ascorbic acid. Complemetation by the addition of histidine restored the active AMP deaminase and protection againt hydroxyl radical produced by the copper plus hydrogen peroxide by Fenton reaction. Whereas, cysteine showed less effective protection against oxidative inactivation in comaparision with other amino acids which exhibited little or no effect.

3. Copper and Iron connection:

The connection between iron and copper was first shown by wintrobe et.al in 1950s. Hart, Elvehjem and coworkers were also credited for the experimentation of the copper/iron field for their paper in 1928 to initiate this exciting research (69), many other scientists and researchers contributed significantly in this field to unravel the connection between iron and copper and continued still as a major focus. Copper acquisition, distribution, utilization and the genes responsible to encode these functional proteins have been extensively investigated during the last few decades leading to the discovery of copper ATPases (6,7), copper chaperones (8,9) and iron transporters (70,71,72). These regulatory and homeostatic proteins being highly conserved from microbes to humans, it would be interesting to know how the changes in one metal concentration affect the homeostasis of the other.

3.1 Copper and Iron connection in Yeast.

Copper in *Saccharomyces cerevisiae* plays a major role in re-oxidation of the Fe^{2+} to Fe^{3+} on the membrane by multicopper oxidase (MCO)/Fet3P (42). In *S. cerevisiae* and mammals, copper chaperones like ATX1 and ATOX1 which are significantly homologus bacterial metal transport CopZ from *B.subtilis*, deliver copper to copper transporting ATPase Ccc2P or Wilson (WND)/Menkes (MNK) proteins (73,74). This transported copper once gets over into

the lumen or secretory pathway vesicles is incorporated into several proteins like apo-Fet3P, apoceruloplasmin in liver (75). This secreted multicopper-ferroxidase ceruloplasmin catalyse the oxidation of Fe^{2+} to Fe^{3+} in serum facilitating iron loading onto transferrins and subsequently systemic iron distribution (76,77). Fet3P and apoceruloplasmin processes Fe^{3+} can be readily accepted by the iron permeases (Ftr1p) due to the fact that Fe^{3+} may provide increase substrate specificity for the high affinity iron uptake into the cell under iron limiting conditions (78). Further, it might be possible that ferric iron bound to chelators (siderophores) is reduced by cell surface metallo reductases (Fre) and subsequent re-oxidation to Fe^{3+} by MCO favours high affinity iron uptake (79). Therefore in *S.cerevisiae* and in mammals, copper deficiency results in iron deficiency. Whereas the unicellular green algae *Chlamydomonas reinhardtii,* which served as a valuble model for studying metal metabolism in photosynthetic background led to the identification of several components in iron assimilation pathway which are copper dependent. These include multicopper ferroxidase (Fox1), copper chaperone (Atx1), an iron permease (Ftr1), a copper transporting ATPase and an iron storage encoding *Fer1*. Further in *C. Reinhardtii,* copper limitation did not result in secondary iron deficiency which is likely to be observed in yeast and mammals extending the possibility of copper-independent iron assimilation pathway (80).

3.2 Copper and Iron connection in *E.coli*.

In model organism gram negative bacterium *E.coli*, iron acquisition is mediated by catecholic siderophores (enterobactin) and its glucosyl derivatives (salmochelins) which are produced by enteric bacteria *Salmonella* spp (81, 82). Enterobactin is produced by non ribosomal peptide synthesis (NRPS) and is negatively regulated by Ferric Uptake Regulator (FUR), when iron concentrations fall below 1µM (83). Recently it was shown that the multicopper oxidase (CueO), a laccase like enzyme from *E.coli* transported to periplasm via TAT pathway is able to protect the cells from copper mediated toxicity. It was shown that CueO oxidise cuprous

Cu^{+} to Cu^{2+} and helps in maintaining the cuprous – cupric redox balance in aerobic environments similar to other MCO (Fet3) from S.cerevisiae and ceruloplasmin (humans) (84). The broad specificity of CueO to oxidise wide variety of substrates interestingly includes ferrous iron, and catecholate siderophores suggesting a link between copper and iron. Further, it was shown that a *fur* mutant over producing enterobactin subsequently facilitates Cu^{+} uptake by reduction of Cu^{2+} (79). Conversely, the reactive oxygen species generated by copper stress could further oxidize Fur-bound Fe^{2+}, releasing Fe from Fur and relieving Fur-mediated repression. This could be a possible reason for an increase in the *fur* transcript during oxidative stress conditions (84, 54).

3.3 Copper and Iron connection in *B.subtilis*.

In *B.subtilis,* iron uptake pathways have been shown to utilize three different types of siderophores hydroxamate, ferrichromes/ferrioxamines and shizokinen (85). Later the identification of catecholate siderophore bacillibactin in *B.subtilis* subsequently led to identification of both export (MFS- type) and import (FeuABC, YusV) bacillibatin transporters (86). In some cases, the elemental iron uptake is also facilitated by ywbLMN, which is orthologus to the copper dependent Fe^{3+} uptake system of *S.cerevisiae* (85). Under low oxygen concentrations, activation of a subset of genes dhbABCEF which were primarily regulated by transcriptional regulator Fur were found to be upregulated in Fnr dependent manner (87), since oxygen limitation and NO may synergistically activate the ResDE regulon facilitating to adapt in an anaerobic environment by changing its metabolic activity. A synergistic regulatory cascade and coordinated regulation of genes was observed while shifting from aerobic to anaerobic conditions (88). Phosphorylation of a putative oxygen sensor kinase ResD phosphorylated by ResE in response to oxygen or redox signal activates the transcription of *nasDE* which encodes nitrite reductase (89), *hmp*, a flavohemoglobin gene and a gene encoding redox regulator Fnr (90). Fnr induces *narGHJI* operon and *narK*,

encoding a nitrate reductase and a nitrite extrusion protein (91). Under reduced cellular conditions, nitrite is spontaneously converted to nitric oxide(NO), this NO can lead to the nitrosylation of ion center in Fur and thereby derepressing the Fur regulon leading to an indirect regulation of iron homeostasis caused by the imbalance in redox state of the cell (92,93).

3.4 Tasks of this work.

The current tasks of this work mainly include characterization of copper homeostasis genes in gram positive bacteria *B.subtilis* using genetic, biochemical and microarray approaches, since copper homeostasis in *B.subtilis* was until now mainly confined to one particular aspect i.e, detoxification or copper efflux. The first task of this study was to indentify and characterize the potential transcriptional regulators involved in copper homeostasis.This would include characterization of known copper responsive transcriptional regulators and also to identify the unknown copper homeostasis processes. The unknown copper homeostasis pathways mainly include copper import in *B.subtilis*, which was not known till date and also to present a model which could clearly outline the copper import and export mechanisms. The second task of this study was to analyse the microarray data, where, possible secondary effects elicited in iron homeostasis due to presence of the copper excess and copper limiting conditions using copper efflux mutants. The second part of the work should give further detailed insights about copper and iron connection in *B.subtilis* which was not clearly documented until now.

4. Materials

4.1 General chemical and reagents.

Agilent Technologies	2,5-dihydroxybenzoate matrix
Ambion Incorporated (Woodward, USA), Ambion Europe Ltd., Huntingdon, UK	T7-RNA-polymerase kit for DIG-UTP-labeled riboprobes, Ambion SlideHyb #1 hybridization buffer
Amersham Biosciences (Braunschweig)	various restriction endonucleases, ampicillin, IPTG,kanamycin, yeast extract, coomassie brilliant blue G and R250, HiTrapTM desalting columns, ECF, chemiluminescence substrate
Böhringer Mannheim (Mannheim)	Expand long template polymerase
Campro Scientific B.V.	RNAzol B(CS 105) reagent for RNA Isolation,
Difco (Detroit, USA)	Yeast extract
Eurogentech (Seraing, Belgien)	agarose, electro poration cuvettes
Fluka (Neu Ulm)	SDS, TEMED, DMF, Amberlite CG-50-II (cation exchange material)
Fermentas	10kD molecular weight protein marker.
Invitrogen	Platinum® Pfx DNA Polymerase, SuperScript™ III Reverse Transcriptase
Millipore (Bedford, USA)	Amicon® Ultra-4 Centrifugal Filter Devices
New England Biolabs (Schwalbach)	deoxyribonucleotides (dATP, dTTP, dGTP, dCTP), restriction endonucleases
Promega	pGEM -T and pGEM -T Easy Vector Systems
Qiagen (Hilden)	QIAquick-spin PCR purification kit, Ni2+-NTAagarose, QIAexpress vector kit ATG, QIAEXII extraction kit
Roche (Mannheim)	anti-DIG Fab-fragment with coupled alkaline phosphatase, High Pure RNA isolation kit
Roth (Karlsruhe)	EtBr, β-mercaptoethanol, acrylamide for SDS-PAGE, 2,3-dihydroxybenzoic acid,
Schleicher & Schüll (Dassel)	Whatman-3MM paper
Serva (Heidelberg)	APS
Sigma (Deisenhofen)	EDTA
Stratagene (Heidelberg)	PfuTurbo DNA polymerase

4.2 Equipment

Agilent Technologies	Agilent 2001 bioanalyzer, ICP-MS
Antair BSK	Clean-Bench
Bio-Rad Laboratories GmbH	Dot blot apparatus, electrophoresis, electroporation-pulse control
Avestin (EmulsiFlex-C5)	Emulsifier (for cell disruption)
Cybertech CS1, video camera,Mitsubishi Video Copy Processor	DNA-gel dokumentation
Thermodrucker	Documentation
Eppendorf Thermomixer Compact	Thermal shaker
Peqlab NanoDrop ND-1000, Ultrospec® 3100, Jasco (FP-6500)	Spectrophotometers
Molecular Dynamics	Storm860 fluorescence imager
New Brunswick Scientific Series 25 Incubator Shaker, NewBrunswick Scientific Innova 4300 Incubator Shaker, Heidolph Instruments GmbH	Shakers.
Olympus	Binocular microscope
Pharmacia FPLC-Biotechnology FPLC-System 250.	FPLC system
Perkin-Elmer Thermal Cycler 480, Perkin Elmer Gene Amp PCR System 2400,Perkin Elmer Gene Amp PCR System 9700	Thermo-Cycler
Tuttnauer 5075 ELV	Autoclave
Sartorius	Weighing balance
Savant Speed Vac Concentrator,Uniequip Univapo 150	Speed-Vac
Schott CG 840	pH-Meter
Sorvall RC26 plus,Heraeus Biofuge pico, Eppendorf	Centrifugation
Seral Seralpur Pro90CN	Distilled wated

4.3 Vector systems

4.3.1 pET28a(+)

The pET28a(+) vector system (Novagen) was used for cloning of the desired genes and overexpression of recombinant proteins Fig (2.1). This expression vector facilitates Ni^{2+}-NTA chromatography purification of the proteins by adding a His_6-tag as a translational fusion to the N- or C-termini of the protein. The transcription of the cloned genes in this vector system is dependent on the T7 RNA polymerase and can be induced by the addition of IPTG. *E. coli* BL21 was serves as an expression host.

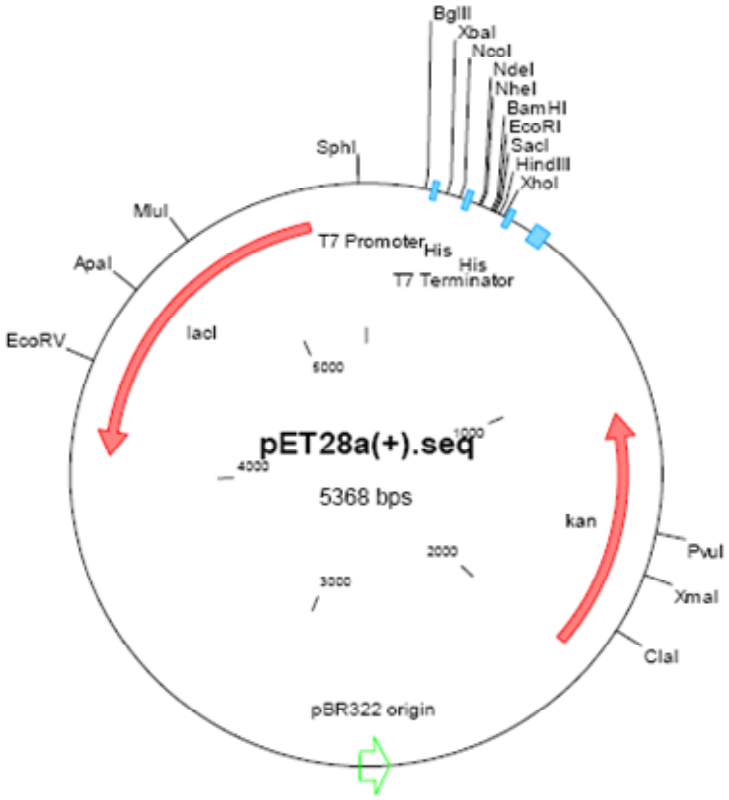


Fig. 2.1 Physical maps of pET28a(+)

4.3.2 pMUTIN, pUS19

The pMUTIN and pUS19 vector contains an erythromycin, and spectinomycin resistance, respectively. These vectors were used for the amplification of the resistance markers during long flanking homology PCR techinique to generate fusion PCR products. These resistance cassettes were used to replace the gene of interest during homologous recombination event.

4.4 Primers (Table 1)

Primer used in this study are listed in Table

Primer name	Primer sequence 5' to 3'
Csp100-Us-FP	CCA CAT GAC GAA GCA ACT TCG TAC AG
Csp100-Us-RP	GCA AGT CAG CAC GAA CAC GAA CC GCT TTT ATG GTT TAA TGT TTT ATG TTC GTT ATG CTT TTC CAT
Csp100-Ds-FP	GTC TAT TTT TAA TAG TTA TCT ATT ATT TAA CGG GAG GAA A TAA GGG GAA CAG GCC ATT TCT GAG C
Csp100-Ds-RP	GGC TTC CCG TTT GTC ACG GTT CC
Csp101-Us-FP	GGC AAG GAG CAG GCA AAA GTG G
Csp101-Us-RP	CTC TTG CCA GTC ACG TTA CGT TAT TAG GGC CTG ACC GGC GAC TTT AAC G
Csp101-Ds-FP	CTA TAA ACT ATT TAA ATA ACA GAT TAA AAA AAT TAT AA GCA GAA GGA TGA TCC ACC ATC TGT TTC G
Csp101-Ds-RP	CGC TCA AAT AAC TCC CAA AGC GTT GC
Csp102-Us-FP	CCG ATC CTA CAA TCA CCC CAA TTG C
Csp102-Us-RP	GCA AGT CAG CAC GAA CAC GAA CC CAG CCA CTT CAG TAT GTG TTG CTG TC
Csp102-Ds-FP	CTA TAA ACT ATT TAA ATA ACA GAT TAA AAA AAT TAT AA GCA GAA GGA TGA TCC ACC ATC TGT TTC G
Csp102-Ds-RP	GAG AGC AGC AAA ACG AGT GCC G
Csp103 ΔycnK-Us-FP	CCG ATC CTA CAA TCA CCC CAA TTG C
Csp103 ΔycnK-Us-RP	CTC TTG CCA GTC ACG TTA CGT TAT TAG CAG CCA CTT CAG TAT GTG TTG CTG TC
Csp104 ΔFur-US-FP	GGC AGC ACA CCA TCT TTC AGC AC
Csp105 ΔFur-US-RP	CTC TTG CCA GTC ACG TTA CGT TAT TAG CTT TCC CTC CTA CGC CGC ATT CC
Csp106 ΔFur-DS-FP	CTA TAA ACT ATT TAA ATA ACA GAT TAA AAA AAT TAT AAGCT GTA ACG GAA AAG AAA CTG AAT AGA CG
Csp107 ΔFur-DS-RP	GAT GAT TTA AAA AGA GGG CGT CAG ATA C
Csp108 CopA-US-FP	GCT CAT GTA CAA CCT CAG CAT CTG G
Csp109 CopA-US-RP	CTC TTG CCA GTC ACG TTA CGT TAT TAG CAA CAT ACT CAC TCC TTT ATA TAC ACC TGG
Csp110 CopA-DS-FP	CTA TAA ACT ATT TAA ATA ACA GAT TAA AAA AAT TAT AA GGC TAT ATG CCG GTT TTT GTT TTT CAT TGA CAC
Csp110 CopA-DS-RP	GCA GAA CGC CGT TTT GAT TGA TAA AGCC
csoR pET28a+FP	ATA TAC CAT GGA AAA GCA TAA CGA ACA TAA AAC ATT AAA CCA TAA AAG C
csoR pET28a+RP	ATA TAC TCG AGT GAT TTT GTG AAC TTT TTA AAT ACG TCC AAA AGC TCA G
ycnJ pET28a+ FP	ATA TAT CCA TGG ACA TGA AGC GAA ACA GAT GGT GG
ycnJ pET28a+ RP	TAT ATA CTC GAG TGA ATC GGC TGC TTT TTG GC
ycnK pET28a+FP	ATA TAC CAT GGA CAT GCT TCC G AT TAA TAG ACA GCA ACA C
ycnK pET28a+RP	TAT ATC TCG AGT TTC TTG GTG CAG GTG CAG CG
fur pET28a+ FP	
fur pET28a+ FP	

4.4.1 Primer used for dotblot analysis (Table 2)

Primer name	Primer sequence 5' to 3'
FP RP ycnJ	AGC TCG TCA AAC GGA CGA GAC G
RP RP ycnJ	TAA TAC GAC TCA CTA TAG GGG AAA TCC ATC AAA ATG CCG ACC G
FP RP dhbB	GCG CGT TTA AGA GAA CGA ATC TGC
RP RP dhbB:	TAA TAC GAC TCA CTA TAGG GTT TGC TGA CGT TTT TTG AAC GTC TGC
FP RP feuB	CGG TGT TAG TAT TCG GCC TTG C
RP RP feuB	TAA TAC GAC TCA CTA TAGGGGC ACT GCC GGT TAG AAT GAT G

(T7 promoter extensions are underlined).

4.5 Microorganisms

4.5.1 *E. coli* TOP10

The *E.coli* Top10 strain was obtained from Novagen and was generally used for sequencing and transformation purposes. The genotype is as follows: F- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) φ80*lacZ*ΔM15 Δ*lacX*74 *deoR nupG recA*1*araD*139 Δ(*ara-leu*)7697 *galU galK rpsL*(StrR) *endA*1 λ-.

4.5.2 *E. coli* BL21(DE3)

The *E. coli* strain BL21(DE3) was obtained from Novagen and the genotype of this strain is as follows F– *ompT gal dcm lon hsdS*B(rB – mB -) λ(DE3). This is mainly used as a host for expression of plasmid DNA. This strain was characterized by the absence of the Lon protease and protease of the outer membrane (OmpT). The strain carries the lambda DE3 lysogen for the expression of T7 RNA polymerase from the lacUV5 promoter which can be induced by IPTG..

4.5.3 Bacillus subtilis ATCC 21332

The *B.subtilis* ATCC 21332 is a gram positive organism and this strain was used for the current studies which includes all vivo studies in identification of copper homeostasis pathways. In contrast to the widely used *B. subtilis* 168, *B.subtilis* ATCC 21332 is prototroph for all amino acids and possesses a functional *sfp* gene that allows production of NRPS-assembled secondary metabolites.

4.5.4 Strains used in this work.(Table 3)

The relevant *B. subtilis* strains used in this study are listed below.

Strains / Plasmids	Genotype or description	Function	Source
B.subtilis:			
ATCC 21332	wild type	-	Cooper *et al.* (1981)
CSP100	Δ*csoR*::*erm*	Copper efflux transcriptional regulator.	This Study
CSP101	Δ*ycnJ*::*spc*	Copper homeostasis	This study
CSP102	Δ*ycnK*::*erm*	Transcriptional regulation	This study
CSP103	Δ*csoR*::*erm*/Δ*ycnK*::*spc*	Transcriptional regulation	This study
CSP104	Δ*copA*::*spc*	P-type copper efflux Pump	This study
CSP105	Δ*csoR*::*erm*/Δ*copA*::*spc*	Copper efflux regulator/copper efflux Pump	This study
CSP106	Δ*ycnI*::*spc*	Unknown	This study
CSP107	Δ*fur* ::*spc*	Iron sensing transcriptional regulation	This study
CSP108	Δ*dhbC* ::*spc*	Bacillibactin synthesis	*Miethke et al* .
CSP109	Δ*yhdQ*::*spc*	Transcriptional regulation	This study
CSP110	Δ*csoR*::*erm*/Δ*yhdQ*::*spc*	Copper efflux transcriptional regulation.	This study
E.coli :			
TOP10	F^- mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80lacZΔM15 ΔlacX74 deoR	Transformation	Invitrogen
	nupG recA1 araD139 Δ (ara-leu)7697 galU galK rpsL (Str^R) endA1 λ^-		
BL21(DE3)	F^- ompT gal dcm lon $hsdS_B(r_B^- m_B^-)$ λ(DE3)	Over expression	Novagen
Plasmids :			
pUS 19	Spc^R	Antibiotic resistance cassette Spc^R	Benson and Haldenwang(1993)
pMUTIN	Erm^R	Gene disruption vector; antibiotic resistance cassette Erm^R	Vagner *et al.* (1998)
pET28a+	Kan^R	Expression vector	Novagen
pCSP01	pET28a+ containing N-terminal 135 codons of *ycnJ* as C-terminal His_6-tag fusion .	Possible copper import	This study
pCSP02	pET28a+ containing *csoR*(*yvgZ)* as a C-terminal His_6-tag fusion.	Copper transcriptional regulation	This study
pCSP03	pET28a+ containing *ycnK* as a C-terminal His_6-tag fusion	Possible copper import transcriptional regulator	This study
pCSP04	pET28a+ containing *fur* as a C-terminal His_6-tag fusion	Iron transcriptional regulation	This study

4.6 Buffers

4.6.1 General buffers:

1 × TE Buffer (adjust to pH8.0) 1000ml	
Tris-HCl	10 mM
EDTA	1 mM

Used for resuspending pellets for chromosomal DNA isolations, resupsending the pellets for test expression of recombinant proteins

10 × TB Buffer (pH8,3) 1000ml	
Tris	108 g
Boric acid	55 g

TB buffer was used for Agarose gel electrophoresis

10 × SDS running Buffer (pH8,3) 1000ml	
Tris	30 g
Glycine	144 g
SDS	10 g

Used for poly acrylamide gel electrophoeresis

4 × SDS loading buffer for denaturing PAGE (20ml)	
Tris-HCl	5 ml from a 0,5 M stock; pH 6,8
Glycerol	8 ml
SDS	8 ml from a 10 % (w/v) stock
2-mercaptoethanol	2 ml
Bromphenolblue	2 ml from a 0,5 % (w/v) stock

Used for denaturing poly acrylamide gel electrophoeresis

4 × SDS loading buffer native gel electrophoresisfor 20ml	
Tris-HCl	5 ml from a 0,5 M stock; pH 6,8
Glycerol	8 ml
Bromphenolblue	2 ml from a 0,5 % (w/v) stock

Used for native poly acrylamide gel electrophoeresis

Coomassie staining solution 1000ml	
Coomassie Brillant Blue R	0,5 g
Methanol	450 ml
Acetic acid	100 ml

filter the solution

4.6.2 Buffers for dot blot

20 × SSC buffer (adjust to pH7.0) 1000ml	
Na-citrate × 2H$_2$O	88,23 g
NaCl	175,32 g

10 × Buffer 1 (adjust to pH7.5) 1000ml	
maleic acid	116,07 g
NaCl	87,66 g
NaOH	72,0 g

10 % Blocking buffer
10 g blocking reagent in 100 ml 1 × Puffer1 (cook in microwave), then autoclave

Ethidium bromide solution	
MOPS buffer 1X	1 volume
ethidium bromide	5 µg/ ml

Prehybridizing solution (for 2 blots)	
ddH$_2$O	1,52 ml
SDS	80 µl from a 10 % (w/v) stock solution
N-laurylsarcosin (Na-salt)	400 µl from a 10 % (w/v) stock solution
blocking Buffer 8 ml from a	10 % (w/v) stock solution
20 × SSC	10 ml
formamide	20 ml

Buffer 2 (for 2 blots) for 80ml	
Blocking buffer	10 ml from a 10 % (w/v) stock solution
10 × Buffer 1	10 ml

Washing buffer (B1)	
Buffer 1	1 volume
Tween 20	0,3 % (v/v)

Buffer 3 (detection buffer; pH9.5)
Tris HCl pH 9.5

4.7 General media

4.7.1 Full media:

The composition of the LB media includes the following components

LB medium	
Bactotrypton	10g/L
Yeast-extract	5g/L
NaCl	10g/L

This media was used for the standard growth of *E.coli, B.subtilis*.

4.7.2 2XYT medium with $MgCl_2$

The composition of the 2XYT media includes the following components

2XYT medium with $MgCl_2$	
Bactotrypton	16 g/L
Yeast-extract	10 g/L
NaCl	10 g/L
$MgCl_2$	10 mM

This medium was alternatively used to LB medium to obtain higher yields of protein expression.

4.8 Defined media

4.8.1 Belitsky minimal medium (BOC – Ground media)

Belitsky minimal medium (BOC – Ground media)	
BOC-Base, pH 7,5	(2-fold!):
$(NH_4)_2SO_4$	30 mM
$MgSO_4 \times 7\ H_2O$	16 mM
KCl	54 mM
Tris-HCl	100 mM

Belitsky minimal medium was used for *B.subtilis*growth under defined copper concentrations.

4.8.1.1 Supplement Solution (BOC-Supplements):

Supplement Solution (BOC-Supplements):	
KH_2PO_4	1,2 mM
$CaCl_2 \times 2\ H_2O$	4 mM
$FeSO_4 \times 7\ H_2O$	1 µM
$MnSO_4 \times 4\ H_2O$	20 µM
glucose	1 % (w/v)
Na-glutamate	9 mM

An equal mixture (v/v) of ground medium (2-fold) and supplement solution (2-fold) gave the final BOC-MM.

4.8.2 Chrome azurol sulfonate (CAS) agar

Semi quantitative estimation of siderophore secretion of *B.subtilis* strains was detected on plate with the help of CAS assay.

CAS-HDTMA stock solution:	
CAS (605 mg) dissolved in ddH_2O	(500 ml)
HDTMA (729 mg) dissolved in ddH_2O	(400 ml)
FeCl3 (1 mM) dissolved in 10mMHCl	(100ml)

The resulting CAS-HDTMA (dark blue color) was autoclaved.

CAS agar (200 ml)
2 × BOC-Base
1.2 % (w/v) agar-agar
74.92 ml ddH2O

Addition of filter-sterilized supplements and CAS-HDTMA stock solution:

CAS-HDTMA Supplements	
0.2 M KH_2PO_4	0.6 ml
1.0 M $CaCl_2 \times 2\ H_2O$	0.4 ml
25 mM $MnSO_4 \times 4\ H_2O$	0.08 ml
0.5 % (w/v) glucose	2 ml
0.45 M Na-glutamate	2 ml
CAS-HDTMA stock solution	20 ml

Add CAS-HDTMA stock solution slowly under continous shaking

For plates add 15ml in each petrilplates under strerile hood and allow them to solidify

4.8.3 *B. subtilis* transformation medium:

For transformation and homologous recombination of B. subtilis ATCC 21332 , cells were grown in high-salt medium (HS) overnight and subsequently, grown in freshly prepared low-salt (LS) medium. The components for the following media includes.

10 × S-Base	
$(NH_4)_2SO_4$	20 g/L
$K_2HPO_4 \times 3H_2O$	140 g/L
KH_2PO_4	60 g/L
Na_3-citrate × $2H_2O$	10 g/L
1 M $MgSO_4$	1 ml/l

All the components were autoclaved and stored for further use

HS(High salt) medium(100ml)	
10 × S-Base	10 ml
50% (w/v) glucose	1 ml
10% (w/v) yeast extract	1 ml
2% (w/v) casein hydrolysate	1 ml
8% (w/v) arginine 0,4% (w/v) histidine	10 ml
ddH_2O	77 ml

The solution was filter-sterilized.

LS(Low salt) medium(20ml)	
10 × S-Base	2 ml
50% (w/v) glucose	200 µl
10% (w/v) yeast extract	200 µl
50 mM spermine	200 µl
2% (w/v) casein hydrolysate	100 µl
1 M $MgCl_2$	50 µl
ddH_2O	17.25 ml

This solution was prepared freshly and filter sterilized

5. Methods

5.1 Bacterial growth in liquid culture conditions:

Bacterial strains and plasmids used in this study are listed in Table 1. *Bacillus subtilis* ATCC 21332(wild type) was grown in Belitsky mimimal media (BMM) supplemented with 0.5% (w/v) glucose as a carbon source and with all essential nutrients required (94). Freshly prepared CuCl(0.5mM) was used to maintain the copper excess conditions and 0.25 mM bathocuprione disulphonate (BCS; Sigma-Aldrich) was used as a Cu(I)-specific chelator for maintaining copper limiting conditions. Unless otherwise indicated, liquid media was inoculated from an overnight pre-culture in LB media incubated at 37°C with constant shaking at 225 rpm. All glassware was washed with 0.1 M HCl and double distilled water before autoclaving. Antibiotics erythromicin (1 μg ml^{-1}) and lincomycin (25 μg ml^{-1}), both for testing macrolide-lincosamide-streptogramin B resistance (MLS^R), and spectinomycin (100 μg ml^{-1}) were used for the selection of various *B. subtilis* mutants after construction. For selection of *E. coli* Top10 strains with transformed plasmids, antibiotic kanamycin (50 μg ml^{-1}) was used. Strain *E. coli* BL-21 was used for protein overexpression.

5.1.1 Bacterial growth on solid LB media and CAS media:

Medium for the preparation of LB plates and CAS plates was achieved by adding 1.2 % (w/v) of agar to the LB liquid medium and 1x BOC base medium supplemented with the CAS-HDTMA solution respectively , which was sterilized at 121°C and 15 bar for 30 min. Sterilized medium was poured in sterile petriplates with approximately quarter an inch thickness under clean bench with or without antibiotic, allowed them to cooldown. After solidification strains with respective antibiotic resistance markers were streaked onto the respective antibiotic LB plates and incubated at 37°C overnight. *B.subtilis* strains were stored at room temperature and the *E.coli* at 8°C. Freshly streaked bacillus strains from LB plates

were spotted onto the CAS plates using a sterile tip and grown for 16-24 hrs at 30°C for the CAS assay.

5.2 DNA manipulations and genetic techniques:

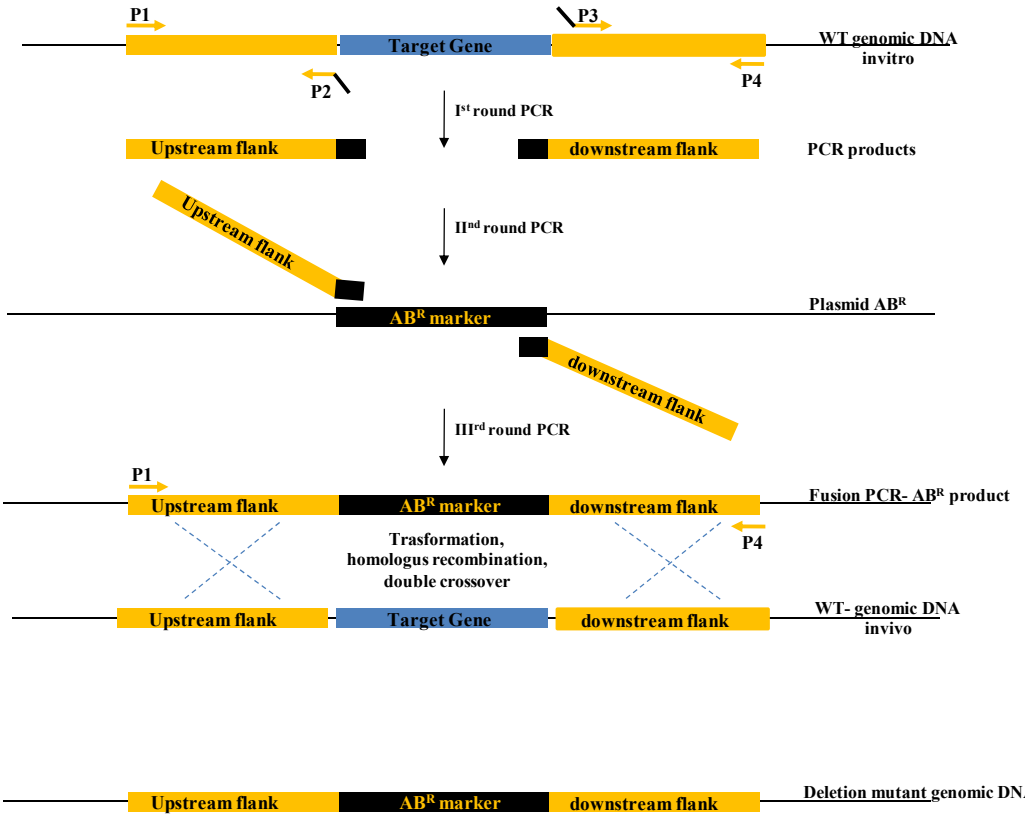
DNA preparations and transformations were carried out as described previously (100, 101). Electroporation was used for the transformation of plasmids into *E. coli* Top10 cells. Homologous recombination was used for transforming the *B. subtilis* ATCC 21332 strain for the mutant construction. Restriction enzymes, T4-DNA ligase and calf intestinal phosphatase were used according to the manufacturer's instructions (New England Biolabs).

5.2.1 *B.subtilis* transformation and mutant construction:

Deletion mutants were generated by long flanking homology PCR method (102). In the first round PCR, long flanking homologous PCR fragments were amplified from upstream and downstream regions of the gene to be deleted. The 3'-ends of the resulting homologous PCR products were designed to be complimentary to the selected resistance cassette, and were used in the second round PCR, generating a fusion construct. These fusion products were used for transformation either directly or gelpurified when more than one bands were seen after PCR amplification. This fusion construct after transformation and homologus recombination displaces of the gene of interest with the selected resistance marker to facilitate the selection and growth of mutant on antibiotic plates. The primers used in generating the mutants are listed in **Table 2**. All PCR reactions were performed using Platinum®*Pfx* DNA polymerase (Invitrogen). Chromosomal DNA of *B. subtilis* ATCC 21332 was used as a template to amplify the corresponding upstream and downstream flanking regions. Expand Long Template PCR System was used to fuse the homologous flanks with the corresponding resistance markers. *B. subtlis* ATCC 21332 strain was used to generate the Δ*csoR*, Δ*yhdQ,* Δ*ycnJ,* Δ*ycnK,* Δ*ycnL,* Δ*copA,* Δ*fur,* mutants. The Δ*csoR* strain was used for the generation of Δ*csoR*/Δ*yhdQ,* Δ*csoR*/Δ*ycnK* and Δ*csoR*/Δ*copA* double mutants. Transformation in *B. subtilis*

ATCC 21332 was achieved by making the competent ATCC 21332 cells using the high-salt low-salt method. The cells to be transformed were grown in HS-medium overnight at 37 °C and 300 rpm. Then, this preculture was inoculated to an OD_{600} of 0.05 into freshly prepared LS-medium and cells were grown at 30°C with continuous shaking at 110 rpm until an OD_{600} of 0.6 - 0.7 was reached. Then, 1 ml each of this culture was transferred into 2 ml eppendorf tube and was incubated with 10-15 µl of prepared DNA for 2 h at 37 °C with constant shaking at 110rpm. Approximately 250 µl of these samples were plated on selective antibiotic resistance plates and incubated at 37°C overnight. Obtained transformants were restreaked onto the corresponding antibiotic containing LB plates. Chromosomal DNA was isolated from all the transformants and the recombination event was further confirmed by PCR. The erythromycin resistance cassettes used for the construction of the Δ*csoR,* Δ*ycnK,* Δ*ycnL* single mutants were amplified from the pMUTIN vector. To generate Δ*ycnJ*, Δ*copA, and* Δ*fur* mutants, the spectinomycin resistance cassette was amplified from the pUS19 vector, while the resistance cassettes for the Δ*csoR*/Δ*yhdQ,* Δ*csoR*/Δ*ycnK* and Δ*csoR*/Δ*copA* double mutant were amplified from pMUTIN and pUS19, respectively.

Fig 2.2 A schematic view of deletion mutant construction



5.3 Purification of CsoR, YcnK , 135 aa N-terminal part of YcnJ and Fur:

The open reading frames of corresponding genes *csoR*, *ycnK*, *ycnJ* and *fur* were amplified by PCR using chromosomal DNA as template derived from *B. subtilis* ATCC 21332 strain. The amplified PCR fragments were then digested using the restriction sites NcoI and XhoI which are incorporated by the primer pair to facilitate the directional cloning into the desired expression vector. Subsequently the desired expression vector pET28a+ is also linearised using NcoI and XhoI restriction sites to facilitate the cloning. The linearised vector and the digested PCR fragment was mixed in 1:2 and 1:4 ratio respectively and ligated using T4 DNA ligase overnight at 16°C. The ligated plasmids were then dialysed against water in a petridish containing water across the floating 0.25µM nitrocellulose membrane for 15-30 min. The dialysed sample was then carefully collected using pipette and then transformed into approximately 40µl of electrocompetent *E. coli* DH5α cells using electroporation with a short pulse for 2 seconds at 0.25volts. Transformed cells were then transferred immediately to a 700µl of prewarmed (37°C) LB media without any antibiotic in it and incubated for 30 to 45min at 37°C in thermal shaker maintained at 600rpm. Approximately 250µl was platted and incubated at 37°C overnight. Plasmid was obtained from the resulting colonies and confirmed by restriction analysis for the integration of the fragment. Confirmed plasmids were then transformed into *E.coli* BL21strain for over expression. Overnight cultures were grown by inoculating an isolated colony of the transformed *E.coli* BL21cells from a plate into 5ml LB media containing kanamycin (50µg ml^{-1}). The overnight cultures were used to inoculate 2L of LB media containing kanamycin (50µg ml^{-1}) with a starting OD_{600} of 0.05. Inoculated cultures were grown under continuous shaking at 225 rpm at 35°C and the temperature was decreased to 30°C after one hour. When the cells reach and OD_{600} of 0.5-0.6, isopropyl-**β**-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1mM in the culture flasks and growth was continued for four hours. Cells were harvested by centrifugation (Sorvall RC 5B plus), and resulting pellets were resuspended in Hepes-A buffer

(50mM Hepes, 300mM Nacl (pH 7.5), 1mM DTT) and lysed by EmulsiFlex-C5 Avestin. The obtained lysate was clarified by centrifugation (Sorvall - RC 26 plus) at 17000 rpm for 30min and a Ni-NTA column was used to purify the protein using a linear gradient with Hepes-B buffer [50mM Hepes, 300mM Nacl, 250mM Imidazole (pH 7.5), and 1mM DTT]. Purified protein was dialysed against Hepes-A buffer containing 100mM NaCl. Protein purity above 95% was observed by coomassie stained SDS-PAGE gel for all the proteins. CsoR protein tends to precipitate at high concentrations, when the pure fractions were dialyzed against Hepes-A buffer containing 100mM Nacl and concentrated by using Amicon Ultra-15 Centrifugal Filter Units with a 5,000 MW cutoff, hence a different buffer system [20mM Tris HCl(pH8.0), 100mM Nacl, 1mM EDTA, 1mM DTT and 5%(w/v) glycerol] was used for dialysis and to stabilize the protein. While the other purified proteins YcnK , 135 aa N-terminal part of YcnJ and Fur are quiet stable when dialysed in Hepes-A with 100mM NaCl and concentrated by using Amicon Ultra-15 Centrifugal Filter Units.

5.3.1 Native gel electrophoresis:

Native gels were casted much similar to non denaturing SDS gels without any SDS in it. The running buffer and the loading buffer used for the native gels do not contain any SDS. Purified protein samples ~100µM were incubated with or without 200µM Cu(I) or Cu(II), respectively, for about 15 to 30 min at room temperature and loaded onto 6% continuous native polyacrylamide gels. Samples were run at 100V/ 10 milliamp for about 4 to 5 hours until the bromophenol blue ran out of the gel. Gels were stained using coomassie blue and destained using 10% acetic acid.

5.4 RNA isolation:

The *B. subtilis* strain ATCC 21332 was grown in BOC minimal medium (supplemented with 0.45 % [w/v] glucose and 3.5 mM sodium glutamate). An overnight grown preculture from LB media was inoculated into fresh BOC minimal medium with an starting OD_{600} of 0.05.

CuCl (0.5mM) was added to the media to create copper excess conditions or bathocuprione disulphonate(BCS) (0.25mM) was added to achieve copper deficient conditions in the media. Cells were grown with a constant shaking of 300 rpm and harvested at an OD_{600} of 0.35, and the total RNA was prepared as described **(48).** In addition to the normal purification method, High Pure RNA isolation kit purification method including DNase-I treatment was also used. RNA purity was tested using Agilent 2001 bioanalyzer and the concentrations were estimated by using NanoDrop spectrophotometer.

5.5 cDNA synthesis, cDNA labeling and slide hybridization:

To obtain cDNA, 2 µl (1.6 µg/µl) of random nonamers was added to 10 to 20 µg of total RNA and final volume was adjusted to 18µl using RNase free water. The following mixture was mixed gently by pipetting up and down and incubated for 5 min at 70 °C. Annealing of this mix was allowed by cooling down to room temperature for 10 min. The annealed sample was added to 12µl of reverse transcription mix, which makes upto a total volume of 30µl and incubated at 42 °C for 16 hours. The reverse transcription mix contains the following components 6 µl 5 × Superscript III buffer, 3 µl 0.1 M DTT, 1.2 µl 25× amino-allyl-modified dUTP/ nucleotide mix and 1.8 µl reverse transcriptase (SuperScript III) which was kept as short as possible outside –20°C. The RNA degradation was achieved by the addition of 3 µl of 2.5 M NaOH and incubating at 37 °C for 15 min. The solution was neutralized by adding 15 µl of 2 M HEPES (free acid). The cDNA purification was perfomed according to the protocol of GFX columns with an exception that 80% ethanol was used as wash buffer and eluted using elution buffer containing 0.1 M sodium carbonate pH 9.0. These CyScribe ^{TM} GFX^{TM} columns (GE Healthcare) use a glass fiber matrix packed into a spin column format for highly efficient purification of labelled cDNA with yielding concentrations between 30 to 120 ng/µl. Labeling of the amino-allyl-modified cDNA was attained by using Cy3 and Cy5 fluorescence dyes and subsequently purified using CyScribe ^{TM} GFX^{TM} columns where the

aminoallyl-labelled and fluorescent-labelled cDNA probes are captured by the glass fiber matrix, while the unincorporated Cy dye and primers are removed by washing. Bound probes are eluted with a quick spin in elution buffer provided in the kit with yielding concentrations of 0.5 - 2.0 pg/µl of labeled cDNA. Three independent biological replicates of RNA samples were used for the preparation of cDNA and labelled with Cy3 and Cy5, respectively with an additional fourth independent preparation of cDNA used as "dye swap". The labeled cDNA samples that should be used for a competitive hybridization were mixed in equal amounts and dried under vacuum rotation. The dried Cy3 and Cy5 cDNA sample pellets were dissolved in 7µl water, gently pipetting up and down. Dissolved pellets were incubated at 94°C for 2 min in a water bath followed by addition of 35µl Ambion SlideHyb #1 which was pre warmed at 68 °C. for 5 min. The hybridized samples were then incubated at 68°C for 5 min followed by short spin to get rid of the precipitates, 35µl of the sample was applied to the preheated microarray slides covered with a lifter slip, where the white straps of the lifter slips face the microarray slide by providing the room for the hybridized solution to sneak between the slides. The loaded slides were transferred into the hybridization chamber maintained at 48 °C for at least 16 h, after which hybridization took place in the dark. The slides were washed with 40 ml 2 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate; pH 7.0) and 0.5 % SDS for 5 min, then twice with 40 ml 1 × SSC and 0.25 % SDS for 5 min, then with 40 ml 1 × SSC and 0.1 % SDS for 5 min. The slides were dried by centrifugation (at 805 × g for 2 min at room temperature) and were subsequently applied to a confocal laser scanner for signal detection and measured the gain in the intensity of the Cy3 and Cy5 dye at different wavelengths. Care should be taken that there should not be more than three saturated white spots.

5.6 Microarray data analysis:

Hybridized DNA arrays were read using a GenePix autoloader 4200AL (Axon Instruments), and data obtained were processed with ArrayPro 4.5 (Media Cybernetics Inc., Silver Spring, Md., USA) ArrayVision software. Expression levels were processed and normalized (Lowess method) using both prep and Micro-Prep software (28,29). Duplication of the genomic fragments on the spotted microarray slide led to sixteen normalized signals from eight microarray slides used (duplicates of Cy3 and Cy5 measurements for three regular and a dyeswap). The ln-transformed ratios of the expression levels of mutant versus wild type were subjected to a t-test using the Cyber-T analysis which calculates the mean induction ratios and the Bayesian standard deviations (Bayes.p-values) of all normalized signal intensities (27).

The results obtained were averaged and the raw data was processed to Cyber-T web interface software for the calculation of the expression ratios and the data was exported to Microsoft Excel.Genes which corresponding to the homogeneous expression pattern with Bayes.p < 10^{-3} values were selected to identify significantly overrepresented functional categories using the FIVA software. The up and down regulation of genes were set to 1.5 and 0.7 respectively, due to the fact that the stringent conditions and parameters which were set in the earlier stages of microprep and cyber-T analysis.

5.7 Dot blot analysis:

B. subtilis strains were grown in normal (Belitsky minimal medium), copper limited (Belitsky minimal medium + addition of 0.25mM BCS) and copper repleted (Belitsky minimal medium + 0.5mM copper in excess) conditions. Overnight cultures were inoculated into fresh medium to an initial OD_{600} of 0.05 and cells were harvested at an OD_{600} of 0.25. Total RNA was isolated from these cells using RNAZol method (33). RNA concentrations were measured using the nanodrop at 260nm:280nm and denaturing gel electrophoresis was run to test the quality and compare the quantity of 16S and 23S RNA. The RNA to protein concentrations

were above 1.65 in all the samples.. 2µg RNA from each sample was subsequently dotted onto the nylon membrane using a dot blot apparatus and fixed by UV crosslinking. The fixed RNA samples were subsequently hybridized with a UTP-11- digoxigenin-labelled antisense RNA ribo probes specific for *ycnJ, dhbB and feuB*.. The riboprobe was synthesized by *in vitro* transcription using T7 RNA polymerase. The T7 promotor sequence was introduced into the PCR product of the respective ribo probes used for the hybridization listed in table (3). After hybridization overnight at 68°C in the rotating hybridizing chamber the membranes were subjected to several washing steps, the filters were treated with a digoxigenin-specific antibody fragment conjugated with an alkaline phosphatase (Roche) and washed again and treated with and AttoPhos (Amersham Biosciences) as an enhanced chemifluorescence (ECF) substrate to analyse the hybridization pattern of different dotted samples. The hybridization signals were detected with a Storm860 fluorescence imager, and relative signal quantification was performed with ImageQuant software.

5.8 Estimation of copper concentrations inside the cells:

Total cytoplasmic copper concentrations were measured using Inductively Coupled Plasma Mass Spectrometry (ICPMS). *B. subtilis* strains ATCC 21332 and Δ*csoR* were grown in Belitsky minimal media overnight. Fresh 100 ml Belitsky minimal media with either 0.5mM copper in excess, or copper limiting conditions achieved by addition of 0.25mM BCS, were inoculated with overnight cultures with a starting OD_{600} of 0.05 and the cells were harvested in mid-log phase. Cells were centrifuged at 13,000 rpm (18000 **g**) for 5 min, pellets were washed three times with buffer containing 10mM Tris/HCl (pH 7.5), 1mM EDTA and finally with milliQ water. Cells were dried overnight at 85 °C and the total copper concentration was determined by breaking the cells using nitric acid.

5.9 Crystallization studies:

Purified recombinant protein was used for the crystallization studies. The protein was additionally purified using filtered columns before crystalization to get rid of the precipitates or aggregates if any which could block the pippeting robot. The initial screening was done by using the pipetting robot, using standard pipetting volume. Protein samples with or without metal was set to 300nl and 300nl of the corresponding buffer was pipetted into flat-well plates for 96 different conditions and 2 or 3 parallels per each condition. The buffer reservoirs were filled manually with 100 µl (Innovazyme-Plates with 80 µl) of the corresponding crystallization buffer.

CsoR-His_6 was used at a concentration of 2.2 mg/ml in the following screens:

1) SIGMA Basic

2) SIGMA Extension

3) SIGMA Cryo

4) SIGMA Basic Membrane

5) NEXTAL Classic

7) NEXTAL Anion

8) NEXTAL Membrane I

The protein was subjected to crystallize both with and without copper that was used in equimolar concentrations. All pipetted screens were stored in dark at 18°C. The screens were examined manually using a binocular microscope for the crystals. Further, diffraction of the crystals were investigated by attaching a diffraction lens to the microscope. Intial examinations for the crystal formation was tested for once in a week in the the first month and twice in a week after crystal formation to check the growth of the crystal.

5. 10 Bacillibactin quantitation:

B. subtilis wild type and Δ*csoR* mutant strain were grown in standard belitzsky minimal medium without iron for 16 hours. 50 ml from each culture were harvested and cells and culture supernatant were separated by centrifugation at 4,000 g for 20 min at 4 °C. The supernatant was extracted three times with equal volumes of ethyl acetate. The organic fractions were pooled and the solvent was evaporated by using Roti vacuum centrifugation at 40 °C . The obtained pellets were dissolved in 400µl of 50% methanol and were shaken overnight at 37°C. The dissolved pellets were centrifuged briefly for 2 min at 13000 rpm and the supernatants were analysed by using LC-MS and quantified by using known concentrations of the purified bacillibactin together with the test samples.

5.11 Quantification of NAD and NADH.

To estimate the nicotinamide levels in the cells, the deletion mutant Δ*csoR* strain was grown in the desired conditions along with wild type as a control. Cells were grown till mid log phase where the OD_{600} corresponds to 0.6-0.8 and harvested the cells in a cooling centrifuge. Pellets were either shock frozen in liquid nitrogen for later use or continued for estimation. Cells were washed once with ice cold 50mM phosphate buffer (KH_2PO_4 + KOH) pH 7.0 and then resupsended in 5-7 ml of 0,1M Tris buffer pH 8.2. To estimate the total nicotinamide levels the suspension was divided into 2 equal portions of 3ml each to proceed in two different pathways. First portion was used to extract the oxidized forms (NAD^+), 1.5ml of 0.33N HCl was added to 3ml of the resuspended pellet suspension and second portion was used to extract reduced form (NADH), 1.5ml 0.33N NaOH was added to 3ml of cell suspension. Both the samples were incubated in a water bath maintained at 50°C for 10 min and cooled down to 0°C. Both the samples were neutralized by adding 0.45ml of 1N NaOH to the portion 1 and 0.45ml 1N HCl to portion 2, Care should be taken to avoid the local concentrations of acid or alkali and hence it should be added drop wise with continuous

shaking. Spin down the samples at 23000g for 15 min. Further, reoxidation of NAD^+ was performed to the second portion by adding 10µl of 2-oxoglutarate + NH_4Cl + 10µl of glutamic dehydrogenase and incubate at room temperature for 15min. The reoxidised forms of NAD^+ from second portion was performed by the addition of 100µl 5N HCl and incubated again for 15min at 50°C followed by neutralization by addition of 90µl 5M NaOH. The samples were centrifuged at 23000 for 15 min to rid of the precipitates. To analyse the nicotinamide levels from the acid and alkali extracted portions, 2.0ml pyrophosphate+ semicarbazide buffer was added to 1ml of the extract and 10µl ethanol was added as a substrate. After measuring the blank at E_{340} without the addition of enzyme, 10µl of alcohol dehydrogenase was added. Incubated it at room temperature of 5-10 minutes and measured the conversion at $E_{340.}$

6. Results:

6.1 Characterization of copper efflux mutants.

Copper homeostasis mechanism in *B.subtilis* is primarily achieved by sensing the intracellular copper levels by the corresponding transcriptional regulators CsoR (YvgZ) and CueR(YhdQ) which activate the copper efflux operon *copZA*.

Fig.3: Genomic organization of copper efflux components

Copper efflux is carried out mainly by heavy metal exporters which belong primarily to the integral membrane protein family of P-type ATPases, whose expression is controlled mainly at the level of transcription. These P-type ATPases are functional in translocating Cu(I) across the cytoplasmic membrane. The recently discovered copper-specific repressor CsoR in *Mycobacterium tuberculosis* belongs to an entirely new set of copper-responsive repressors, whose homologs are widely spread in all major classes of eubacteria (16). CsoR from *Bacillus subtilis*, which is encoded upstream of the *copZA* operon, is 37% homologous to *M.tuberculosis* CsoR, and elevated copper levels in *B. subtilis* are sensed by CsoR, which leads to derepression of the *copZA* copper efflux operon. As previously described, the YhdQ (CueR) protein also binds to the copZA regulatory region, but genetic experiments indicate that this protein is not responsible for the copper-dependent regulation of this operon (49).

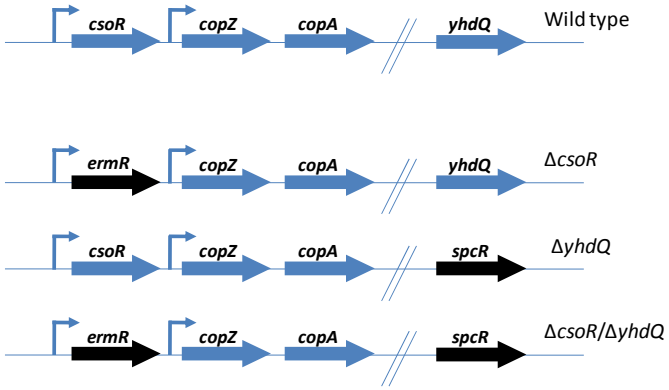


Fig.4: Deletion and replacement of the *csoR, yhdQ, and both with* gene by an erythromycin resistance cassette, spectinomycin and both respectively.

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The deletion mutants of *ΔcsoR(yvgZ),* Δ*yhdQ(cueR)* and *ΔcsoR(yvgZ)/*Δ*yhdQ(cueR)* double mutant were generated by using long flanking homology PCR method and followed by homologus recombination event in *B.subtilis.* The recombination event was further confirmed by PCR and antibiotic resistance integration by streaking on corresponding antibiotic plates.

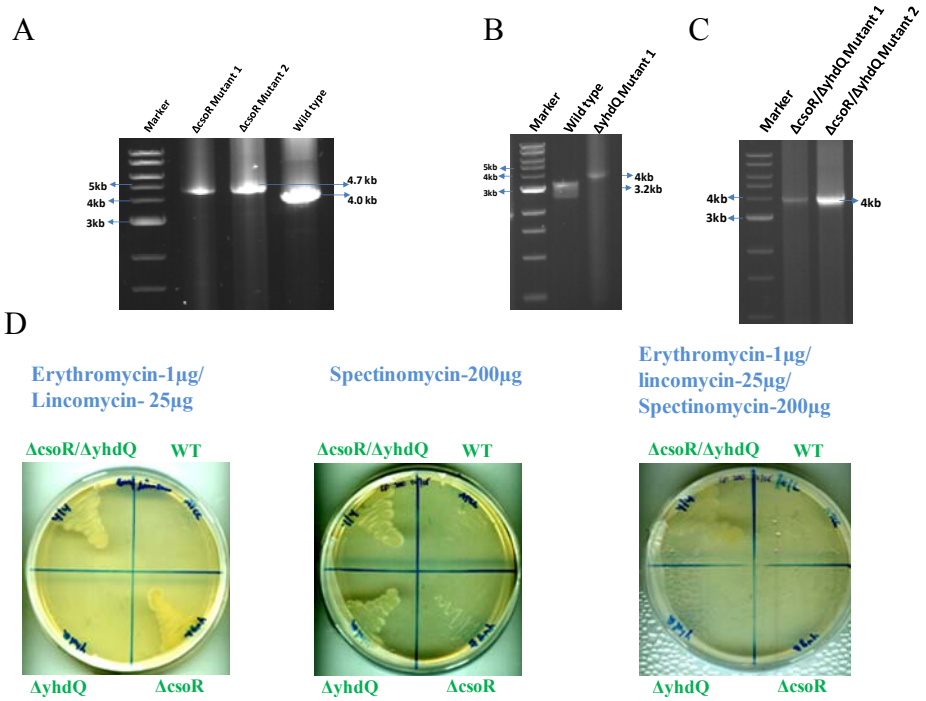

Fig.5 : A) PCR confirmation of *ΔcsoR* mutant where wildtype was used as a control. B) PCR conformation of Δ*yhdQ* where wildtype was shown as a control. C) PCR confirmation of *ΔcsoR (yvgZ)/*Δ*yhdQ(cueR)* mutant where *ΔcsoR* mutant was used for control of double mutant and control was shown in fig:B. D) Confirmation of the generated mutants by streaking them on the respective antibiotic plates where *ΔcsoR* mutant and *ΔcsoR(yvgZ)/*Δ*yhdQ(cueR)* were seen growing confirming the integration of erythromycin resistance cassette. While, Δ*yhdQ and ΔcsoR (yvgZ)/*Δ*yhdQ (cueR)* were grown on spectinomycin plates confirming the integration of spectinomycin resitance cassette. Only *ΔcsoR (yvgZ)/*Δ*yhdQ (cueR)* was grown on double antibiotic plate confirming the double mutant.

The generated mutants were tested for their ability to grow aerobically in the presence of 0.5mM copper in excess and subsequently grown in copper depleted medium achieved by the addition of 0.25mM Bathocupriondisulphonic acid disodium salt (BCS). All these constructed mutants exhibited a growth defective phenotype under copper excess conditions in comparision with wild type. Whereas, under copper limiting conditions, in contrast to other mutants Δ*csoR* and Δ*csoR(yvgZ)/*Δ*yhdQ(cueR)* mutants grew better in comparision to wild

type. The increased growth of these mutants was in a way intresting to know, what makes this deregulated copper efflux grow better than the wildtype.

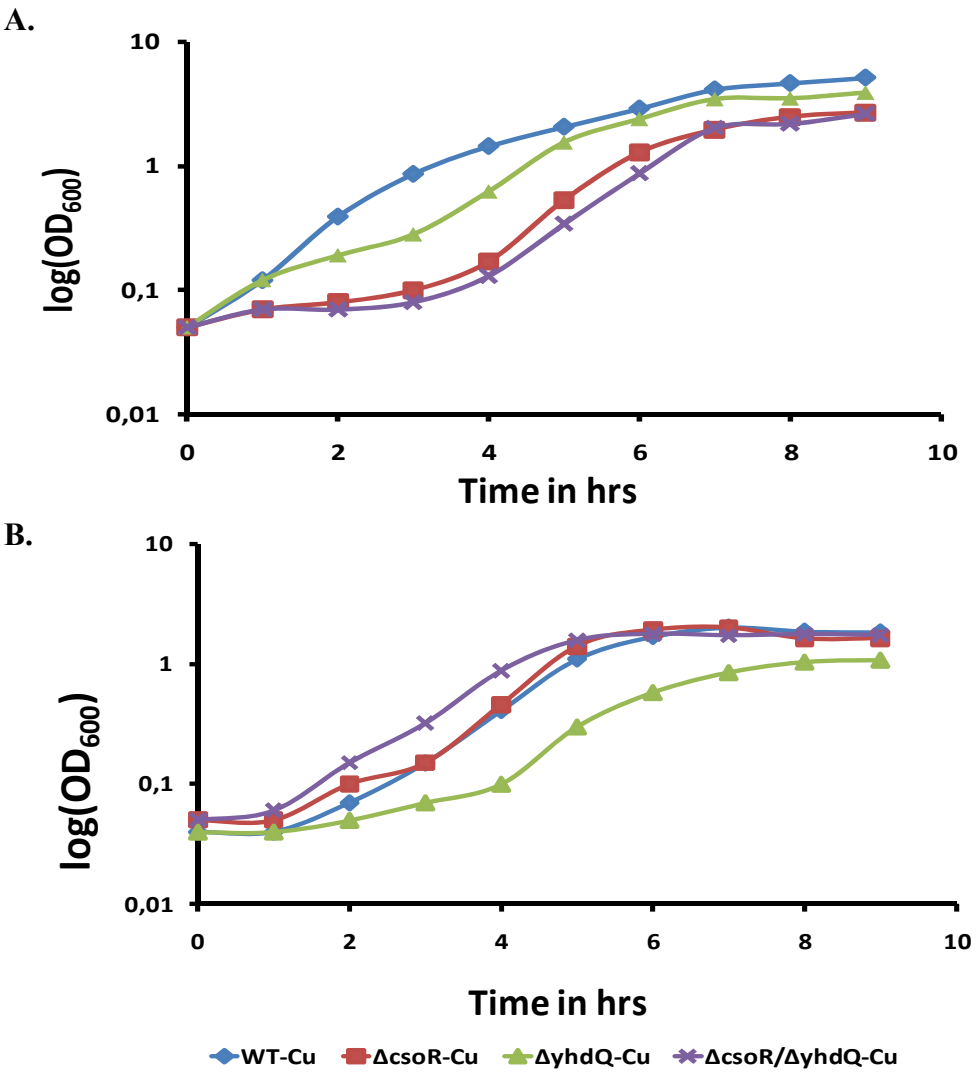

Fig.6: Growth of wild type, Δ*csoR*, Δ*yhdQ* and *ΔcsoR* Δ*yhdQ* in minimal medium. A) Supplemented with 0.5mM copper(copper repleted). B) supplemented with 0.25mM BCS (copper depletion)

Since the components for copper uptake in *B. subtilis* have not been reported so far, it was speculated that components of copper uptake might be responsible for this increased growth under copper limiting conditions. Hence we initiated the microarray studies under copper limiting conditions using *B.subtilis* ATCC 21332 (wild type) strain along with Δ*csoR(yvgZ),* and Δ*csoR(yvgZ)*/Δ*yhdQ(cueR)* to identify the probable copper importing targets.

6.1.2 Copper-induced oligomerization of CsoR.

In order to determine the specificity of copper with CsoR, the gene encoding CsoR was amplified by PCR from *B.subtilis* chromosomal DNA and cloned in pET28a+ vector which translate the corresponding gene with a His6 tag fusion protein. The overexpressed CsoR and the cytosolic domain (Nterminal 135 aa) of YcnJ were examined for the possible formation of oligomeric complexes in the presence of copper by using native gel electrophoresis. Polyacrylamide gels (6%) were loaded with assay mixtures containing protein samples that were incubated with and without 0.1 mM CuCl and 0.1 mM DTT. DTT was added to minimize the conversion of Cu (I) to Cu (II). A gel shift of the CsoR protein sample was observed in the presence of 0.1 mM CuCl and 0.1 mM DTT (Fig. 4B). Binding was also tested in the presence of the strong metal chelator EDTA. While incubation of a protein sample with 0.5 mM EDTA alone resulted in no shift, the shift was not abolished in the presence of EDTA and copper (Fig. 4B), indicating that CsoR has higher affinity to Cu(I) than EDTA. A similar observation has been made previously in binding competition experiments with CsoR and BCS (16).

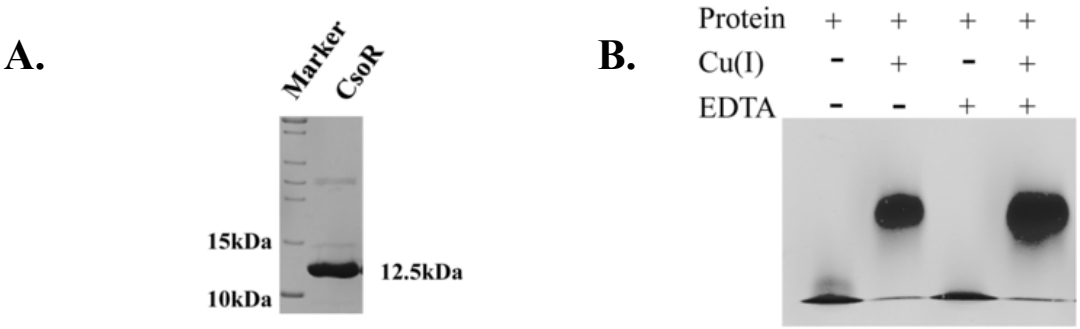

Fig.7: A) CsoR recombinant protein, purified using Ni-NTA chromatography, was loaded on a denaturing 17% gel along with markers, and the molecular mass of the protein was observed to be 12.5 kDa, including the His6 tag. B) Copper binding studies were performed in a 6% native gel, in which lanes were loaded with 80 µg purified protein incubated without or with 200 µM Cu(I) and/or 100 µM EDTA as indicated .

6.1.3 Copper binding charecteristics of CsoR

To define the half-maximal copper concentration (Cu0.5), it was necessary to saturate the CsoR protein; different copper concentrations of between 10 and 80 µM were added to the assay mixture, keeping the protein concentration constant at 80 µM. The formation of cysteine thiolate-copper complex was measured at 240 nm (Fig. 5), and the calculated Cu0.5 (half maximal copper concentration required to saturate the CsoR protein) was found to be at 35.5 µM, suggesting a Cu(I)-CsoR complex stoichiometry of 1:1.

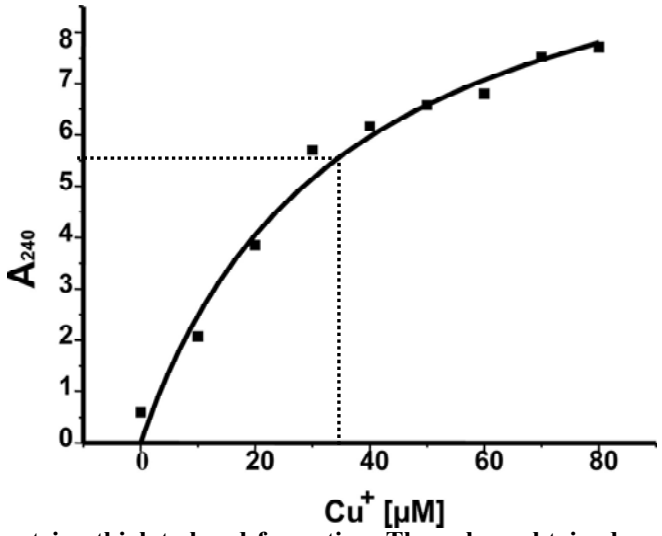

Fig.8: Estimation of cysteine thiolate bond formation. The values obtained were plotted on a graph, and the Cu0.5 was determined.

6.1.4 Crystalization studies with copper binding transcriptional regulator CsoR

The recombinant copper binding protein CsoR was subjected to crystallization studies for crystal structure resolution if diffracting crystals are obtained. Since CsoR belongs to new genre of copper binding transcriptional regulators, it was interesting to see how these proteins remodel in the presence of copper and sense the toxic levels of copper inside the cell. The purified protein obtained from Ni-NTA purificataion was dialysed against Hepes-A dialysis buffer containing 50mM HepesA, 100mM NaCl and 0.1mM DTT. Oligomerising activity was tested prior to the utilization for crystallization studies. For this purpose, the above mentioned copper binding studies were performed and analysed by native gels. Prelimnary crystallization studies were successful and formation of crystals in different conditions was observed in the initial screens that were pipetted automatically. The time taken for the initial crystal to appear

when observed under microscope was one week. Conformation of the crystals for their structure resolutions were tested if diffracting crystals were obtained. The results from diffractions revealed that the obtained crystals were protein crystals but the diffraction was very weak '8-angstroms'. Further screening and optimization of these crystals would give more insight into the structural details about the copper dependent regulation further co-crystalization with DNA would be more interesting to know how the metal dependent derepression of the transcriptional regulator is occurred. The following are the conditions where CsoR protein crystals were observed.

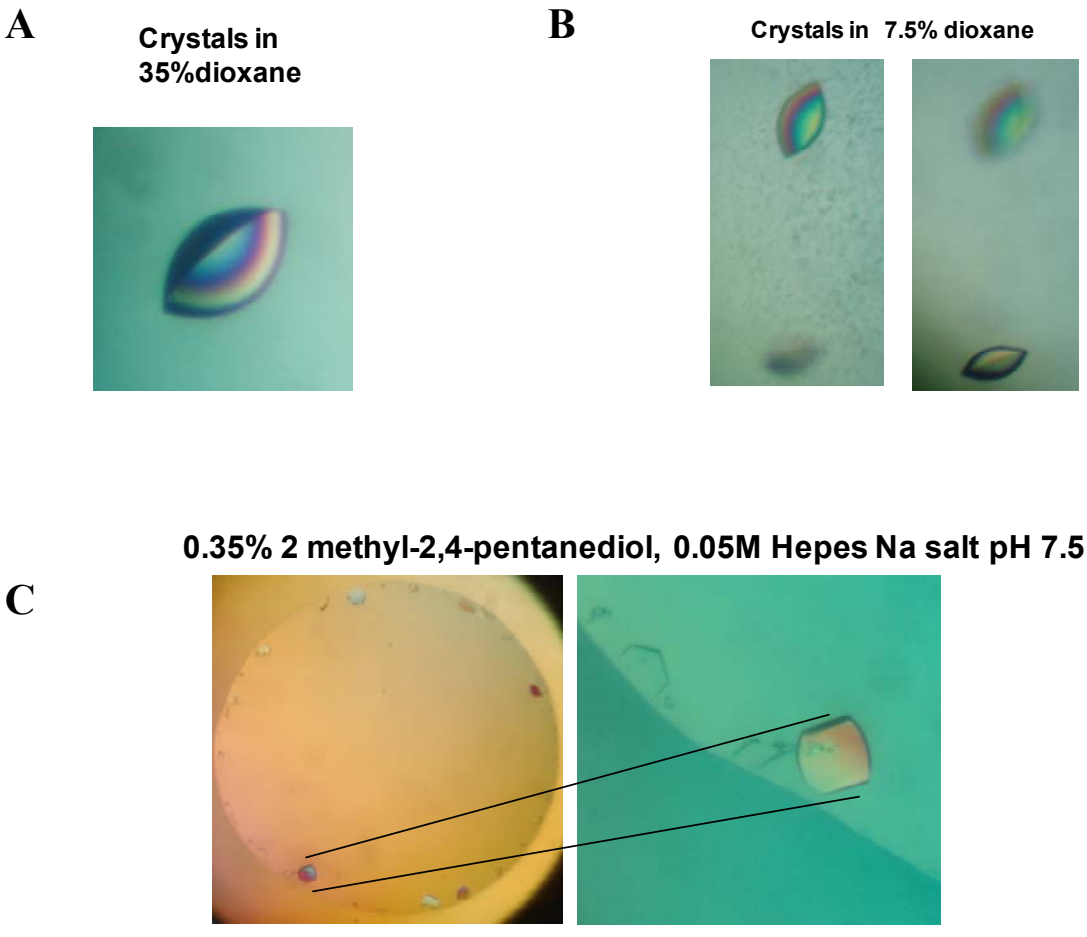

Fig.9: Crystals observed during CsoR crystallization studies. A) CsoR (2.2 mg/ml) crystals observed without copper in 35% dioxane. B) CsoR (2,2 mg/ml) crystallized without copper 17.5% dioxane. C) CsoR (2.2 mg/ml) 0.35% 2methyl-2,4-pentane diol, 0.05M Hepes Na salt pH 7.5

6.2 Microarray analyses of *B.subtilis* wild type(WT) and Δ*csoR* mutant in Belitzky minimal media (BMM).

In order to gain insights into the transcriptional response under various copper conditions, microarray experiments were performed with the following combinations: (i) WT (BMM without Cu) compared with WT (BMM), (ii) Δ*csoR* mutant (BMM without Cu) compared with WT (BMM without Cu), (iii) WT (BMM plus Cu) compared with WT (BMM), and (iv) Δ*csoR* mutant (BMM plus Cu) compared with WT (BMM plus Cu). The essential results from these experiments are summarized in Table (4). *B. subtilis* wild type cultures grown under copper-limiting conditions (BMM-Cu) show a significant increase in the upregulation of the copper-responsive genes *ycnI*, *ycnJ*, *ycnK*, and *ycnL*. In contrast, in the wild type the same genes are significantly downregulated in copper-replete medium (BMM+Cu). Thus, we speculated that these genes play a possible role in copper acquisition. The results from microarray studies revealed *ycnJ* gene from *B. subtilis* is highly induced under copper limiting conditions. Apart from these genes, the copper-inducible *copZA* operon, encoding a copper chaperone and an efflux ATPase, was found to be strongly upregulated in the Δ*csoR* mutant, suggesting a considerable role of CsoR in copper efflux as recently described (26).

Genetic background Expression level	WT (BMM-Cu) / WT (BMM)	ΔcsoR (BMM-Cu) / WT (BMM-Cu)	WT (BMM+Cu) / WT (BMM)	ΔcsoR (BMM+Cu) / WT (BMM+Cu)
copA	0.90	7.80	0.93	4.20
copZ	0.98	16.64	2.61	6.53
ycnJ	8.17	0.93	0.40	2.31
ycnI	6.36		0.29	
ycnK	10.9		0.34	
ycnL	1.77		0.68	

Table4: Expression of genes which were found to be significantly upregulated is shown in green and those with significant down regulation in red. While the combination used for the microarray studie was shown on the top and the names of the genes were shown in the complete left

6.2.1 Characterization of Δ*ycnJ* mutant.

To test the results obtained from the microarray results, and to verify if *ycnJ* plays a major role in copper uptake or copper homeostasis under copper limiting conditions, a Δ*ycnJ* deletion mutant was constructed.

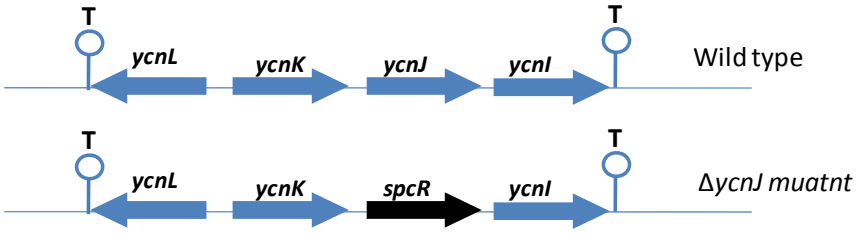

Fig.10: Deletion and replacement of the *ycnJ* gene with an spectinomycin resitance cassette.

The Δ*ycnJ* deletion mutant when grown in liquid belitzsky minimal media exhibits a strong growth defective phenotype under copper-limiting conditions.

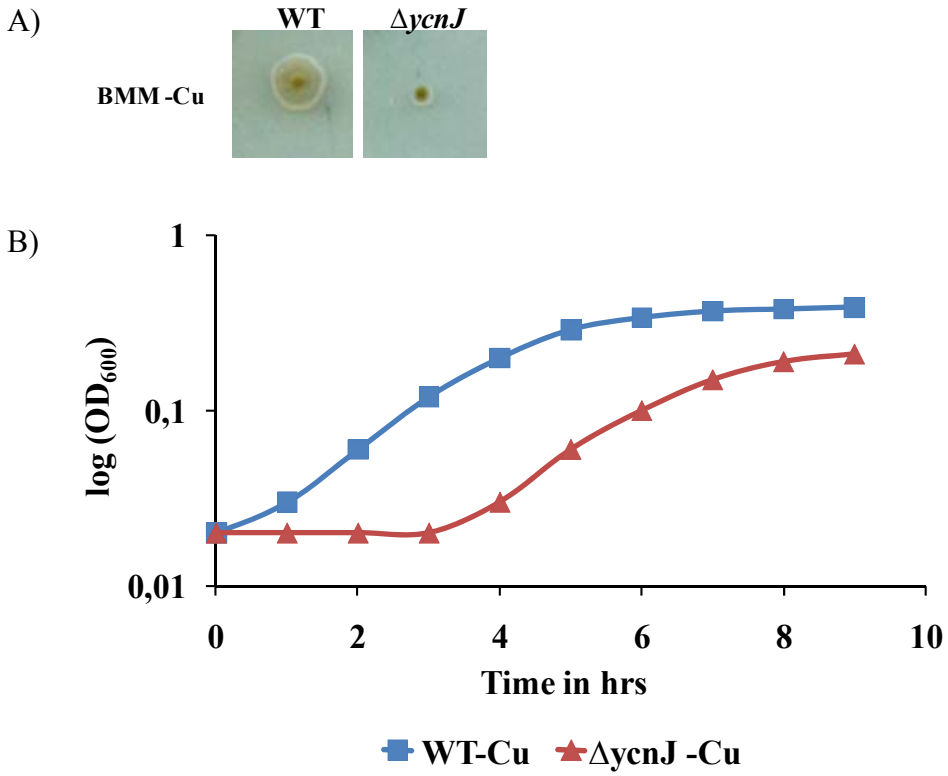

Fig.11: A) Comparison of phenotypes of wild type and Δ*ycnJ on plates* where copper is depleted by addition of copper specific chelators. B) Comparison of phenotypes of wildtype and Δ*ycnJ* in belitzsky minimal medium under copper limitingconditions achieved by addition of copper specific chelators.

Hence, we investigated the role of *B.subtilis* YcnJ in copper import, as blast analysis revealed YcnJ as a close homologue of *P. syringae* CopCD, which mediates copper uptake functions. The CopC and CopD gene products of *P. syringae* exhibit a high sequence identity (28%) with YcnJ. The N-terminal region of YcnJ (135 aa) is homologous to the periplasmic protein CopC, a copper binding protein which is involved in copper uptake in *P. syringae* (1, 5). The putative copper binding amino acid residues (His-24, Glu-51, Asp-108, and His-110) identified within the N-terminal region of YcnJ are highly homologous to the Cu(II) binding sites in CopC (1). The Cterminal region of YcnJ (401 aa) represents a transmembrane domain that is homologous to the inner membrane copper transport protein CopD of *P. syringae* (1, 5). Alignments of the N- and C-terminal regions of YcnJ with CopC and CopD are shown below.

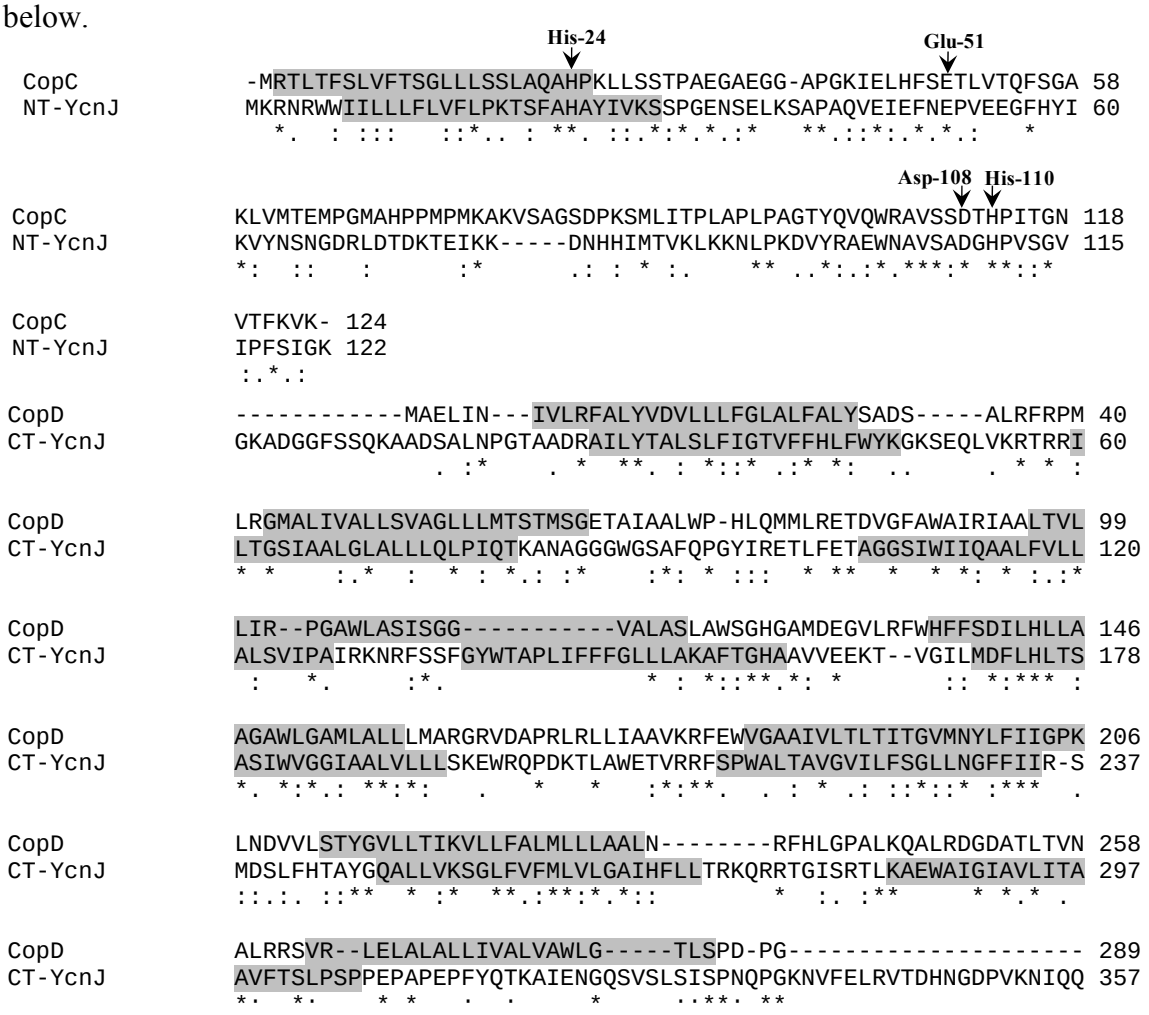

Fig.12: Alignment of amino acid sequences of *P. syringae* CopC and CopD and *B. subtilis* YcnJ (ClustalW). NT, N terminal; CT, C terminal. Arrows indicate conserved copper binding residues. Highlighted amino acids represent the predicted transmembrane domains.

6.2.2 Copper-induced oligomerization of YcnJ

Experiments performed with the recombinant periplasmic domain of YcnJ (N-terminal 135 aa) exhibited oligomerization specifically in the presence of Cu(II), which resulted in a clear shift of the preincubated protein under these conditions (Fig. 4A). The shift observed upon incubation with Cu(I) was rather weak and might also result from partial oxidation of Cu(I) to Cu(II). However, these findings suggest that YcnJ recruits into an oligomeric state if copper sensing and/or transport into the cell is mediated. The specific response of the predicted periplasmic N-terminal domain of YcnJ to Cu(II) further supports its role in uptake of extracellular oxidized copper, whereas copper-responsive regulators such as CsoR are specific for the intracellular copper in its reduced state.

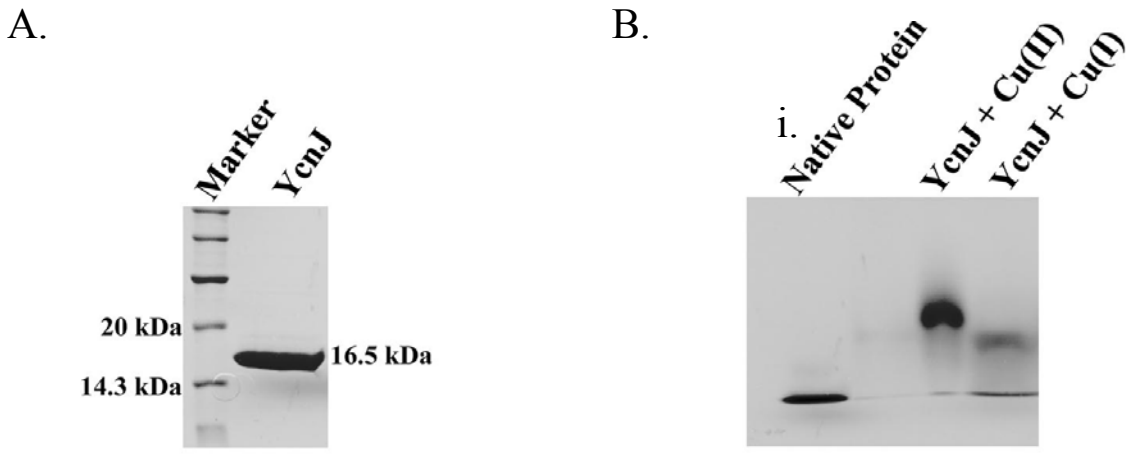

Fig.13: Copper-induced oligomerization. A) The N-terminal part (135 aa) of recombinant YcnJ protein, purified using Ni-NTA chromatography, was loaded on denaturing 17% sodium dodecyl sulfate gel along with markers. The molecular mass of the protein was observed to be 16.5 kDa, including the His6 tag and two additional amino acids before the start codon. B) Native 6% polyacrylamide gel was loaded with 15 µg of purified protein either without metal preincubation as a control (first lane) or after incubation with either 0.2mMCuCl or 0.2mMCuSO4.

6.2.3 Regulation of *ycnJ* by YcnK and CsoR.

To further investigate how the regulation of ycnJ was achieved in response to copper we had a closer look into the corresponding upstream genes and found two intresting candidate genes. Genes *ycnK* and *ycnL*, which are encoding for a putative transcriptional regulator and a putative signal permease, respectively, several metal binding sites (Cys-x-x-Cys and Cys-x-Cys) were identified. YcnK was identified as a putative transcriptional regulator with a

conserved helix-turn helix motif in its N-terminal region and a C-terminal region with a sensor function. The sensor function is defined by a NosL superfamily motif which indicates specific binding of Cu(I) (17). Further to verify the role of YcnK in regulating *ycnJ* in response to copper , we have generated a Δ*ycnK* mutant was generated by long flanking homology PCR method and verified by PCR. The Δ*ycnK* mutant showed no or little difference in growth under copper-limiting conditions. In contrast, it exhibited enhanced growth under copper excess conditions.

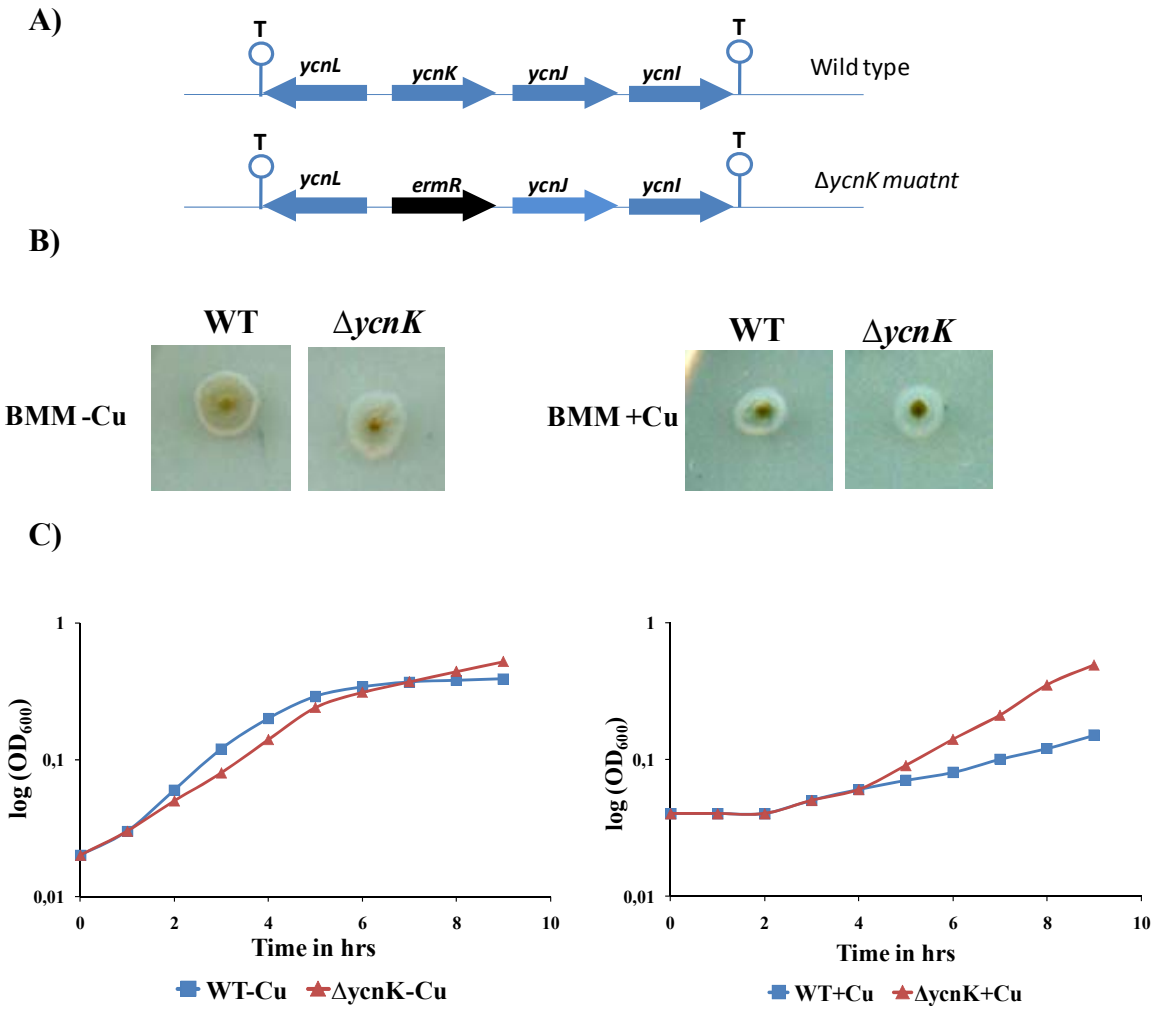

Fig.14: A) Deletion and replacement of the *ycnK* gene with an erythromycin resitance cassette. B) Comparison of growth phenotypes of wild type and Δ*ycnK on plates* with and without copper. C) Comparison of growth phenotypes of wild type and Δ*ycnK* in belitzsky minimal medium(BMM) under copper limitingconditions achieved by addition of copper specific chelators and copper excess conditions (0.5mM CuCl).

6.2.4 Metal binding affinity of YcnK.

In the case of YcnK, which was also recombinantly expressed and tested for copper-induced oligomerization, no such oligomerization in the presence of copper was observed (Fig. 4C). However, since oligomerization may also take place in the absence of copper and might additionally become further stabilized but not altered by addition of the metal, as already suggested for *M. tuberculosis* CsoR (16), there is yet no indication that YcnK does not oligomerize or does not bind copper at all.

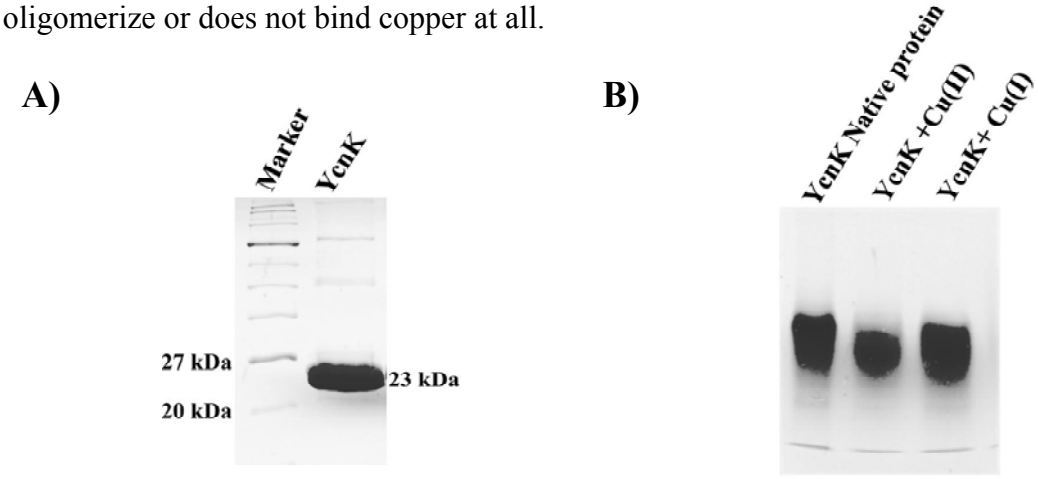

Fig.15: A) YcnK recombinant protein, purified using Ni-NTA chromatography, was loaded on a denaturing 17% gel along with markers, and the molecular mass of the protein was observed to be 23 kDa, including the His6 tag. B) Metal binding studies with purified proteins were performed in 6% native gels. Lane1 is loaded with 100 µg purified protein. Protein samples incubated with 200 µM Cu(I) and 200µM Cu(II) are loaded in lanes 2 and 3.

6.2.5 Transcriptomic analysis to identify the possible regulators of *ycnJ*.

The initial transcriptome studies were followed by a closer examination of *ycnJ* regulation with respect to different copper concentrations and possibly involved transcription factors. As a first candidate, we addressed the putative transcriptional regulator-encoding gene *ycnK*, which is located directly upstream from *ycnJ*. A *ycnK* deletion mutant was constructed and grown under different copper conditions along with the wild type. Total RNA was isolated at mid-log phase, and *ycnJ* gene expression was estimated semi quantitatively by use of dot blots (Fig. 3). As expected, transcription of *ycnJ* in the wild type was elevated under copper-limiting conditions. In comparison to the wild type, the *ycnK* mutant showed an upregulation of *ycnJ* expression, especially under copper excess conditions (Fig. 3A). Further in this

context, the effect of increasing copper concentrations on *ycnJ* gene expression was tested, and the Δ*ycnK* mutant was found to upregulate *ycnJ* expression during copper excess approximately twofold compared to the wildtype (Fig. 3B). This points to a function of YcnK in which it acts as a negative regulator of *ycnJ*. Since this function is present mainly under conditions of high copper concentrations, YcnK is predicted to use copper as a corepressor.

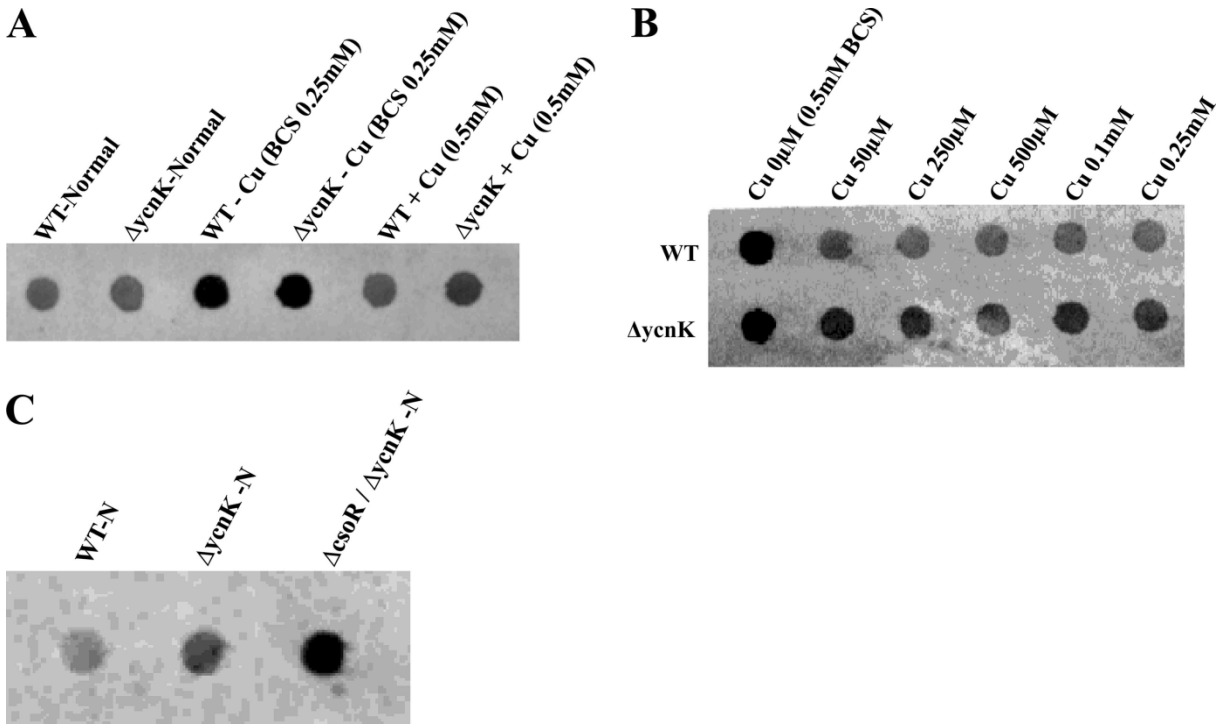

Fig.16: Transcriptional analysis of *ycnK*- and *csoR*-dependent *ycnJ* expression. Two micrograms of total RNA isolated from the wild type and mutants under normal, 0.25 mM batho cuprione di sulphonete (BCS), and 0.5 mM copper excess conditions was blotted onto a nylon membrane by dot blotting and hybridized with a digoxigenin-UTP-labeled antisense riboprobe specific for the *ycnJ* transcript. Hybridization signals were detected by using a digoxigeninspecific antibody fragment conjugated with alkaline phosphatase and AttoPhos as chemifluorescence substrate. A) *ycnK*-dependent expression of *ycnJ* with different copper concentrations. B) *ycnK*-dependent expression of *ycnJ* under increasing copper concentrations. C) Expression of *ycnJ* in the wild type and the Δ*ycnK* and Δ*csoR* Δ*ycnK* mutants under normal conditions.

In addition, enhanced growth of the *ycnK* mutant under copper excess conditions was observed (Fig. 1A). Thus, as long as toxic copper concentrations are compensated for by copper efflux detoxification systems such as CopZA, induction of the predicted copper uptake system YcnJ is not detrimental for cell growth without carbon and energy source limitation.

As a second putative candidate for *ycnJ* regulation, we examined the recently described copper efflux regulator CsoR (16, 26), according to the finding of *ycnJ* upregulation in the

Δ*csoR* background during the microarray studies. Since absence of the CsoR repressor leads to strong derepression of the *copZA* copper efflux system (26), effects of CsoR on *ycnJ* expression might be regarded as rather indirect via modulating the intracellular copper concentration and, in the course of that, also YcnK activity. However, when the Δ*csoR* and Δ*ycnK* backgrounds were combined in a Δ*csoR* Δ*ycnK* double mutant which was tested for *ycnJ* expression, an additional elevation of *ycnJ* expression compared to that in the Δ*ycnK* mutant was observed (Fig. 3C), suggesting also a direct participation of CsoR in *ycnJ* regulation.

6.2.6 Estimation of intracellular copper content.

To further verify the predicted roles of *ycnJ* and *ycnK* as a copper uptake mediator and corresponding regulator, respectively, total intracellular copper contents were measured by using inductively coupled plasma mass spectrometry analysis. These studies revealed that the Δ*ycnK* mutant contains approximately double the amount of cytoplasmic copper (511.1 ppb) in the wild type (278.87 ppb) and the Δ*ycnJ* mutant (205.0 ppb). These high values are a result of copper accumulation under copper excess conditions. In agreement with the dot blot results, these findings suggest that YcnK may act as a negative transcriptional regulator of copper uptake that is active in the presence of copper. In addition, the entire copper contents within the wild type and Δ*ycnJ* cells under normal and copper-limiting conditions were determined. The results revealed that the amount of copper in wild type cells grown in the presence of the copper specific chelator BCS is approximately twofold higher (3.58 ppb) than that in cells those grown without the addition of BCS (1.84 ppb). This might be a possible consequence of *ycnJ* upregulation under copper-limiting conditions, where the Δ*ycnJ* mutant under normal conditions holds only 1.2 ppb (Fig. 3A). The total copper content measured in the Δ*ycnJ* mutant when grown in the presence of BCS was found to be 1.57 ppb and thus more than twofold less than that in the wild type under these conditions. Together with the

growth experiments, this supports the supposed role for YcnJ in copper acquisition under copper-limiting conditions.

7. Copper and Iron connection:

7.1 Characterization of iron acquisition genes mutants in varying copper concentrations:

Since we observed the possible link between iron and copper in *B.subtilis,* in connection with bacillibactin, it was interesting to check the behavior of deletion mutants of iron responsive genes, Δ*dhbF* and Δ*dhbC,* in varying copper concentrations, as these genes are involved in bacillibactin synthesis. Growth experiments performed with these mutants and wildtype as control revealed the importance of dhbF gene in copper homeostasis.

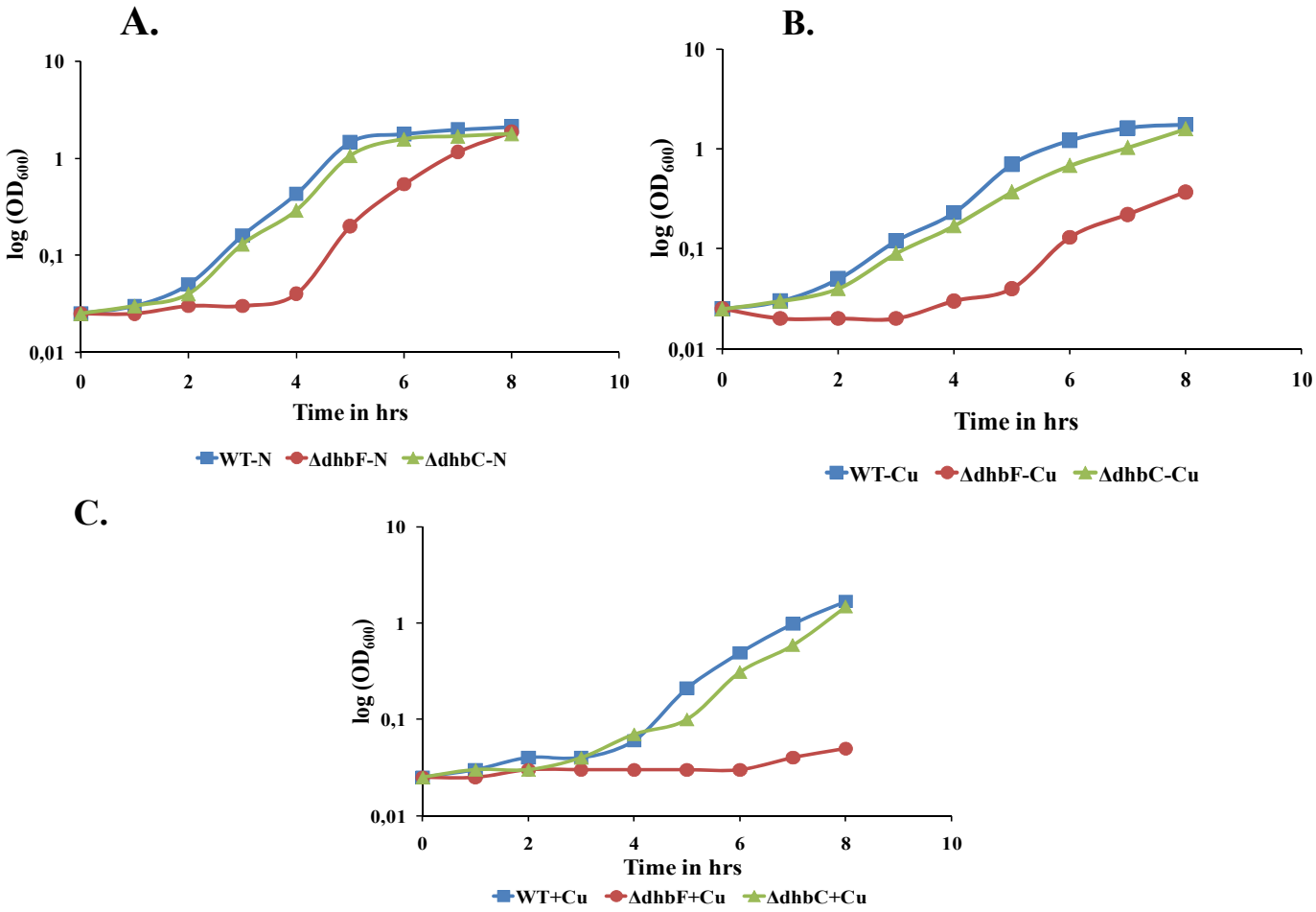

Fig.17: Comparison of growth phenotypes of wild type, Δ*dhbF* and Δ*dhbC* in belitzsky minimal medium. A) (BMM) under normal (only BMM). B) copper limiting conditions (BMM-Cu) achieved by addition of copper specific chelators. C) copper excess conditions (BMM+Cu) 0.5mM copper in excess was added.

Under normal conditions (only BMM) of growth, the Δ*dhbF* mutant exhibited an extended lag phase in its growth in comparison to wild type and Δ*dhbC*. Whereas, Δ*dhbF* exhibited a growth defective phenotype in both copper depletion and copper repletion media, while the effects observed were stronger in the presence of copper repletion.

7.1.2 Characterization of copper homeostasis genes mutants in varying iron concentrations:

Similar experiments were performed in order to investigate how the deletion mutants ΔcsoR and ΔcopA are responsible for copper detoxification and how these copper responsive genes will adapt or respond to varying iron responsive conditions. Growth experiments with these mutants performed under normal and iron depletion and repletion conditions did not show any significant differences in the growth in comparision to wildtype.

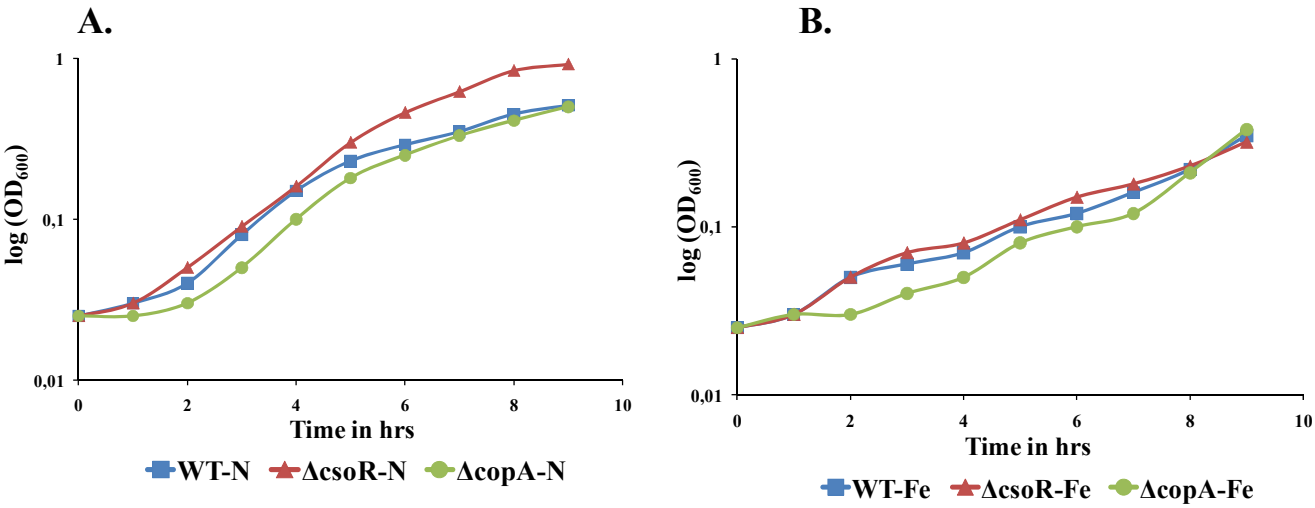

Fig.18: Comparison of growth phenotypes of wild type, Δ*csoR* and Δ*copA* in belitzsky minimal medium(BMM). A) Under normal(only BMM). B) Iron limitingconditions (BMM-Fe) achieved by addition of iron specific chelators.

7.1.3 Transcriptomic data mining for copper induced iron response.

In addition to perform copper homeostasis by CsoR, microarray studies performed with Δ*csoR* and Δ*csoR*/Δ*yhdQ* mutants under copper excess conditions showed a significant down regulation of iron acquisition genes which are under the control of negative transcriptional regulator Fur. Fur regulated genes *dhbABCEF* mediate siderophore (bacillibactin) mediated iron acquisition when iron concentration inside the cell falls below 1µM. The genes which

encode for bacillibactin production are *dhbE, dhbB* and *dhbF* which comprise bacillibactin NRPS (Non ribosomal peptide synthesis). Genes *feuABC* encode for an ABC transporter which is involved in sensing the iron loaded bacillibactin and also the transport of bacillibatin across the membrane. After translocation of bacillibactin into the cells *besA* and *btr* mediates hydrolysis, cleavage and subsequent release of iron from bacillibactin. Conversely, in wild type under copper excess conditions these genes are up regulated.

Genes	WT (BMM+Cu) / WT-Normal(only BMM)	ΔcsoR (BMM+Cu)/ WT(BMM+Cu)	ΔcsoR&ΔyhdQ (BMM+Cu) / WT(BMM+Cu)
dhbA	1.53	0.41	0.35
dhbB	2.71	0.29	0.18
dhbC	1.88	0.36	0.20
dhbE	2.16	0.3	0.17
dhbF	2.28	0.32	0.14
feuA	2.79	0.63	0.32
feuB	2.3	0.69	0.55
feuC	1.24	0.7	0.65
besA	2.11	0.29	0.33
btr	1.55	0.78	0.85

Table 5. Expression patterns of the iron responsive genes in strains under the conditions mentioned. Upregulation is shown in green and down regulation in red.

The increased copper concentration in the cell can lead to unspecific binding of the copper to sulfhydryl groups in proteins, which results in enzyme inactivation or altered protein structure. This unspecific binding of copper to Fur protein led to the derepression and resulted in increased transcription of the Fur regulon. While in Δ*csoR* and Δ*csoR*/Δ*yhdQ* mutant, constitutive copper efflux resulted in the decreasing toxic copper concentrations inside the cell. Hence in these mutants, under copper excess conditions, the copper homeostasis controls the unspecific binding of copper to the Fur, there by not resulting in altered expression of the iron regulated genes, which are responsible for maintaining iron homeostasis and protecting cellular components from damage. Down regulation of iron acquisition genes in copper efflux mutants attribute to a protection mechanism by the organism. This synergy between copper and iron homeostasis is possibly to avoid bacillibactin mediated toxicity inside the cell. When

copper along with bacillibactin is present inside the cell, it could further elevate the toxicity, since catecholates can act as Cu(II) reductants.

7.1.4 Copper induced oxidative stress.

Redox cycling of copper ions has been reported to influence many cellular processes. It is the most likely catalyst for conversion of H_2O_2 to $OH^{\cdot}$, the formation of reactive oxygen species via a Haber-Weiss or Fenton-like reaction. Since it was shown in *E.coli* that increased oxidative stress inside the cell could lead to the derepression of Fur, we checked the genes which are involved in protection against oxidative stress. The inspection of the anti-oxidative genes which were up regulated for protection during oxidative stress included *mrgA, katA* and *dps.* As expected, in Δ*csoR* mutant under copper excess conditions, these genes were found to be up regulated in comparison with wild type under the same conditions.

Genes	WT(BMM+Cu)/WT-Normal(only BMM)	ΔcsoR(BMM+Cu)/WT(BMM+Cu)	WT(BMM-Cu)/WT-Normal(only BMM)
mrgA	0.68	1.58	0.53
katA	0.83	1.17	1.21
dps	1.11	2.30	2.2

Table 6. Expression patterns of the anti oxidative stress responsive genes in strains under the conditions mentioned. Upregulation is shown in green and down regulation in red.

The CsoR-mediated induction of copper detoxification genes is thought to be a critical mechanism involved in protection of the cells from various challenges induced by an imbalance in the redox status of the cell, electrophiles and reactive oxygen species (ROS). Thus, the ability of redox cycling copper transition metal is critical to activate the correct metal homeostasis pathway. Transcription through the copper mediated oxidative stress response has not been fully charecterised in this study. In gram negative bacterium *E.coli*, a gene encoding the transcriptional regulator of iron regulon ``*fur*´´ was expressed significantly under oxidative stress which is mediated by transcriptional regulators OxyR and SoxRS. Homologues of SoxR in *B.subtilis* include YhdQ; although not highly homologous, blast analysis reveals a SoxR motif in YhdQ. Microarrays performed with the double mutant Δ*csoR*/Δ*yhdQ* under copper excess conditions further showed a significant down regulation of

the genes involved in regulation of iron acquisition pathway. Although the binding of transcriptional regulator YhdQ is unspecific to the promoter regions of CopZA operon, there could be an additional function involved in sensing the copper induced oxidative stress inside the cell. This could lead to derepression of *copZA* operon and thus protecting the cell from copper induced oxidative stress, leaving a speculation that YhdQ might have a possible protective role in copper induced oxidative stress. In the prevailing oxidative stress conditions induced by copper excess conditions, wild type and Δ*csoR* mutant might have different behaviour in response to the variable oxygen content. Under low oxygen concentrations, *cydABCD* genes are highly upregulated by a redox-sensing transcriptional regulator YdiH (Rex), that responds to the NADH/NAD(+) ratio inside the cell. *B. subtilis* YdiH functions as a repressor not only of the *cydABCD* operon but also of the *ldh lctP* operon and of a putative formate-nitrite transporter gene, *ywcJ* (96, 97).

Genes	WT(BMM+Cu)/WT-Normal(only BMM)	ΔcsoR(BMM+Cu)/ WT(BMM+Cu)	WT(BMM-Cu)/WT-Normal(only BMM)
cydA	0.66	1.98	1.17
cydB	0.25	4.88	4.87
cydC	0.55	2.35	11.54
cydD	0.88	0.94	1.08

Table 7. Expression patterns of the oxygen stress response genes in strains under the conditions mentioned. Upregulation is shown in green and down regulation in red.

cydABCD genes are involved in the assembly of one of the three distinct terminal oxidases (cytochrome *bd* oxidase). Since copper is an essential cofactor for most of the terminal oxidases, its restricted bioavailability interferes with cellular respiration. *cydABCD* genes were found to be down regulated in wild type under copper excess conditions and are significantly up regulated in copper scarcity suggesting a probable requirement for copper.

Genes	WT(BMM+Cu)/WT-Normal(only BMM)	ΔcsoR(BMM+Cu)/WT(BMM+Cu)
ldh	0.28	7.16
lctP	0.34	5.89

Table 8. Expression patterns of the oxygen stress response genes in strains under the conditions mentioned. Upregulation is shown in green and down regulation in red.

Conversely, these genes were found upregulated under copper excess conditions in Δ*csoR* mutant, while no futher significant changes were observed in Δ*csoR*/Δ*yhdQ* double mutant, suggesting a probable role for CsoR and its significance in cellular respiration under different oxygen availability conditions imposed by the presence of copper. Cellular NADH levels increase when the cytoplasmic copper concentrations are low. This increase in NADH levels inturn inhibits the ydiH binding to the *cydABCD* promoter region (95). In addition to this, increased NADH levels are toxic to the cells and LDH is the key enzyme involved in reoxidation of the NADH formed by glycolysis during fermentation. Here in Δ*csoR* mutant, we have also observed a strong upregulation of *ldh* and *lctp* genes, the physiological function of these enzymes was thought to be the in vivo regeneration of NAD in aerobic metabolism of the bacteria.

7.1.5 Copper mediated regulation of iron responsive genes.

In order to validate the gene expression pattern observed in microarray experiments, RNA dotblots were performed. Genes *dhbB* and *feuB* which were found to be down regulated are involved in siderophore biosynthesis and import and are regulated by negative transcriptional regulator, Fur.

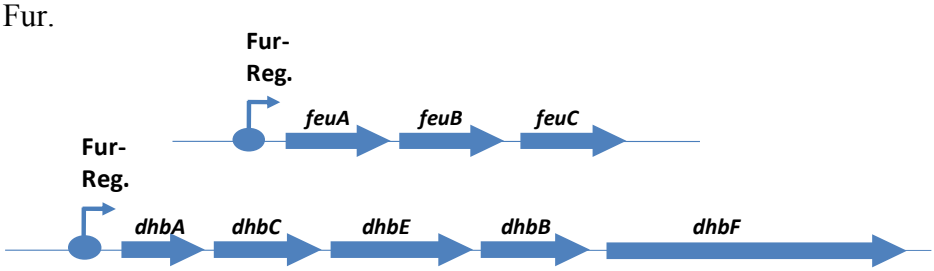

Fig.19: Genomic organization of bacillibactin (BB) biosynthesis (*dhbACEBF*), ferri-BB uptake (*feuABC*)

RNA dot blots were performed with the RNA isolated from wild type and Δ*csoR* mutants which were grown till mid log phase under normal and copper excess conditions. Gene expression results obtained from RNA dot blots were similar to the microarray data. Wildtype plus copper when compared to wildtype under normal conditions showed an increase in the gene expression. Wildtype plus copper when compared with Δ*csoR* plus copper showed down

regulation, although copper is in excess in deregulated copper efflux Δ*csoR* mutant. The genes *dhbB* and *feuB* showed a significant down regulation when compared to wild type due to the fact that copper inside the cell is continuously drained out which leads to no unspecific interaction of excess copper with alternate iron regulatory transcriptional regulators.

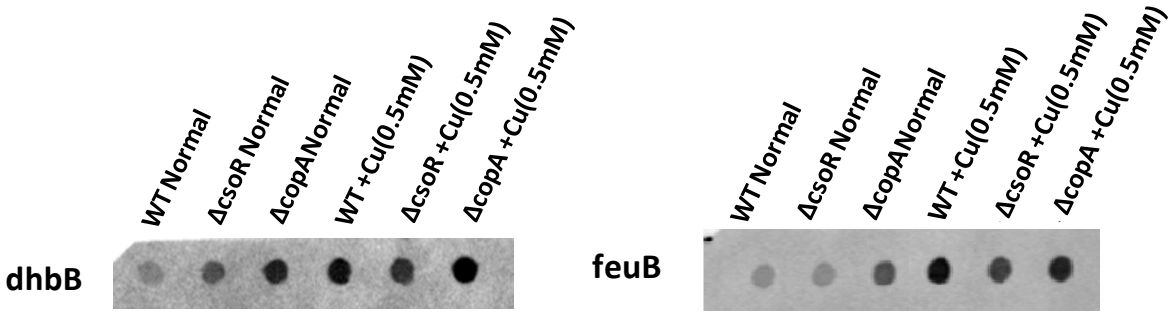

Fig.20: Transcriptional analysis of *csoR*- and *copA*-dependent *dhbB and feuB* expression. Two micrograms of total RNA isolated from the wild type and mutants under normal and 0.5 *mM* copper excess conditions was blotted onto a nylon membrane by dot blotting and hybridized with a digoxigenin-UTP-labeled antisense riboprobe specific for the *dhbB and feuB* transcript. Hybridization signals were detected by using a digoxigeninspecific antibody fragment conjugated with alkaline phosphatase and AttoPhos as chemifluorescence substrate. (A) *csoR*- and *copA*-dependent *dhbB* expression with different copper concentrations. (B) *csoR*- and *copA*-dependent expression of *feuB* under different copper concentrations.

7.2 Deletion of *copA*, a cross validation of copper mediated derepression.

To test if copper accumulation inside the cells can reverse the *dhbB* and *feuB* gene expression pattern in contrast to Δ*csoR* mutant and wildtype, a Δ*copA* copper efflux deletion mutant was constructed.

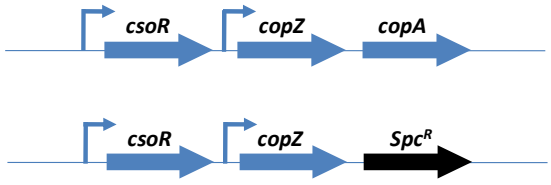

Fig.21: A) Deletion and replacement of the *copA* gene with an spectinomycin resitance cassette.

The Δ*copA* deletion mutant exhibited a growth defective phenotype in comparision to wildtype under both normal and copper excess conditions possibly due to the fact that the defects in copper efflux gene resulted in increased cellular cytoplasmic copper concentrations under both normal and copper excess conditions in comaprision to wildtype, which was futher confirmed by ICPMS. RNA was extratcted from Δ*copA* mutant and wildtype cells which

were grown under both normal and copper excess conditions till mid log phase and RNA dot blots were performed. The results exhibited a strong upregulation of the *dhbB* and *feuB* genes in Δ*copA* mutant in comparision with wildtype and Δ*csoR* mutant. These results further strengthen the above findings and support the probable role of unspecific binding of copper to alternate transcriptional regulators when copper is accumulated in excess inside the cells.

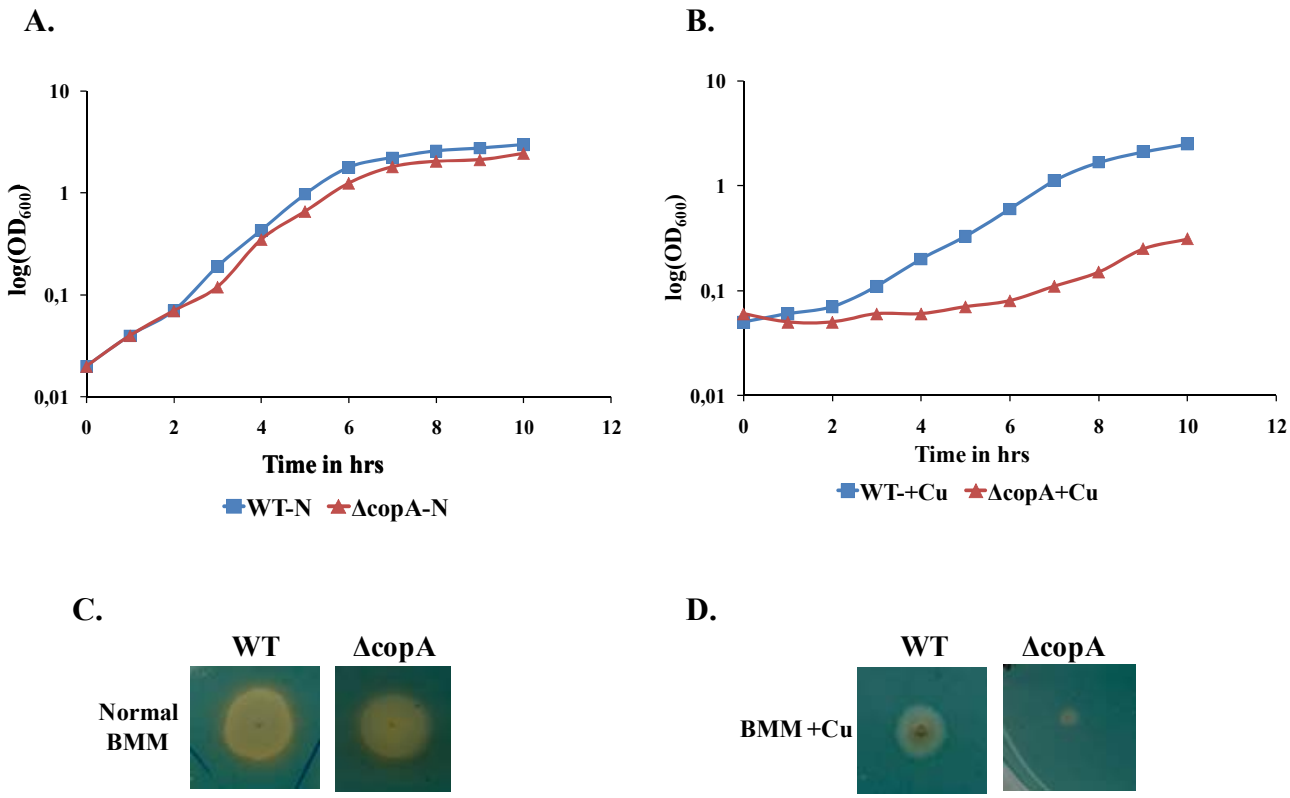

Fig.22: (A) Comparison of growth phenotypes of wild type and Δ*copA* in normal (only belitzsky minimal medium). (B) copper excess conditions achieved by 0.5mM CuCl. (C) Comparison of growth phenotypes of wild type and Δ*copA* on normal(only BMM) plates D) Comparison of growth phenotypes of wildtype and Δ*copA* on plates with 0.5mM copper in BMM.

It was shown in *E.coli* that copper in excess can bind to the iron response transcriptional regulator Fur and subsequently derepress the *fur* regulon. Further, there is clear evidence in up regulation of *fur* transcript during oxidative stress which is not the case as seen in microarrays. Results obtained from ICPMS strongly support the data that the unspecific binding of copper in excess inside the cell might lead to the derepression of the *fur* regulon.

7.2.1 Estimation of total copper in strains under normal conditions.

Estimation of the total cellular metal concentrations and to determine the metal binding specificity of the purified protein was performed by using ICPMS. Determination of the total cellular copper concentrations inside different mutants when grown under normal conditions exhibited significant differences. Mutants Δ*csoR*, Δ*copA*, Δ*csoR*/Δ*copA*-N and *B.subtilis* wildtype were grown under normal conditions in belitzky minimal media (BMM-N) until mid log phase and harvested was shown to contains the following amounts of copper.

Strains	Cu(ppm)
WT(*B.subtilis*)-N	0.00520
ΔcsoR-N	0.00253
copA-N	2
ΔcsoR/ΔcopA-N	4

Table 9. Estimation of total copper content in the strains mentioned under normal growth conditions (only Belitzky minimal media). The values obtained were shown in parts per million (PPM)

In Δ*csoR* mutant, the deregulated copper efflux operon *copZA* continuously pumps the copper out of the cell which resulted in low copper amounts in comparision with the wildtype, while the copper efflux deletion mutant Δ*copA* which is defective in pumping copper out of the cell corresponds to contain high copper amounts in the cell. Further, the deletion of both the genes exhibited high copper concentrations inside the cells suggesting a dominant copA phenotype.

7.2.2 Estimation of total copper in strains-copper excess conditions.

Copper contents measured in the strains Δ*csoR*, Δ*copA*, Δ*csoR*/Δ*copA*-N and *B.subtilis* wildtype were grown copper excess conditions, maintained by addition of 0.5mM CuCl in belitzky minimal media (BMM+Cu). Strains which were grown until mid log phase and harvested was shown to contains the following amounts of copper.

Strains	Cu(ppm)
WT(B.subtilis)+Cu	0.493
ΔcsoR+Cu	0.488
ΔcopA+Cu	892
ΔcsoR/ΔcopA+Cu	607

Table 10. Estimation of total copper content in the strains mentioned under copper excess (0.5mM copper) conditions . The values obtained were shown in parts per million (PPM).

7.2.3 In vitro metal binding characterization of FUR.

Metal binding affinity studies were performed with the purified protein under strict anaerobic conditions to prevent the oxidation of metals. To determine the specificity of the Fur protein towards different metals, purified recombinant Fur protein which can bind different metals was incubated with 3 fold excess of the following metals Fe(II), Zn(II) and Cu(I). Before estimation, an additional purification step was performed to separate the protein from the metal using PD10 size exclusion columns and protein fractions were identified using Bradford. The metal content of the purified protein after size exclusion was determined by using ICPMS. The results obtained exhibited a strong unspecific binding of the copper to the Fur protein as shown below.

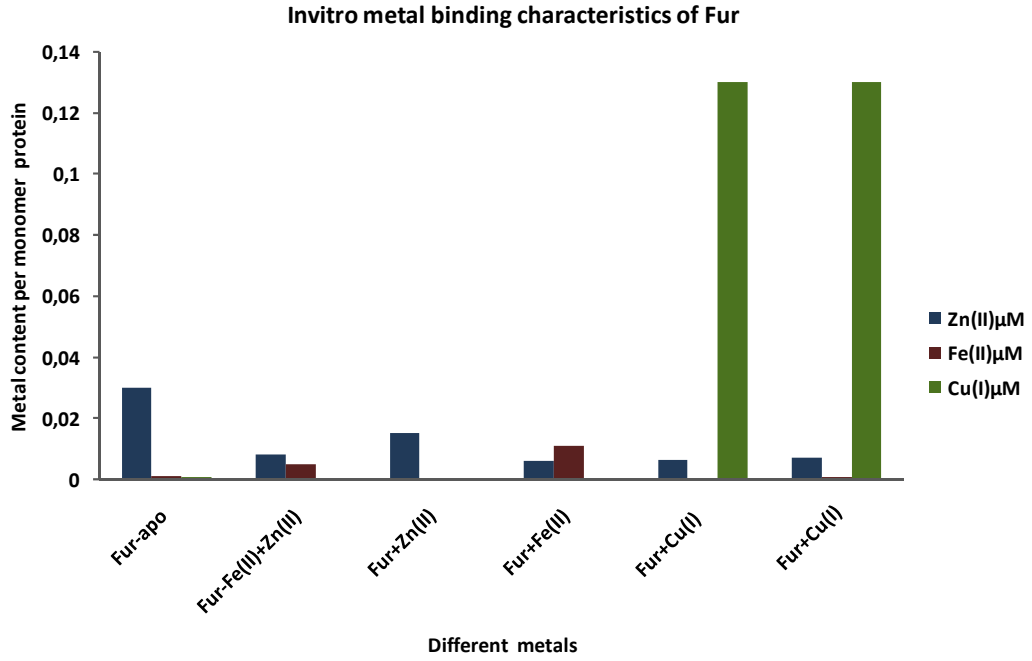

Fig.23: Invitro estimation of metal bidning specificity of recombinant Fur when incubated with different metals as metioned. The binding specificity was calculated to amount of metal monomer bound to monomer of protein. The different metals were indictaed with different colurs as mentioned above.

The purified Fur apo protein was observed to contain high zinc concentrations, which is in agreement with the known metal binding conditions of the Fur protein. Crystalization studies performed with Fur protein from *Pseudomonas aeruginosa* in the presence of ZnSO4, revealed a homodimeric state with the two metal binding sites per monomer fully occupied by Zn(II). In the presence of Fe(II) and Zn(II) it appears that there is strong shift in the metal

binding specificity towards Fe(II), which support the functional role of Fur in sensing the Fe-levels inside the cell. Previous studies suggested that the binding site I which was observed in the Zn (II)-Fur structure might substitute for the regulatory Fe(II) binding site *in vivo*. The apo-protein samples incubated with either of this metals Fe(II) or Zn(II) exhibited their dominance over the other metal. Intrestingly, protein samples when incubated with 3 fold excess of each metal together in the assay resulted to contain high copper amounts. The high specificity of copper binding with Fur protein was not addressed in this work. To conclude, the high unspecific binding of copper to the Fur protein might result in a conformational change of the protein which further negatively influence the binding of this protein to DNA and subsequently ending up in derepression of *fur* regulon.

7.2.4 Bacillibactin estimation.

As observed in the microarray results, in ΔcsoR mutant down regulation of *dhbABCEF* genes under increased copper conditions should result in no or less bacillibactin production. CAS plate assay as a semi quantitative approach was performed to test the BB secretion by emerging halos around the colonies. Wild type cells and Δ*csoR* mutant cells were applied onto normal CAS plates (no addition of copper or copper specific chelator was added) and CAS plates supplemented with 0.5mM copper. Δ*csoR* mutant produced significant halos after 16 hrs in comparison to wild type on normal CAS plates, whereas no significant halos were observed for wild type and Δ*csoR* mutant on copper supplemented CAS plates supporting the down regulation of bacillibactin synthesis genes from microarray data.

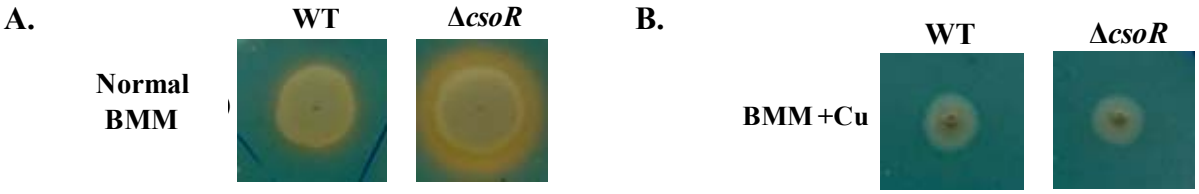

Fig.24: (A) Comparison of wild type and Δ*csoR* bacillibactin productions on CAS normal (only Belitzsky minimal media) plates. (B) Comparision of wild type and Δ*csoR* bacillibactin production on CAS plates containing 0.5mM copper in excess.

Subsequently, quantitative estimation of bacillibactin from the wildtype and Δ*csoR* mutant, after 16 hrs of inoculation in normal belitzky minimal media without iron showed significant differences. Increased bacillibactin amounts were observed, 1x 10^7 in the Δ*csoR* mutant compared to wild type 4.5x10^6, iron was omitted from the media to induce the bacillibactin production in the wild type cells. This could be possibly due to the fact that under normal conditions, there is a constitutive expression of copper efflux leading to decreased copper concentrations in the cell. This continuous copper outflow will lead to the defects in the assembly of terminal oxidases. The genes *ctaA* and *ctaB* which encode for one of these terminal oxidases involves Heam A and Heam O are located immediately upstream of the ctaCDEFG gene cluster, a highly specialized variant of heme required for the cellular aerobic respiration and energy conversion in animals, plants, and microorganisms. Heam A is also essential as a prosthetic group in the mitochondrial cytochrome *c* oxidase and in similar oxidases of bacterial aerobic respiratory chains(98).

Genes	WT(BMM-Cu)/WT-Normal(BMM only)	Genes	WT(BMM-Cu)/WT-Normal(BMM only)
cydA	1.17	*ctaA*	3.43
cydB	4.87	*ctaB*	2.41
cydC	11.54	*ctaC*	4.03
cydD	1.08	*ctaD*	2.12
		ctaE	5.03
		ctaF	8.73
		ctaG	1.2
		ctaO	2.57

Table 11. Expression patterns of the oxygen stress response genes and cytochrome genes in wild type under the conditions mentioned. Upregulation is shown in green.

The significant induction of cytochrome genes *cydABCD* and *ctaABCDEFGO* were observed in wild type cells when grown in copper limiting conditions, indicating a probable requirement of the copper in respiration, while the Heam A and Heam O which acts as a prosthetic group in the assembly of cytochrome *caa*3 requires iron. This synergy between iron and copper might be responsible for the increased upregulation of *dhb* genes in deregulated copper efflux mutant where there is continous out flow of copper from the cells. This

increased production of bacillibactin could supplement the iron needs of cells and subsequently the assembly of cytochromes involved in the respiration.

7.2.5 Estimation of total cellular copper content.

The total cellular copper content was measured by using ICPMS in wild type and Δ*csoR* mutant under normal conditions and also under copper excess conditions. The results obtained show a clear difference under normal condition. These measurements revealed that the wild type contains approximately double the amount of cytoplasmic copper (5.20 ppb) in comparision to the Δ*csoR* mutant (2.53 ppb), this could lead to the upregulation of cytochrome genes as was observed in wild type under copper limiting conditions (Table.8). In contrast, no significant differences in the total cytoplasmic copper were observed when cells were grown in copper excess conditions.

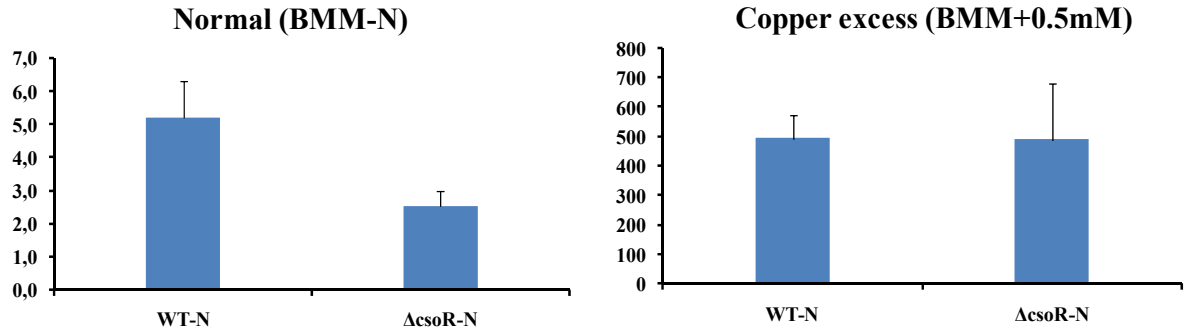

Fig.25: Estimation of total copper content in wild type and ΔcsoR mutant under normal and copper excess concentrations. The values obtained were represented in parts per billion (ppb).

7.2.6 Estimation of total cellular iron content.

Further the total cellular iron content in the same samples exhibited slight differences under normal conditions and copper excess conditions. The results revealed that the amount of iron content in wild type cells (77.88ppb) under normal conditions were slightly low in comparision to Δ*csoR* mutant (92.66ppb), the increase in the iron content might be a possible consequence of derepressed iron regulon which exhibited increased bacillibactin production under normal conditions which was verified by both semi quantitatively (CAS assay) and

quantitatively as well (LCMS), whereas under copper excess conditions the iron content in wild type (104.01ppb) was slightly higher than Δ*csoR* mutant (65.28ppb), a possible consequence of down regulation of iron acquisition genes.

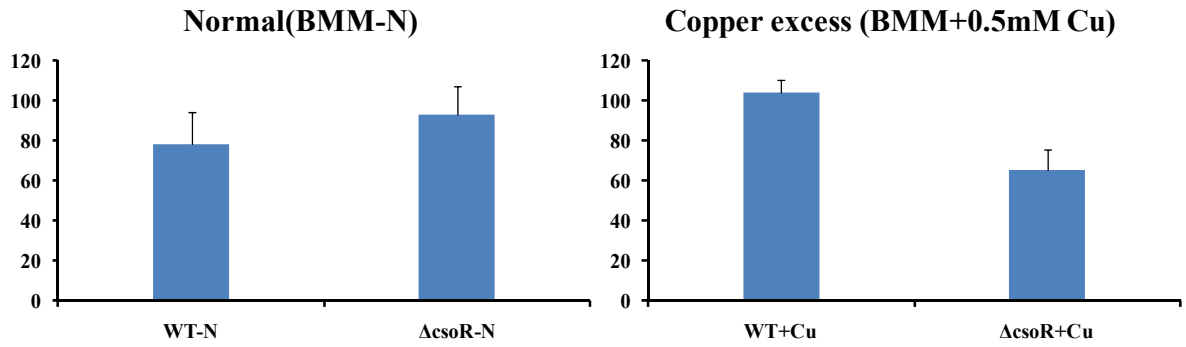

Fig.26: Estimation of total iron content in wild type and ΔcsoR mutant under normal and copper excess concentrations. The values were represented in parts per billion (ppb).

7.3 Role of Fur in copper homeostasis.

In order to investigate the probable role of Fur in copper uptake a fur deletion mutant was constructed.

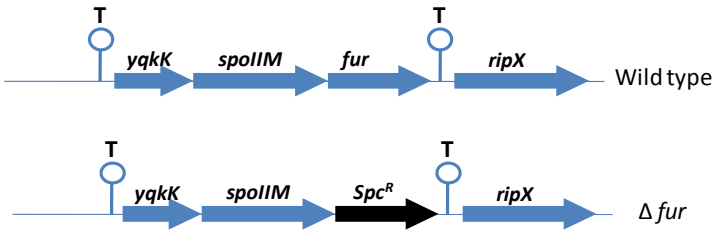

Fig:

Fig.27: Deletion and replacement of *fur* gene with spectinomycin resistance.

Since it was shown in *E.coli* that *fur* deletion mutants producing enterobactin constitutively might lead to enhanced copper uptake through FeoB, MntH and ZupT transporters and become copper hypersensitive and a subsequent deletion of entC (gene responsible for enterobactin porduction) in *fur* mutant preventing enterobactin biosynthesis restored the copper tolerance. The deletion mutant Δ*fur* when grown in belitzky minimal media under normal and copper excess conditions did not show any difference in their growth phenotype.

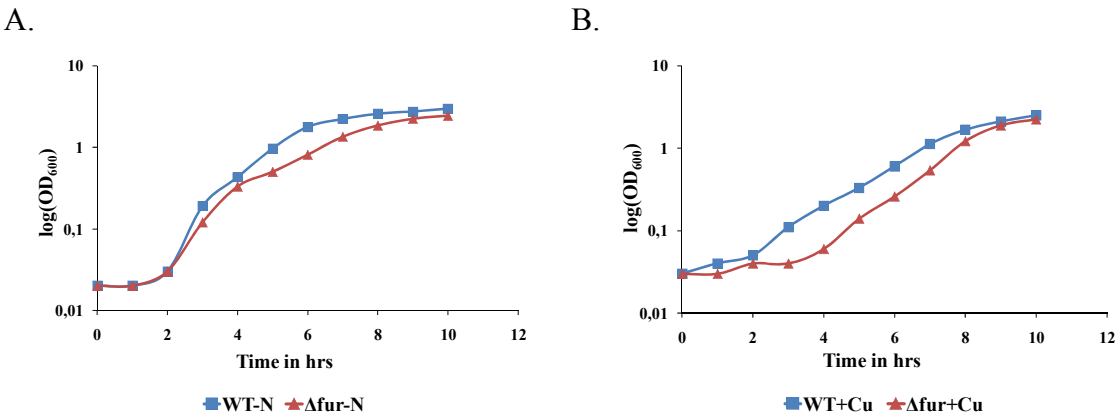

Fig.28: Comparision of growth phenotypes of wild type and Δ*fur* in A). BMM-normal conditions and B).BMM- copper excess conditions

Increased production of bacillibactin found in Δ*csoR* mutant under normal conditions might help in copper uptake where bacillibactin might subsequently reduce of Cu(II)-Cu(I). To verify if the constitutive production of bacillibactin leads to increased copper content inside the cell, a Δ*fur* mutant was constructed. Deletion of *fur* was confirmed by PCR and then tested on CAS plate assay for the production of bacillibactin.

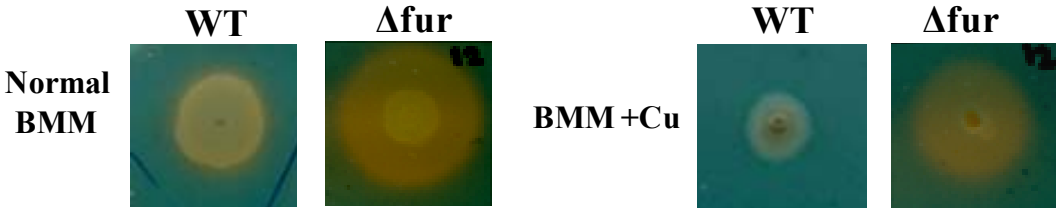

Fig.29: A.Comaparison of bacillibactin productions on CAS normal plates B. Comparision of bacillibactin production on CAS plates containing 0.5mM copper in excess

The resulting mutants show a constitutive production of bacillibactin which can be visualised by the emerging halos around the colonies on CAS plates. Wild type and Δ*fur* mutant were grown in normal BMM liquid media and BMM + 0.5mM copper liquid media till mid log phase followed by harvesting and determining the total cytoplasmic copper concentrations using ICPMS. The results revealed that the amount of copper in Δ*fur* mutant was significantly high in comparison with wildtype under both normal and copper excess conditions.

Strains	Cu(ppm)	Strains	Cu(ppm)
WT(*B.subtilis*)-N	0.005	WT(*B.subtilis*)+Cu	325
Δfur-N	0.2	Δfur+Cu	450

Table 12. Estimation of total copper content in the wild type and Δ*fur* strains under the conditions used. N= BMM-Normal, +Cu= BMM+ 0.05mM copperexcess. The values obtained were shown in parts per million (PPM).

7.4 Estimation of iron content

To extend this analysis and to determine whether these changes in the gene expression levels of iron acquisitions and distribution encoding proteins are responsible for the change in metal concentrations levels, we used Inductively Coupled Argon Plasma Mass Spectrometry (ICPMS). Increased iron levels (99.6 ppb) were observed in the Δ*csoR* mutant under nomal conditions in comparision with wild type (77.88 ppb) which is probably a consequence of increased production of bacillibactin. Although we have discussed above the role of increased bacillibactin production in Δ*csoR* mutant supporting the uptake of Cu^{+} during deregulated copper efflux situation, increased iron levels in parallel to increase in bacillibactin accounts to its primary function as an iron importing target and secondly might support the reduction of Cu^{2+} to Cu^{+} and subsequent uptake. Unlike under normal conditions, in Δ*csoR* mutant where deregulation of copper efflux resulted in increased bacillibactin and subsequently the iron amounts were found to be less or suppressed by the supplementation of copper (0.5mM) in the growth media, suggesting a possible link between iron and copper in *B.subtilis*.

7.5 Effect of copper on Iron-Sulphur clusters.

In this study, mutants of *B.subtilis* that lack a putative transcriptional regulator involved in copper homeostatic system Δ*csoR* were used to identify the primary intracellular targets which were affected by the disturbances in copper homeostasis. Further, to validate the hypothesis that copper toxicity involves in generation of reactive oxygen species, it was shown that low copper concentrations were sufficient to inhibit the growth of both wild type and Δ*csoR* mutant strains. Micro array studies performed with Δ*csoR* mutant in copper excess

conditions when compared against normal conditions revealed that the addition of copper treatment rapidly exhibited an up regulation of genes encoding iron-sulfur cluster enzymes, while other enzymes belong to this iron-sulfur family were also similarly affected. It was recently shown that the iron sulphur clusters are damaged with the displacement of iron atoms from the solvent exposed cluster, suggesting that Cu (I) damages these proteins by liganding to the coordinating sulfur atoms (51).

4Fe-4S cluster	Function	**ΔcsoR-N WT-N**	**ΔcsoR+Cu ΔcsoR-N**
narG	nitrate reductase (alpha subunit)	**0.61**	**2.04**
yrhE	similar to formate dehydrogenase	**0.53**	**2.04**
glcF(ysfD)	glycolate oxidase,	**0.96**	**2.28**
yhbA	4Fe-4S cluster binding putative ferredoxin	**0.8**	**2.6**
yqeV	Fe-S oxidoreductase or methyl thio transferase	**2.68**	**0.27**
fnr	transcriptional regulator of anaerobic genes	**1.18**	**1.72**
leuC	3-isopropylmalate dehydratase (large subunit), Superoxide inducible protein	**0.7**	**2.2**
yrhE	Formate dehydrogenase Chain A	**0.54**	**2.04**
yvgQ	sulfite reductase (NADPH) hemoprotein, beta-component	**1.79**	**0.42**
yoaE	Putative formate dehydrogenase	**0.67**	**2.66**
nadA	Quinolinate synthase	**1.12**	**0.49**
skfB(ybcP)	similar to coenzyme PQQ synthesis protein	**0.75**	**4.64**
yfjO	unknown; similar to RNA methyltransferase	**0.67**	**4.14**
sdaAB	probable L-serine dehydratase (beta chain)	**4.01**	**0.18**
citB	aconitate hydratase	**1.01**	**0.29**
albA	antillisterial bacteriocin subtilosin biosynthesis production	**0.78**	**2.02**
hemZ	oxygen independent coproporphyrinogen III oxidase 2	**2.04**	**0.34**
yotD	Rubrerytrin	**1.06**	**11.09**

		ΔcsoR-N WT-N	**ΔcsoR+Cu ΔcsoR-N**
2Fe-2S cluster			
yrhE	putative formate dehydrogenase	**0.54**	**2.04**
nasE	assimilatory nitrate reductase (NADH-dependent)	**1.16**	**0.39**
nsrR(ydhE)	HTH-type transcriptional regulator	**1.2**	**0.36**
prpD(mmgE)	2-methylcitrate dehydratase	**1.13**	**1.54**
pyrK	dihydroorotate dehydrogenase (electron transfer subunit)	**0.65**	**2.61**
qcrA	menaquinol:cytochrome c oxidoreductase (iron-sulfur subunit)	**0.7**	**1.52**
yjgC	formate dehydrogenase alpha subunit homolog	**1.26**	**0.5**

Table13 : The following table shows the upregulation of the iron-sulphur clusters in the Δ*csoR* mutant.

7.6 Estimation of nicotinamide nucleotide coenzymes.

During aerobic respiration, electrons from reducing equivalents are transferred to molecular oxygen by terminal oxidases. NADH produced during the catabolism of glucose via glycolysis and tri carboxylic cycle should be oxidized to NAD^+ which will provide the oxidizing power to the cellular machinery by catalyzing the oxidation of NADH by transferring electrons to ubiquinone which establishes proton motive force across the cell membrane. It was reported that the redox sensor YdiH acts as a regulatory loop between redox sensing of NADH/NAD^+ ratio which negatively regulates the expression of *ndh* operon which encodes NADH dehydrogenase, while the increase in the NAD^+ enhances the binding of YdiH (Rex) to *ndh* operon and as well to the *cydABCD* operon. Microarray results obtained from the deletion mutant Δ*csoR* when subjected to copper excess conditions when compared with wild type under the same copper excess conditions exhibited a significant up regulation of *cydABCD,* genes which encodes for cytochrome *bd* terminal oxidase, and *ldh, lctP and ywcJ* which encodes lactate dehydrogenase, lactate permease and putative formate nitrate transporter respectively. It was already shown that a lower binding affinity of YdiH for *cyd* operon when the NADH/NAD^+ ratio was high. Hence YdiH was supposed to decrease the temporarily increased NADH/NAD^+ ratio in the cytoplasm under aerobic conditions by up regulating the *ldh*.

In order to determine the NADH/ NAD^+ ratios and to examine this hypothesis, NADH and NAD^+ levels in the wild type and Δ*csoR* mutant were estimated by a sensitive technique using London & Knight method. Wild type and Δ*csoR* mutant were grown till mid log phase and harvested, subsequently continued for the estimation of the NADH/NAD^+ ratios. The results obtained exhibited a significant difference, the Δ*csoR* mutant was 3-4 fold higher when compared to wildtype. The observed where NADH/NAD^+ were ~3.4 for Δ*csoR* mutant and 0.91 for wild type. Hence we concluded that, the siginficant upregulation of the *ldh* in Δ*csoR* mutant seems to be a consequence of increased NADH/NAD^+ levels.

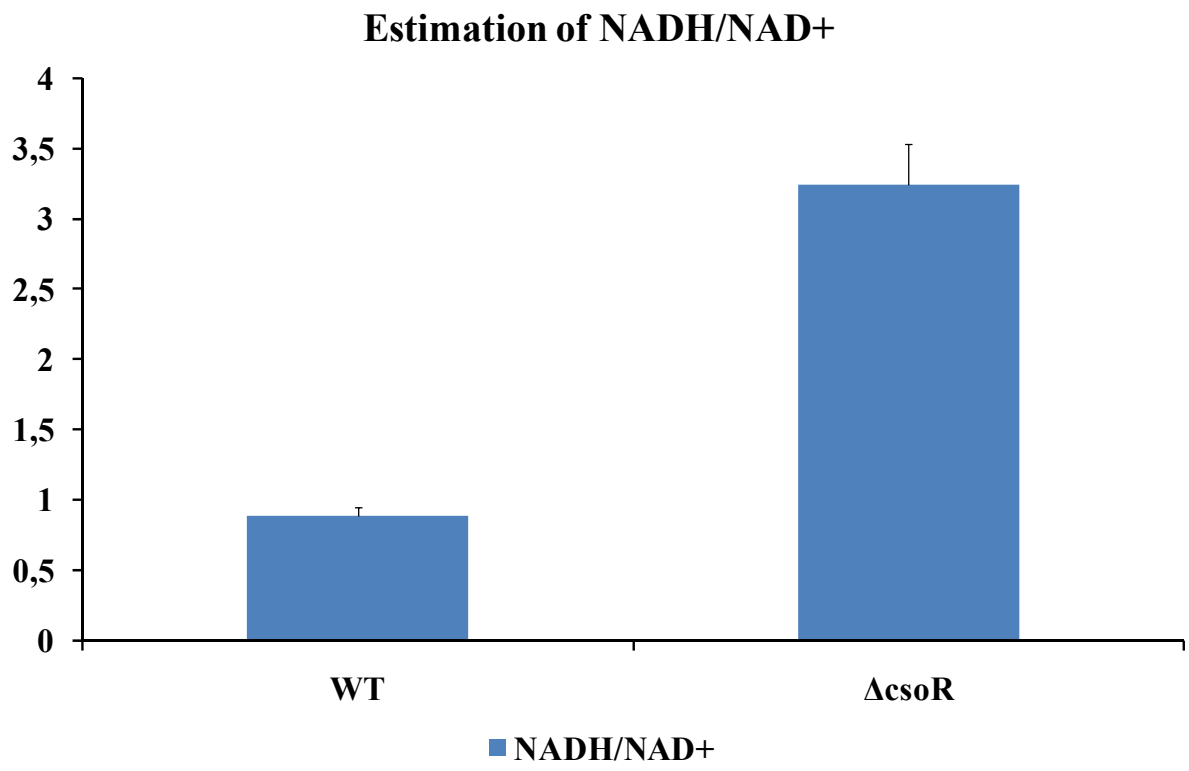

Fig.30: Estimation of NADH/NAD$^+$ ratios in the wildtype and Δ*csoR* mutant.

8. Discussion.

The conclusions which were drawn out of the results from this current work led us propose the current model for copper homeostasis for *B. subtilis.* The following model illustrates the novel components presented here and indicates the regulatory connection between copper uptake and efflux systems.

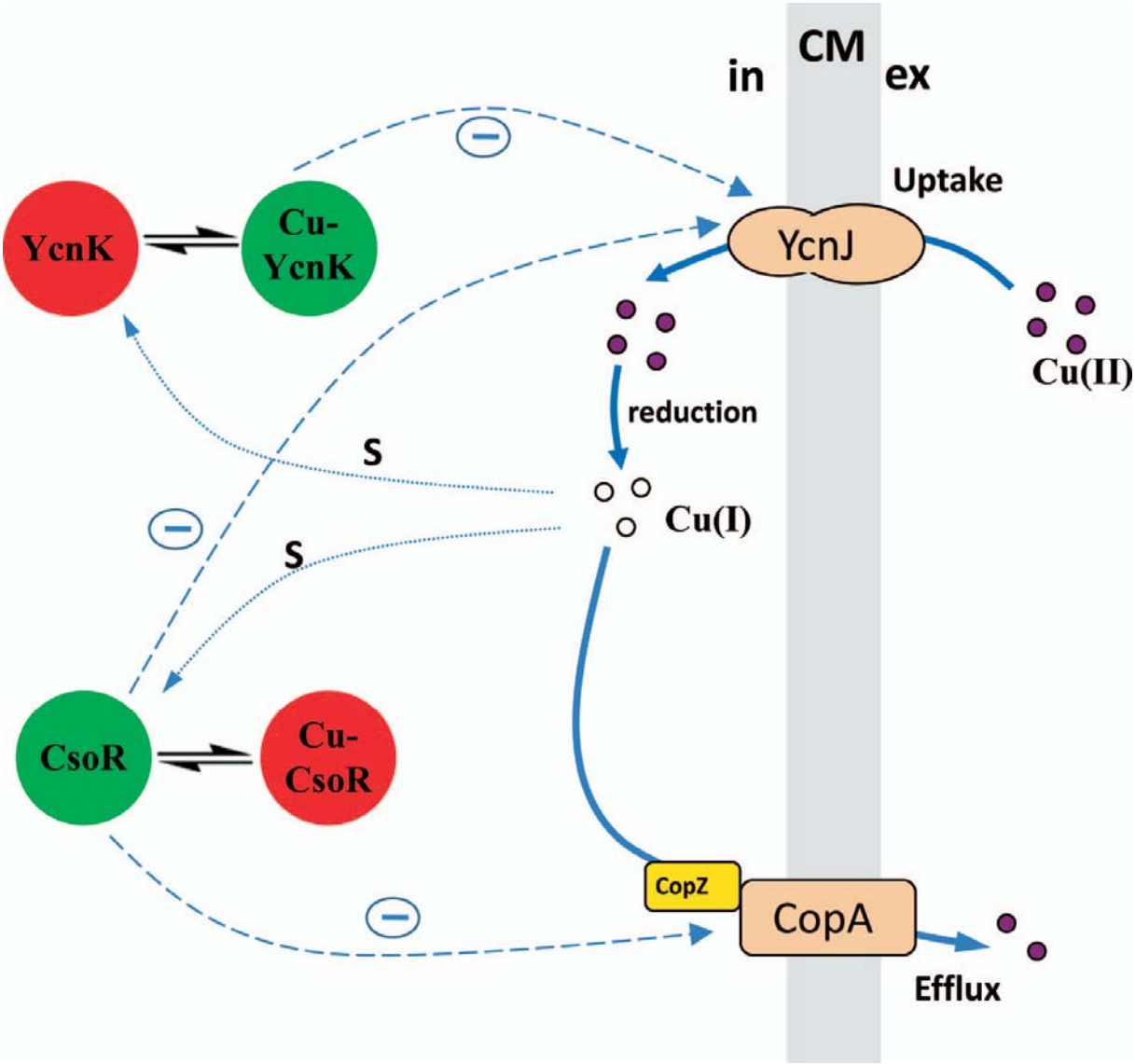

Fig.31: Current model of copper homeostasis in *B. subtilis*, including functional components for copper uptake and efflux as well as their cognate repressors. Depending on their association with copper, the active state of transcriptional repressors YcnK and CsoR is shown in green, and the inactive state of transcriptional repressors is shown in red. The negative regulation of components (-) is indicated with dashed arrows. Copper sensing (s) is indicated with dotted arrows. Cu,copper; CM, cytoplasmic membrane; in, intracellular; ex, extracellular.

So far, copper transport in *B. subtilis* is known only as a function of efflux, which is mediated by the CsoR-dependent CopZA system and the nonspecific cation diffusion facilitator CzcD, which is repressed by the ArsR homolog CzrA (18, 26). Components and mechanisms of copper uptake have not yet been identified in *B.subtilis*. Transcriptome studies were performed to identify genes involved in copper homeostasis in *B. subtilis using* Δ*csoR* mutant and wild type ATCC 21332 strain.

In our approach, we have identified genes that were upregulated by copper deprivation and downregulated under copper excess. The *ycnJ* gene was especially highly upregulated under copper-limiting conditions. The sequence homology studies reveal that YcnJ is highly homologous at its N terminus to CopC and at its C terminus to CopD from *P. syringae.* Unlike *P. syringae*, in which copper uptake is assisted by two distinct proteins (CopC and CopD), *B. subtilis* YcnJ is organized in a single polypeptide chain that facilitates the direct transfer of copper ions across the membrane.

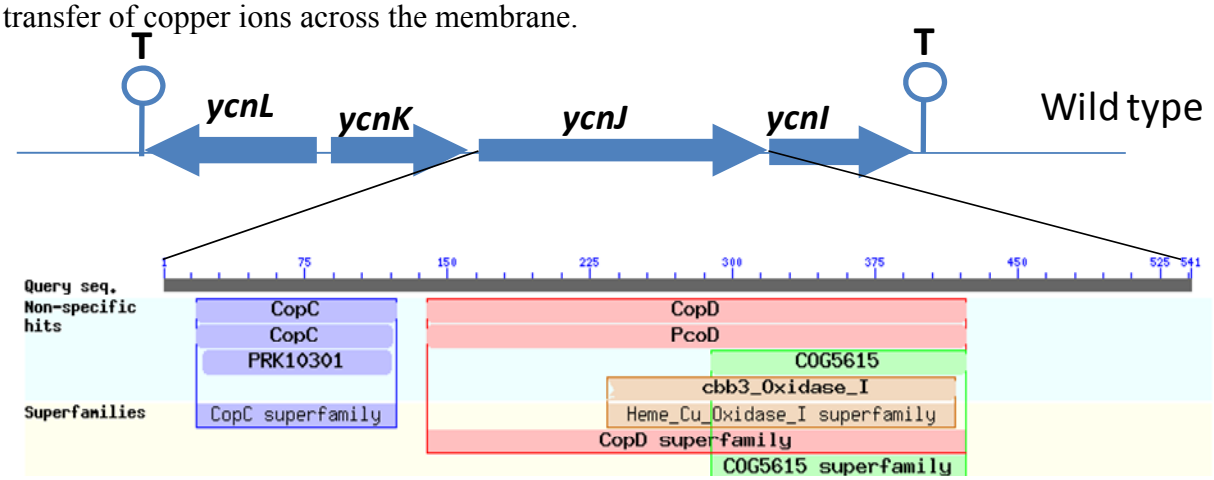

Fig.32: Homology of YcnJ with CopC and CopD of *P.syringae*

Studies undertaken with *P. syringae* demonstrate either that the CopC protein could interact with CopA and perhaps the outer membrane protein CopB to perform copper sequestration or that CopC along with CopD may function in copper uptake. Disruption of the *ycnJ* gene in *B. subtilis* resulted in a growth defective phenotype under copper-limiting conditions and a reduced intracellular copper content. Thus, these findings indicate that the primary role of YcnJ in *B. subtilis* is associated with copper uptake. A putative resistance function as

observed for CopC in *P. syringae* cannot be excluded. However, since this resistance was described for the periplasmic compartment, this might be a rather secondary function in *B. subtilis*. Sequence homology of putative Cu(II) binding amino acid residues (His-24, Glu-51, Asp-108, and His-110) of CopC from *P.syringae* are conserved in N-terminal domain of YcnJ. The copper binding studies with the recombinant purified N-terminal domain of YcnJ strengthen its role as a extracellular periplasmic Cu(II) binding protein and its role in uptake of extracellular oxidized copper.

8.1 YcnK and CsoR regulates the expression of ycnJ.

In an attempt to find the possible transcriptional factors that regulate the expression of *ycnJ*, mutants with a deletion of the unknown transcriptional regulator gene *ycnK* which is located upstream to *ycnJ* demonstrated an increase in the transcription of *ycnJ*. While, increased upregulation of *ycnJ* under copper limiting conditions accounts for the copper uptake functions and subsequently compensate the copper demands of the cell under copper scarcity. Increased copper content in Δ*ycnK* mutant when compared to wild type and the Δ*ycnJ* mutant under copper excess conditions implies YcnK acts as a negative regulator of *ycnJ* using copper as a corepressor under copper excess conditions. The Δ*ycnK* mutant showed elevated expression of *ycnJ* compared to the wild type, especially under copper excess conditions which is in accordance with the excess copper levels found inside the cell. YcnK in combination with a deletion of the recently identified copper-sensing repressor gene *csoR* were constructed and the *ycnJ* expression was quantified using dot blots. Expression was further elevated in the background of the Δ*csoR* Δ*ycnK* double mutant, suggesting that both regulators participate in *ycnJ* expression control.

As far as this interplay has been investigated, it points out the demands on a system that is developed to maintain the essential accurate levels of copper inside the cell and, at the

same time, to avoid passage over the critical threshold of copper toxification in order to allow proper physiological function.

8.2 Copper induced imbalance in Iron regulation.

In the course of identifying possible copper responsive genes under varying copper conditions, alterations in iron regulatory genes were observed. Previous transcriptomic studies conducted to identify the copper responsive genes were also shown to exhibit an upregulated Fur regulon under copper excess conditions, while the possible reasons underlying the same were not experimentally shown in *B.subtilis*. The results obtained while addressing the possible reasons underlying copper induced alteration of iron responsive genes led us to extend the previous model with the following model.

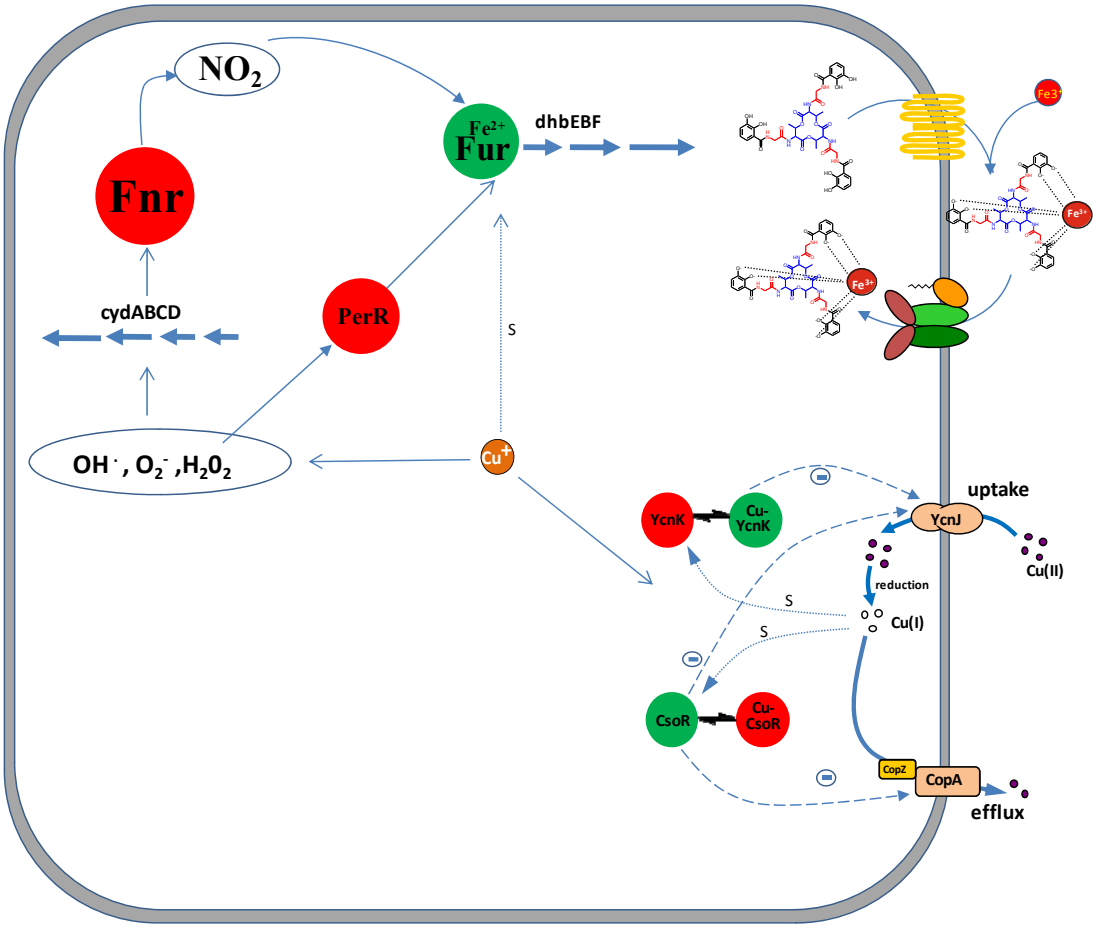

Fig.33. Current model of copper homeostasis in *B. subtilis*, including functional components for copper uptake and efflux as well as their cognate repressors. The active state of transcriptional repressors YcnK , CsoR and Fur is shown in green, and the inactive state of transcriptional repressors is shown in red. The negative regulation of components (–) is indicated with dashed arrows. Copper sensing (s) is indicated with dotted arrows.

Microarray studies and transcriptome studies performed using wild type under copper excess conditions exhibited an upregulation of Fur regulon whereas the microarray results obtained from the deregulated copper efflux mutant when copper in excess showed a siginficnat downregulation of the Fur regulon. The results underlying this shift in the gene regulation in connection to copper underlies several possibilities. One of the possible reasons for this upregulation is due to the fact that copper in excess inside the cell could lead to the unspecific binding of copper to the iron responsive transcriptional regulator Fur resulting in derepression of Fur in wildtype under copper excess condition. While, the constitutive copper efflux in the Δ*csoR* mutant resulted in no unspecific copper binding and thus exhibited a downregulation pattern in comparision to wildtype under the same conditions. Invitro experiments performed using the recombinant Fur protein when incubated with several metals alone and in combinations, exhibited a strong unspecific binding of copper among other metals. Since it is a known fact that iron and copper behaves as a more potential candidates for Fenton reaction, tight regulation of these metals and their coupling with defence against the oxidative stress response regulators has become essential in most forms of life. In the presence of oxygen, defects in these metal homeostasis pathways would still worsen the situation of the cell leading to increased oxidative stress. Hence it is important to tightly control the metal homeostasis pathways. Fur-like proteins are associated to coordinately regulate the iron assimilation with defense against oxidative, acid stresses and with other general metal imbalances. Growth experiments performed with copper homeostasis defective mutant Δ*csoR* exhibited partially better growth phenotype in iron depletion conditons when compared to wildtype and a copper efflux deletion mutant Δ*copA*. The above observation drives us to conclusion that Δ*csoR* mutant under iron-deficient conditions, could lead to the excess sequestration and distribution of copper inside the cells, since it is imperative for the cell to satisfy the copper demands. The *copZ* chaperone and *copA* are constitutively expressed in Δ*csoR* mutant which not only detoxify the copper but could also induce unknown copper

sequestration mechanisms, which leads to unspecific distribution and incorporation of copper by these copper proteins. This unspecific copper distribution pattern might probably incorporate copper into many iron storage proteins. One such important class of these incorporations include iron-sulphur clusters, resulting in their destabilization and subsequent damage resulted as a consequence of displacement of iron atoms from the solvent exposed cluster, suggesting that Cu(I) damages these proteins by liganding to the coordinating sulfur atoms (51). The destruction of iron sulphur clusters could subsequently lead to the iron deficient conditions inside the cell and triggers the iron acquisition pathways which include bacillibactin synthesis as observed from the CAS plate assay and bacillibactin quantification experiments. Results obtained from the other copper efflux deletion mutants Δ*copA* are quiet contrasting in response to iron acquisition pathways. The Δ*copA* mutant exhibited a strong growth defective phenotype and increased copper accumulation which in principle should result in damaging the iron sulphur clusters more extensively in comparision to Δ*csoR* mutant. In contrast, the results indicated that the constitutive copper efflux systems exhibited more significant iron responsive stress situations which were restored by inducing the iron acqusition pathways when compared to Δ*copA* mutant where, either the copper accumulation might potentially damage the iron sulphur clusters and further leading to copper induced stress conditions which appears to be quite harsh in comparison to copper efflux conditions.

These disturbances in the metal homeostasis arose during aerobic respiration results in differences in the total cellular NADH/NAD^+ ratios. The differences in these ratios inside the cell would certainly lead to altered metabolic pathways including respiration. The estimation of the NADH/NAD^+ ratios exhibited a 3 fold increase in the in the Δ*csoR* mutant when compared to wild type. The difficulties associated with estimating the total intracellular free metal content led to contradictory results, not currently allowing us to clearly support or nullify the genetic and transcriptomic evidence.

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Acknowledgements

My first and foremost acknowledgement goes to my principle investigator, Prof. Dr. Mohamed Marahiel. I am greatly indebted to him for his immense belief in me and his all time support and encouragement during the bad and good times of my Ph.D career. I have started my research career in his lab and it is only because of his able guidance, that I have reached this phase of my Ph.D career. “Thank you” is a very small word to extend my gratitude to Prof. Marahiel.

I extend my sincere gratitude to Dr. Marcus Miethke, who is more a friend than my colleague and who was constantly guiding me through out my Ph.D with his stimulating discussions and interesting ideas.

I sincerely thank Prof. Dr. Oscar Kuipers, for his fruitful co-operation and allowing me to perform microarrays in his lab. I also thank Dr. Hein Trip for his help in performing the arrays. Without their co-operation, the microarray study would not have been possible.

I would like to thank Prof. Saubert group for extending their help in ICPMS measurements and I would also like to thank Prof. Erhard Bremer group for chemiluminescence detection in his lab.

I would like to thank Prof. Michael Bölker, Prof. Erhard Bremer for reviewing this thesis and for helpful and encouraging discussions.

I would like to extend my sincere thanks to all the lab members for their constant encouragement and inspiring discussions and for creating an excellent professional atmosphere in the lab.

I thank all the lab technicians, for their all time help. My sincere thanks to Roswitha Roller-Müller, from whom I received unforgettable help in administrative issues.

Last but not least, I would like to thank my wife who is my best friend and always stood by me with a lot of patience, understanding and caring, and also my family members who always encouraged me constantly all through out my career. I dedicate my thesis to my parents and it goes without saying that my mom, dad, my brother, sister, and inlaws ever deserve my deepest gratitude.

Curriculum Vitae

- Born in Manthani, India, on 30th January 1980

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- Finishing PhD thesis with disputation in October 2009.

Erklärung

ich versichere, daß ich meine Dissertation

Characterization of copper mediated transcriptional responses in *Bacillus subtilis.*

selbständig, ohne unerlaubte Hilfe angefertigt und mich dabei keiner anderen als der von mir ausdrücklich bezeichneten Quellen und Hilfen bedient habe.

Die Dissertation wurde in der jetzigen oder einer ähnlichen Form noch bei keiner anderen Hochschule eingereicht und hat noch keinen sonstigen Prüfungszwecken gedient.

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(Ort/Datum) (Unterschrift mit Vor- und Zuname)