New components of copper homeostasis in *Lactococcus lactis* and *Escherichia coli*

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PhD Thesis

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Accepted by the Faculty of Medicine, the Faculty of Science and the Vetsuisse Faculty of the University of Bern at the request of the Graduate School for Cellular and Biomedical Sciences

Bern, Dean of the Faculty of Medicine

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Life is not about waiting for the storms to pass...

it's about learning how to dance in the rain!

(Anonymous)

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Abbreviations

ABC	ATP-binding cassette
A domain	actuator domain
ATCC	American Type Culture Collection
ATP	adenosine-5'-triphosphate
BC	before Christ
BLAST	basic local alignment search tool
bp	base pair
CA	California
cAMP	cyclic adenosine monophosphate
CCD	charge-coupled device
cDNA	complementary DNA
CFU	colony forming unit
CPx	intramembranous CPC or CPH motif in heavy metal ATPases
CRP	cAMP response protein
C-terminus	carboxy terminus
DCPIP	2,6-dichlorophenolindophenol
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EDTA	2,2',2'',2'''-(ethane-1,2-diyldinitrilo)tetraacetic acid
EMSA	electrophoretic mobility-shift assay
EXAFS	extended X-ray absorption fine structure
FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide
GSNO	S-nitrosoglutathione
GW9662	2-chloro-5-nitro-N-phenylbenzamide
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
his	histidine
INRA	Institut national de la recherche agronomique
k_{cat}	unimolecular rate constant, turnover number
k_d	DNA dissociation rate constant
kDa	kilodalton
K_m	Michaelis constant
kT	kiloton
LAB	lactic acid bacteria
LA Taq	Long and Accurate Taq

LB	Luria-Bertani
Mb	mega base pairs
MBD	heavy metal binding domain
Me	metal
MI	Michigan
MOCO	molybdenum cofactor
mRNA	messenger RNA
NAD(P)H	nicotinamide adenine dinucleotide (phosphate)
N domain	nucleotide binding domain
NONOate	diethylamine NONOate sodium salt
NaP_i	sodium phosphate
NMR	nuclear magnetic resonance
NQO	4-nitroquinoline-N-oxide
Nramp	natural resistance-associated macrophage protein
NTA	2,2',2''-nitrilotriacetic acid
N-terminus	amino terminus
OD	optical density
PCR	polymerase chain reaction
PDB	protein database
P domain	phosphorylation domain
Pit	inorganic phosphate transport
qPCR	real-time quantitative PCR
re	rectus
RMS	root mean square
RNA	ribonucleic acid
RND	resistance/nodulation/cell division
ROS	reactive oxygen species
rTEV	recombinant tobacco etch virus
SDS	sodium dodecyl sulfate
si	sinister
SOD	superoxide dismutase
TAT	twin-arginine translocation
Tris	2-amino-2-hydroxymethyl-propane-1,3-diol
tRNA	transfer RNA
UV	ultraviolet
V5	part of the sequence of Simian virus 5
XDS	X-ray detector software

Abstract

Copper (Cu) is an essential micronutrient for all living organisms. The two oxidation states, Cu^{+} and Cu^{2+}, allow its participation as cofactor in many biological processes. However, its high reactivity can also cause damage to the cell and implies a tight regulation of copper import, intracellular chaperoning, detoxication, and removal by special pumps. Two bacterial models, *Lactococcus lactis* and *Escherichia coli*, were used in the course of this study to characterize new components of copper homeostasis. In *L. lactis*, copper stress activates a set of 14 genes, which are under the control of the copper-responsive regulator CopR. The genes encoding copper pumps, the copper chaperone or the copper responsive regulator itself have already been described in detail. The physiological role and relevance of most of the remaining genes are, however, still unknown. One of these genes, *cinD*, was further characterized. CinD is a flavoprotein and exhibits nitroreductase activity towards the oxidative agent 4-nitroquinoline-*N*-oxide. CinD also showed significant catalase activity and may thus support the copper detoxication machinery by attenuating toxic effects caused by oxidative stress inducers like hydrogen peroxide, nitroaromatic compounds or copper. Copper is not only toxic to the cell, but also an essential component of many copper-containing proteins. Nevertheless, the way copper enters the cell is still unknown. In a second project, a *lux*-based biosensor was used to monitor intracellular copper levels *in situ* and to identify components involved in copper entry in *E. coli*. YcfQ, a TetR-like transcriptional regulator, and its target, *ycfR*, were found to be involved in this process. Increased levels of YcfR in the outer membrane appear to make the membrane less permeable to copper. Mechanisms of copper homeostasis are highly conserved from bacteria to humans and allow the extrapolation of knowledge from one cellular system to another. Findings in prokaryotic systems may pave the way towards better understanding of the role of copper deficiency or copper toxicity in physiological and pathological conditions in humans.

Chapter I: Introduction

The transition metal copper

Copper (Cu) is the 29th element of the periodic table and forms the first secondary group together with silver and gold. The most important oxidation states of copper are +1 and +2 (2). In aqueous solutions, copper persists in its most stable form as Cu^{2+}, whereas the prevailing form in the reducing cytosolic environment of a cell is Cu^{+} (3). Pure copper is ductile and malleable and shows a reddish-orangish luster (Fig. 1) owing to a layer of Cu_2O on its surface. A freshly exposed surface of copper has a pinkish or peachy color.

Due to its lustrous appearance and good malleability copper has been used for jewelry and tool-making soon after its first discovery around 9000 BC (4) and is still used for household products, handcraft and coinage today. Copper is the metal with the highest electrical and thermal conductivity after silver and finds a large application in any aspect of engineering and high technologies (wiring, electronics, computer technologies, automotive industry) (5). Different alloys of copper exist, the most familiar being brass (with zinc) and bronze (with tin), whose discovery established the Bronze age around 3000 BC (4). Copper ions show antimicrobial properties. For this reason, copper salts find different applications in agriculture (fungicides in vineyards), water ecology (algae control in lakes) and medicine (5). Copper therapy is first mentioned 1550 BC in the Middle Empire in Egypt. Today, the antiinflammatory effects (arthritis) of copper salts is not questioned (6), even tough not clearly explained. Copper surfaces in hospital equipments receive growing attention due to the same characteristics: Bacteria are rapidly killed on copper surfaces, but remain viable for days on surfaces made from stainless steal (7).

Fig. 1: Natural copper nugget. Native copper shows a reddish-orangish luster. Taken from (1).

Copper is mostly found in the form of compounds and very rarely as native copper. Sulphide ores like chalcopyrite ($CuFeS_2$) chalcosine (Cu_2S), bornite (Cu_5FeS_4), cuprite (Cu_2O), malachite ($CuCO_3$), azurite ($Cu(OH)_2$) and others are among the most significant ones. The most important manufacturer of copper is Chile, followed by Peru and the USA (2). Copper is 100 % recyclable without any loss of quality whether in a raw state or contained in a manu-

factured product. It is the third most recycled metal after iron and aluminum, and it is estimated that 80 % of the copper ever mined is still in use today (5).

Copper as bioelement

Biological systems started to use copper only after the advent of oxygen in the atmosphere less than $3x10^9$ years ago. The action of oxygen-producing cyanobacteria transformed the highly insoluble copper sulfides (Cu^+) to soluble and thus bioavailable Cu^{2+}. Many organisms adapted to the new conditions by acquiring an oxidative metabolism and by utilizing this newly available metal, whose redox properties were just ideally suited to exploit the oxidizing power of dioxygen. Concomitant with the arrival of oxygen, multicellular organisms developed which had extracellular cross-linked matrices capable of resisting attacks by free oxygen radicals (6).

The ability of copper to exist in different oxidation states, makes it a central player in cellular biology. Copper acts as a cofactor in over 30 enzymes of higher organisms (3) playing an important role in processes such as mitochondrial respiration (cytochrome *c* oxidase), protection against free radicals (superoxide dismutase) or crosslinking of collagen and elastin (lysyl oxidase) (8, 9). Besides its function as cofactor, copper proteins are also involved in dioxygen transport in some invertebrates (hemocyanin) or can act as electron carriers, such as plastocyanins or azurines (10, 11). In bacteria, eleven cuproenzymes have so far been characterized (Table 1). Copper is transported to the enzymes that require it by passing from one copper protein site to another, exploiting gradients of increasing copper-binding affinity (13).

Table 1. Known bacterial copper-containing enzymes. Adapted from (12).

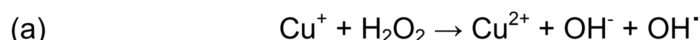
Enzyme	Function	References
Cytochrome *c* oxidase	Terminal oxidase	(14)
NADH dehydrogenase 2	Electron transport, Cu reduction	(15, 16)
Nitrosocyanin, cuproredoxin-like	Electron transfer, other?	(17)
Plastocyanins	Electron transfer	(14)
Cu-containing nitrite reductases	Nitrite reduction	(18)
Cu-containing nitrous oxide reductases	Nitrous oxide reduction	(19)
Tyrosinase	Phenol oxidation, melanin synthesis	(20, 21)
Cu amine oxidases	Oxidation of primary amines	(22)
Particulate methane monooxygenase	Methane oxidation	(23)
Cu,Zn-superoxide dismutase (cuprein)	Defense during infection?	(24)
Cu-containing laccase	Polyphenol oxidase	(25)

Almost all of the characterized eukaryotic copper proteins are extracellular; exceptions are cytochrome *c* oxidase, which is located in the inner membrane of mitochondria and the copper-zinc superoxide dismutase found in the cytosol (6). It is not clear, if bacteria as well have a requirement of cytosolic copper. All currently documented bacterial copper proteins are either located in the periplasm or embedded in the plasma membrane facing the periplasmic space. Copper loading of these proteins could occur via the periplasmic space (3). The only described system that suggests a need for cytoplasmic copper is the biosynthesis of the molybdenum cofactor, MOCO (26). MOCO forms the active site of molybdenum enzymes found in animals, plants and microorganisms. It consists of molybdenum bound to two sulfur atoms of a unique pterin referred to as molybdopterin. It seems that copper is bound to the sulfur atoms to hold the place for the subsequent insertion of molybdenum. This finding suggests a link between molybdenum and copper metabolism and would require cytoplasmic copper (27).

The homeostasis of copper is closely linked to the homeostasis of other trace metals, especially iron. The human protein ceruloplasmin, the main copper carrying protein in the blood, shows ferroxidase activity and participates in iron homeostasis. It oxidizes Fe^{2+} to Fe^{3+} and promotes thereby the rate of Fe^{3+} incorporation into the iron storage protein transferrin (28). A low copper level and as a result lower amount of functional ceruloplasmin in the blood were shown to provoke iron metabolism disorders leading to anemia and liver iron accumulation (29). In bacteria, investigations on the relationship between iron and copper homeostasis have only recently started with the discovery of a novel pathway of copper toxicity (30).

Mechanisms of copper toxicity

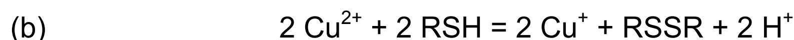
The fact that copper undergoes redox chemistry makes this metal not only essential for biological processes, but also potentially toxic at higher concentrations. Eukaryotic cells treated with high amounts of copper show typical damages which arise from the generation of reactive oxygen species (ROS) in the cell. ROS include hydroxyl radicals ($OH^{\bullet}$), hydrogen peroxide (H_2O_2), and superoxide (O_2^-) and damage tissue and DNA (31-33). *In vitro* studies showed that copper reacts with endogenous H_2O_2 in a process analogous to the Fenton reaction (a) producing highly reactive hydroxyl radicals (3).



(a) $$Cu^+ + H_2O_2 \rightarrow Cu^{2+} + OH^- + OH^{\bullet}$$

In the analogous reaction *in vivo*, which is driven by ferrous ions, Fe^{2+}, the hydroxyl radicals are potent oxidants of DNA and cause both mutagenesis and lethality (34).

As alternative cell damaging mechanism of copper, sulfhydryl-depletion by reactions (b) and (c) has been proposed (3).



(b) $2\ Cu^{2+} + 2\ RSH = 2\ Cu^{+} + RSSR + 2\ H^{+}$

(c) $2\ Cu^{+} + 2\ H^{+} + O_2 = 2\ Cu^{2+} + H_2O_2$

Nevertheless, the discovery that free copper or iron in the cell is extremely low or even non-existent makes Fenton chemistry and sulfhydryl depletion rather unlikely mechanisms (34), and recent findings suggest that primary copper toxicity might occur by alternative mechanisms. *In vivo* studies in *Escherichia coli* showed that copper-loaded cells were less sensitive to killing by H_2O_2 and showed less DNA damage than cells grown without copper. Excessive intracellular copper even eliminated iron-mediated oxidative killing. Based on these observations the authors suggested that copper exerts its toxicity by other mechanisms than oxidative stress (35). The authors could show in further experiments that copper displaces the iron atom in the solvent-exposed iron-sulfur cluster of the enzyme isopropylmalate dehydratase, thereby blocking the biosynthesis of branched-chain amino acids. Addition of these branched-chain amino acids could restore growth of sensitive strains (29).

Microarray analyses in *Bacillus subtilis* presented a connection between copper stress and the expression of genes coding for iron-sulfur cluster biogenesis and associated pathways, such as cysteine or branched-chain amino acid biosynthesis and genes coding for iron-sulfur cluster proteins (36). Further work will be necessary to obtain more insights into the processes of metal-induced iron-sulfur cluster destabilization and reconstitution and to establish whether this mechanism is a general route of copper toxicity.

Given that copper is both, essential and toxic, a tight regulation of copper uptake, distribution and export is necessary. Cells have evolved homeostatic control mechanisms which include chaperones for proper distribution of copper, transmembranous pumps to remove excess copper from the intracellular space, oxidases to change the oxidation state of copper, and special regulators that control gene expression in response to the intracellular copper concentration.

Copper homeostasis in humans

Copper is the third most abundant essential trace mineral in the body, after iron and zinc. In humans, copper is usually absorbed through the diet rather than water. Copper content of different foods vary considerably. Shellfish, oysters and organ meats (liver) are the richest sources of copper followed by chocolate and cocoa products, nuts (mostly cashew) and seeds. Unpolluted fresh water has very little copper, but leaching from copper piping in

houses and buildings might as well have an effect on copper exposure and intake (9, 37, 38). The World Health Organization recommends a dietary allowance of 0.9 mg copper per day for the general population, the upper limit being 10 mg. For pregnant and nursing women the recommended intake is a bit higher, around 1.3 mg (39). Due to the lack of good copper biomarkers the setting of dietary recommendations remains difficult and recommendations vary from source to source.

The way copper usually enters mammals is through the alimentary tract (Fig. 2). The site of maximal copper absorption in humans is not known, but is assumed to be the stomach and upper intestine (40). After absorption of copper across the brush border membrane into the cells of the intestinal mucosa, it is subsequently transferred across the basolateral membrane into interstitial fluid and blood. In the portal blood, copper is bound to albumin or transcuprin; a small amount may be chelated by peptides and amino acids, especially histidine (38, 41). Via the blood, copper is routed directly to the liver where it is incorporated into copper-requiring proteins. These are thereafter secreted back into the blood and transported to extrahepatic tissues (42, 43). Ceruloplasmin is the primary copper transport protein in systemic circulation and contains around 75 % of the plasma copper (44). Besides the liver and body fluids, copper is mostly found in the heart, the brain, the kidneys and skeletal muscles (42). Excess copper is not stored in the body, but eliminated over the bile. Only a tiny fraction leaves the body over the urine. Normal physiological levels of copper in mammals are regulated by both duodenal absorptions and endogenous excretion of copper into the gastrointestinal tract (45).

Defects in copper homeostasis result in severe diseases

The importance of maintaining mechanisms for proper copper homeostasis in the liver is underscored by the existence of two well characterized human genetic diseases in copper transport, Menkes and Wilson diseases (46-48). Patients with Wilson disease have an inability to secrete copper into the bile. The genetic defect is caused by either a mutation or a deletion of a gene encoding a P-type ATPase, ATP7B, making it impossible for the copper to enter the bile. As a result, these patients accumulate copper in the liver and the brain and suffer from hepatic abnormalities (cirrhosis and chronic hepatitis, culminating in progressive liver failure), neurological defects (parkinsonian features, seizures) and psychiatric symptoms (personality changes, depression, psychosis) (49). Wilson disease is treated by reducing copper absorption through the diet and removing excess copper from the body by copper chelation (50, 51). But nevertheless, if treatment is ineffective, a liver transplant is occasionally required.

Patients suffering from Menkes syndrome, an X-chromosome linked disorder, fail to transfer copper from the cells of the intestinal mucosa into the blood. The syndrome is caused, like in Wilson disease, through the lack of a P-type ATPase (46). Without this transporter, called ATP7A, copper cannot travel to the liver and its subsequent destinations and most enzymes that depend on copper for their function will be inactive or have a reduced activity. The symptoms apparent for Menkes syndrome are versatile and include profound mental retardation, and connective tissue (collagen) abnormalities that result in soft bones and cartilage and weakened artery walls. Baby boys with the disease show characteristic steely or kinky hair (38) and very few of them survive beyond the age of three. Treatment with daily copper injections to bypass its lack of absorption by the intestine may improve the outcome in Menkes disease (52), however, the negative effects already occurred during gestation cannot be prevented.

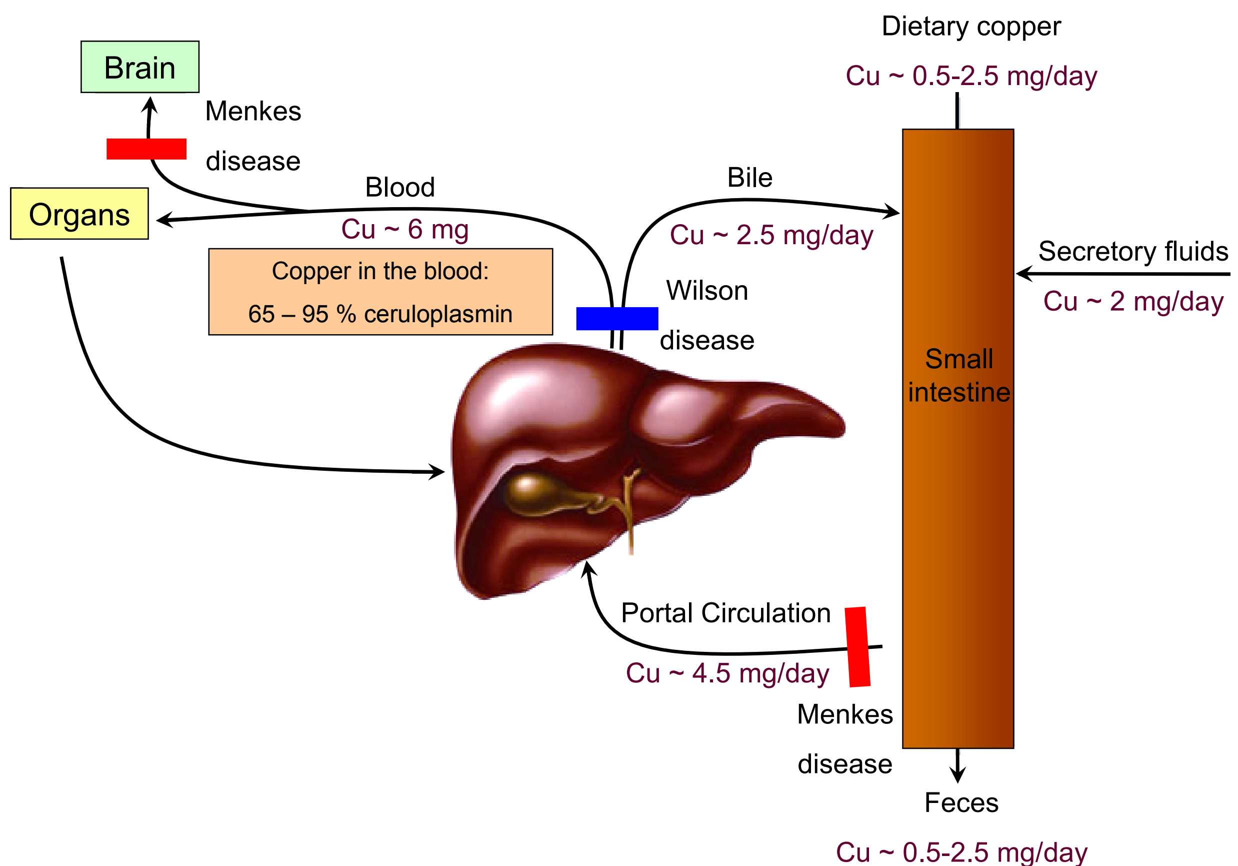


Fig. 2. Overview of body copper homeostasis. Dietary copper is absorbed in the duodenum and small intestine. It is transported to the portal venous circulation where it binds to serum proteins and is directly routed to the liver. Patients with Menkes disease have a block in copper uptake, caused by mutations in ATP7A, and suffer from copper deficiency. In the liver, copper is incorporated in cuproproteins and subsequently excreted back into the blood. Excessive copper in the hepatocyte is excreted by the liver into the bile via the copper ATPase ATP7B, and this copper is discarded from the body via defecation. Patients with Wilson disease suffer from copper toxicosis in the liver due to mutations in ATP7B. Adapted from (53).

Wilson and Menkes ATPases share high sequence homology with copper pumps in the Gram-positive bacterium *Enterococcus hirae* (54). Investigations on the copper regulation machinery in *E. hirae* started in 1992 with the discovery of two copper ATPases (55), and today this organism is regarded as one of the best studied bacterial models for copper homeostasis. *Lactococcus lactis* is a close relative of *E. hirae* and bioinformatical studies suggest a very similar mechanism for the regulation of intracellular copper levels (56). The availability of the genomic sequence of *L. lactis* makes this organism the perfect choice to study new components involved in copper homeostasis (57).

Copper homeostasis in prokaryotes

Lactococcus lactis

L. lactis is a Gram-positive, aerotolerant bacterium that grows in pairs or short chains. In nature, *L. lactis* is found on plant surfaces and in the gastrointestinal tract of humans and animals. The cells are non-sporulating and non-motile and have a homo-fermentative metabolism. The production of lactic acid during carbohydrate fermentation is one of the reasons why *L. lactis* belongs to the most important microorganisms involved in dairy industry. The generation of a very acidic environment during growth, often accompanied by the secretion of bacteriocins, such as nisin, efficiently inhibits growth of competing bacteria and explains the wide use of *Lactococci* in food production (buttermilk, yoghurt, cheese) and preservation (58). However, the acidic ambient conditions can lead to the solubilization of complexed metal ions, thereby generating unfavorably high metal ion concentrations. In traditional cheese making, for example, the cells are challenged by copper released from the copper kettles (59). While this process is important for flavor development (60), it also puts stress on the bacteria.

There is currently no prove of a copper-containing protein in the species *Lactococci* (12). Nevertheless, defense mechanisms to avoid copper intoxication have evolved. Copper homeostasis in *L. lactis* appears to resemble the well studied copper homeostatic system of *E. hirae* (61). Details regarding copper homeostasis of *E. hirae* as well as additional information about the structural properties of the single copper homeostasis components can be found in the review article in chapter II, section 3 or in the book chapter in section 4.

Similar to *E. hirae*, *L. lactis* possesses a *cop* operon encoding a putative P-type copper export ATPase, CopA, a copper chaperone, CopZ, and the repressor CopR (56, 62). CopR regulates the expression of the *copRZA* operon dependent on the copper content of the cell. A second putative copper ATPase is encoded by the unlinked, monocistronic *copB* gene, which is also under the control of CopR (Fig. 3) (56).

CopR is a zinc-containing regulator which binds to the *cop*-box, a conserved TACAnnTGTA motif in the promotor region of the operon (63). If cytoplasmic copper is in excess, the CopZ copper chaperone delivers Cu^{+} to CopR. This results in the exchange of the Zn^{2+} in CopR by two Cu^{+} and the concomitant release of the repressor from the promoter, allowing transcription of the *cop*-genes to proceed (3, 61). CopA and CopB are transmembranous ATPases which transport Cu^{+} ions across the cytoplasmic membrane (64, 65). CopA was shown to complement a copper-sensitive phenotype of an *E. coli* strain deficient in cytoplasmic copper export and thus clearly serves in copper extrusion (56). The monocistronic *copB* gene shows 55 % sequence identity to the copper export pump CopB of *E. hirae* (65). Cu^{+} and Ag^{+} transport by the enterococcal enzyme has been directly demonstrated with radioisotopes in membrane vesicle and from whole cells loaded with silver (65, 66). However, a function of *L. lactis* CopB in copper export has not yet been directly demonstrated.

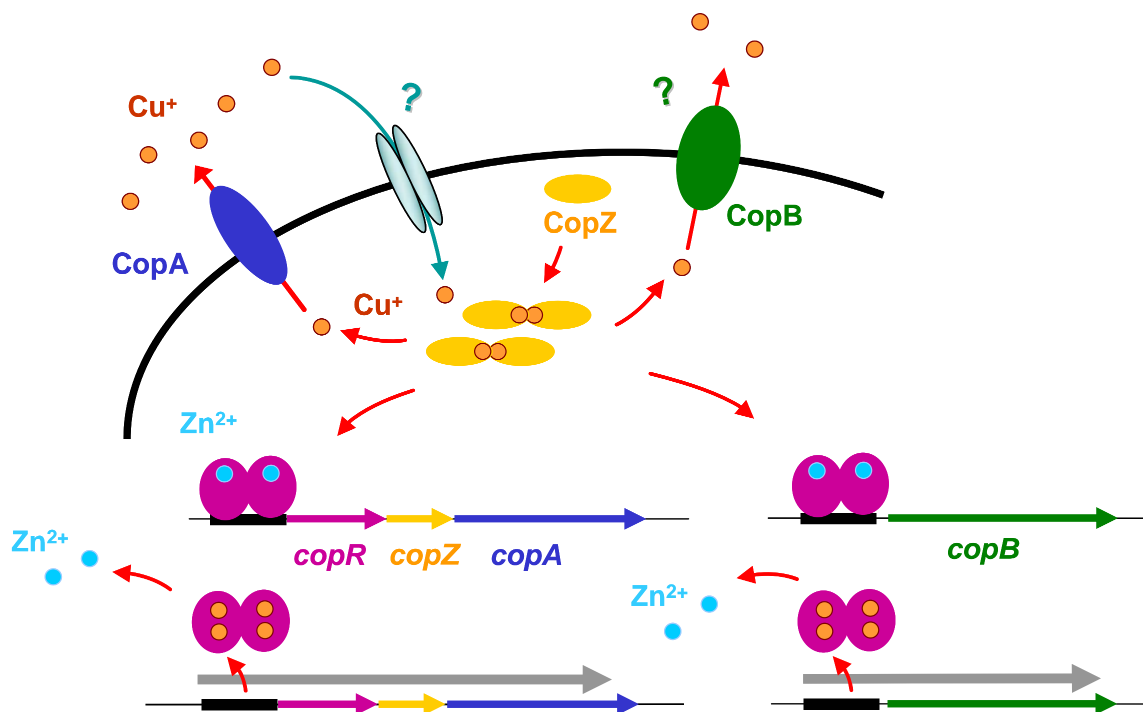


Fig. 3. Copper homeostasis in *L. lactis*. Copper enters the cell by unknown ways. In the cell, copper binds to the chaperone CopZ, which can then donate Cu^{+} to either the copper ATPases for export or the CopR repressor to induce transcription. In low-copper conditions, a CopR dimer in the zinc form is bound to the *cop* box in front of the *copRZA* operon and the *copB* gene. When CopZ donates Cu^{+} to CopR, one Zn^{2+} per CopR monomer is replaced by two Cu^{+}, with concomitant release of CopR from the promoters and induction of transcription of the downstream genes. CopA and CopB then accomplish copper export from the cytoplasm.

It must be noted that *E. hirae* CopB is encoded by the *copYZAB* operon (CopY is the repressor in *E. hirae*), while CopB of *L. lactis* is encoded by a monocistronic gene. Whether these different gene organizations in *L. lactis* and *E. hirae* are a consequence of functional

differences remains an open question. Copper secretion by copper ATPases for the rapid export of excess copper out of the cytosol is common, if not ubiquitous, and is the basic mechanism of bacterial copper resistance. The process has been documented in many eukaryotic and bacterial systems.

A genome wide search for *cop*-boxes, the binding site of the CopR repressor, revealed the existence of another ten genes, which together with *copRZA* and *copB* encompass the CopR regulon of *L. lactis* (Fig. 4) (56). These genes were shown to be induced by copper *in vivo* and direct interaction between consensus sequences and the repressor could be verified *in vitro*. These new genes include *lctO*, encoding a lactate oxidase, and the *ydiDE*, *yahCD-yaiAB*, and *ytjDBA* operons of unknown functions. Of all the genes and operons constituting the CopR regulon, the *ytjDBA* operon was most strongly induced by copper (56). Based on sequence comparison, the first gene of this operon, *ytjD*, encodes an oxygen-insensitive nitroreductase, which was renamed *cinD* for copper-induced nitroreductase. The biochemical characterization of CinD and a possible role in the copper homeostasis machinery of *L. lactis* are discussed in chapter II, section 1.

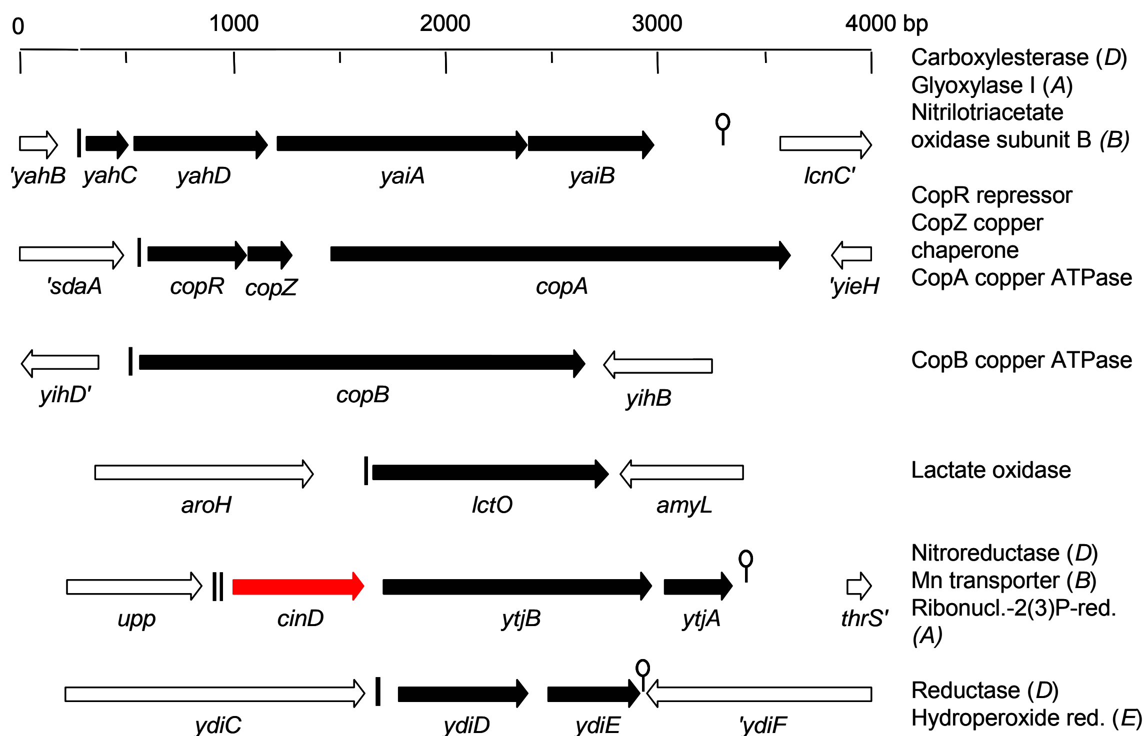


Fig. 4. Copper-induced genes in *L. lactis*. The genes in black (red) are regulated by CopR. Vertical lines indicate the location of *cop*-boxes and the *lariats* those of ρ-independent transcriptional terminators. The predicted functions of the genes are indicated on the right.

Escherichia coli

E. coli is a Gram-negative, rod-shaped bacterium with peritrichously arranged flagella. The cells do not form spores and are usually found in the lower intestine of humans and other warm-blooded organisms. *E. coli* is a facultative anaerobe and survives when released to the natural environment, allowing widespread dissemination to new hosts. Most *E. coli* strains are harmless, but some can cause serious food poisoning while others are responsible for infections of the enteric, urinary, pulmonary and nervous system (67, 68). *E. coli* is the historical workhorse of bacterial genetics and has proven to be an excellent model to study copper homeostasis in Gram-negative bacteria.

In *E. coli*, different systems are involved in avoiding the accumulation of too high copper concentrations which may be generated through proteolytic and acidic degradation of food in the animal gut: a P-type ATPase, CopA, a multicopper oxidase, CueO, and the *cus* determinant (69).

Excess copper in the cytoplasm of the cell is sensed by CueR, a MerR-like transcriptional activator. These proteins bind to the promoters of their target genes in the presence and absence of their effector metals (70). In the presence of elevated metal ions, they allosterically activate transcription initiation by RNA polymerase by realigning abnormally spaced consensus RNA polymerase recognition sequences, while in the absence of metal ions these proteins may cause slight repression (71-73). CueR controls the expression of the ATPase CopA which pumps Cu^{+} out of the cytoplasm to the periplasmic space (Fig. 5) (74, 75). At the same time, CueR also activates the expression of the multi-copper oxidase CueO which is exported to the periplasm via the TAT pathway for prefolded proteins (74). Multicopper oxidases are copper containing enzymes (usually four copper ions) that couple the oxidation of a substrate to the reduction of dioxygen to water. CueO has a broad substrate specificity and oxidizes numerous compounds *in vitro*, including ferrous iron, catechols, and iron-chelating siderophores (76). Oxidized siderophores sequester copper and contribute to the detoxification of the periplasm (77). CueO also shows cuprous oxidase activity and can oxidize Cu^{+} to Cu^{2+}, thus rendering the metal less toxic (78, 79). Unlike other multicopper oxidases, CueO requires a fifth copper adjacent to the substrate binding site for activity. The crystal structure displays a methionine rich helix that lies over the active site and it was hypothesized that this helix may function in copper sensing and may activate the enzyme under excess copper (80, 81).

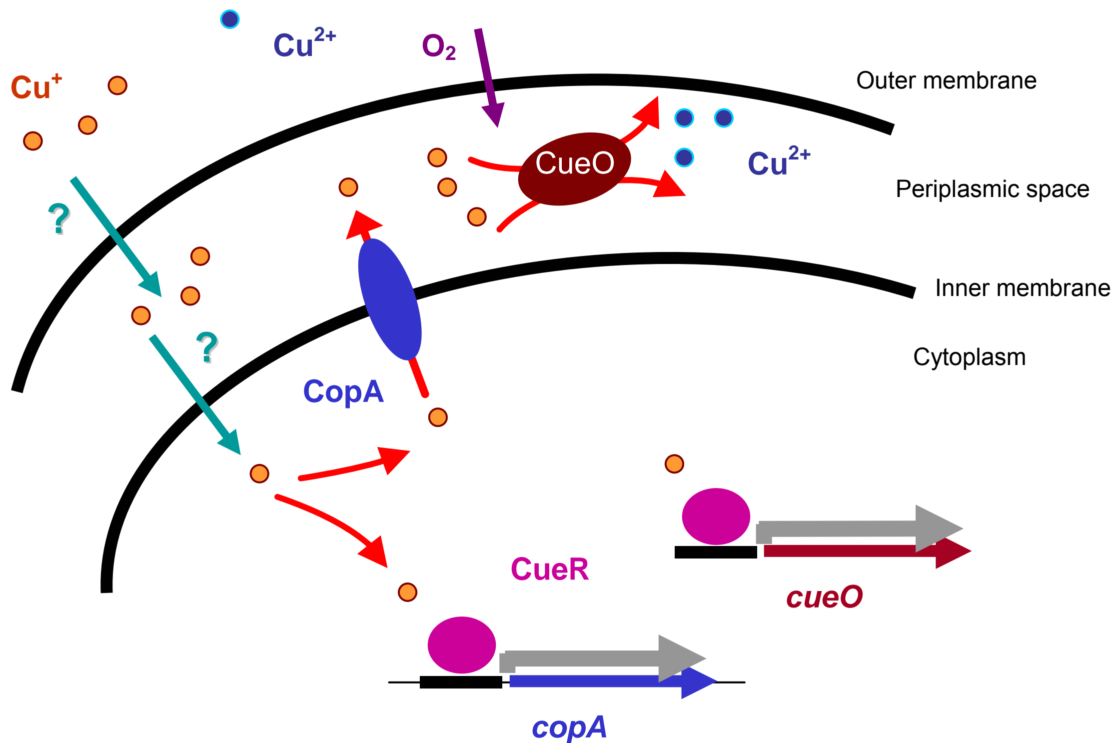


Fig. 5. The *cue* system of *E. coli*. The CopA ATPase and the multi copper oxidase CueO are both regulated by the CueR transcription activator in response to increasing intracellular copper concentrations. CueR activates transcription initiation by realigning abnormally spaced consensus RNA polymerase recognition sequences. CopA is an ATP-driven copper pump that pumps Cu^{+} from the cytoplasm into the periplasm. Periplasmic Cu^{+} is oxidized by the CueO multi-copper oxidase to less toxic Cu^{2+}. Adapted from (3).

Multicopper oxidases only work under aerobic conditions and *E. coli* has to rely on another copper homeostatic system to safeguard the periplasm under anaerobic conditions: The *cus* determinant is formed by two operons, *cusRS* and *cusCFBA*, which are transcribed in opposite directions (Fig. 6). The *cusRS* operon encodes a histidine kinase, CusS, and a response regulator, CusR. This so called two component regulatory system initiates the transcription of *cusCFBA* in a copper dependent manner (82, 83). The components of *cusCFBA* form a proton-cation antiporter complex spanning both membranes and the periplasmic space. CusA is an inner membrane protein belonging to the resistance nodulation cell division family of proteins. It functions as a proton-substrate antiporter and is responsible for substrate specificity. CusB is a membrane fusion protein and serves as an adaptor to link CusA to the outer membrane factor CusC (3). CusF is a 10-kDa periplasmic protein. Yeast two hybrid experiments suggested that CusF interacts with CusB and CusC and may function as a periplasmic copper chaperone (83). The CusCFBA system works under aerobic as well as under anaerobic conditions (84).

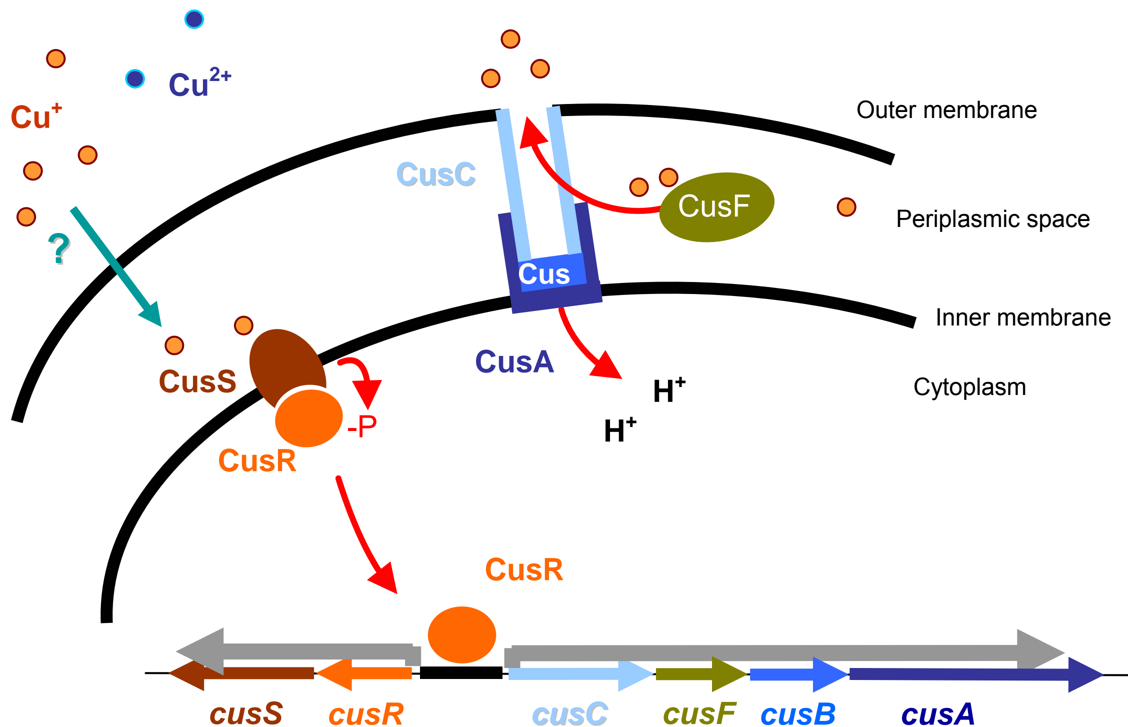


Fig. 6. The *cus* system of *E. coli*. The two component regulatory system CusRS senses periplasmic copper and activates the transcription of the *cusCFBA* and *cusRS* operons. The CusCBA complex forms a channel bridging the periplasmic space. The chaperone CusF delivers copper ions to this complex via the periplasmic space. Copper is expelled to the extracellular space in exchange for a proton. Adapted from (3).

Some *E. coli* strains possess additional plasmid encoded genes to prevent intoxication by copper. The *pco* determinant encodes genes that detoxify copper in the periplasm and support the chromosomally encoded homeostatic mechanisms (85).

E. coli possesses several copper containing proteins. Copper-zinc superoxide dismutase and amine oxidase in the periplasm and NADH dehydrogenase 2 and *bo*$_3$-type cytochrome oxidase located in the cytoplasmic membrane (86). Nevertheless, it is still unknown by which pathways copper enters the cells. This question was experimentally addressed by using a plasmid borne biosensor which responds to cytoplasmic copper levels. The biosensor consisted of the *lux* gene cluster of *Vibrio fischeri* and was placed under the control of the *E. coli copA* promoter (87). An increase in luminescence therefore directly reflected a higher level of intracellular copper. An *E. coli* mutant showing decreased luminescence under copper stress was further analyzed. The mutant strain contained a transposon insertion in the *ycfQ* gene which encodes a regulator for the *ycfR* gene. The interaction of YcfQ with *ycfR* and a possible role for the predicted outer membrane protein YcfR in the process of copper entry are discussed in chapter II, section 2.

Chapter II: Publications and Manuscripts

1) Structure and Function of CinD (YtjD) of *Lactococcus lactis*, a Copper-Induced Nitroreductase Involved in Defense Against Oxidative Stress

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Abstract

In *Lactococcus lactis* IL1403, 14 genes are under the control of the copper-inducible CopR repressor. This so-called CopR regulon encompasses the CopR regulator, two putative P-type copper ATPases, a copper chaperone, and 10 additional genes of unknown function. We addressed here the function of one of these genes, *ytjD*, which we renamed *cinD* (copper-induced nitroreductase). Copper, cadmium, and silver induced *cinD in vivo*, as shown by real-time quantitative PCR. A knockout mutant of *cinD* was more sensitive to oxidative stress exerted by 4-nitroquinoline-*N*-oxide and copper. Purified CinD is a flavoprotein and reduced 2,6-dichlorophenolindophenol and 4-nitroquinoline-*N*-oxide with k_{cat} values of 27 and 11 s^{-1}, respectively, using NADH as a reductant. CinD also exhibited significant catalase activity *in vitro*. The X-ray structure of CinD was resolved at 1.35 Å and resembles those of other nitroreductases. CinD is thus a nitroreductase which can protect *L. lactis* against oxidative stress that could be exerted by nitroaromatic compounds and copper.

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Structure and Function of CinD (YtjD) of *Lactococcus lactis*, a Copper-Induced Nitroreductase Involved in Defense against Oxidative Stress[▽]

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In *Lactococcus lactis* IL1403, 14 genes are under the control of the copper-inducible CopR repressor. This so-called CopR regulon encompasses the CopR regulator, two putative CPx-type copper ATPases, a copper chaperone, and 10 additional genes of unknown function. We addressed here the function of one of these genes, *ytjD*, which we renamed *cinD* (copper-induced nitroreductase). Copper, cadmium, and silver induced *cinD in vivo*, as shown by real-time quantitative PCR. A knockout mutant of *cinD* was more sensitive to oxidative stress exerted by 4-nitroquinoline-*N*-oxide and copper. Purified CinD is a flavoprotein and reduced 2,6-dichlorophenolindophenol and 4-nitroquinoline-*N*-oxide with k_{cat} values of 27 and 11 s^{-1}, respectively, using NADH as a reductant. CinD also exhibited significant catalase activity *in vitro*. The X-ray structure of CinD was resolved at 1.35 Å and resembles those of other nitroreductases. CinD is thus a nitroreductase which can protect *L. lactis* against oxidative stress that could be exerted by nitroaromatic compounds and copper.

Lactococcus lactis IL1403 is a Gram-positive lactic acid bacterium which is used for the manufacture of food and dairy products but also for an increasing number of biotechnological applications. Given the economical importance of this microorganism, it is often used as a model for molecular studies. Its genome has been sequenced (4), and its proteome has been extensively characterized (11). When applied to industrial processes, this bacterium has to face various stress conditions, such as low pH, high temperature, osmotic shock, and metal stress (44). For instance, in traditional cheese making in Switzerland, *L. lactis* is exposed to copper released from the copper vats.

Copper is an essential micronutrient for both prokaryotes and eukaryotes. The two oxidation states of copper, Cu^{+} and Cu^{2+}, allow its participation in many important biological functions. More than 30 enzymes are known to use copper as a cofactor, such as superoxide dismutase (SOD), cytochrome *c* oxidase, or lysyl oxidase (20). The redox activity of copper can also lead to the generation of free radicals, which cause cellular damage (42, 43). Recently, alternative copper toxicity mechanisms have been demonstrated in bacteria in which copper interferes with the formation of catalytic iron-sulfur clusters (6, 22). Whatever the mechanism of copper toxicity, maintenance of copper homeostasis by controlling the uptake, accumulation, detoxification, and removal of copper is critical for living organisms.

Copper homeostasis in *L. lactis* has not yet been investigated in great detail but appears to resemble the well-characterized copper homeostatic system of *Enterococcus hirae* (34). *L. lactis* possesses a *copRZA* operon, which provides copper resistance. It encodes the CopA copper export ATPase, the CopR copper-inducible repressor, and the CopZ copper chaperone (23). CopR regulates not only the *copRZA* operon but also an additional 11 genes. This so-called CopR regulon also includes *copB*, encoding a second putative copper ATPase; *lctO*, encoding lactate oxidase; and the *ydiDE*, *yahCD-yaiAB*, and *ytjDBA* operons of unknown function. Of all the genes and operons constituting the CopR regulon, the *ytjDBA* operon was most strongly induced by copper (23). Based on sequence comparison, the first gene of this operon, *ytjD*, encodes an oxygen-insensitive nitroreductase, which we renamed *cinD* for copper-induced nitroreductase.

Nitroreductases are called oxygen insensitive when they can catalyze the two-electron reduction of nitro compounds in the presence of oxygen. Such enzymes are widespread in nature and are able to reduce a wide range of substrates, such as furazones, nitroaromatic compounds, flavins, and ferricyanide, using NADH or NADPH as the reductant. They are flavoproteins of 22 to 24 kDa and form homodimers with one flavin mononucleotide cofactor per monomer. Although oxygen-insensitive nitroreductases have been extensively studied, their *in vivo* function remains largely unknown. The closest relative of CinD, which has functionally been studied, is FRP of *Vibrio harveyi*, with 29% sequence identity to CinD. FRP is not a typical nitroreductase but appears to function as an NADH flavin oxidoreductase which provides reduced flavin to luciferase (19). The next closest relative of CinD, NfsA of *Escherichia coli*, with 23% sequence identity, exhibits the broad substrate specificity typical of most nitroreductases (48). The structure of this enzyme has been solved at a resolution of 1.7 Å (17). It closely resembles the structures of other enzymes which belong to the oxygen-insensitive nitroreductase family. NfsA has re-

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cently been shown to participate in the degradation of 2,4,6-trinitrotoluene (10). This suggests that an important function of nitroreductases could be the metabolism of xenobiotics.

We investigated here the structure and function of CinD of *L. lactis*. CinD was induced by copper, cadmium, and silver and protected *L. lactis* from oxidative stress exerted by 4-nitroquinoline-*N*-oxide (NQO). The purified enzyme is a flavoprotein and exhibited nitroreductase activity on NQO and a variety of other substrates, using NADH as the reductant. CinD also possesses catalase activity and is thus able to defend cells against oxidative stress exerted by hydrogen peroxide, xenobiotics, or copper. The three-dimensional structure of CinD was resolved at a 1.35-Å resolution and exhibits a typical nitroreductase structure.

MATERIALS AND METHODS

Chemicals. Medium components were obtained from Axon Lab AG, Dättwil, Switzerland. Chemicals for protein extractions and enzymatic assays were supplied by Merck (Darmstadt, Germany), DNA nucleases were supplied by Roche Biochemicals (Switzerland), the plasmid pProExHTa and recombinant tobacco etch virus (rTEV) protease were supplied by Invitrogen, Carlsbad, CA, and the Ni-nitrilotriacetic acid-agarose (Ni-NTA-agarose) chromatographic matrix was supplied by Qiagen. Oligonucleotides were synthesized by Microsynth (Balgach, Switzerland). Proofreading DNA polymerases were obtained from Takara Bio Inc. (Otsu, Japan), Roche Biochemicals (Switzerland), and Stratagene (La Jolla, CA). NQO (Sigma-Aldrich, St. Louis, MO) was dissolved in dimethyl sulfoxide (DMSO) at 100 mM and stored at −20°C. Working solutions were diluted from this stock in DMSO on the day of use.

Bacterial strains and culture conditions. *L. lactis* IL1403 was obtained from Emmanuelle Maguin (INRA, Jouy-en-Josas, France) and was grown semi-anaerobically (air-saturated media in sealed bottles), in M17 media (39) at 30°C or on plates containing M17 media with 1.5% agar (AppliChem, Darmstadt, Germany). Milk for growth of *L. lactis* was prepared by autoclaving a 10% solution of milk powder (Difco) at 121°C for 15 min. Growth in milk was followed either by plating and assessing the number of CFU or by clarifying samples by the addition of 4 volumes of 15 mM Na-EDTA, pH 12, and measuring the absorption at 600 nm. *E. coli* Top10 (Invitrogen) or DH5α (Stratagene, La Jolla, CA) cells used for cloning were transformed according to manufacturer's instructions. *E. coli* strains were cultivated aerobically at 37°C in LB media (32) with appropriate antibiotics. To determine growth inhibition zones, 200 μl of stationary-phase cultures was spread on M17 plates, and 1.5-cm-diameter cellulose filter disks with the required chemicals were applied to the plates. Plates were incubated at 30°C, and the growth inhibition zones were measured after 16 h. To determine the growth rates of *L. lactis* in liquid cultures, 1 ml of M17 media in capped, disposable spectrophotometric cuvettes was inoculated with a 1/50 volume of cells which had been frozen in the logarithmic growth phase in 17% glycerol at −70°C. After growth for 1 h at 30°C, growth inhibitors were added, and growth was monitored at 600 nm with a Lambda 16 spectrophotometer (PerkinElmer Life Sciences). For competition assays between *L. lactis* strains, 25-ml cultures in M17 media were grown for 26 generations by four successive 100-fold dilutions into fresh media every 24 h. After four transfers, the numbers of CFU of wild-type and Δ*cinD* cells were determined by plating serial dilutions on M17 plates with and without erythromycin and counting the numbers of CFU.

Real-time quantitative PCR. For RNA isolation, fresh mid-log cultures of *L. lactis* IL1403 in M17 medium were induced as indicated below in Results for 45 min at 30°C. Cells from 1 ml of culture were harvested by centrifugation, and the pellet was used immediately for RNA isolation with the RNeasy kit from Qiagen, according to the manufacturer's instructions. The optional treatment with DNase I (RNase free) for 30 min at room temperature was included. RNA concentrations were measured with a NanoDrop ND-1000 spectrophotometer. RNA quality was assessed on 1.2% agarose-formaldehyde gels stained with ethidium bromide. RNA was stored at −70°C. cDNA was reverse transcribed from 1 μg of total RNA per reaction using the iScript cDNA synthesis kit (Bio-Rad), according to the manufacturer's protocol. Real-time quantitative PCR was performed on the LightCycler (Roche), using SYBR Premix Ex *Taq* (Takara Bio Inc., Otsu, Japan), according to the manufacturer's instructions. Quantitative PCR conditions used were as follows: 1 cycle at 95°C for 10 s plus 40 cycles of denaturation at 95°C for 6 s, annealing at 60°C for 20 s, and extension at 72°C for 45 s. After the LightCycler PCRs, the products were analyzed for specificity and homogeneity by a melting curve analysis and additionally by electrophoresis on 1.2% agarose gels. For quantitative PCR, primers ytjD3 (5′-GCACAAGGTGTTCC TGAATC) and ytjD4 (5′-TCAGCAAGAGCAGTCCATAC) were used to measure *cinD* gene expression. 16S RNA was determined with primers dm7 (5′-GT GGCTCAACCATTGTATGC) and dm8 (5′-AGCCTCAGTGTCAGTTAC AG). Real-time quantitative PCR results were expressed relative to 16S RNA.

Construction of the CinD expression vector pHYDSA1. All plasmids were purified from *E. coli* DH5α using the large-scale NucleoBond AX plasmid isolation kit (Macherey-Nagel, Oensingen, Switzerland). The *cinD* gene of *L. lactis* IL1403 was cloned by PCR amplification of genomic *L. lactis* IL1403 DNA with the primers fm1 (5′-ATATGGCGCCATGTCATTCATTAAATCATTAG) and fm2 (5′-GCATGCAGCTTTTAAGAGCTCAACTTTAC). The resultant PCR product was cut with NarI and SacI and ligated into the histidine tag expression vector pProExHTa, resulting in pHYD2. This construct contained an A69S mutation in the *cinD* gene, which was mutated back to the wild-type sequence with the Chameleon mutagenesis kit (Invitrogen), as detailed by the manufacturer, resulting in pHYDSA2. The vector expressed a CinD fusion protein with an N-terminal six-histidine tag and a cleavage site for rTEV protease to remove the His tag. Cleaved CinD had the N-terminal methionine replaced by the sequence Gly-Ala-Arg.

CinD expression and purification. *E. coli* DH5α (Invitrogen) transformed with expression plasmid pHYDSA2 was grown at 37°C in Luria-Bertani broth containing 100 μg/ml ampicillin to mid-log phase, cooled to 30°C, and induced with 1 mM isopropyl-β-D-thiogalactoside. Growth was continued for 4 h before the cells were harvested by centrifugation at 5,000 × *g* for 10 min at 4°C. Cell pellets were suspended and washed twice with 140 mM NaCl and 20 mM NaP_i, pH 7. All buffers used in subsequent steps were supplemented with 10 μM flavin mononucleotide (FMN) to prevent loss of the cofactor. The final pellet was resuspended in buffer A (50 mM Na-HEPES, 50 mM K_2SO_4 at pH 7.5, 1 mM β-mercaptoethanol), and a 1/100 volume of a protease inhibitor cocktail (100 mM *N*-α-*p*-tosyl-L-lysine-chloromethyl ketone, 100 mM *N*-*p*-tosyl-L-phenylalanine-chloromethyl ketone, 100 mM *p*-aminobenzamidine, 100 mM phenyl-methyl-sulfonyl-fluoride dissolved in dimethyl sulfoxide) was added. Cells were broken by three passages through a French press at 40 MPa. Debris was removed by centrifugation for 10 min at 3,000 × *g* in the cold. The supernatant was centrifuged at 90,000 × *g* for 30 min at 4°C, filtered, and loaded onto a 2- by 8-cm Ni-nitrilotriacetic acid (Ni-NTA)-agarose column, preequilibrated with buffer A. The column was washed with 5 column volumes of buffer A containing 20 mM imidazole, and CinD was eluted with 10 column volumes of a linear 0- to 200-mM imidazole gradient in buffer A. The fractions were analyzed by electrophoresis on 12% sodium dodecyl sulfate (SDS)-polyacrylamide gels and stained with Coomassie blue as described previously (18). The CinD-containing fractions were pooled, dialyzed against 50 mM Na-HEPES and 50 mM K_2SO_4, pH 7.4, concentrated 10-fold with a Centricon concentrator, and frozen at −70°C. For removal of the histidine tag, 600 μg of purified CinD was incubated with 100 U of rTEV protease for 4 h at 30°C. The proteolysis reaction mixture was passed through an Ni-NTA-agarose column in 50 mM Na-HEPES and 50 mM K_2SO_4, pH 7.0, and the flowthrough containing cleaved CinD was collected. For crystallization, CinD was further purified by gel filtration on a Superdex S75 column (GE Healthcare, Switzerland) in 300 mM NaCl, 25 mM Tris-Cl at pH 7.5, and 5 mM dithiothreitol. The purified protein was concentrated to 20 mg/ml and stored at −70°C. Samples for crystallization trials were supplemented with 1 mM FMN. Protein concentrations were determined with the Bio-Rad protein assay, using bovine serum albumin as a standard, except for that of pure CinD, which was estimated spectrophotometrically at 280 nm, using the extinction coefficient ε of 1.66 mg^{-1} cm^{-1}.

Construction of the Δ*ytjD* mutant strain. An internal fragment of the *cinD* gene was PCR amplified from plasmid pHYD2 using primers fm33 (5′-GGCG GAAGCTTCACCAACAGCTTTCAATTC) and fm34 (5′-CGCGACTAGTTA GCAACAGCTTCGTCAATC). The resultant 399-bp product was digested with HindIII-SpeI and ligated into plasmid pTE1 (3), digested with the same enzymes. The resulting plasmid, pTEYD1, was propagated in *E. coli* DH5α in LB media with 40 μg/ml of kanamycin or 500 μg/ml of erythromycin. Plasmid integrity was checked by restriction analysis. For gene knockout, *L. lactis* IL1403 was transformed with pTEYD1 by electroporation as described previously (9). Transformants were selected at 30°C on agar plates with brain heart infusion media (Difco Laboratories, Detroit, MI), containing 5 μg/ml of erythromycin. Clones were screened for Δ*cinD* mutants by PCR amplification of genomic DNA, using primer fm18 (5′-GCTGCACCAGAAGGAGTGAAAGC), located upstream of the *cinD* gene, and primer fm23 (5′-AAGCTAAGGGCGAATTCCAGCAC), located on the pTEYD1 plasmid. The correctness of the junction between plas-

mid and genomic DNA was verified by restriction analysis of the resultant PCR fragments.

Western blot analysis of CinD expression. Antibodies to CinD were raised by injecting rats with an initial subcutaneous injection of 60 μg of gel-purified, cleaved CinD, followed by two intravenous booster injections of 60 μg CinD each. Serum samples were coagulated overnight at 4°C, and the clear supernatant was used for Western blotting. Cultures in 10 ml of M17 media were grown to an optical density at 546 nm (OD_{546}) of 0.6 to 0.8, followed by induction with 1 mM $CuSO_4$ for 90 min when required. Cell lysates were prepared by centrifuging the cultures and treating the cell pellets with 200 μl of 10 mg/ml lysozyme, 1 mM EDTA, and 10 mM Tris-Cl at pH 8 on ice for 15 min. The lysates were treated with 40 μg of DNase I in 15 mM $MgCl_2$ for 10 min at 0°C and centrifuged. The supernatants were denatured in SDS sample buffer, and 10 μg of each extract was resolved by electrophoresis on a 15% Tricine-SDS-polyacrylamide gel (33). Western blots were prepared as previously described (41), using a horseradish peroxidase-coupled goat anti-rat IgG secondary antibody (Santa Cruz). Bands were visualized by chemiluminescence using homemade reagents. Chemiluminescence signals were captured with a Fuji LAS-1000 imaging system (Fuji Photo Film, Tokyo, Japan).

Nitroreductase assay. The reductase activity of purified CinD was measured spectrophotometrically at 25°C. The reaction mixture contained in 1 ml of 50 mM Tris-Cl at pH 7.5, 0.05 mM NADH, and 0.05 mM substrate. The reaction was started by the addition of enzyme, and the change in absorption of either NADH or substrate was monitored over time. The following molar absorption coefficients were used: 6,220 M^{-1} cm^{-1} for NADH at 340 nm, 20,600 M^{-1} cm^{-1} for 2,6-dichlorophenolindophenol (DCPIP) at 600 nm, and 1,000 M^{-1} cm^{-1} for $K_3[Fe(CN)_6]^{3-}$ at 420 nm. Self-absorption at 340 nm of substrates was corrected as follows: $\varepsilon_{NADH}/|[\varepsilon_{NADH} + (\varepsilon_{Sox} - \varepsilon_{Sred})]|$, where ε_{Sox} and ε_{Sred} are the extinction coefficients of the oxidized and reduced substrates, respectively.

Oxygen measurements. The catalase activity of CinD was measured by recording the production of oxygen from hydrogen peroxide with a Clark electrode. The reactions were performed at 25°C in a 1-ml chamber containing 50 mM Tris-Cl at pH 7.5 and various amounts of H_2O_2. Reactions were started by adding 3 μg of purified CinD.

Crystallization and structure determination. Crystals were grown at 20°C by vapor diffusion with hanging drops, mixing 2 volumes of protein to 1 volume of crystallization buffer (100 mM Tris-Cl at pH 8, 200 mM $MgCl_2$, 20% [wt/vol] polyethylene glycol 3350, 2% 2-methyl-2,4-pentanediol). Crystals were cryoprotected using the crystallization buffer supplemented with 20% 2-methyl-2,4-pentanediol and flash cooled in liquid nitrogen. Diffraction data were collected at 100 K at the Swiss Light Source (Paul Scherer Institute, Villigen, Switzerland) at beamline X06DA to a 1.35-Å resolution and processed using XDS (15). The structure was solved by molecular replacement using the program PHASER (25) and the nitroreductase structure from *Streptococcus mutans* as the search model (Protein Data Bank [PDB] accession number 2IFA) (Northeast Structural Genomics Consortium, unpublished data). Data collection and refinement statistics are given in Table 1.

Protein structure accession number. The CinD structure was deposited in the PDB under accession number 2WQF.

TABLE 1. Data collection and refinement statistics

Parameter	Value(s)[a]
Space group properties	
Space group	C222$_1$
a, *b*, *c* (Å)	54.10, 122.22, 69.05
α, β, γ (Å)	90.0, 90.0, 90.0
No. of molecules/ASU[b]	1
Data collection statistics	
Beamline	X06DA
Wavelength (Å)	1.000
Resolution range (Å)	50.0–1.35
No. of observations	433,802
No. of unique reflections	48,865
Completeness (%)	99.2 (97.3)
R_{merge} (%)[c]	9.4 (58.4)
$I/\sigma(I)$	12.36 (1.9)
Refinement statistics	
Resolution range (Å)	32.13–1.35
No. of reflections, working set	47,384
No. of reflections, test set	1,466
R/R_{free} (%)	13.15/15.55
No. of atoms	
Protein	3,253
Ligand	51
Water	300
RMS deviations	
Bond length (Å)	0.007
Bond angle (°)	0.863
Ramachandran plot (%)	
Most favored	98.33
Allowed	1.67
Disallowed	0.00
B-factors (Å)	
Main chain	19.1
Side chain and water	27.7

[a] The values in parentheses for the resolution range, completeness, R_{merge}, and $I/\sigma(I)$ correspond to the outermost-resolution shell. Data collection and refinement statistics values were measured using XDS and Phenix, respectively.
[b] ASU, asymmetric units.
[c] $R_{merge} = \Sigma_{hkl}\Sigma_j |I(hkl; j) - \{I(hkl)\}|/[\Sigma_{hkl}\Sigma_j \{I(hkl)\}]$, where I(hkl; j) is the jth measurement of the intensity of the unique reflection (hkl) and {I(hkl)} is the mean overall symmetry-related measurement.

RESULTS

Regulation of CinD expression by metal ions. A global proteomics and bioinformatics analysis of the response of *L. lactis* IL1403 to copper exposure led to the identification of a copper-inducible nitroreductase, encoded by the *ytjD* gene (GenBank accession number NP_268070), which we here rename *cinD* (23). To learn more about the function of CinD in copper homeostasis and stress response, we analyzed the induction of the *cinD* gene in more detail. By real-time quantitative PCR, dose-dependent upregulation of *cinD* expression by copper was observed. Maximal induction was measured in cells induced with 1 mM Cu^{2+} (Fig. 1A). Among other metals tested, 200 μM Cd^{2+} induced *cinD* to an extent similar to that of copper, while Ag^{+} at the same concentration induced *cinD* to about 60% of these levels (Fig. 1B). Zn^{2+}, Fe^{2+}, Ni^{2+}, Mn^{2+}, and Ca^{2+} failed to stimulate *cinD* expression. The *cinD* gene is under the control of CopR (23), a repressor homologous to CopY of *E. hirae*. Induction of genes under the control of CopR proceeds via formation of a binuclear Cu(I)-thiolate cluster which displaces the bound Zn^{2+} and releases the repressor from the DNA. Other soft metals with a preponderance to form thiolates, like Ag^{+} and Cd^{2+}, were also found to induce the closely related CopY repressor of *Enterococcus hirae* (30). Induction of *cinD* by copper, silver, and cadmium is thus in line with the expectations for a gene under the control of the CopY-like repressor CopR. No significant or marginal induction of *cinD* by nitrosative (*S*-nitrosoglutathione [GSNO]) or oxidative stress (paraquat, H_2O_2) was observed, suggesting that oxidative or nitrosative stress does not directly induce *cinD*.

Overexpression and purification of CinD. To determine the catalytic activity of CinD, an *E. coli* expression system was set up by constructing the plasmid pHYDSA2. It contained an isopropyl-β-D-thiogalactoside-inducible fusion of CinD with a cleavable N-terminal 6×His tag. This allowed for the expression of 6×His-CinD in copious amounts. It was purified by Ni-NTA-agarose affinity chromatography, followed by cleavage of the 6×His tag with rTEV protease. CinD could be purified to better than 95% purity in a single affinity purifica-

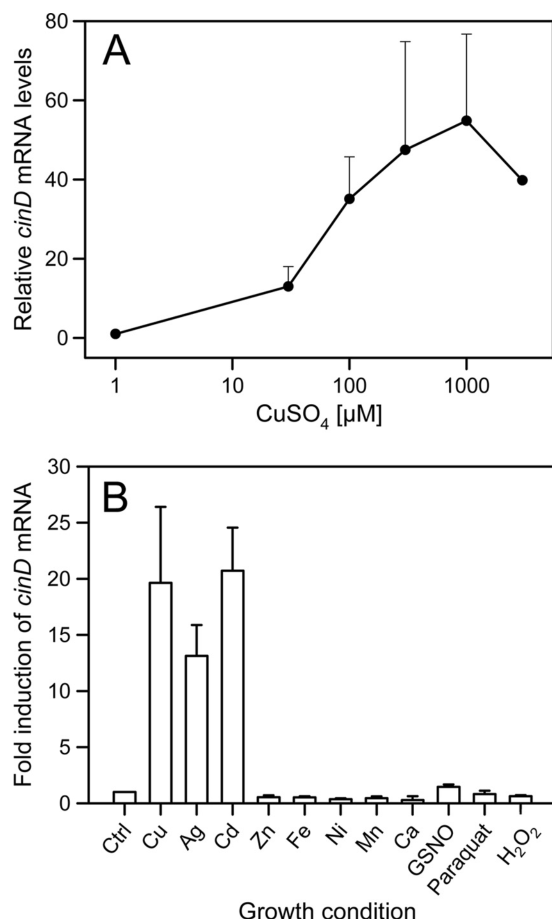

FIG. 1. Real-time quantitative PCR analysis of *cinD* mRNA expression. (A) The induction of *cinD* mRNA was measured as a function of the copper concentration in media. Cells were grown semianaerobically and induced for 45 min with copper at the concentrations indicated on the abscissas. The error bars show standard deviations based on three independent experiments. (B) Induction of *cinD* mRNA was measured in response to different stress conditions. Cells were grown semianaerobically and induced for 45 min with the chemicals indicated on the abscissas. The following metal salts were used at 200 μM: $CuSO_4$, $AgNO_3$, $CdSO_4$, $ZnSO_4$, $FeCl_2$, $NiSO_4$, $MnCl_2$, and $CaSO_4$. GNSO, paraquat, and H_2O_2 were used at concentrations of 4 mM, 20 mM, and 1 mM, respectively. The error bars show standard deviations based on three independent experiments. All values are statistically different ($P < 0.01$) from each other (Kruskal-Wallis one-way analysis of variance on ranks).

tion step, with an average yield of 10 mg of recombinant protein per liter of culture (Fig. 2). Purified CinD was highly soluble and had the expected molecular mass of 22.6 kDa. CinD solutions were yellow and exhibited a typical FMN absorption spectrum (Fig. 3). Based on an extinction coefficient of 12,500 M^{-1} cm^{-1} for FMN, purified CinD contained 0.91 FMN molecules per monomer. This is reasonably close to the expected stoichiometry of one FMN per monomer.

Characterization of the activity of CinD. The sequence similarity between CinD and bacterial flavoproteins of the nitroreductase family suggested that CinD could have a similar function. A range of substrates typical for this class of enzymes was tested, using NADH as the reductant. The highest k_{cat} value, 27 s^{-1}, was measured with DCPIP. The K_m value for this

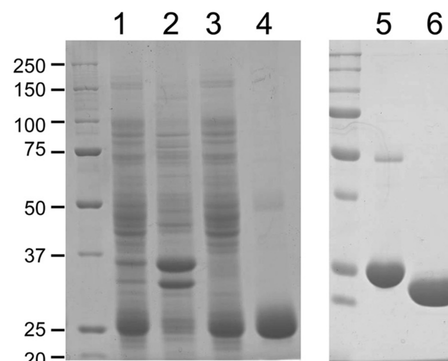

FIG. 2. Affinity purification of CinD and His tag cleavage. Proteins were resolved on 12% sodium dodecyl sulfate gels and stained with Coomassie blue. Lane 1, 10 μg of crude bacterial lysate; lane 2, 10 μg of protein from the pellet obtained by ultracentrifugation of the lysate; lane 3, 10 μg of protein from the supernatant from ultracentrifugation; lane 4, 6 μg of protein eluted from a Ni-NTA-agarose column with 200 mM imidazole; lane 5, 6 μg of uncleaved 6×His-CinD; lane 6, 6 μg of rTEV-cleaved CinD after dialysis. The sizes of molecular mass markers are indicated in kDa on the left.

reaction was 79 μM, and the V_{max} value was 383 μmol min^{-1} mg^{-1} (Fig. 4). A substantial k_{cat} value of 11 s^{-1} was also measured with NQO, while activities below 10% of the maximal activity for a range of other substrates were recorded, including methylene blue (2.6 s^{-1}), 4-chloro-7-nitrobenzofurazan (0.83 s^{-1}), and $K_3[Fe(CN)_6]$ (0.69 s^{-1}). No substrate reduction in the presence of 50 μM FMN but without enzyme was observed. CinD failed to reduce many known substrates of nitroreductases, like nitrofurazone, 4-nitroanisol, 4-nitrophenol, chloramphenicol, GW9662, nitroprusside, methyl-3-nitro-1-nitrosoguanidin, 2,4,6-trinitrophenol, a range of differently halogenated nitrobenzenes, nitroaniline, 3-nitro-L-tyrosine, sulfafurazol, nitrosocysteine, nitrosoglutathione, nitrophenylphosphate, flavin adenine dinucleotide (FAD), and FMN. Both NADH and NADPH could serve as electron donors, but NADH resulted in four times higher specific activities. Thus, CinD is an oxygen-insensitive nitroreductase and exhibits rather narrow substrate specificity. CinD was also found to possess significant catalase activity (Fig. 5). This reaction was NAD(P)H independent, was not catalyzed by FMN alone, and exhibited a K_m of 31 mM and a V_{max} of 68 μmol min^{-1} mg^{-1}.

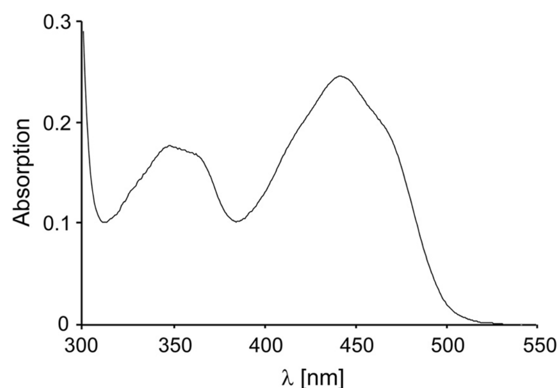

FIG. 3. Absorption spectrum of CinD. The spectrum was recorded with 23 μM of purified CinD in 50 mM Tris-Cl, pH 8, at 25°C.

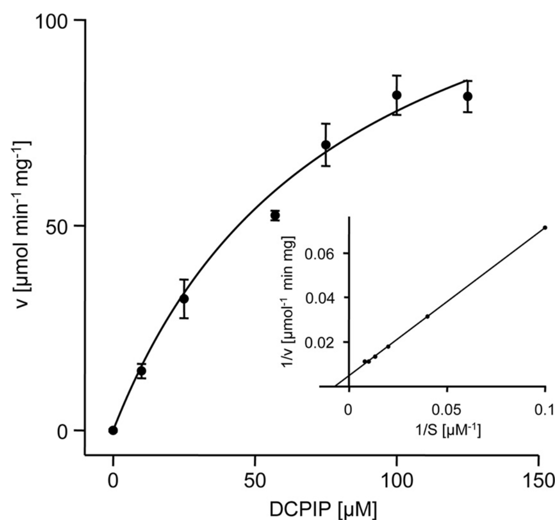

FIG. 4. Kinetic analysis of DCPIP reduction by CinD. DCPIP reduction by 3 µg of CinD was monitored by following the oxidation at 340 nm of NADH. Reduction rates were fitted with Michaelis-Menten kinetics using SigmaPlot. (Inset) Lineweaver-Burk plot of the same data.

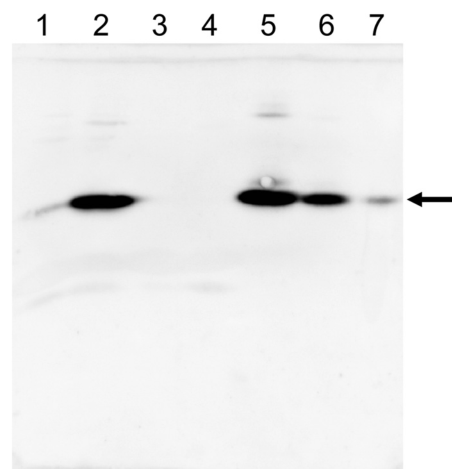

FIG. 6. CinD expression in the wild type and the Δ*cinD* strain and induction by copper. CinD expression was assessed by Western blotting, developed with a CinD antibody. Lane 1, uninduced wild type; lane 2, wild type induced with 1 mM $CuSO_4$; lane 3, uninduced Δ*cinD*; lane 4, Δ*cinD* induced with 1 mM $CuSO_4$; lanes 5 to 7, 60, 6, and 0.6 ng of purified CinD, respectively. Induction in all cases was for 90 min. The arrow points to the position of CinD.

While the K_m for this reaction is relatively low, the activity may still be biologically relevant; *L. lactis* and some related organisms do not possess catalase and accumulate hydrogen peroxide in millimolar concentrations (4, 45).

Effect of *cinD* gene inactivation. To obtain information about the function of CinD *in vivo*, a Δ*cinD* strain was constructed by homologous, Campbell-like recombination with plasmid pTEYD1, carrying a fragment of the *cinD* gene. Insertion of pTEYD1 into the genome at the correct site was confirmed by PCR, and the absence of the CinD protein in the knockout mutant was demonstrated by Western blotting with an antibody against CinD (Fig. 6). The Δ*cinD* strain exhibited increased sensitivity toward NQO (Fig. 7). There was complete growth inhibition of the Δ*cinD* mutant by 300 µM NQO, while wild-type cells still grew at a growth rate of 35% of that observed in the absence of NQO. The increased sensitivity of the Δ*cinD* strain to NQO was even more apparent on solid growth media by the large zone of growth inhibition around an NQO-soaked filter disk (Fig. 7, inset). NQO causes oxidative stress and is a good substrate of CinD *in vitro*; NQO reduction apparently diminishes the oxidative stress exerted by this agent. DCPIP, which was the best substrate of CinD *in vitro*, was essentially nontoxic to both the wild type and the Δ*cinD* mutant. NQO, like paraquat and hydrogen peroxide, did not induce expression of CinD (not shown).

Although NQO primarily causes oxidative stress, we also investigated a possible role of CinD in defense against nitrosative stress. Protein nitrosylation was indistinguishable in the wild type and the Δ*cinD* mutant, as assessed with an anti-nitrosocysteine antibody and the biotin switch detection method for nitrosylated proteins (8). There was also no phenotypic difference between the wild type and the *cinD* mutant strain when grown in the presence of agents that cause nitrosative stress, such as nitrosoglutathione, nitrosocysteine, ni-

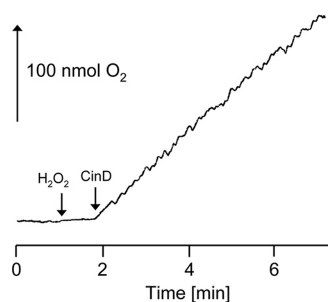

FIG. 5. Catalase activity of CinD. Purified CinD (3 µg) was incubated with 10 mM H_2O_2, and oxygen evolution was measured with a Clark electrode. The arrows indicate the additions of H_2O_2 and CinD.

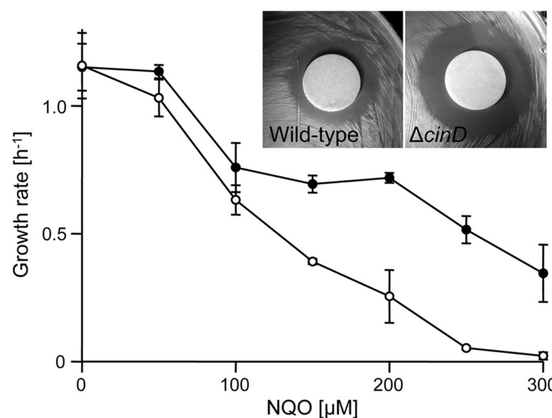

FIG. 7. Effect of *cinD* inactivation on the sensitivity toward 4-nitroquinoline-*N*-oxide. *L. lactis* wild-type (●) and Δ*cinD* (○) cells were grown in 1-ml cuvettes containing M17 medium supplemented with 0 to 300 µM NQO. Standard deviations calculated from three independent experiments are depicted with error bars. (Inset) Phenotype of Δ*cinD* under oxidative stress. Filter disks soaked with 25 mM NQO were applied to bacterial lawns of *L. lactis* wild-type and Δ*cinD* cells. The plates were incubated overnight at 30°C.

trosocysteinyl-glycine, diethylamine, NONOate, and nitrite (not shown). So, a role of CinD in the defense against nitrosative stress appears unlikely.

Since CinD expression is regulated by copper, we examined the copper sensitivity of the Δ*cinD* strain. In standard growth experiments, growth inhibition of the wild type and the Δ*cinD* mutant by copper appeared to be the same. A greater sensitivity of the Δ*cinD* mutant toward copper could, however, be observed in growth competition experiments. Cultures in M17 media were inoculated with a 100-fold excess of the Δ*cinD* mutant over the wild type (1.5×10^9 and 1.5×10^7 CFU, respectively) and grown in the presence or absence of 0.5 mM copper for 26 generations by serial transfers. In the final cultures, the ratio of the wild type to Δ*cinD* was $(5.82 \pm 0.06) \times 10^6$ CFU when grown with copper and $(1.8 \pm 1.4) \times 10^3$ CFU when grown without copper. This clearly shows a growth advantage of the wild type over the Δ*cinD* mutant, which is strongly accentuated by copper. We also performed a series of growth experiments in milk to mimic a natural environment of *L. lactis*. In growth media prepared from milk powder, wild-type cells invariably exhibited a growth advantage and better survival over the Δ*cinD* mutant in the presence of copper. Unfortunately, these experiments were difficult to quantify and are thus not shown. The main problem was the different cell morphologies observed in milk. While the wild type preferentially grew as cell pairs, the Δ*cinD* mutant grew in chains of 8 to 12 cells. Copper shifted these morphologies to more single cells for the wild type and spherical clumps of many cells for the Δ*cinD* mutant. This made it difficult to enumerate living cells reliably, either by optical density or by plating and counting of the number of CFU. Yet, it appears clear that expression of CinD provides wild-type cells with a growth advantage under stress conditions exerted by copper and oxidative stress-inducing chemicals.

Crystal structure of CinD. The crystal structure of the CinD-FMN complex was refined at a 1.35-Å resolution, with good statistics (Table 1). The structure is very well defined; only residues 75 to 86, forming a flexible loop, have very weak electron densities. A DALI search (13) revealed a hypothetical protein, SMU.260, with *S. mutans* (50% sequence identity, root mean square [RMS] deviation of 1.2 Å for 200 residues; PDB accession number 2IFA) and *Bacillus cereus* ATCC 14579 (41% sequence identity, RMS deviation of 1.3 Å; PDB accession number 1YWQ) having the most similar entries in the PDB. Other database entries classified as similar have sequence identities of 23% and less.

Like other nitroreductases, CinD forms a homodimeric structure in which the monomer possesses an α+β fold and the FMN is positioned at the dimer interface (Fig. 8A), i.e., the active site is built by residues of both subunits. A total of 2,521 $Å^2$ of solvent-accessible surface per monomer becomes buried upon dimer formation, with a total monomer surface of 10,891 $Å^2$. Both termini of the CinD monomers extend into the direction of the dimer partner. The central β-sheet is formed by four antiparallel strands together with the parallel β5 strand from the C terminus of the other subunit. The five-stranded β-sheet is shielded by two and three α-helices, respectively.

The dimer interface is built up by hydrophobic interactions formed by the short α1-helices (Phe3-Glu8) and the α6-helices (Ala119-Ala141) from both subunits. Residues from the N-terminal-extending α1-helix (Phe3, Ser6, Leu7, and Glu8) are buried in a hydrophobic cleft of the neighboring subunit (formed by residues Phe3, Ser6, Phe36, Asn136, Thr139, Ala140, and Glu143), and the α6-helix from each subunit is placed in a hydrophobic cleft of the other subunit. As mentioned above, the C-terminal part (Glu188-Ala202) composed

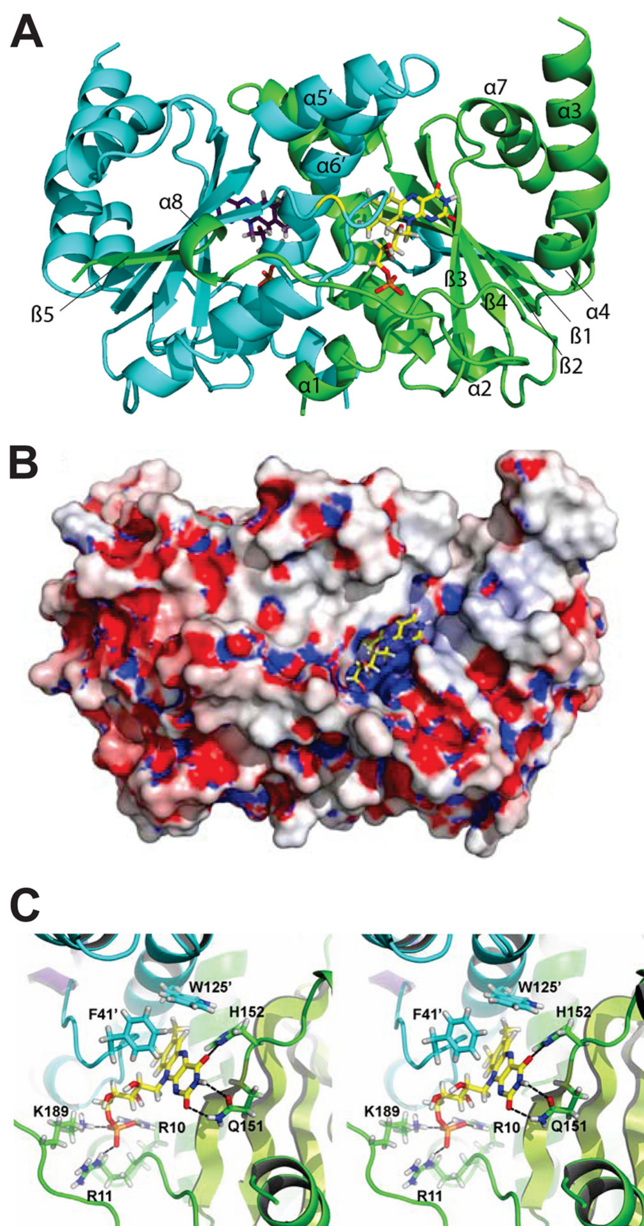

FIG. 8. Structure of CinD. (A) Cartoon representation with one subunit shown in light blue and purple and the other in green and lime. FMN molecules are depicted as stick models, and key residues discussed in the text are indicated. (B) Molecular surface representation with mapped electrostatic potential. The potential is drawn from −15 kT (red) to +15 kT (blue). Adaptive Poisson-Boltzmann Solver was used for the electrostatics calculation, assuming an ion concentration of 0.15 M (2). (C) Stereo view of the FMN binding site. Hydrogen bonds are indicated as black dashed lines, nitrogen atoms are colored in dark blue, oxygen atoms are in red, hydrogen atoms are in gray, and carbon atoms are in yellow.

of the short α8-helix (Asp194-Arg197) and the β5 strand (Phe198-Ala201) extends over the surface to the neighboring monomer, where the β5 strand contributes to the central pleated β-sheet of the other monomer. Seven hydrogen bonds and two salt bridges (between Arg35 and Asp194 of the other subunit) contribute to the stability as well.

The cofactor FMN is bound in a deep cleft that is positively charged (Fig. 8B). The local electrostatic potential originates mainly from Arg10, Arg11, and Lys189, which coordinate the phosphate group (Fig. 8C). The *si* face of the isoalloxazine ring is backed by strand β1. From this side, the carboxylamide of Gln151 forms hydrogen bonds to O_2 and HN_3 of the pyrimidine moiety of FMN. A further hydrogen bond is donated by the side chain of His152. All residues mentioned belong to the same subunit. The *re* face of the isoalloxazine ring is covered by Phe41′ and Trp125′ of the other subunit. They are further apart and form part of the substrate binding site. The heterocycle of FMN is nonplanar, with a dihedral angle of about 18.5 degrees between the planes of the dimethylbenzoide and pyrimidine ring. This bend has been implied in favoring the reduction of FMN, owing to the fact that reduced FMN possesses a dihedral angle of about 25 degrees (12). Bent cofactors have been observed in many nitroreductases, but exceptions have also been described, namely, for *E. coli* YdjA (7) and *Streptococcus mutans* SMU.260 (PDB accession number 2IFA). The latter was the initial search model and shares 40% amino acid identity with CinD. Closer inspection of SMU.260 revealed a clear indication of a bent cofactor in this nitroreductase as well, but the bending was less pronounced than that in CinD. While the overall crystal structure of CinD resembles a typical nitroreductase, there is one profound difference between CinD and the majority of the reported nitroreductases. Two prominent α-helices that usually shield the active site on the *re* face of FMN are substantially shortened in CinD and *Streptococcus mutans* SMU.260 and are even completely missing in *E. coli* YdjA. These helices are believed to have an influence on substrate specificity and recognition (7).

Taken together, the present work shows the structure and the function of CinD nitroreductase of *L. lactis*. We showed that this enzyme can protect cells against oxidative stress by NQO but also function as a catalase. In a natural setting, CinD probably protects *L. lactis* against a range of xenobiotics and oxidative stress conditions.

DISCUSSION

CinD had been identified previously by proteomics as the most strongly copper-induced protein of the CopR regulon of *L. lactis* IL1403 (23). In the present study, we characterized CinD (YtjD) as a copper-induced oxygen-insensitive nitroreductase with a role in defense against oxidative stress. Purified CinD was most active in the reduction of DCPIP and NQO, a strong inducer of oxidative stress. The enzyme also exhibited significant catalase activity.

By quantitative PCR as well as by Western blotting, it was shown that copper induced *cinD* in the order of 20- to 50-fold, in line with the 35-fold induction determined by proteomics (23). *CinD* was also induced by silver and cadmium, as would be expected for a gene under the control of CopR, which is homologous to CopY of *E. hirae* (30). Oxidative stress by NQO, paraquat, or hydrogen peroxide did not induce *cinD* expression, which contrasts with the oxidative stress-dependent activation of some other nitroreductases. *nfsA* of *E. coli* and *nfrA* of *Staphylococcus aureus* are nitroreductases that are under the control of SoxR (21) and PerR (37), respectively, which are regulators that respond to oxidative stress. The NfrA nitroreductase of *Bacillus subtilis*, on the other hand, is induced by paraquat and hydrogen peroxide (28). Oxidative stress and nitrosative stress are apparently interconnected, since overlapping sets of genes are induced by transcription factors sensing oxidative stress, like OxyR and SoxR, and by those sensing nitrosative stress, like NorR and Fur (29). In contrast, *cinD* induction in *L. lactis* was observed only with heavy metal ions. Chemicals which induce nitrosative stress, such as nitrosoglutathione, nitrosocysteine, or L-arginine, did not induce genes under the control of CopR. This provides support to the notion that *cinD* expression is governed solely by the copper-responsive CopR repressor. Further support for this comes from the promoter structure of CinD, which features two copies of the so-called "*cop* box" of consensus TACANNTGTA, which is a universal DNA binding site for CopR-like repressors (31). The *cop* box is present in one or two copies in all seven promoters of copper-regulated genes and operons we have studied so far (23, 36), and copper, silver, and cadmium are the only inducers of these genes/promoters which could be identified.

Oxygen-insensitive nitroreductases have been studied in microorganisms as different as *Enterococcus cloacae* (5), *E. coli*, and *Vibrio harveyi* (48), *Vibrio fischeri* (49), *Bacillus subtilis* (27), *Staphylococcus aureus* (37), and *Synechocystis* spp. (38). Nitroreductases catalyze the reduction of a large range of nitroaromatic compounds; therefore, they are generally assumed to be involved in detoxification processes. Indeed, the first enzyme described was isolated from bacteria growing in a weapons storage dump and could reduce the explosive trinitrotoluene (5). Because of their broad substrate specificity, nitroreductases have also received attention for biotechnological applications (26, 47). However, the biological function of this class of enzymes remains unclear. It has been proposed for *Bacillus subtilis* that nitroreductases are responsible for the maintenance of the cellular redox state, thereby protecting from oxidative stress. Indeed, we showed here that CinD can protect *L. lactis* from oxidative stress exerted by NQO. This chemical is quite toxic to *L. lactis* and is a good substrate for CinD, second only to DCPIP, which does not appear to be toxic to *L. lactis*. Detoxification of NQO by CinD is supported by the observations that it is reduced by CinD *in vitro* and that the Δ*cinD* strain is more sensitive to NQO than the wild type. In its natural environment, *L. lactis* may not encounter NQO but may encounter similar compounds with oxidative stress potential. If copper elicits or acerbates the oxidative stress potential of such chemicals, it would make sense that copper induces the CinD defense system.

Alternatively, or in addition, the catalase activity of CinD could offer an important protection against oxidative stress by decomposing hydrogen peroxide to oxygen and water. Fast-growing *L. lactis* produces considerable quantities of H_2O_2. This can occur by at least two mechanisms, namely, by NADH oxidase (45) and by the LctO lactate dehydrogenase. LctO converts lactate to pyruvate, under production of hydrogen peroxide. It has been argued that these two activities serve in

the scavenging of molecular oxygen to reduce oxidative stress (3, 24). Since *L. lactis* is devoid of a "normal" catalase and of SOD, alternative mechanism dealing with hydrogen peroxide and oxidative stress must be present. NADH peroxidase can convert hydrogen peroxide to water, but this activity is apparently very low (45). Interestingly, LctO belongs to the CopR regulon, is upregulated by copper, but does not contribute to increased copper tolerance (3). The induction of LctO by copper appears counterproductive since this would lead to the production of H_2O_2, which in turn, could undergo a Fenton reaction with copper, leading to the generation of toxic $OH^{\bullet}$ radicals. However, pyruvate (generated by LctO) can nonenzymatically react with hydrogen peroxide according to the following reaction: pyruvate + $H_2O_2 \rightarrow$ acetate + CO_2 + H_2O. Such a mechanism is supported by the accumulation of up to 4 mM pyruvate by *L. lactis* strain NZ2007, which is deficient in lactate dehydrogenase and does not produce detectable hydrogen peroxide during logarithmic growth (45). The induction of LctO under copper stress may thus serve in the elimination of hydrogen peroxide by nonenzymatic reaction with pyruvate. Pyruvate was also shown to exert a protective effect against copper-induced cysteine autoxidation in astrocytes (46) and to protect motor neurons expressing mutant forms of SOD1 (which cause amyotrophic lateral sclerosis) against copper toxicity (16).

CinD (YtjD) is the first gene of an operon originally designated *ytjDBA*. *ytjB* is predicted to encode a manganese transporter, and *ytjA* is predicted to encode a ribonucleotide-2(3)-phosphate reductase. Copper-induced manganese uptake by YtjB could provide an additional level of protection against oxidative stress. Although *L. lactis* IL1403 is devoid of a Mn-containing SOD, Mn^{2+} has also been claimed to dismutate hydrogen peroxide nonenzymatically (35). The reaction has been studied in detail *in vitro* (40), but recent evidence suggests that it does not play a role *in vivo*. Instead, manganese apparently can metallate mononuclear enzymes in lieu of iron, thereby precluding a Fenton reaction between iron and hydrogen peroxide (1). Induction of manganese uptake by hydrogen peroxide has been reported for many bacteria, and uptake can be accomplished by P-type ATPases, ABC-type permeases, or Nramp-type manganese uptake systems (for a review, see reference 14). YtjB of *L. lactis* resembles an Nramp-type transporter; whether it plays a role in manganese uptake and protection against oxidative stress is currently under investigation in our laboratory.

Clearly, the defense against copper toxicity and copper-associated oxidative stress takes place at several levels. Depending on the bacterial species, it involves copper export by ATPases, detoxification of superoxide by SOD, and scavenging of hydrogen peroxide enzymatically by catalase or maybe nonenzymatically by manganese, pyruvate, and other α-ketoacids. In addition, cytoplasmic manganese may metallate some enzymes in lieu of iron to curb the Fenton reaction. Intracellular glutathione can complex copper and protect against oxidative damage, and cytoplasmic zinc can also protect enzymes from copper toxicity. Finally, a number of regulatory systems respond to oxidative stress and prevent DNA and cell damage by other mechanisms. CinD with its nitroreductase and catalase activities adds yet another protective element to this network. Several more uncharacterized, copper-induced genes of the CopR regulon are likely to contribute to this complex network, and considerably more work is required to fully understand the defense of *L. lactis* against copper and oxidative stress.

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2) The Copper-Inducible YcfQ Repressor Regulates Expression of the Outer Membrane Protein YcfR and Affects Copper Entry into *Escherichia coli*

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Abstract

The mechanism of copper entry into *Escherichia coli* is still unknown. In an attempt to identify components involved in this process, a *lux*-based biosensor was utilized to monitor intracellular copper levels *in situ*. From a transposon-mutagenesis library, strains were selected in which copper entry into the cell was reduced, apparent as clones with reduced luminescence (low-glowers) when grown in the presence of copper. One low-glower strain had a transposon insertion in the *ycfQ* gene, which encodes a TetR-like transcriptional regulator. The mutant strain could be complemented by the *ycfQ* gene on a plasmid, restoring luminescence to wild-type levels. YcfQ did not regulate its own expression, but was required for copper-induction of the neighboring, inversely transcribed *ycfR* gene, as shown by real-time quantitative PCR and with a promoter-*lux* fusion. Purified YcfQ bound to the promoter region of the *ycfR* gene *in vitro* and was released by copper and gold, but not by other metals tested. The binding site was mapped to an 80 basepair region upstream of *ycfR*. By membrane fractionation, YcfR with a C-terminal his-tag was shown to be localized in the outer membrane. These findings suggest that YcfQ controls the expression of YcfR, which lowers the permeability of the cytoplasmic membrane for copper.

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The Copper-Inducible YcfQ Repressor Regulates Expression of the Outer Membrane Protein YcfR and Affects Copper Entry into *Escherichia coli**

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Running Title: Copper Entry into *E. coli*

The mechanism of copper entry into *Escherichia coli* is still unknown. In an attempt to identify components involved in this process, a *lux*-based biosensor was utilized to monitor intracellular copper levels *in situ*. From a transposon-mutagenesis library, strains were selected in which copper entry into the cell was reduced, apparent as clones with reduced luminescence (low-glowers) when grown in the presence of copper. One low-glower strain had a transposon insertion in the *ycfQ* gene, which encodes a TetR-like transcriptional regulator. The mutant strain could be complemented by the *ycfQ* gene on a plasmid, restoring luminescence to wild-type levels. YcfQ did not regulate its own expression, but was required for copper-induction of the neighboring, inversely transcribed *ycfR* gene, as shown by real-time quantitative PCR and with a promoter-*lux* fusion. Purified YcfQ bound to the promoter region of the *ycfR* gene *in vitro* and was released by copper and gold, but not by other metals tested. The binding site was mapped to an 80 basepair region upstream of *ycfR*. By membrane fractionation, YcfR with a C-terminal his-tag was shown to be localized in the outer membrane. These findings suggest that YcfQ controls the expression of YcfR, which lowers the permeability of the cytoplasmic membrane for copper.

Copper is an essential micronutrient for all living cells. Its chemical reactivity is based on the high redox potential and cells harness this property by incorporating copper as a cofactor into the active site of several enzymes involved in fundamental processes such as energy generation, iron uptake and protection against oxidative stress. Due to its ability to accept and donate electrons, copper can also catalyze unwanted, deleterious reactions and damage cellular components such as DNA, proteins and membranes. In a Fenton type reaction highly reactive hydroxyl radicals are generated and oxidize proteins and lipids (1). Copper may also catalyze the production of hydrogen peroxide. Therefore, protective mechanisms against copper toxicity are a necessity..

Cells developed various copper resistance mechanisms, including extracellular sequestration, intracellular routing, and efficient export. All these components of copper homeostasis have to be tightly regulated. In prokaryotic organisms, copper homeostasis is mainly effected by intracellular complexation of copper by specialized metallochaperones and

metallothionein-like proteins, and by copper efflux ATPases (2, 3).

Copper homeostasis in *Escherichia coli* consists of several systems (4). A key component is the Cu(I) transporting ATPase, CopA. It detoxifies the cytoplasm by extruding excess intracellular copper (5, 6). This important pump is induced by copper *via* the action of CueR, a MerR-like transcription activator, which senses copper in the zeptomolar range (7-11). CopA pumps copper from the cytoplasm to the periplasmic space. There, a multi-copper oxidase, CueO, and a copper transport system, CusCFBA, safeguard the periplasm against copper toxicity by exporting periplasmic copper (4, 12). CueO, which is also under the control of the CueR regulator, has cuprous oxidase activity and can oxidize Cu^{+} to Cu^{2+}, thus rendering it less toxic (13). Enterobactin, a catechol siderophore of *E. coli,* is very toxic to the cell in the presence of copper. CueO can oxidize enterobactin which, in its oxidized form, sequesters copper and contributes to the detoxification of the periplasm (14).

The CusCFBA system is essential in providing copper resistance under anaerobic conditions, presumably because the accessory CueO copper resistance mechanism cannot operate in the absence of oxygen (15, 16). The *cusCFBA* operon is induced by copper *via* the CusRS two-component regulatory system. The combined operation of CopA, CueO, CusCBA, and their respective regulators, provide efficient copper homeostasis in *E. coli* (2).

Currently, there is no information about the copper entry mechanisms of *E. coli*. We addressed this question by using a plasmid-borne biosensor which responds to cytoplasmic copper levels. It consisted of the *lux* gene cluster of *Vibrio fischeri*, placed under the control of the *E. coli copA* promoter (17). The biosensor responds to an increase in extracellular copper by an increase in luminescence. It was hypothesized that *E. coli* mutants with reduced copper influx would contain reduced levels of cytoplasmic copper, which would result in reduced luminescence of such clones. A transposon mutant library of *E. coli* was screened for clones with decreased luminescence. One such 'low glower' contained a transposon insertion in the *ycfQ* gene, which encodes a regulator for the *ycfR* gene. YcfR is a 85 amino acid outer membrane protein which appears to control the permeability to copper.

EXPERIMENTAL PROCEDURES

Generation of Transposon Library—E. coli Top10 electrocompetent cells (Invitrogen) were electroporated with a GenePulser (BioRad) at 1,8 kV and a 200 Ω serial resistor with 1 µl of EZ:TN<DHFR-1> transposon (Epicentre Technologies), suspended in 1 ml of SOC medium (2 % trypticase peptone (BBL), 0.5 % yeast extract (BBL), 10 mM NaCl, 2.5 mM KCl; 10 mM $MgCl_2$, 10 mM $MgSO_4$, 0.2 % glucose (added after autoclaving), and incubated for 1 h at 37 ^{o}C with shaking, followed by plating on LB agar plates (18), supplemented with 10 µg/ml of trimethoprim. Following incubated at 37 ^{o}C for 36 hours, 8.3 x 10^3 colonies containing a transposon insert were obtained. The colonies were washed off the plates and

aliquots of this transposon library were frozen in 25 % glycerol at -80 ^{o}C.

Mutant Selection—Of the transposon library, electrocompetent cells were prepared and electroporated with the biosensor pUA615 (17) as described (18). Cells were plated on LB plates containing 50 µg/ml of kanamycin and 3 mM of $CuSO_4$. After overnight incubation at 37 ^{o}C, luminescence of the colonies was recorded with a cooled CCD camera (LAS1000, Fuji). Images were inspected for strongly glowing colonies (high-glowers) and faintly glowing colonies (low-glowers). Clones of interest were subcloned for further use.

Identification of Transposon Insertion Sites—The Wizard Genomic DNA Purification kit (Promega) was used to isolate genomic DNA from 1 ml overnight cultures according to the manufacturer's instructions. Single primer PCR was used to generate PCR products spanning the transposon-genome junction by PCR amplification with a single primer, DHFR RP (5'-GACACTCTGTTATTACAAATCG) or DHFR FP (5'-GGCGGAAACATTGGATGCGG), complementary to the transposon. PCR amplifications were conducted with 5 U of LA Taq polymerase (Takara) according to manufacturers instructions and the following thermal cycling: 95 °C for 5 min, 60 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 3 min, followed by 72°C for 5 min. The PCR products were separated on 0.7 % agarose gels and the DNA extracted from the gels, using the Qiagen gel purification kit as described by the manufacturer. The DNA fragments were submitted to a commercial sequencing service (Microsynth, Balgach, Switzerland), using the primers DHFR RP-1 (5'-ATCGTGCATGCCGTCTATCC) and DHFR FP-1 (5'-CTGCAGGCATGCAAGCTTCA), which were nested relative to the PCR amplification primers.

Cloning of ycfQ—A YcfQ expression vector was constructed by PCR amplification of the *ycfQ* open reading frame from genomic DNA, using the primers ycfQHisF (5'-TAGGCGCCGCAACTGACTCAACACAATGTG) and ycfQHisR (5'-TATTCTAGATTATTTCACCAGTTCGGGCCA). The resultant PCR product was cut with *Xba*I and *Nar*I and ligated into the histidine-tag expression vector pProExHTa (Invitrogen) to yield an N-terminal 6x histidine-*ycfQ* fusion gene containing a cleavage site for rTEV protease for tag removal. The resultant expression vector pProExYcfQ was verified by sequencing. Cleaved YcfQ had the N-terminal methionine replace by gly-ala.

Luminescence Assays—Cultures of 5 ml LB media were grown aerobically at 37 ^{o}C to an OD_{595} of 0.5, and induced for 1 h with $CuSO_4$ as indicated in the experiments. Luminescence was measure in 0.3 ml aliquots of culture in 96-well microtiter plates, using a cooled CCD camera (LAS1000, Fuji) and quantified with the AIDA image analyses software (Raytest).

Purification of YcfQ—Histidine-tagged YcfQ was overexpressed in *E. coli* by growing cells aerobically to mid-log phase in LB at 7 °C, followed by induction with 5 mM isopropyl-β-D-thiogalactoside for 2 h. Cells were collected by centrifugation, disrupted with a French press, and the

cleared cell lysate applied to a Ni-NTA agarose column (Qiagen). The column was washed with 50 ml 20 mM imidazole, 50 mM Na-HEPES, pH 7.4, followed by elution of his_6-YcfQ in 5 ml of 200 mM imidazole in the same buffer. The protein was desalted by dialysis against 50 mM Na-HEPES, pH 7.4. The histidine tag of 1 mg of protein in 1 ml was cleaved with 100 U of rTEV protease (Invitrogen) by incubation for 2 h at 30 °C and overnight at 4 °C and the cleaved tag was removed by ultrafiltration on an Amicon ultrafiltration device, using a membrane with a cut-off of 10 kDa. One hundredth volume of a protease inhibitor cocktail (100 mM N-α-p-tosyl-L-lysine-chloromethyl ketone, 100 mM N-p-tosyl-L-phenylalanine-chloromethylketone, 100 mM p-aminobenzamidine, 100 mM phenyl-methyl-sulfonyl-fluoride dissolved in dimethylsulfoxide) was added to the final preparation to prevent degradation.

RNA Isolation and cDNA Synthesis—Mid-log aerobic cultures in LB media were induced as indicated under Results for 1 h at 37 °C and RNA was isolated from 1 ml aliquots, using the Qiagen RNeasy miniprep column kit according to the manufacturer's procedure, including treatment with DNase I (RNase-free) for 1 h at 37 °C. RNasin (100 U/sample, Promega) was added and the RNA stored at -80 °C. The quality of the RNA was checked on 1.2 % agarose-formaldehyde gels. cDNA was transcribed from 1 μg of RNA using the iScript cDNA synthesis kit (BioRad) according to the manufacturer's protocol.

Real-Time Quantitative PCR [3] *(qPCR)*—qPCR was performed with the SYBR Premix Ex Taq (Takara) in a LightCycler 1.5 (Roche). The PCR products were analyzed for specificity and homogeneity by melting curve analysis and electrophoresis on 1% agarose gels. The primers used were ycfQFq (5'-CGCCCAAAAGTGTTCGACAG) and ycfQRq (5'-CTTTGTTGGTAAATTCCGCG) and ycfRF (5'–AGTTCAGTCAACGCCAGAAG) and ycfRR (5'-ATTCGGACCGGTTACAGAAG). To normalize the qPCR results, the *E. coli* 16 S cDNA was amplified with primers 16SF (5'-TGAAGAGTTTGATCATGGCT) and 16SR 5'-CGTTTCCAGTAGTTATCCCC).
Reaction conditions were as follows: 95 °C for 10 s, followed by 40 cycles of 61 °C for 20 s, 72 °C for 45 s, and 95 °C for 6 s.

Construction of a ycfR promoter-lux Fusion—A 241 bp fragment of the *ycfR* promoter region was amplified by PCR with the primers PycfQF (5'-TAATAGTGGCCTTATGCAGA) and PycfQR (5'-GATGCCGTTGTACCTGGTGA), phosphorylated with polynucleotide kinase, and ligated into pUCD615 (19), which had been cut with *Eco*RI and *Bam*HI, made blunt-ended with Klenow DNA polymerase (Roche) and dephosphorylated with alkaline phosphatase (New England Biolab). The resulting plasmid, pPycfR-lux, had the *ycfR* promoter region cloned in front of the *lux* gene cluster of *Alivibrio fischeri*.

Electrophoretic mobility-shift assays (EMSA)—PCR product of the *ycfQ-ycfR* intergenic region were amplified from *E. coli* genomic DNA using LA Taq polymerase (Takara) and the primers F1 (5'-TAATAGTGGCCTTATGCAGA), F2 (5'-TCGCAAGCAATTACTTAACG-3'), F3

(5'-GTGATCTGGATCACATACAA-3'), F4 (5'-ATGGGATCTGGACTGGTGAA-3'), R1 (5'-GATGCCGTTGTACCTGGTGA), R2 (5'-ATCACCTCCGTTCACCAGTC-3'), R3 (5'-ACCAGTCCAGATCCCATAAA), and R4 (5'-GTGATCCAGATCACATCTAT-3'). EMSA were performed according to Parkhill *et al.* (1993). Briefly, DNA and purified YcfQ were incubated in 10 mM Tris, pH 8.0, 100 mM K-glutamate, 0.1 mM EDTA, 1 mM $CaCl_2$, 5% glycerol, 1 mM DTT, 100 μg/ml BSA for 15 min at 20 °C and then applied to 8% vertical polyacrylamide gels in 40 mM Tris-acetate, pH 8, 1 mM EDTA, pre-run at 150 V for 30 min at 25°C. Gels were stained with SYBR Green I (Invitrogen) according to manufacturers protocol and images recorded with a CCD camera under UV light.

Disruption of ycfR—The *ycfR* gene was inactivated by using the commercial Targetron gene knockout system (Sigma Aldrich) according to the manufacturer's instructions. Targeted disruption was verified by PCR amplification and sequencing of the corresponding genomic region.

Localization of YcfR—A C-terminal *ycfR-V5-6xhis* fusion was generated by PCR amplification of genomic *E. coli* DNA with primer ms100 (5'-CACCATGAAAAACGTAAAAACCCTCATC) and ms101 (5'-TTTATAAATTACTGCTGTTCCATGGAG). The PCR product was ligated into pET101 with the D-Topo kit from Invitrogen according to the manufacturer's instructions, resulting in pETR103. which verified by sequencing and transformed into *E. coli* BL21(DE3)RIL (Invitrogen) as described before. Cells were grown aerobically at 37 °C in 400 ml of LB media to mid-log phase and induced with 1 mM isopropyl-β-D-thiogalactoside for 2 h. Cells were collected, washed once with 100 mM NaCl, 50 mM NaPi, pH 7,4, and disrupted with a French press. Debris was collected by centrifugation for 10 min at 8,000 x g and the membranes were pelleted from the supernatant by centrifugation for 1 h at 100,000 x g. Membranes were suspended in 1 ml of 50 mM Na-HEPES, pH 7, and 5 mg fractionated on a 30 to 60% sucrose gradient as described (20). Inner membrane NADH oxidase was assayed by measuring NADH oxidation at 340 nm and outer membrane phospholipase A was assay with a kit from Shizuoka (Japan). YcfR-V5-6xhis was determined by Western blotting as described (21), using histidine-antibody (Qiagen).

RESULTS

To identify genes involved in copper influx into *E. coli*, we randomly mutagenized cells with a transposon to obtain a random knock-out library. This library was transformed with the biosensor pUA615, which consists of the *lux* operon of *Alivibrio fischeri* under the control of the *E. coli copA* promoter (17). This allowed *in situ* monitoring of intracellular copper concentrations by luminescence. Colonies of a transposon library exposed to 1 mM copper were screened for colonies with reduced luminescence (low-glowers, Fig. 1). Several low-glowers were analyzed for the gene defect. Most of them had transposon insertions in genes which control plasmid

copy number, thus reducing the gene-dosage of the biosensor, or were in genetic regions of undefined function. One low-glower of particular interest had a transposon insertion in the *ycfQ* gene, which encodes a TetR-like transcription regulator. We also isolated two high-glowers which both had a transposon insertion in the *copA* gene, which encodes the CopA copper export ATPase. The Δ*ycfQ* low-glower was studied in more detail here.

The low-glowing phenotype of the Δ*ycfQ* mutant was confirmed in liquid media at different copper concentrations (Fig. 2). Luminescence of the Δ*ycfQ* strain was maximal at 2 mM copper and was 3-fold lower than in the wild-type. The reduced luminescence of the Δ*ycfQ* strain suggests that this mutant has lower cytoplasmic copper levels than the wild-type when exposed to copper.

Inspection of the genomic region around the *ycfQ* gene revealed that it is monocistronic, but is juxtaposed to the divergently transcribed *ycfR* gene. The function of the 85 amino acid protein encoded by *ycfR* is unknown. There is a 241 bp putative promoter region located between *ycfR* and *ycfQ*. The expression levels of *ycfQ* and *ycfR* in response to copper were as assessed by qPCR. Expression of *ycfQ* was not significantly stimulated by copper (Fig. 3). In contrast, *ycfR* was induced approximately 30-fold by 3 mM copper. No significant induction by copper of the other nearby up- and downstream genes *ndh, ycfJ, ycfM, ycfO, ycfP, ycfS*, and *ycfT* was observed (not shown).

To confirm the copper-induction of the *ycfR* promoter, the 241 bp intergenic region upstream of the *ycfR* gene was cloned into a *lux* reporter plasmid and luminescence was recorded as a function of added copper. Copper induced luminescence maximally 30-fold at 2 mM copper (Fig. 4). Induction of luminescence by copper thus qualitatively agrees with copper-induction of the *ycfR* mRNA as measured by qPCR.

To test the interaction of the putative YcfQ repressor with the *ycfR* promoter, YcfQ was expressed in *E. coli* as a his-tagged protein and purified to homogeneity (Fig. 5). YcfQ with the his-tag cleaved with rTEV was used for EMSA with the 241 bp intergenic region. Increasing amounts of YcfQ resulted in an increasing fraction of the DNA forming a YcfQ-DNA complex of low electrophoretic mobility (Fig. 6*A*, band near the top of the gel). At a molar YcfQ/DNA ratio of 64, the DNA was quantitatively shifted to lower mobility. Copper, silver, and gold released YcfQ from DNA, but also formed some protein aggregates of intermediate mobility, apparent as dark smears of intermediate electrophoretic mobility (Fig. 6*B*). Cobalt, cadmium, and nickel did not dissociate YcfQ from the DNA. Thus, copper appears to be the physiological inducer of YcfQ.

The binding region of YcfQ on the 241 bp fragment used in Fig. 6 was narrowed down by testing a set of PCR fragments for the interaction with YcfQ (Fig. 7*A*). This delineated an approximately 40 bp YcfQ binding region extending from -70 to -30 relative to the *ycfR* gene; copper dissociated YcfQ from this binding region (Fig. 7*B*), corroborating that ycfR is under the control of YcfQ and copper.

Taken together, our findings so far suggest that *ycfR* is under the control of the

copper-inducible repressor YcfQ. Knock-out of *ycfQ* results in constitutive expression of YcfR, which is accompanied by reduced copper entry into cells and the low-glower phenotype of the Δ*ycfQ* mutant. To demonstrate a direct effect of YcfR on copper entry into the cytoplasm, a Δ*ycfR* strain was constructed. As expected, growth of the Δ*ycfR* mutant was more sensitive to copper, compared to the wild type (Fig. 8). This supports that YcfR expression directly affects copper entry into cells.

By bioinformatics tools, YcfR is predicted to be an outer membrane protein with a 21 or 22 amino acid N-terminal signal sequence. The membrane localization of YcfR was determined by expressing a fusion protein with a C-terminal V5-6xhis tag from a plasmid. Outer and inner membranes were separated by sucrose gradient centrifugation and YcfR was localized by Western blotting, using an anti-histidine antibody. The band corresponding to YcfR-V5-6his predominantly co-localized with the highest activity of phospholipase A, a marker for outer membranes, at a density of 1.23 to 1.26 g/ml (Fig. 9). However, there was also some phospholipase A activity co-migrating with the NADH oxidase marker for inner membranes at a density of 1.14 to 1.17 g/ml, as there was some NADH oxidase activity co-migrating with phospholipase A. This cross-over of activities indicates incomplete membrane separation; however, better separation could not be achieved in several attempts, possibly due to the formation of mosaic membrane vesicles in the cell disruption process. The presence of small amounts of YcfR in most gradient fractions would support this notion. Also, using F_1F_o-ATPase activity as an alternative inner membrane marker yielded essentially identical results (not shown). Nevertheless, these data strongly support an outer membrane localization of YcfR. An approximately 2 kDa smaller degradation product of unknown nature was also present the fractions. This could represent cleavage of an N-terminal YcfR signal sequence, which was only partial under the high-expression conditions used in this experiment.

Taken together, we here identify YcfR as an outer membrane protein under the control of the novel copper-responsive repressor YcfQ. The YcfRQ system appears to control copper influx into the cytoplasm in response to copper. Future studies will have to address the mechanism of copper-induction of YcfQ. It will also be of interest to investigate if YcfQ only controls *ycfR*, or also other genes, and to what extent the YcfRQ system is involved in other stress phenomena.

DISCUSSION

Since it is still unknown how copper enters *E. coli* cells, we here used transposon mutagenesis in combination with a biosensor to identify mutants with altered copper uptake properties. This led to the identification of a low-glower with a transposon insertion in the *ycfQ* transcriptional regulator. *YcfQ* is predicted to encode a 210 amino acid protein resembling TetR transcriptional regulator. TetR represses transcription by the binding of a homodimer to the promoter. Upon binding of the inducer, Mg^{2+}-tetracyclin, the protein undergoes a conformational change which

lowers its DNA binding affinity and allows transcription to proceed (23).

An obvious candidate gene for regulation by YcfQ was, *ycfR*, a divergently transcribed gene 241 bp upstream of *ycfQ*. *YcfR* mRNA was strongly induced by copper *in vivo,* while *ycfQ* transcription was not significantly affected by copper. Transcription levels of the other nearby up- and downstream genes *ndh, ycfJ, ycfM, ycfO, ycfP, ycfS*, and *ycfT* were not notably influenced by copper. Direct regulation of *ycfR* by YcfQ was substantiated by the binding of YcfQ to an approximately 40 bp region of the *ycfR* promoter *in vitro.* Copper released YcfQ from the DNA, in line with a function of YcfQ as a copper-inducible repressor of the *ycfR* gene. In line with this, copper induced luminescence of a reporter plasmid with a *ycfR* promoter-*lux* fusion. Taken together, these findings provide evidence that YcfQ is a novel, copper-inducible repressor which regulates expression of *ycfR*. Induction of the YcfQ repressor by copper of course suggests that the repressor binds copper. Modeling of the structure of YcfQ with SWISS-MODEL yielded a TetR-like structure with a potential copper binding site formed by Cys-117, Met-119, Cys-123, and Gln-179. Future studies will address the structure and copper binding of YcfQ.

The copper-induction of *ycfR* of course raised the question of what the function of this small, 9 kDa protein is. A C-terminal 6xhis-V5-fusion of YcfR was shown to be primarily localized in the outer membrane, as assessed with an anti-histidine antibody and corresponding inner and outer membrane markers in density gradient-fractionated membranes. Increased levels of YcfR in the outer membrane appear to make the membrane less permeable to copper, as apparent by the low-glower phenotype of the *ycfQ* mutant (constitutive YcfR expression) and the increased copper sensitivity of a Δ*ycfR* knock-out strain.

In various microarray studies, ycfR was found to be induced 25-fold by 25 μM cadmium [(24), copper was not found to induce *ycfR,* although the report relied on a single dataset], during a pH-shift from 8.7 to 5 (25), a heat-shock of 50 °C for 7 min (26), treatment with 5 mM sodium salicylate (27), 1 mM hydrogen peroxide (28). More recently, YcfR was also reported to be involved in biofilm formation (29). It was found that *ycfR*-deletion rendered cell more hydrophobic, increased cell aggregation, and sensitivity to acid, heat, hydrogen peroxide, and cadmium. Deleting *ycfR* also decreased the expression of a range of membrane proteins and made cells to more readily form biofilms in glucose-containing media. It remains unclear to what extent YcfR was involved in these phenotypic changes, as the expression levels of other outer membrane proteins were also affected by the *ycfR* deletion.

Based on bioinformatics, the promoter region of *ycfR* had been predicted to contain a binding site for the cAMP response protein (CRP), which is a global regulator involved in catabolite repression (30). CRP-binding to a 259 bp DNA fragment upstream of ycfR was also shown experimentally (29). In addition, *ycfR* induction by cadmium was shown to be lower in an *rpoE* mutant (24). RpoE of *E. coli* functions in general stress response and controls the extracytoplasmic

function protein family (proteins involved in maintaining the integrity of periplasmic and outer membrane components (24). These findings suggested that *ycfR* expression is modulated by the global regulators RpoE and CRP, in addition to the copper-dependent regulation by YcfQ shown here. However, induction of *ycfR* by copper *via* YcfQ is much more pronounced than the effects described for RpoE and CRP and we assume that copper-induction of *ycfR* is the principal regulation of this gene.

It has been proposed by Zhang et al. to rename *ycfR* to *bhsA*, for influencing biofilm through hydrophobicity and stress response (29). We consider this renaming premature as YcfR influenced the sensitivity of cells to copper in the present study. Conceivably, YcfR functions as a scaffolding or tethering protein in the outer membrane.

In line with a periplasmic function of YcfR, related proteins could not be found in Gram-positive bacteria by a BLAST search, while the genomes of most, if not all, Gram-negative bacteria encode proteins with 50 to 90% sequence identity to YcfR. Thus, YcfR-like proteins may be a typical feature of outer membranes and thus a universal feature of Gram-negative organisms. The wide-spread occurrence of YcfR-like proteins contrasts with the lack of information on the localization, structure, and function of this family of proteins and further work on YcfR-like proteins is required.

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FOOTNOTES

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[3]The abbreviations use are: qPCR, real-time quantitative PCR; EMSA, electrophoretic mobility-shift assay.

FIGURE LEGENDS

FIGURE 1. **Luminescence of colonies.** Transposon-mutagenized cells containing the *lux* biosensor were plated on LB agar plates with 1 mM $CuSO_4$ and grown for 14 h at 37 °C. The luminescence of colonies was recorded with a cooled CCD camera. The left *arrow* points to a high-glower, the right *arrow* to a low-glower colony.

FIGURE 2. **Quantitative luminescence assays.** Luminescence was recorded in liquid media for the Δ*ycfQ* strain (○) and the wild-type (●) at different $CuSO_4$ concentrations. The error bars indicate the standard deviations of three independent experiments.

FIGURE 3. **Induction of *ycfQ* and *ycfR* by copper.** The expression of *ycfQ* (○) and the *ycfR* (●) gene was determined by qPCR at a range of $CuSO_4$ concentrations. The error bars indicate the standard deviations of three independent experiments.

FIGURE 4. **Luminescence assay with a *ycfR* promoter-*lux* reporter plasmid.** *E. coli* DH5α containing the reporter plasmid pPycfR-lux was exposed to different $CuSO_4$ concentrations in LB media and the luminescence was measured with a cooled CCD camera. The error bars indicate the standard deviation of three independent experiments.

FIGURE 5. **Purification of YcfQ. Overexpression of the YcfQ protein.** Coomassie blue-stained SDS gel of the various fractions of the purification of YcfQ. *Lane 1*, marker with the molecular weights indicated in kDa; *lane 2,* cleared lysate of uninduced culture; *lane 3,* cleared lysate of culture induced for 2 h with 1 mM isopropyl-β-D-thiogalactoside; *lane 4,* flow-through of Ni-NTA column; *lanes 5 and 6,* column washes with wash buffer; *lane 7,* column eluate with 200 mM imidazole. The *arrow* indicates the migration of recombinant his-YcfQ.

FIGURE 6. **EMSA of ycfR promoter region with purified YcfQ.** *A*. A 241 bp *ycfR* promoter fragment (0.4 pmol) was incubated with purified YcfQ at the molar YcfQ/promoter ratios indicated in the Figure, separated by polyacrylamide gel electrophoresis, stained with SYBR green and the gel photographed under UV light. *B*. EMSA with the *ycfR* promoter fragment without YcfQ (-*Q*), or with a 24-fold molar excess of YcfQ, but no metal (-Me), or with YcfQ and 50 μM of the metal ions indicated below the lanes. The following salts were used: $CuSO_4$, $AgNO_3$, $AuCl_3$, $CoCl_2$, $CdSO_4$, and $NiSO_4$. Other conditions were as in *A*.

FIGURE 7. **Mapping of the YcfQ binding region.** *A*. Intergenic region between *ycfQ* and *ycfR*, with the starts of these genes indicated by *arrows*. The PCR fragments depicted by bold horizontal lines were generated with the primers listed on the left. Interaction with YcfQ is indicated by + and - signs on the right. *B*. EMSA with the PCR fragment shown in *A*. DNA fragments (0.4 pmol) were incubated either without YcfQ (-), a 24-fold molar excess of YcfQ (+), and without (-) or with (+) 50 μM $CuSO_4$. Other details of the experiment were as in Fig. 6.

FIGURE 8. **Growth inhibition by copper of wild-type and Δ*ycfR* cells.** Wild-type E. coli (*A*) or the Δ*ycfR* mutant (B) was grown in LB media, supplemented with no copper (●), 0.5 mM (○), 1 mM (▼), 1.5 mM (∇), 2 mM (■)or 2.5 mM (□) $CuSO_4$. The error bars show the standard deviations of three independent experiments.

FIGURE 9. **Membrane localization of YcfR.** *A*. *E. coli* membranes were separated on a 30 to 60% (w/v) sucrose gradient and fractionated from the top. Fractions were assayed for the inner membrane marker NADH oxidase (○), the outer membrane marker phospholipase A (●), and the density (Δ). *B*. Western blot showing the presence of YcfR in the fractions of *A*. The *arrow* indicates the position of the YcfR-V5-6xhis fusion protein and the scale on the right indicates the migration of molecular weight standards in kDa.

Figure 1

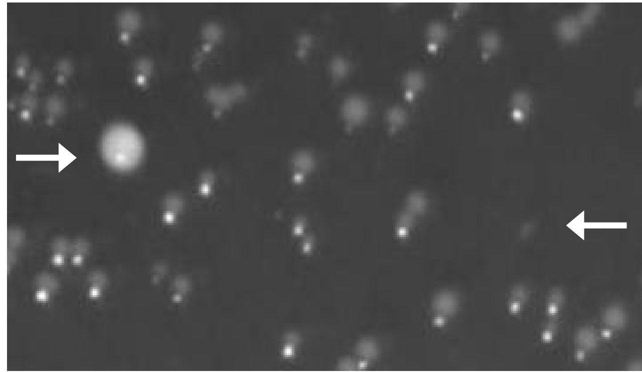


Figure 2

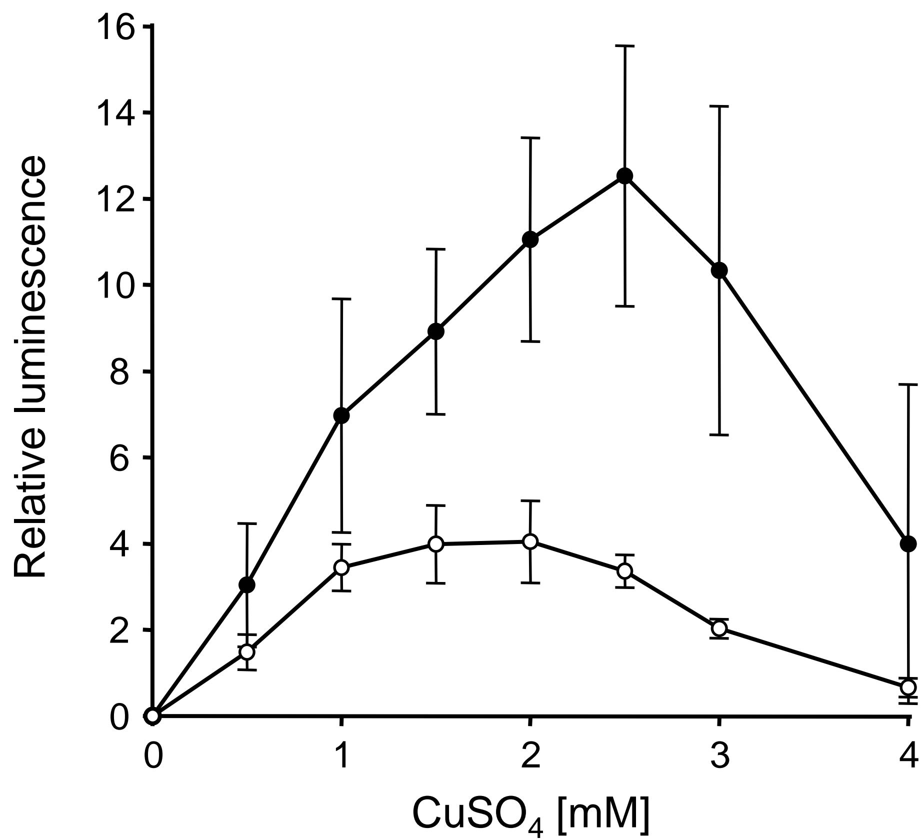


Figure 3

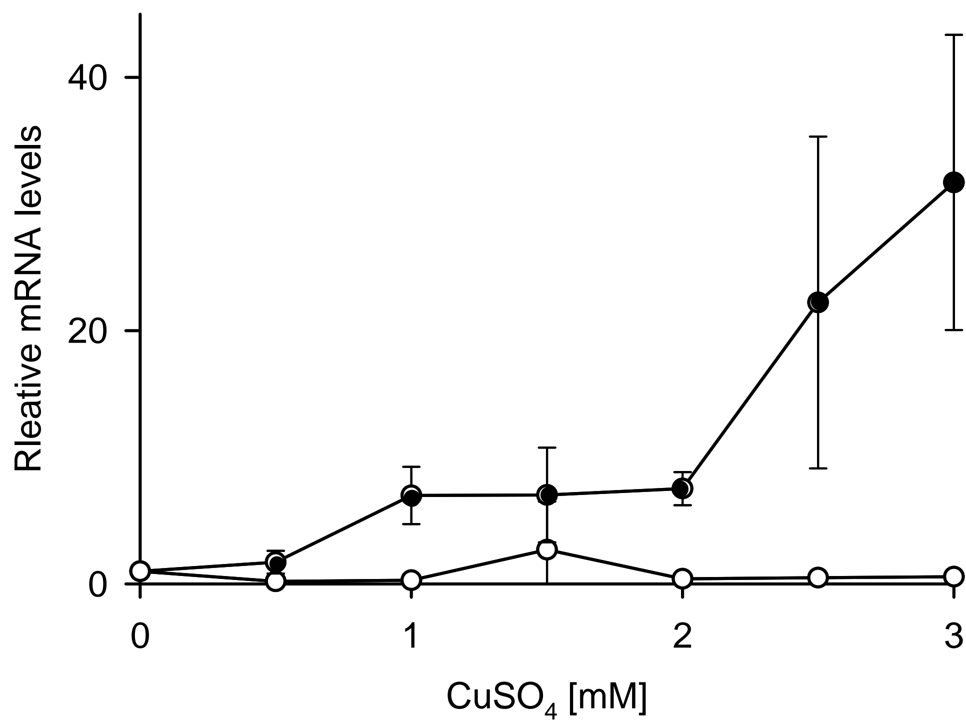


Figure 4

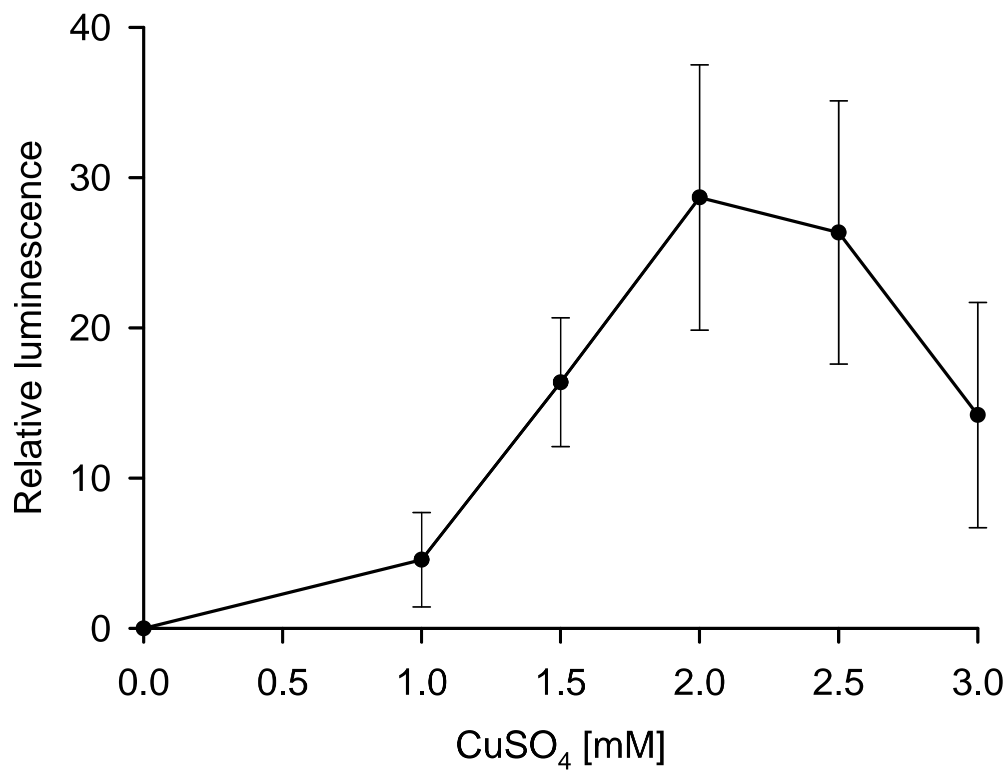
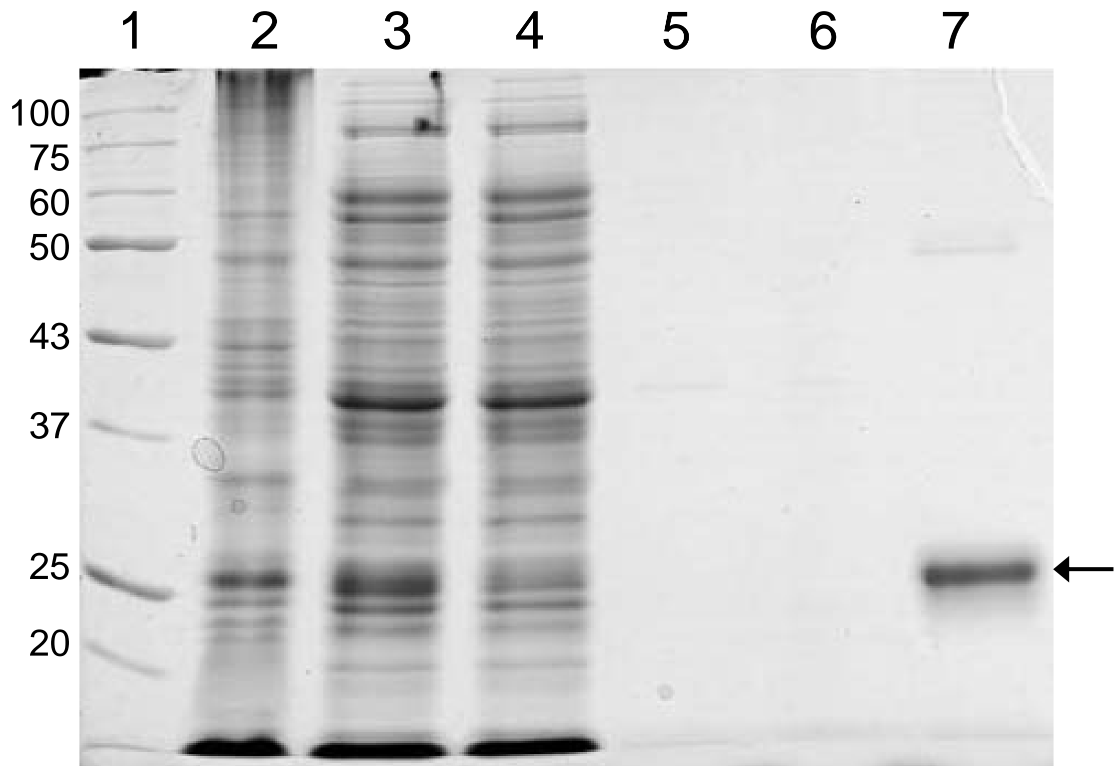


Figure 5



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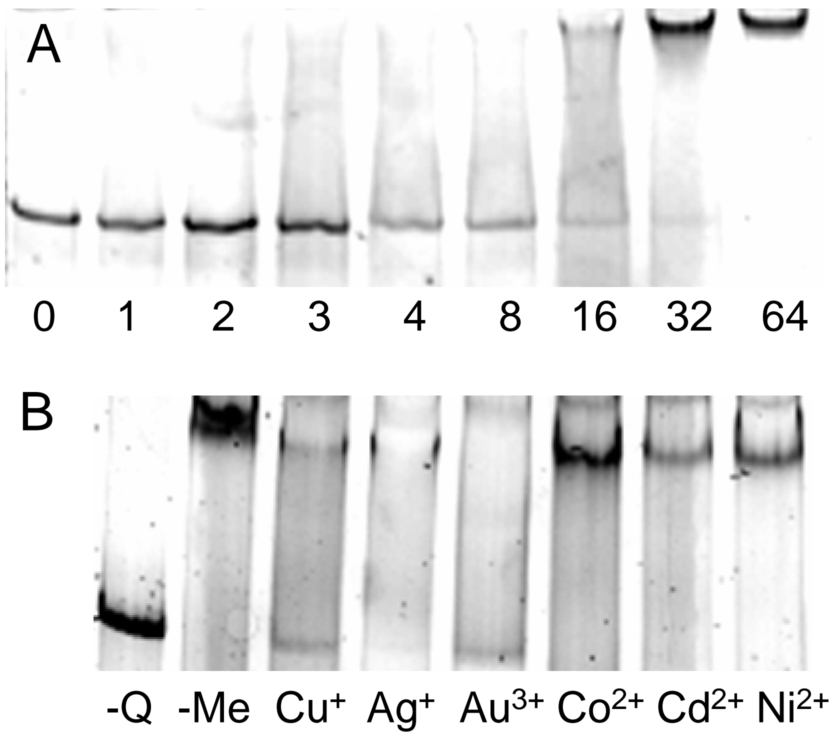
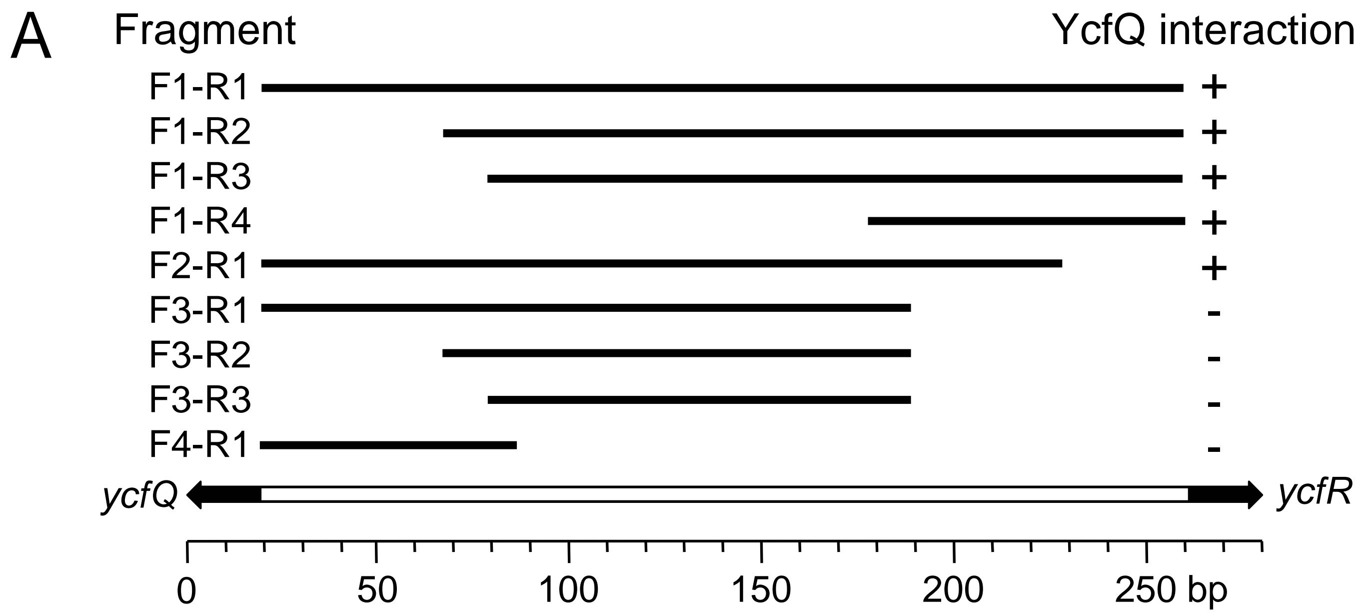


Figure 7



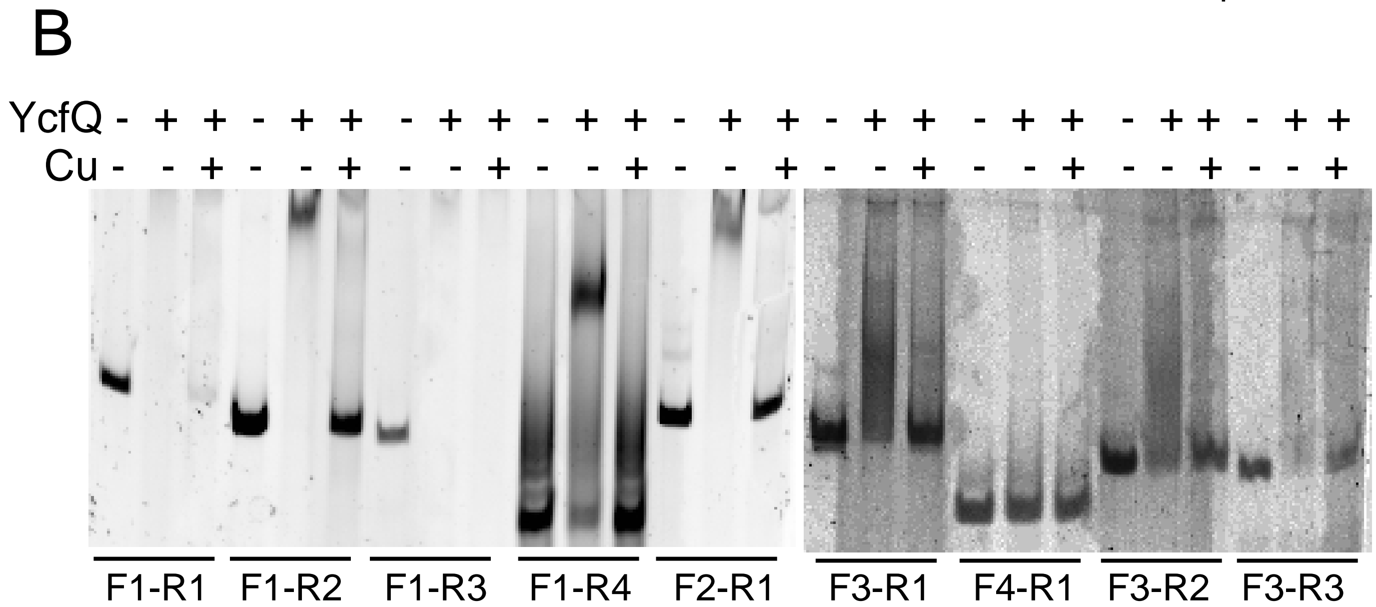


Figure 8

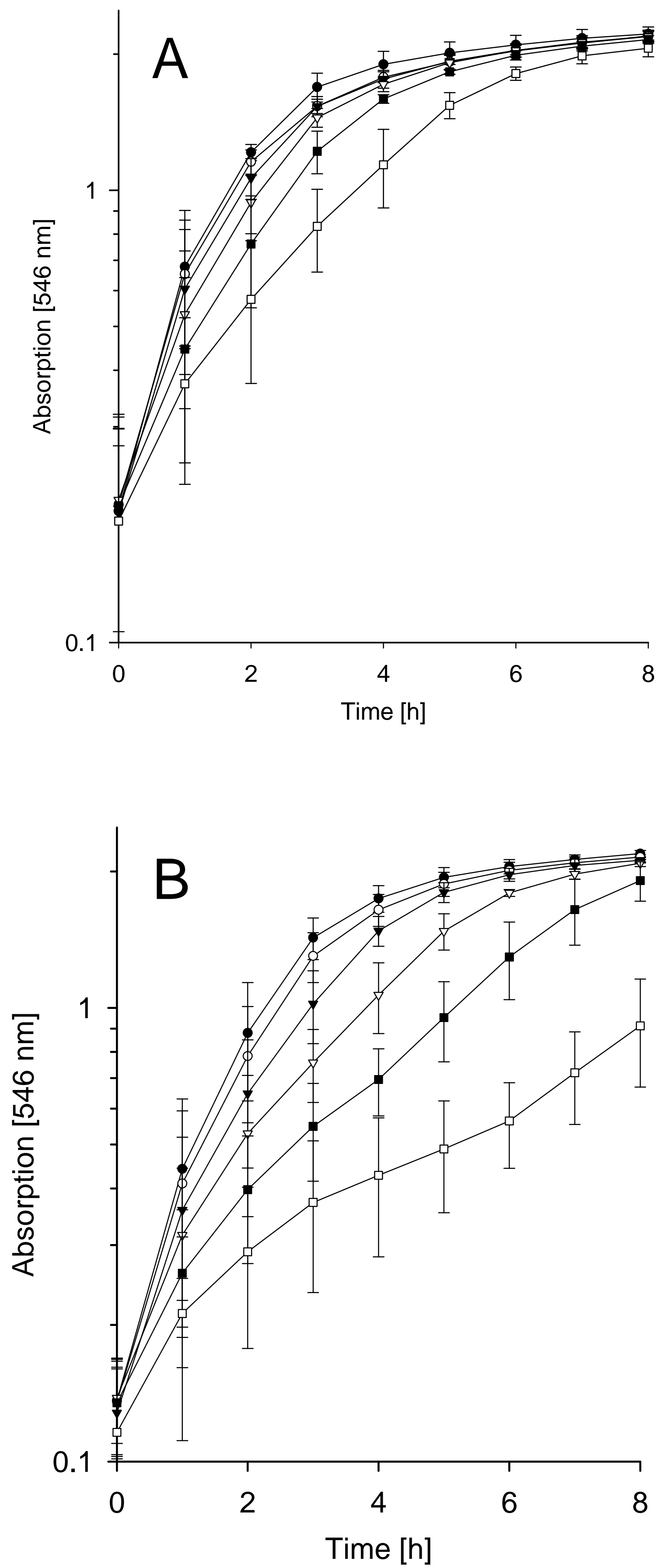
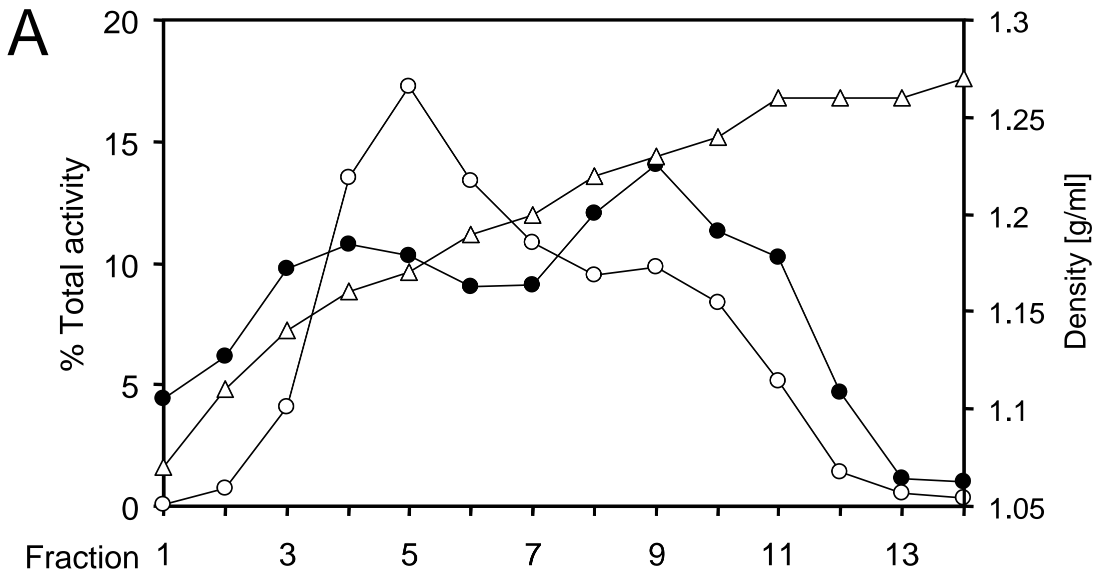
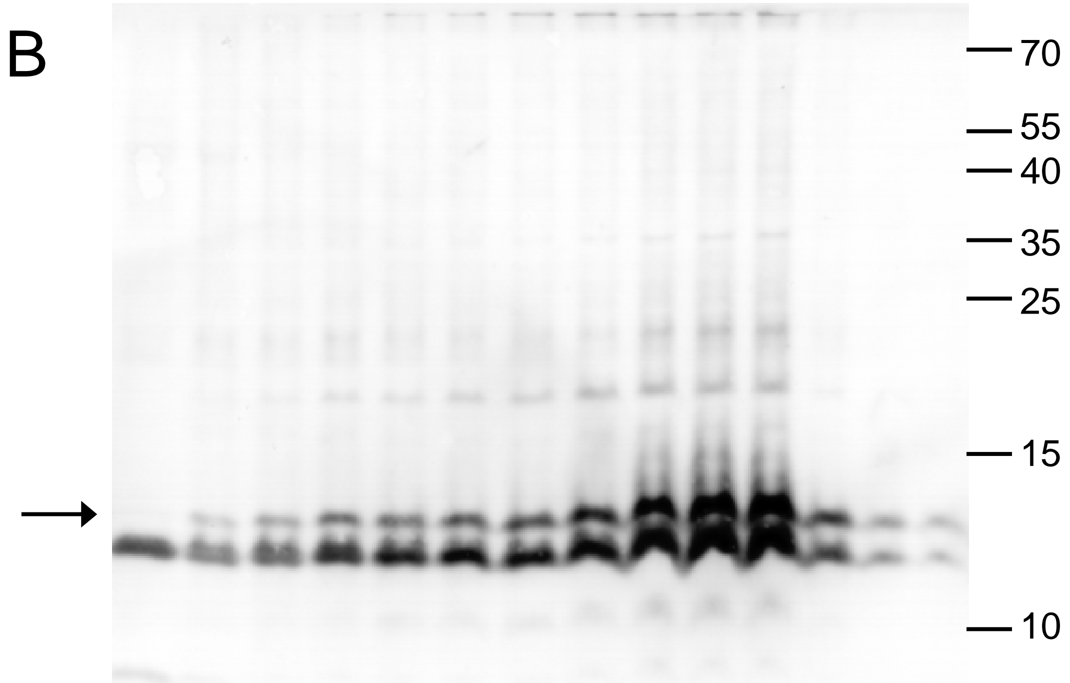


Figure 9





3) Response of Gram-positive bacteria to copper stress

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Abstract

The Gram-positive bacteria *Enterococcus hirae*, *Lactococcus lactis*, and *Bacillus subtilis* have received wide attention in the study of copper homeostasis. Consequently, copper extrusion by ATPases, gene regulation by copper, and intracellular copper chaperoning are understood in some detail. This has provided profound insight into basic principles of how organisms handle copper. It also emerged that many bacterial species may not require copper for life, making copper homeostatic systems pure defense mechanisms. Structural work on copper homeostatic proteins has given insight into copper coordination and bonding and has started to give molecular insight into copper handling in biological systems. Finally, recent biochemical work has shed new light on the mechanism of copper toxicity, which may not primarily be mediated by reactive oxygen radicals.

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MINIREVIEW

Response of Gram-positive bacteria to copper stress

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Abstract The Gram-positive bacteria *Enterococcus hirae*, *Lactococcus lactis*, and *Bacillus subtilis* have received wide attention in the study of copper homeostasis. Consequently, copper extrusion by ATPases, gene regulation by copper, and intracellular copper chaperoning are understood in some detail. This has provided profound insight into basic principles of how organisms handle copper. It also emerged that many bacterial species may not require copper for life, making copper homeostatic systems pure defense mechanisms. Structural work on copper homeostatic proteins has given insight into copper coordination and bonding and has started to give molecular insight into copper handling in biological systems. Finally, recent biochemical work has shed new light on the mechanism of copper toxicity, which may not primarily be mediated by reactive oxygen radicals.

Keywords Copper homeostasis · Toxicity · Copper ATPases · Gene regulation · Copper chaperones

Introduction

The differentiation of bacteria into Gram-positive and Gram-negative organisms by Gram staining, a method developed by the Danish scientist Hans Christian Gram in 1884, has remained alive to this day. This is due to the fact that Gram staining conveniently differentiates between organisms with an inner and an outer cell membrane and a cell wall between (Gram-negative) and those with only a single cell membrane surrounded by the cell wall (Gram-positive). Traditionally, Gram-positive organisms are of the phyla *Actinobacteria* and *Firmicutes*; the latter includes the genera *Bacillales*, *Clostridia*, *Lactobacillales*, and *Mollicutes*. Some *Mollicutes* (e.g. *Mycoplasma*) lack a cell walls and thus cannot be Gram-stained, but phylogenetically belong to the Gram-positive bacteria. Since very little is known about their copper metabolism, they will not be further discussed here. *Actinobacteria* is the other major group of Gram-positive bacteria. In contrast to the members of *Firmicutes*, members of *Actinobacteria* have a high guanosine plus cytosine content in their genomes. Members of *Firmicutes* are acid-tolerant, mostly nonsporulating, and generally facultative anaerobic bacteria. Gram-positive organisms in general occupy a variety of habitats, ranging from soil and water to decomposing plants and mammalian gut or oral flora, thereby also being potentially pathogenic [1].

Of the Gram-positive bacteria, lactic acid bacteria have received the widest attention owing to their extensive use in food production and preservation. The eponymous trait of these organisms, namely, the production of acid during carbohydrate fermentation, generates a very acidic environment [2]. This is often accompanied by the secretion of bacteriocins, such as nisin. Bacteriocins are proteinaceous toxins which inhibit the growth of similar or closely related bacterial strains. The combined action of low pH and bacteriocins efficiently inhibits the growth of competing bacteria, a property which is made use of in food preservation [3]. However, the acidic ambient condition can lead to the solubilization of complexed metal ions, thus

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generating unfavorably high metal ion concentrations. For example, in traditional cheese making, mostly involving members of the genus *Lactococcus*, the cells are challenged by copper released from the copper vats [4]. Copper is one of the metal ions known to exert toxic effects on bacteria and other organisms. Excess copper avidly binds to many biomolecules such as proteins, lipids, and nucleic acids, regardless of its valence state [5]. But in contrast to other toxic metals such as silver and lead, copper is also an essential trace nutrient. Bacteria therefore evolved tight copper homeostatic control mechanisms, involving copper binding and transport and the regulation of gene expression by copper. Work chiefly on *Enterococcus hirae*, *Lactococcus lactis*, and *Bacillus subtilis* has provided profound insight into basic principles of how Gram-positive organisms handle copper. Key aspects of copper handling by Gram-positive organisms will be discussed in this review.

Copper as a bioelement

Because of copper's ability to cycle between Cu^{2+} and Cu^{+} at biologically relevant redox potentials, it has become a cofactor for over 30 known enzymes in higher organisms [6]. Prominent examples are lysyl oxidase, involved in the cross-linking of collagen, tyrosinase, required for melanin synthesis, dopamine β-hydroxylase of the catecholamine pathway, cytochrome *c* oxidase as a terminal electron acceptor of the respiratory chain, and superoxide dismutase, required for defense against oxidative damage. Members of another class of copper proteins, such as plastocyanins and azurins, act as electron carriers. Depending on the type of coordination of the copper to the protein, the redox potential can vary over the range from 200 to 800 mV. Concomitant with the lower complexity of bacteria, only ten cuproenzymes have so far been characterized in microbes (Table 1). However, it is likely that many cuproenzymes have not yet been identified in eukaryotes as well as in prokaryotes.

In the primordial, anaerobic world, copper was in the Cu(I) state in the form of water-insoluble sulfides under neutral pH conditions and was only bioavailable in the acidic waters near hydrothermal vents. The emergence of an oxygen-containing atmosphere by the action of oxygen-evolving microorganisms, probably cyanobacteria, less than 3×10^9 years ago was a dramatic event for most living organisms [17]. It could be considered as an early, irreversible pollution of the earth. Most living organisms adapted to the new conditions by acquiring an oxidative metabolism. Enzymes involved in anaerobic metabolism were designed to operate in the lower portion of the redox spectrum. The arrival of dioxygen created the need for a new redox-active metal that could attain higher redox potentials. The oxidation of insoluble Cu(I) led to soluble and thus widely bioavailable Cu(II), which was ideally suited to exploit the oxidizing power of dioxygen [18]. Copper therefore is a modern bioelement [19]. Concomitant with the arrival of oxygen, multicellular organisms developed.

Interestingly, not all bacteria appear to have acquired cuproenzymes and at the current state of knowledge a distinction can be made between copper "users" and "nonusers" (Table 2). This information has been derived from a bioinformatics analysis of sequenced microorganisms [20]. Strikingly, only about half of the members of *Firmicutes* analyzed appear to be copper users. The definition of "users" here is based on the currently known bacterial cuproenzymes as outlined in Table 1. It cannot be precluded that new functions of copper will emerge that are also found in the nonusers. But this will not change the basic observation that some bacteria make extensive use of copper as a bioelement, whereas others appear to avoid it. Interestingly, members of *Firmicutes*, which are users, have an average genome size of 3 Mb, whereas the average genome size of nonusers is only 2.3 Mb [20]. The reason for this is not known.

Recently, an unexpected link between copper and molybdenum cofactor (MOCO) synthesis was discovered.

Table 1 Known bacterial copper-containing enzymes

Enzyme	Function	References
Cytochrome *c* oxidase	Terminal oxidase	[7]
NADH dehydrogenase-2	Electron transport, copper reduction	[8, 9]
Nitrosocyanin, cuproredoxin-like	Electron transfer, other?	[10]
Plastocyanins	Electron transfer	[7]
Cu-containing nitrite reductases	Nitrous oxide reduction	[11]
Tyrosinase	Phenol oxidation, melanin synthesis	[12, 13]
Copper amine oxidases	Oxidation of primary amines	[14]
Particulate methane monooxygenase	Methane oxidation	[15]
Copper-containing laccase	Polyphenol oxidase	[16]

Table 2 Occurrence of cuproenzymes in Gram-positive bacteria (from [20])

Organisms	Number of genomes	Number of "users"	MOCO synthesis
Actinobacteria	38	34	31
Firmicutes			
Bacillales	19	17	18
Clostridia	17	0	16
Lactobacillales	22	0	4
Mollicutes	17	0	0

MOCO molybdenum cofactor

Plant Cnx1G, a domain of the Cnx1GE protein, catalyzes the adenylation of molybdopterin. Cnx1G-bound molybdopterin was found to have copper bound to the molybdopterin dithiolate sulfurs [21]. The function of this bound copper is presently unknown, but copper might play a role in protecting the molybdopterin dithiolate from oxidation, and/or in presenting a suitable leaving group for molybdenum insertion [22]. It remains currently unclear if the binding of copper to molybdopterin is an essential step in MOCO synthesis, but if so, this pathway generates a copper requirement in addition to those considered in Table 1 [23]. Approximately 70% of the Gram-positive organisms are capable of synthesizing MOCO; of these, 85% are also copper users (Table 2). Only members of *Clostridia* and a few of the members of *Lactobacillales* appear to be copper nonusers and still capable of MOCO synthesis. The cooccurrence of copper use and MOCO synthesis can be observed across most bacterial phyla. Whether this has a biological significance remains open.

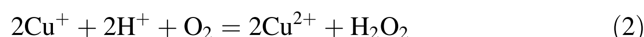
In line with the abundance of apparent copper nonusers, very few bacterial copper importers have been described. All sequenced microorganisms do, however, possess one or several defense systems against copper toxicity. An explanation for this could lie in the evolution of the first life forms in volcanic environments, such as deep-sea volcanic vents, 3.5 billion years ago [24, 25]. Owing to the high temperature and acidity, such environments are rich in dissolved heavy-metal ions [26], making defense mechanisms against these potentially toxic elements an evolutionary priority. Systems for copper defense and copper utilization may thus have evolved independently of each other.

In eukaryotic organisms, there is a clear requirement for copper import into the cytoplasm for the synthesis of cytoplasmic cuproenzymes, but also for enzymes synthesized in organelles, such as cytochrome *c* oxidase in mitochondria, and tyrosinase and ceruloplasmin in the endoplasmatic reticulum [27]. Eukaryotes therefore have copper importers, such as Ctr1, in the plasma membrane. In contrast, bacteria may not have a general requirement for cytoplasmic copper. Cyanobacteria (e.g., *Synechocystis*) are the one bacterial group that has a known demand for cytoplasmic copper for the synthesis of copper-containing, thylakoid-localized plastocyanin and cytochrome oxidase [28]. In other organisms, the cuproenzymes are localized to the cytoplasmic membrane or the periplasm and copper loading of these proteins could take place at the cytoplasmic membrane or in the periplasm. Thus, many bacteria, particular Gram-positive ones, do not appear to have a requirement for intracellular copper, and the copper homeostatic machinery in these organisms may have the sole purpose of keeping copper out. This concept is supported by the complete absence of copper chaperones in many bacteria, whereas copper chaperones are essential in eukaryotes for delivering copper to enzymes such as cytochrome *c* oxidase and superoxide dismutase [29].

Novel copper toxicity mechanisms

The major toxic effect of copper has frequently been claimed to be due to the generation of toxic reactive oxygen species in a Fenton-type reaction [30], leading to the generation of hydroxyl radicals (OH·), hydrogen peroxide (H_2O_2), and superoxide (O_2^-). Alternatively, sulfhydryl depletion by reactions 1 and 2 has been put forth as a cell-damaging mechanism.

$$2Cu^{2+} + 2RSH = 2Cu^+ + RSSR + 2H^+ \quad (1)$$


$$2Cu^+ + 2H^+ + O_2 = 2Cu^{2+} + H_2O_2 \quad (2)$$

Although lipid, protein, and nucleic acid damage by these mechanisms has been demonstrated in vitro in many studies, recent findings suggest an alternative mechanism to be responsible for the primary toxic effects of copper in vivo. First, the discovery that free copper in the cell is at extremely low levels or even nonexistent makes Fenton chemistry and sulfhydryl depletion very unlikely mechanisms [31]. Second, many Gram-positive organisms are rather tolerant to H_2O_2. For example, *L. lactis* IL1403, described in some detail below, generates H_2O_2 by NADH dehydrogenation, but does not possess catalase for H_2O_2 removal [32–34]. Third, Macomber et al. [35] recently showed that copper-loaded *Escherichia coli* was less sensitive to killing by H_2O_2 than *E. coli* cells grown without copper. Also, copper decreased the rate of H_2O_2-induced DNA damage. High intracellular copper levels even impaired iron-mediated oxidative killing by H_2O_2. The authors suggested that copper exerts its toxicity by mechanisms other than oxidative stress.

A novel mechanism of copper toxicity was indeed recently demonstrated. It could be shown in vivo as well as in vitro that copper specifically damaged the iron–sulfur

clusters of isopropylmalate dehydratase of *E. coli* [36]. This enzyme of the branched-chain amino acid biosynthesis pathway contains an iron-sulfur cluster from which the iron can be displaced by copper in the absence of oxygen. Copper efflux systems, chelation by glutathione, and cluster repair by assembly systems all enhance resistance of cells to this type of copper toxicity. To establish whether this mechanism is a general route of copper toxicity in bacteria, including Gram-positive organisms, will require further investigation.

Copper homeostasis in Gram-positive organisms

The copper homeostatic system of *E. hirae* is the best understood of those in Gram-positive bacteria and has served as a model for metal homeostasis in general [37]. The core element is an operon which consists of the four genes *copY*, *copZ*, *copA*, and *copB*. The genes *copA* and *copB* encode copper-transporting ATPases (Fig. 1). These ATPases mark the discovery of ATP-driven transmembranous copper transport in 1992 [38]. The gene product of *copY* encodes a copper-responsive repressor which derepresses transcription of the *cop* operon under conditions of copper excess (see below). The *copZ* gene, finally, encodes a copper chaperone which serves in the intracellular routing of copper. The *cop* operon enables *E. hirae* to grow in up to 8 mM copper and under copper-limiting conditions.

It is assumed that CopA acquires copper under low-copper conditions, whereas CopB extrudes excess copper,

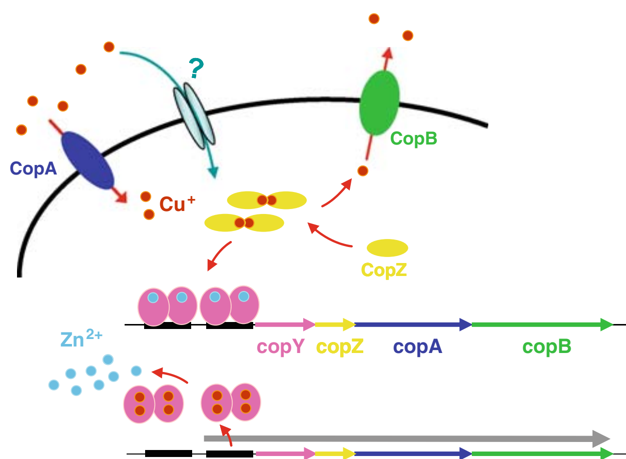


Fig. 1 Copper homeostasis in *Enterococcus hirae*. Copper enters the cell via CopA or by nonspecific leakage. Excess cytoplasmic copper binds to CopZ, which can then donate Cu^+ to CopB for export and to the CopY repressor to induce the *cop* operon. In low copper conditions, two CopY dimers in the zinc form are bound to the two *cop* boxes in front of the *cop* operon. When CopZ donates Cu^+ to CopY, one Zn^{2+} per CopY monomer is replaced by two Cu^+, with concomitant release of CopY from the promoter and induction of transcription of the downstream genes

and also silver [39, 40], when these ions are in excess. In Gram-positive bacteria, cytoplasmic enzymes that require copper are unknown. Nevertheless, specific copper importers that are expressed under copper-limiting conditions have been described in *E. hirae*, *Listeria monocytogenes*, and *B. subtilis* [41–43]. Energy-dependent copper uptake has not yet been directly demonstrated in any of these organisms and copper uptake in other ways such as copper complexed to copper chalkophores [44, 45] or as copper–substrate complexes through substrate transporters remain open possibilities [30].

YcnJ has recently been shown to be a candidate for a copper uptake pump in *B. subtilis*. The *ycnJ* gene showed a strong upregulation under copper-limiting conditions and a Δ*ycnJ* strain grew poorly under copper deprivation. On native gels, the periplasmic N-terminal domain (135 amino acids) of YcnJ oligomerized in the presence of Cu(II), but not Cu(I). Hence, in contrast to CopA of *E. hirae*, YcnJ was suggested to import copper in its divalent state [43]. Further characterization of this import system is, however, still required.

Current evidence that CopA of *E. hirae* is involved in copper import is based on the following observations: (1) Δ*copA* strains grow poorly in media where copper is limited by complexation with copper chelators and (2) Δ*copA* strains are more resistant to Ag^+ than the wild type, presumably because CopA can be a route for entry of silver into the cell [40]. Purified CopA was shown to catalyze ATP hydrolysis and to form an acylphosphate intermediate, which was inhibited by vanadate, a characteristic inhibitor of P-type ATPases. Inhibition was also detected in the presence of Cu(I) chelators, but not with Cu(II) chelators, supporting a role of CopA in the transport of Cu^+ ions [42]. However, the postulate that CopA of *E. hirae* serves in copper import still awaits rigorous experimental confirmation.

Copper secretion by copper ATPases for the rapid export of excess copper out of the cytosol is common, if not ubiquitous, and is the basic mechanism of bacterial copper resistance. The process has been documented in many eukaryotic and bacterial systems. In *E. hirae*, CopB is responsible for copper export. Cu^+ transport and Ag^+ transport by this enzyme have been directly demonstrated with radioisotopes in membrane vesicles and in whole cells loaded with silver [39, 40]. CopB features, unlike other copper ATPases, a histidine-rich N-terminus instead of a CxxC motif. Similar histidine repeat structures were found in two *Pseudomonas syringae* proteins which were demonstrated to be periplasmic copper-binding proteins [46]. In *B. subtilis*, the copper export pump CopA features two N-terminal domains, each containing a Cu^+-binding motif, CxxC. It was shown that these motifs play a role in dimerization of CopA under high copper concentrations (more than one copper ion per protein; [47, 48]).

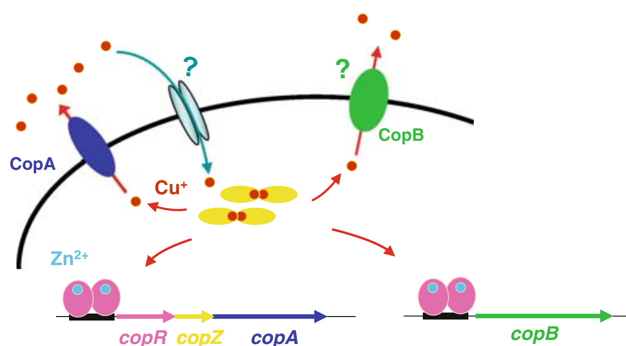


Fig. 2 Copper homeostasis in *Lactococcus lactis*. How copper enters the cell is unknown. Excess cytoplasmic copper binds to CopZ, which can then donate Cu^{+} to either the copper ATPases for export or the CopR repressor to induce transcription. In low-copper conditions, a CopR dimer in the zinc form is bound to the *cop* box in front of the *copRZA* operon and the *copB* gene. When CopZ donates Cu^{+} to CopR, one Zn^{2+} per CopR monomer is replaced by two Cu^{+}, with concomitant release of CopR from the promoters and induction of transcription of the downstream genes. CopA then accomplishes copper export from the cytoplasm. The function of CopB is unknown

In *L. lactis* IL1403, the copper-inducible *copRZA* operon encodes the CopR repressor, a CopY-type repressor, the CopZ copper chaperone, and the CopA copper ATPase (Fig. 2). The latter exhibits 45% sequence identity to CopA of *E. hirae*. This enzyme has been proposed to serve as a copper importer [37, 40, 49] under copper-limiting conditions. The nomenclature of CopA ATPases is thus confusing: with the exception of CopA of *E. hirae*, CopA copper ATPases export copper and have a role in copper resistance in all other organisms. Also in *L. lactis*, CopA clearly serves in copper extrusion [50]. The CopR repressor of *L. lactis* regulates the CopR regulon in a fashion analogous to CopY in *E. hirae*. The CopZ-like copper chaperone can be assumed to function in intracellular copper routing like CopZ of *E. hirae* [51, 52].

A second putative copper ATPase in *L. lactis* is encoded by the unlinked, monocistronic *copB* gene, which is also under the control of CopR. CopB features a histidine-rich N-terminus and shares 55% sequence identity with *E. hirae* CopB. However, a function of *L. lactis* CopB in copper export has not yet been demonstrated. It is notable that *E. hirae* CopB is encoded by the *copYZAB* operon, whereas CopB of *L. lactis* is encoded by a monocistronic gene. Whether these different gene organizations in *L. lactis* and *E. hirae* are a consequence of functional differences remains an open question.

Copper-responsive repressors

Copper-responsive transcriptional regulators detect excessive copper ions in the cell and modulate the transcription of genes and operons with roles in copper homeostasis, thereby ensuring a proper balance of copper ions in the cell. In Gram-positive bacteria, two families of copper-responsive transcriptional regulators appear to regulate copper homeostasis (Table 3). These are, named by their founding members, the CopY- and the CsoR-type regulators [53]. CopY-type regulators have experimentally been associated with gene regulation by copper in *E. hirae* [54], *Enterococcus faecium* [55], *L. lactis* IL1403 [50], *Streptococcus mutans* [56, 57], and *Streptococcus gordonii* [58].

Table 3 Distribution of copper-responsive regulators in Gram-positive bacteria and proteobacteria

Organisms	CopY-type	CsoR-type	CueR-type
Actinobacteria	0	43	0
Firmicutes			
Bacillales	3	30	7
Clostridia	0	25	0
Lactobacillales	39	3	0
Proteobacteria	0	54	215

CsoR-type regulators have only recently been described, although their occurrence is more widespread, and they are abundant not only in Gram-positive organisms, but also in members of *Proteobacteria* [53]. CsoR-related proteins may in fact be the primary copper sensors in prokaryotes which lack CueR-type regulators. In over 70% of the identified CsoR-type repressors, all three copper binding ligands were conserved. Also, many of the repressor genes were adjacent to either putative copper ATPase or copper chaperons. In more distantly related CsoR homologous, not all three copper ligands were conserved and some of these genes are adjacent to permease genes, homologous to the major facilitator superfamily. These efflux proteins may mediate multidrug resistance, thereby raising the intriguing hypothesis that some CsoR-encoding genes may have evolved to sense organic molecules [53].

CueR-type regulators, which regulate copper homeostatic genes in *E. coli* [59], occur in a few species of *Bacillales*. However, the initial claim that CueR of *B. subtilis* regulates the expression of the *copZA* operon in this organism [60] was later refuted and it was shown that a CsoR-type regulator controls *copZA* expression [61]. It thus remains unclear if CueR-type regulators have a role in copper homeostasis by Gram-positive organisms. CueR-type regulators appear to be a typical feature of the Gram-negative proteobacteria and will not be discussed further.

CopZ-type copper chaperones

Since excess copper can produce cellular damage, cells need to keep the intracellular concentration of free copper ions very low. On this account, specific copper chaperones

have evolved that tightly bind copper ions and escort them from the point of entry to target enzymes. There is a range of copper chaperones in all eukaryotes to deliver copper to copper ATPases, cytochrome *c* oxidase, or superoxide dismutase [29]. The 8-kDa Atx1-like chaperones, first described in yeast [62], have been found in all organisms, including mammals, plants, insects, fungi, and bacteria [63]. In bacteria, these copper chaperones are usually called CopZ, based on the founding member, CopZ of *E. hirae* [64]. However, many bacterial species, including *E. coli*, do not appear to possess a *copZ* gene. Among the Gram-positive bacteria, *Actinobacteria* are devoid of CopZ, whereas most, if not all other Gram-positive bacteria possess CopZ. Since the gene is small and does not always start with methionine, it may not have been discovered in all sequenced genomes.

Bacteria may be devoid of copper chaperones, or may feature only a CopZ-like or a Sco-like chaperone [65]. Sco-like proteins can be found in Gram-positive organisms [66, 67] and exhibit a thioredoxin-like fold [68]. *B. subtilis* possesses a Sco-like chaperone, YpmQ or BSco, with a proposed function in delivering copper to cytochrome *c* oxidase [69]. In contrast, a new periplasmic protein, PCu_AC, was recently shown to selectively insert Cu(I) into the Cu_A site of the ba_3 oxidase of *Thermus thermophilus* [70]. Sco1 was unable to deliver copper to the oxidase, but worked as a thiol disulfide reductase to maintain the correct oxidation state of the Cu_A cysteine ligands. This finding and the fact that some organisms possess Sco-like proteins but no cytochrome *c* oxidase [71] suggests diverse roles for Sco-like proteins in the assembly of cuproenzymes.

Many structures for Atx1- or CopZ-like metallochaperones have been solved (see [72] for a review). They all share the same $\beta\alpha\beta\beta\alpha\beta$ ferredoxin-like structure, with two cysteines of a CxxC motif located in a loop between $\beta1$ and $\alpha1$ (Fig. 3). There is still uncertainty as to how Cu^+ is complexed by the chaperone in vivo. Cu^+ can in principle bind to the two solvent-exposed cysteines, located at one end of the molecule, in a near-linear S–Cu–S bonding. However, X-ray structures of Hah1, the human CopZ-like copper chaperone, have revealed structures where a single Hg^{2+} or Cu^+ ion is complexed by the four cysteines of two chaperones in a dimeric arrangement [73]. Cu^+–CopZ of *E. hirae*, on the other hand, appeared to be dimeric in solution, with trigonally bound copper the most likely structure (Fig. 4) [74]. The prevalence of homodimeric Cu^+–CopZ was also demonstrated by biochemical and light-spectroscopic techniques [75, 76]. A three-coordinate metal center is also supported by extended X-ray absorption fine structure measurements of Cu^+–thiol bonds [74, 77]. Glutathione was shown to inhibit dimer formation in vitro and could, in principle, be a ligand to monomeric Cu^+–CopZ inside the cell, where glutathione concentrations are high. It is also conceivable that there is an equilibrium between monomeric, dimeric, and even trimeric CopZ in the cell, but this will be very difficult to assess. How CopZ interacts with CopY-type repressors and copper ATPases will be discussed in detail in the following sections.

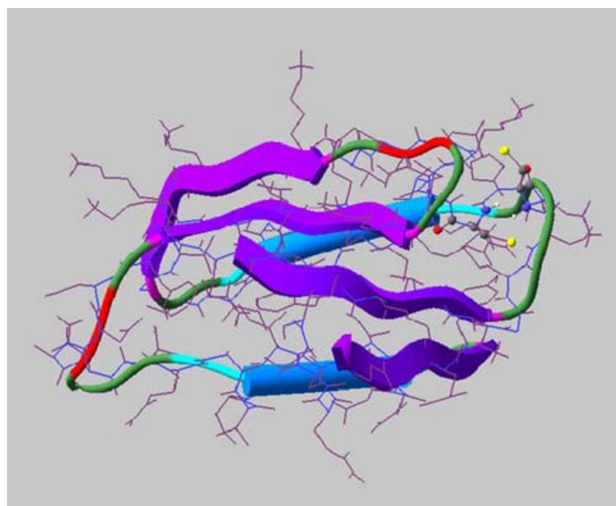


Fig. 3 Structure of CopZ of *E. hirae*. The protein is folded in a $\beta\alpha\beta\beta\alpha\beta$ structure. Note the exposed cysteines (*yellow*) which serve to bind Cu^+

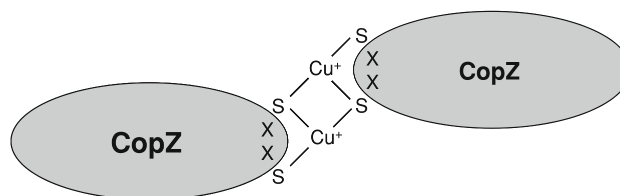


Fig. 4 Model of Cu^+–CopZ dimer formation. Each Cu^+ ion is coordinated by three sulfur atoms of the cysteine ligands of two CopZ molecules

At high intracellular copper levels CopZ appears to be degraded through a proteolytic pathway [78]. This observation led to the proposal that high levels of CopZ may be toxic to the cell; however, the mechanisms of toxicity and degradation are still unclear.

Function of CopY-type repressors

CopY and related repressors modulate the expression of genes in response to copper in most, if not all, species of *Lactobacillales*. Like many bacterial regulators, CopY-type repressors have a bipartite structure. The N-terminal domain is responsible for the interaction with DNA, and the C-terminus interacts with zinc or copper. The N-terminus of CopY of *E. hirae* shows extensive sequence similarity to BlaI, MecI, and PenI, which are repressors involved in the regulation of β-lactamase in Gram-positive

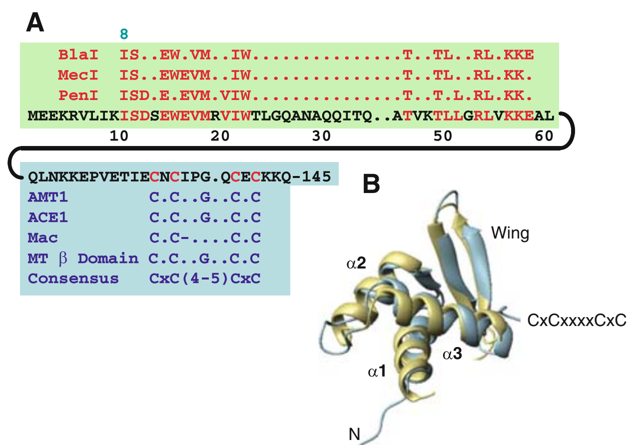


Fig. 5 **a** Alignment of the protein sequence of CopY of *E. hirae* with those of β-lactamase regulators in the N-terminal region and fungal transcriptional regulators and metallothionein in the C-terminal region. **b** Overlay of the N-terminal DNA binding domain of *L. lactis* CopR (*blue*) and the BlaI β-lactamase regulator of *Bacillus licheniformis* (*gold*)

bacteria (Fig. 5a) [79–82]. The structure of the N-terminus of CopR of *L. lactis*, a CopY homologue, has been solved by solution NMR [83] and in fact is nearly superimposable on the structure of BlaI of *Bacillus licheniformis* (Fig. 5b). The C-termini of the latter type of repressors and their mode of induction by proteolysis are entirely different from those of CopY-type repressors [84]. The C-terminus of CopR exhibits sequence similarity to the yeast copper-inducible repressors AMT1, ACE1, and Mac, and to the β-domain of metallothioneins [85]. All these proteins feature the consensus motif $CxCx_{4–5}CxC$. In the newly synthesized CopY-type repressors, this site is occupied by a single Zn^{2+}, which is coordinated by four sulfur atoms in a tetrahedral fashion.

At low ambient copper concentrations, CopY is present as a Zn(II)-containing homodimer and is bound to the operator–promoter region of the operon [54]. The CopY dimer binding sites feature the so-called *cop* box of consensus TACAnnTGTA, a motif which is widely conserved in members of *Lactobacillales*. The DNA–CopY interaction has been assessed in quantitative terms by surface plasmon resonance analysis [57]. It was found that the CopY-type repressors of *L. lactis, E. hirae*, and *S. mutans* had very similar affinities for either their native promoters or heterologous promoters, as long as they contained the *cop*-box. It could also be shown that the induction of the CopY repressor by copper resulted in a relatively moderate change of the DNA dissociation rate constant, k_d, from 1×10^{-7} to 5×10^{-7} s^{-1} [86]. Interestingly, the β-lactamase regulators which feature an N-terminal DNA binding domain essentially identical to CopY-like repressors also recognize a “*cop* box” [87]; the possible consequences of this have not been investigated.

For unknown reasons, there are two *cop* boxes upstream of the *E. hirae cop* operon, but the majority of CopY-controlled genes or operons feature only a single *cop* box. Under low-copper conditions, a CopY dimer is bound to each *cop* box and prevents transcription. When the level of medium copper is raised, Cu^+–CopZ donates Cu(I) to CopY. This displaces the bound Zn(II) and CopY is released from the DNA, allowing transcription to proceed (Fig. 6) [51, 88]. Cu^+ in CopY is trigonally bound by cysteines and solvent-shielded. This makes the protein luminescent, a typical property of solvent-shielded copper thiolates. The induction mechanism of CopY is

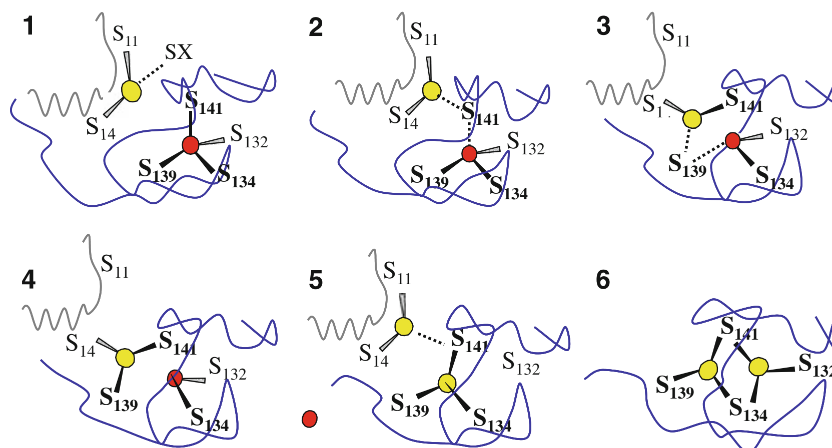


Fig. 6 Model of Cu^+ transfer from Cu^+–CopZ to Zn^{2+}–CopY. *1* Cu^+ bound to the sulfur atoms of the cysteines of C11 and C14 (S11, S14) of CopZ and probably a third ligand (e.g., glutathione) approaches CopY. *2* S141 of CopY interacts with the Cu^+, thereby destabilizing the corresponding S–Zn bond in CopY. *3, 4* a second Cu–S bond with CopY is made, further destabilizing the zinc binding. *5* Zn^{2+} is released from CopY and one Cu^+ is now bound to CopY in a triagonal $Cu–S_3$ coordination, thermodynamically aided by a second, incoming Cu^+. *6* CopY in the final $(Cu^+)_2$-CopY form

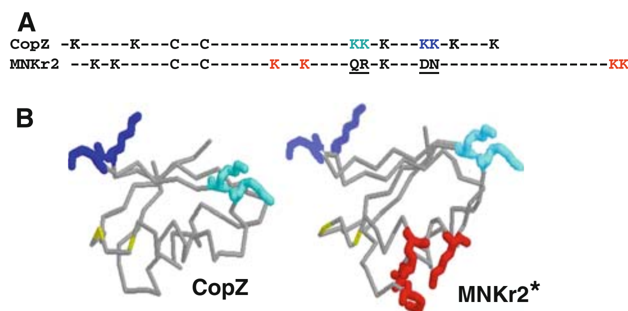


Fig. 7 Gain-of-function engineering of MNKr2. **a** Positions of lysine residues and the two copper-binding cysteines along the polypeptide chain of CopZ, compared with the positions of the corresponding residues in MNKr2. The four residues which were mutated to lysine in MNKr2* are *underlined*. **b** Arrangement of the lysine residues of CopZ which is critical for interaction with CopY and these lysine residues modeled into the MNKr2 structure. The native lysines of MNKr2 which were not mutated are shown in *red*

experimentally well supported [51, 89, 90] and protein–protein interaction between CopZ and CopY was measured by surface plasmon resonance spectroscopy [91]. This interaction appeared to involve mainly R29, R30, R36, and R37 on one face of CopZ.

The second metal binding domain of the human Menkes ATPase, MNKr2, exhibits essentially the same structure as CopZ, but cannot donate copper to CopY, presumably because it lacks the four prominent surface lysines; insertion of four corresponding lysine residues into MNKr2 resulted in a gain-of-function mutant protein which could donate copper to CopY (Fig. 7) [51]. This further supports the CopZ–CopY interactions and it will be interesting to see if the structure of CopY features the expected negative surface patch which could interact with CopZ.

Function of CsoR-type copper sensors

CsoR from *Mycobacterium tuberculosis* represents the founding member of a new and large class of prokaryotic Cu(I) regulators and its structure has recently been solved [53]. CsoR is tetrameric, with two monomers each forming a stable homodimer that adopts an antiparallel four-helix-bundle architecture (Fig. 8). This represents a novel DNA-binding fold because it lacks the obvious candidate DNA binding domains present in winged-helix-type metalloregulators. Each CsoR homodimer binds two Cu^+ such that they bridge the two subunits. By X-ray absorption spectroscopy, it was shown that Cu^+ adopts a planar trigonal coordination involving two cysteines and a histidine residue [53].

CsoR has been shown to regulate the *copZA* operon of *B. subtilis* by copper-dependent derepression [92]. The

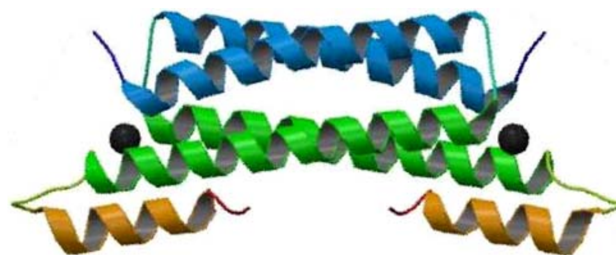


Fig. 8 Structure of a CsoR dimer from *Mycobacterium tuberculosis*. The *two dark spheres* represent bound Cu^+ (Protein Data Bank accession code 2hh7)

operon encodes a CopZ-type copper chaperone and a copper-efflux ATPase. Two tetramers of apo-CsoR were shown to bind to a 30-bp DNA region overlapping the promoter of the *copAZ* operon. The CsoR–DNA binding was weakened upon Cu^+ binding, thereby acting as a copper-inducible repressor [53]. Clearly, CopY-type and CsoR-type repressors have very different structures and activation mechanisms to fulfill essential the same role. From an evolutionary point of view, it is interesting why and how such diverse mechanisms for copper regulation of gene expression evolved.

Global responses to metal stress by *Lactococcus lactis* IL1403

The CopY-type repressors of three Gram-positive bacterial species have been shown to recognize the TACAnnTGTA consensus motif, the *cop* box [57]. By performing a genomewide search for this conserved motif in *L. lactis* IL1403, Barré et al. found 28 genes whose operator regions harbor the *cop* box. Seven of these *cop* boxes were shown to interact with CopR, the CopY-type repressor of *L. lactis*, in a copper-responsive manner in vitro. The genes and operons associated with these *cop* boxes were termed "CopR regulon," which encompasses a total of 14 genes, organized into four operons and two monocistronic genes [50]. Three proteins of the CopR regulon, namely, YaiA, a glyoxylase, YtjD, a nitroreductase, and LctO, a lactate oxidase, were independently identified by two-dimensional gel electrophoresis and mass spectrometry as copper-induced proteins [4]. For other genes of the CopR regulon, induction by copper was verified by real-time quantitative PCR.

What is the function of these genes in copper homeostasis? As described above, the *copRZA* operon functions in the defense against copper toxicity. The function of the other genes, with the exception of *lctO*, remains unclear. The *lctO* gene encodes an NAD-independent, flavin-containing lactate oxidase which converts lactate to pyruvate, using molecular oxygen. Induction of LctO was observed

when *L. lactis* cells were challenged with copper under microaerobic growth conditions, whereas copper exposure under anaerobic growth conditions failed to induce *lctO*. Since LctO requires oxygen for function, this makes biological sense, but suggests that another regulatory mechanism is also involved. Barré et al. [4] proposed that induction of lactate oxidase serves in the elimination of molecular oxygen, thereby attenuating formation of reactive oxygen radicals that could form under copper stress. Similarly, an oxygen-consuming NADH oxidase has been proposed to be involved in the defense against oxidative stress in *Lactobacillus delbrueckii* subsp. *bulgaricus* by removing oxygen and thereby preventing the generation of H_2O_2 and its reaction products [33].

Copper ATPases

All bacterial cells appear to feature copper-exporting ATPases to remove excess cytoplasmic copper. This function is accomplished by CopB in *E. hirae* and by CopA in *L. lactis* and other bacteria. Copper-importing ATPases, on the other hand, have only been described in *E. hirae* (CopA) and *Synechocystis* (CtaA). Whereas the role of *E. hirae* CopA in cell physiological function is still unclear, CtaA of *Synechocystis* has been shown to import copper for plastocyanin, a copper-containing thylakoid protein which functions in the photosynthetic electron transport chain [93].

Copper ATPases belong to the superfamily of P-type ATPases, a group of ATP-driven transport proteins characterized by unique signature motifs. The most prominent feature of this family of pumps is the formation of an acylphosphate intermediate (hence the name P-type ATPases), whereby the γ-phosphate of ATP phosphorylates the aspartic acid residue in the conserved motif DKTGT during the reaction cycle [94]. Other conserved features include consensus domains for ATP binding and energy transduction and a conserved, intramembranous proline residue with a function in ion transport [95, 96].

A subgroup of the of P-type ATPases, the CPx-type ATPases (also named heavy-metal ATPases or P1B-type ATPases [97, 98]), catalyzes the transport of transition-metal or heavy-metal ions across the membrane [99]. The range of transported substrates is wide, including monovalent (Cu^+, Ag^+) as well as divalent (Co^{2+}, Zn^{2+}, Cd^{2+}, Hg^{2+}, Pb^{2+}) cations [37, 100]. CPx-type ATPases differ from non-heavy-metal ATPases in several ways: (1) they feature only eight transmembrane helices, compared with non-heavy-metal ATPases, which feature ten [98, 101], (2) they contain one to six metal binding domains (one or two in bacteria) with a CxxC motif or a histidine-rich region at their N-terminus, (3) they possess a conserved HP

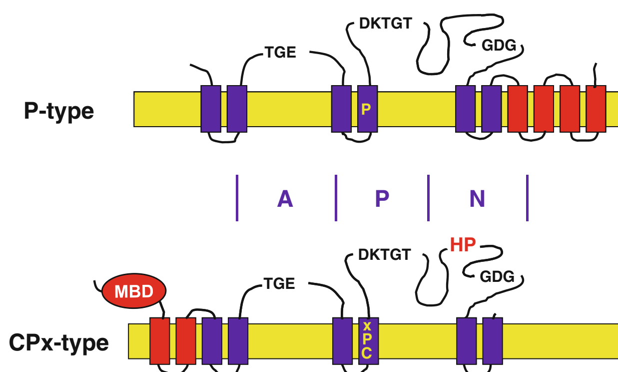


Fig. 9 P-type and CPx-type ATPases. The proteins are organized into three domains: *A* actuator domain, *P* phosphorylation domain, and *N* nucleotide binding domain. Membranes are in *yellow*, and membrane helices common to both types of ATPases are shown in *blue*, additional membrane helices in *red*. The following sequence features are also indicated: MBD, heavy-metal binding domain with either a CxxC motif or a histidine-rich region, TGE, "kinase" motif; P, intramembranous proline in non-heavy-metal ATPases; CPx, intramembranous CPC, or CPH motif in heavy-metal ATPases; TKTGT, phosphorylation motif; GDG, ATP binding region; HP, conserved motif of unknown function in heavy-metal ATPases

sequence 34–43 residues C-terminal to the CPx motif, and (4) they possess a highly conserved CPx (x is C or H) motif in the sixth transmembrane helix (Fig. 9) [98]. The CPx motif is located in the most conserved core structure of the ATPases and includes the proline characteristic to all P-type ATPases.

So far, no complete structure of a CPx-type ATPase is available, but two models were proposed, based on either cryoelectron microscopy or intramolecular cross-linking [102, 103]. Both models integrate partial structures available for some of the soluble domains and alignment with the structure of Serca1 [101]. Figure 10 shows the model for CopA by Lübben et al. [103]. The key differences from the model of Wu et al. [102] are the arrangement of the transmembrane helices and the N-terminal metal binding domain. The metal binding domain of *E. hirae* CopA is arranged such that the CxxC metal binding motif is facing away from the bulk of the ATPase and is accessible to chaperones. The respective interacting surfaces exhibited a complementary electrostatic fit. Other orientations of the metal binding domain could be ruled out because they would violate the cross-linking geometry, resulting in false lengths. This contrasts with the model of Wu et al. [102], in which the metal binding domain has the opposite orientation, with the Cu^+-binding CxxC domain facing the ATPase. The *E. hirae* CopA model shown in Fig. 10 also results in a more favorable arrangement of the conserved residues of the membrane ion channel (Fig. 11). There are two sites in the membrane domain of the copper ATPase which can be titrated with Cu^+. According to Gonzales-Guerrero et al. [104], site I is formed by two cysteines in

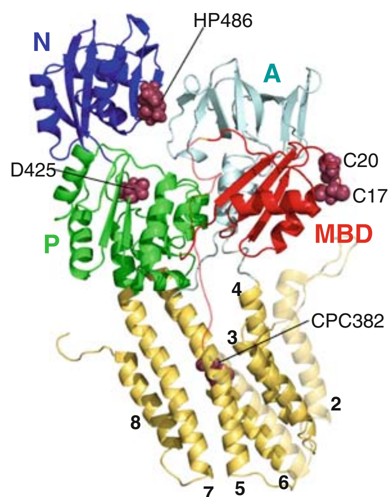


Fig. 10 Structure of the *E. hirae* CopA ATPase, modeled on the basis of intramolecular cross-linking data and known partial structures [103]. The metal binding domain is colored in *red*, the A-domain in *grayish blue*, the N-domain in *dark blue*, the P-domain in *green*, and the transmembranous domain in *ochre*, with helices 1 and 2 colored in a lighter shade owing to uncertainty in the position. Characteristic residues discussed in the text are shown in *brown* space-filling representation and are labeled

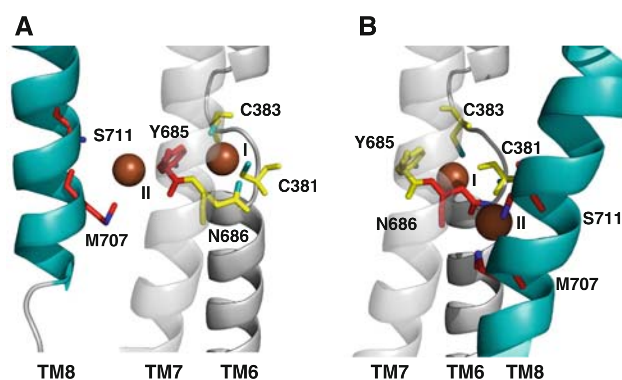


Fig. 11 Enlarged view of Cu^{+} binding sites I and II located in transmembrane helices 6, 7, and 8. Ligands are placed as in the model of *E. hirae* CopA shown in **a** or the model of *Archaeoglobus fulgidus* CopA by Wu et al. [102]. **b**. Cu^{+} site I (*yellow residues*), C381 and C383 in transmembrane helix 6 and N686 in transmembrane helix 7; Cu^{+} site II (*red residues*), Y685 in transmembrane helix 7 and M707 and S711 in transmembrane helix 8 (cartoon courtesy of Gerd Kock and Mathias Lübben, Ruhr University)

transmembrane helix 6 and a tyrosine in transmembrane helix 7 and site II is formed by asparagine in transmembrane helix 7 and methionine and serine in transmembrane helix 8. In the *E. hirae* CopA model, the copper site I is formed by C381 and C383 in transmembrane helix 6, and N686 in transmembrane helix 7, and the copper site II is formed by Y685 in transmembrane helix 7 and M707 and S711 in transmembrane helix 8. This arrangement appears sterically much more favorable. Clearly, final information on the structure of the ion channel will have to await a high-resolution X-ray structure of a copper ATPase.

CopZ of *E. hirae* has been shown by surface plasmon resonance to interact with CopA [91]. It is assumed that Cu^{+} imported by CopA is transferred to the CopZ copper chaperone, which subsequently delivers copper to the CopY repressor for induction of the *cop* operon or to other sites requiring copper. An interaction of CopZ of *E. hirae* with the copper-exporting ATPase CopB has also been shown (unpublished observation). In yeast, it has been shown that the CopZ-like chaperone, Atx1, delivers copper to the Ccc2 copper ATPase [105], which transfers copper across the membrane into the *trans*-Golgi network. Interaction of CopZ with the copper-exporting ATPase was also demonstrated in *B. subtilis* [106]. Although copper transfer from chaperones to the N-terminal metal binding domains of CPx-type ATPases is now well documented, it has never been shown that this copper can actually be transported across the membrane. Rather, it has been suggested that the N-terminus regulates the activity of the ATPase by domain interactions. Copper transport may thus require a separate copper-donation event to the membrane region of the ATPases [107].

There has been discussion of how it is mechanistically possible for copper ATPases of very similar primary structure to pump copper out of the cell in some cases (most copper ATPases), but into the cell in others (CtaA of *Synechocystis*, CopA of *E. hirae*). It is frequently ignored, even in textbooks, that the calcium ATPases of the eukaryotic plasma membrane and the sarcoplasmatic reticulum both catalyze calcium–proton antiport [108]. The ubiquitous eukaryotic NaK-ATPase catalyzes the exchange of three Na^{+} for two K^{+}, and the gastric KH-ATPase exchanges K^{+} for H^{+}. There is still debate about the stoichiometry of these exchange mechanisms because proton movements are difficult to measure in biochemical experiments, but on the basis of structural and mechanistic considerations, it appears likely that cation antiport is an obligatory feature of P-type ATPases [108]. Copper ATPases would thus exchange Cu^{+} for H^{+}. The direction of transport of an ion by a P-type ATPase is not per se a property of the E1–E2 reaction cycle. From which side of the membrane an ion has access to the high-affinity or low-affinity binding site of the enzyme determines the direction of transport. A change in the transport direction probably requires relatively minor structural alterations to reverse the affinities for the incoming and the leaving ion at the respective side of the ion gate. The slow turnover of copper ATPases makes it difficult to study their transport properties in vitro and many open questions about copper transport will remain challenges for the future.

Acknowledgments Some of the work described in this review was supported by grant 3100A0_122551 from the Swiss National Foundation and a grant from the International Copper Association.

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4) The lactic acid bacteria response to metal stress

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Abstract

Lactic acid bacteria are of great importance in the manufacture of food and dairy products, but also for an increasing number of biotechnological applications. When applied to industrial processes, these bacteria face various stress conditions, such as low pH, high temperature, osmotic shock, and metal stress. Of the latter, exposure to copper has received wide attention and detailed mechanistic insight is available. We thus have a comprehensive understanding of copper extrusion by ATPases, gene regulation by copper and intracellular copper chaperoning. Structural work on copper homeostatic proteins has given insight into copper coordination and bonding and has started to give molecular insight into copper handling in biological systems in general. Also, recent biochemical work has shed new light on the mechanism of copper toxicity. The response of lactic acid bacteria to metals other than copper has received only little attention and will be discussed for other organisms to the extent that it could be relevant for lactic acid bacteria.

Chapter 8

The lactic acid bacteria responses to heavy metal stress

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Abstract

Lactic acid bacteria (LAB) are of great importance in the manufacture of food and dairy products, but also for an increasing number of biotechnological applications. When applied to industrial processes, these bacteria face various stress conditions, such as low pH, high temperature, osmotic shock, and metal stress. Of the latter, exposure to copper has received wide attention and detailed mechanistic insight is available. We thus have a comprehensive understanding of copper extrusion by ATPases, gene regulation by copper and intracellular copper chaperoning. Structural work on copper homeostatic proteins has given insight into copper coordination and bonding and has started to give molecular insight into copper handling in biological systems in general. Also, recent biochemical work has shed new light on the mechanism of copper toxicity. The response of lactic acid bacteria to metals other than copper has received only little attention and will be discussed for other organisms to the extent that it could be relevant for LAB.

8.1 Introduction

Lactic acid bacteria (LAB) belong to the order *Lactobacillales* and produce lactic acid as a result of carbohydrate fermentation. They are widely used in the production of fermented food, such as yogurt (*Streptococci* and *Lactobacilli*), cheeses (*Lactococci*), sauerkraut (*Leuconostoc*), wine (*Oenococci*), or cured sausages like Salami (*Pediococci, Lactococci*). They are heterotrophic and generally have complex nutritional requirements because they lack many biosynthetic capabilities. Because of this, LAB are generally abundant only in environments where these requirements can be provided, such as animal oral cavities and intestines (e.g. *Enterococci*), plant leaves (*Lactobacillus, Leuconostoc*), decaying plant or animal matter, feces, compost, etc.

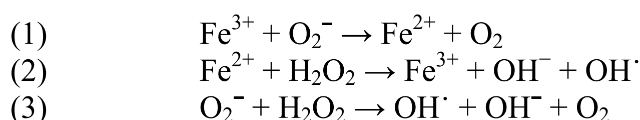
LAB are used in the food industry for several reasons. Their growth lowers both the carbohydrate content of the foods that they ferment, and the pH due to lactic acid production. This is often accompanied by the secretion of bacteriocins, such as nisin. Bacteriocins are proteinaceous toxins which inhibit the growth of similar or closely related bacterial strains. The combined action of low pH and bacteriocins is very important in food preservation to efficiently inhibit the growth of competing bacteria, including the most common human pathogens (Galvez et al., 2007). This bestows prolonged shelf lives on these foods. The acidity also changes the texture of the foods due to precipitation of some proteins. In addition, the biochemical conversions involved in growth greatly enrich the flavor of fermented food. The acidic ambient generated by the secreted lactic acid can lead to the solubilization of complexed metal ions. For example in traditional cheese making, the cells are challenged by copper released from the copper kettles (Kiermeier and Kyrein, 1971). While this process is important for flavor development (Steffen et al., 2009), it also puts stress on the bacteria.

A distinction should be made between metal ions which are required by LAB for certain enzyme functions and are thus vital, and metals that are only toxic without a benefit for life. Of the trace metals known to function in biochemical processes, iron, zinc, and magnesium are probably used by all bacteria, while nickel, cobalt, selenium, or molybdenum are only used by some. No function for copper or selenium has been identified in any member of the *Lactobacillales*, while only a few organisms of this order have an apparent requirement for nickel or cobalt (Table 8.1).

Some trace metals, like iron, selenium, molybdenum, manganese, and copper are often scarce in the environment and it can be assumed that cells requiring these metals possess corresponding uptake systems. However, few of these have been characterized to date. For essential metals which can occur in widely different bioavailable concentrations in the environments, like copper, nickel, cobalt, and zinc, bacteria must have homeostatic control mechanisms which can deal with excess as well as with deficiency. For purely toxic metals without a known function in biology, like lead, silver, or cadmium, specialized defense mechanisms have evolved in many bacterial species (a biological function for cadmium has been described in a marine diatom, but this may represent an exceptional case (Lane and Morel, 2000)). Unfortunately, the knowledge of metal homeostasis and defense against metal stress by LAB is still very limited. Of all the biologically relevant metals, copper has by far received the widest attention. Copper homeostasis and the response to copper stress have been studied in detail in *Lactococcus lactis* and *Enterococcus hirae* and will be a major focus of this chapter. Stress responses to other metals, which have received little interest in LAB, will also be discussed for related bacteria such as *Bacillus subtilis*, to the extent that such work could be relevant to LAB on the basis of the known gene complements. Vanadium, molybdenum, and tungsten, which serve as cofactors in a variety of bacterial enzymes, will not be discussed as they are generally rare in the ambient and have not received any attention in LAB.

8.2 Metal toxicity mechanisms

Several reactive oxygen species (ROS) and one thiyl radical ($RS^{\cdot}$) can be formed in cells and can exert toxicity by modifying biomolecules (see (Miyoshi et al., 2003) for review). Metal ions can catalyze some of the reactions which lead to their formation, which is one of the underlying mechanisms of metal-induced stress. Superoxide radicals (O_2^-) are formed when oxygen takes up one electron. It is a product of "leaks" in the mitochondrial electron transport chain, but it can also be produced by macrophages in the 'oxidative burst', which is an important bactericidal action by these cells. O_2^- can be directly toxic, e.g. by oxidizing and displacing iron from Fe-S clusters (Fig. 8.1), whereby the released iron can catalyze additional toxic reactions (see below). Alternatively, superoxide can be converted by catalase to less toxic hydrogen peroxide (H_2O_2). However, the combination of iron, H_2O_2, and superoxide leads to the generation of hydroxyl radicals ($OH^{\cdot}$) by a combination of the Fenton reaction (2) and the Haber-Weiss reaction (3).



(1) $Fe^{3+} + O_2^- \rightarrow Fe^{2+} + O_2$

(2) $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\cdot}$

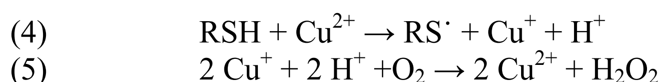
(3) $O_2^- + H_2O_2 \rightarrow OH^{\cdot} + OH^- + O_2$

$OH^{\cdot}$ are very reactive and damage biomolecules by hydroxylation. In the overall ROS-scheme, iron, copper, and other redox-active metal ions exert their effects by stimulating the Fenton reaction.

H_2O_2 is mainly produced by enzymatic reactions, such as the dehydrogenation of NAD(P)H. Since most *Lactococci* do not possess catalase, H_2O_2 can reach levels of 1-2% (Rochat et al., 2006) and may thus be considered not very toxic *per se*. It can leave the cells by diffusion through the membrane. H_2O_2 is also produced by dismutation of O_2^- by superoxide dismutase. The combination of superoxide dismutase and catalase provides an efficient antioxidant mechanism.

Nitric oxide is naturally formed in activated macrophages and endothelial cells and is considered as an active agent in several pathologies based on inflammation and organ reperfusion and may also play an important role in atherosclerosis. It has a poor oxidizing power and is even antioxidant under physiological concentrations (up to 100 nM). It does, however, react rapidly with oxygen to yield nitrogen dioxide ($NO_2^{\cdot}$) which in turn may react with $NO^{\cdot}$ to yield nitrogen trioxide, N_2O_3. The rapid reaction of O_2^- with $NO^{\cdot}$ gives the extremely reactive peroxynitrite, $ONOO^-$, which mediates oxidation, nitrosation, and nitration reactions.

Aliphatic thiols, RSH, are contained in living organisms in high concentrations. Typical levels of intracellular glutathione are 5 to 10 mM and a similar level of RSH is provided by cysteines in proteins. RSH can be oxidized in the presence of redox-active metal ions like iron or copper ions according to reactions (4) and (5).



(4) $RSH + Cu^{2+} \rightarrow RS^{\cdot} + Cu^+ + H^+$

(5) $2\ Cu^+ + 2\ H^+ + O_2 \rightarrow 2\ Cu^{2+} + H_2O_2$

The thiyl radicals have strong reactivity towards oxygen (5).

(5) $RS^{\cdot} + O_2 \rightarrow RSO_2^{\cdot}$

Furthermore, thiyl radicals are able to oxidize NADH to $NAD^{\cdot}$, ascorbic acid, and to generate various free radicals such as $OH^{\cdot}$ and O_2^-. There can also be thiol-depletion by reaction (6), in cyclic combination with reaction (5).

(6) $2\ Cu^{2+} + 2\ RSH \rightarrow 2\ Cu^+ + RSSR + 2H^+$

While lipid and protein damage by the above mechanism have been demonstrated *in vitro* in many studies, recent findings suggest that alternative mechanisms of metal toxicity may be responsible for the primary toxic effects of copper, iron, and related metals *in vivo*. First, the

discovery that free copper or iron in the cell is extremely low or even non-existent makes Fenton chemistry and sulfhydryl depletion very unlikely mechanisms (Changela et al., 2003). Second, most *Lactobacillales* are rather tolerant to H_2O_2. For example *L. lactis* IL1403, described in some detail below, generates H_2O_2 by NADH dehydrogenation, but does not possess catalase for H_2O_2 removal (Bolotin et al., 2001; Marty-Teysset et al., 2000; Rochat et al., 2006). Third, Macomber et al. recently showed that copper-loaded *Escherichia coli* was less sensitive to killing by H_2O_2 than cells grown without copper. Also, copper decreased the rate of H_2O_2-induced DNA damage. High intracellular copper levels even impaired iron-mediated oxidative killing by H_2O_2 (Macomber et al., 2007). Based on these observations the authors suggested that copper exerts its toxicity by mechanisms other than oxidative stress.
A novel mechanism of copper toxicity was indeed recently demonstrated. It could be shown *in vivo* as well as in *vitro* that copper specifically damaged the iron-sulfur clusters of isopropylmalate dehydratase of *E. coli* (Macomber and Imlay, 2009). This enzyme of the branched-chain amino acid biosynthesis pathway contains an iron-sulphur cluster from which the iron can be displaced by copper in the absence of oxygen. Copper efflux systems, chelation by glutathione, and cluster repair by assembly systems all enhance resistance of cells to this type of copper toxicity. To establish whether this mechanism is a general route of copper toxicity in bacteria, including LAB, will require further investigation.

8.3 Response to copper and silver

8.3.1 Copper as a bioelement

In the primordial, anaerobic world, copper was in the Cu(I) state in the form of water-insoluble sulphides under neutral pH conditions and was only bioavailable in the acidic waters near hydrothermal vents. The emergence of an oxygen-containing atmosphere by the action of oxygen-evolving microorganisms, probably cyanobacteria, less than 3×10^9 years ago was a dramatic event for most living organisms (Kasting and Siefert, 2002). Most of them adapted to the new conditions by acquiring an oxidative metabolism. The "old" enzymes involved in anaerobic metabolism were designed to operate in the lower portion of the redox spectrum. The arrival of dioxygen created the need for a new redox active metal that could attain higher redox potentials. The oxidation of insoluble Cu(I) led to soluble and thus widely bioavailable Cu(II), which was ideally suited to exploit the oxidizing power of dioxygen (Crichton and Pierre, 2001). Copper therefore is a modern bioelement (Kaim and Rall, 1996). Concomitant with the arrival of oxygen, multi-cellular organisms developed.
Because of copper's ability to cycle between Cu^{2+} and Cu^{+} at biologically relevant redox potentials, it has become a cofactor for over 30 known enzymes in higher organisms (Karlin, 1993). Prominent examples are lysyl oxidase involved in the crosslinking of collagen, tyrosinase required for melanin synthesis, dopamine β-hydroxylase of the catecholamine pathway, cytochrome *c* oxidase as a terminal electron acceptor of the respiratory chain, and superoxide dismutase required for defense against oxidative damage. Another class of copper proteins, such as plastocyanins or azurins acts as electron carriers. Depending on the type of coordination of the copper to the protein, the redox potential can vary over the range of + 200 to + 800 mV. Concomitant with the lower complexity of bacteria, only ten cuproenzymes have so far been characterized in microbes (Table 8.2).
Strikingly, none of the sequenced *Lactobacillales* appear to be copper users based on bioinformatics analysis of known copper enzymes. The definition of 'users' is obviously based on the currently know bacterial cuproenzymes summarized in Table 8.2. However, not all functions of copper in LAB are known. It was, for example, observed that *Lactococcus lactis* subsp. *lactis* 3022 produced more biomass when grown aerobically with hemin and copper (Kaneko et al., 1990). The activity of diacetyl synthase was greatly stimulated by the addition of hemin or copper, and the activity of NAD-dependent diacetyl reductase was very high. Pyruvate formed *via* glycolysis was converted to diacetyl, which in turn was converted to ace-

toin by the NAD-dependent diacetyl reductase to reoxidize NADH. This suggest that hemin or copper stimulates acetyl coenzyme A formation from pyruvate, but the nature of this mechanism remains unknown. At any rate, some bacteria make extensive use of copper as a bioelement, while others, like the *Lactobacillales*, use it for only a few functions, if at all. It might be speculated that there is a connection between the small average genome size of *Lactobacillales* of only 2.3 Mb and those of copper-using Gram-positive organisms, with an average genome size of 3 Mb (Ridge et al., 2008).
Recently, an unexpected link between copper and molybdenum cofactor (MOCO) synthesis was discovered. Plant Cnx1G, a domain of the Cnx1GE protein, catalyzes the adenylation of molybdopterin. Cnx1G-bound molybdopterin was found to have copper bound to the molybdopterin dithiolate sulfurs (Kuper et al., 2004). The function of this bound copper is presently unknown, but copper might play a role in protecting the molybdopterin dithiolate from oxidation, and/or in presenting a suitable leaving group for molybdenum insertion (Schwarz and Mendel, 2006). It remains currently unclear if the binding of copper to molybdopterin is an essential step in MOCO synthesis, but if so, this pathway generates a copper requirement in addition to those considered in Table 8.1 (Zhang and Gladyshev, 2008). If one looks across the bacterial phyla, a co-occurrence of copper use and MOCO synthesis strongly prevails. In the *Lactobacillales*, only three of 22 sequenced organisms appear to be capable of MOCO synthesis. Conceivably, this could create a need for copper for these apparent copper non-users. In fact, the make-up of the copper homeostasis machinery of *E. hirae* strongly argues for a copper requirement in this organism (see below).

8.3.2 Copper homeostasis in *Enterococcus hirae*

In LAB, copper homeostasis has most extensively been studied in *E. hirae* and this system has served as a model for metal homeostasis in general (Solioz and Stoyanov, 2003). The core element is an operon which consists of the four genes, *copY*, *copZ*, *copA*, and *copB*. *CopZ* encodes a copper chaperone, *copY* a copper-responsive repressor, and *copA* and *copB* encode copper transporting ATPases (Fig. 8.2). The *cop* operon enables *E. hirae* to grow in up to 8 mM copper and under copper limiting conditions. The function of the four Cop-proteins will be described below.

8.3.2.1 The CopZ copper chaperone

The identification of copper chaperones marked the emergence of a new concept in the handling of metal ions by cells, namely the escorting of the metal by a protein to prevent nonspecific, damaging interactions. There is a range of different copper chaperones in eukaryotes to deliver copper to cytochrome *c* oxidase, superoxide dismutase, or copper ATPases (Kim et al., 2008), but only two types of copper chaperones have so far been described in bacteria. Sco-like chaperones deliver copper to cytochrome oxidase; they appear to be absent in LAB. CopZ-like chaperones, on the other hand, transport copper to ATPases and transcriptional regulators and are ubiquitous in LAB (Huffman and O'Halloran, 2001). Interestingly, no CopZ-like copper chaperones have been described in Actinobacteria or in *E. coli* and related organisms and it remains unknown how copper is escorted in the cytoplasm of those organisms.
CopZ of *E. hirae* is an 8 kDa protein and the structure of CopZ and other CopZ-like proteins have been solved (see (Davis and O'Halloran, 2008) for review). They all share the same βαββαβ ferredoxin-like structure, with two exposed cysteines of a CxxC motif located in a loop between β1 and α1. There is still uncertainty as to how Cu^+ is complexed by the chaperone *in vivo*. Cu^+ can in principle bind to the CxxC motif in a near-linear S-Cu-S bonding. However, X-ray structures of Hah1, the human CopZ-like copper chaperone, have revealed structures where a single Hg^{2+} or a Cu^+ ion is complexed by the four cysteines of two chaperones in a dimeric arrangement (Rosenzweig, 2001). Cu^+-CopZ of *E. hirae* on the other hand

appeared to be dimeric in solution with triagonally bound copper to be the most likely structure (Fig. 8.3) (Wimmer et al., 1999). The prevalence of homodimeric Cu^+-CopZ was also demonstrated by biochemical and light-spectroscopic techniques (Kihlken et al., 2002; Kihlken et al., 2008). A three coordinate metal center is also supported by EXAFS measurements of Cu^+-thiol bonds (Pufahl et al., 1997; Wimmer et al., 1999). Glutathione was shown to inhibit dimer formation *in vitro* and could, in principal, be a ligand to monomeric Cu^+-CopZ inside the cell, where glutathione concentrations are high. It is also conceivable that there is an equilibrium between monomeric, dimeric, and even trimeric CopZ in the cell, but this will be difficult to assess.
CopZ of *E. hirae* was shown by surface plasmon resonance to interact with the CopA copper ATPase and the CopY repressor (Multhaup et al., 2001; Portmann et al., 2004). It is assumed that Cu^+ imported by CopA is transferred to the CopZ copper chaperone, which subsequently delivers copper to the CopY repressor for induction of the *cop* operon (discussed below) or to other sites requiring copper. An interaction of CopZ with the CopB copper exporting ATPase has also been shown (unpublished observation), suggesting that CopZ, in addition, has a role in copper export from the cell. CopZ-interaction with the copper exporting ATPase was also demonstrated in *B. subtilis* (Radford et al., 2003). In eukaryotes, the primary function of CopZ-like copper chaperones (Hah1, Atx1, Atox1) is in fact the delivery of copper to copper ATPases (Huffman and O'Halloran, 2001).

8.3.2.2 Copper ATPases

The two *E. hirae* copper ATPases mark the discovery of ATP-driven copper transport across cell membranes in 1992 (Odermatt et al., 1992). Before that time, there was no concept and no serious discussion of how copper could cross cell membranes. According to the current model, CopA serves in the uptake of copper when copper is limiting, while CopB serves in copper extrusion under conditions of copper excess (Odermatt et al., 1994; Solioz and Odermatt, 1995) (the nomenclature is confusing: copper export is accomplished by CopB in *E. hirae*, but by enzymes called 'CopA' in most other bacteria).
Copper ATPases belong to the superfamily of P-type ATPases, classically represented by eukaryotic Ca- and NaK-ATPases. The most prominent feature of this family of pumps is the formation of an acylphosphate intermediate (hence the name P-type ATPases) whereby the γ-phosphate of ATP phosphorylates the aspartic acid residue in the conserved motif DKTGT during the reaction cycle (Pedersen and Carafoli, 1987). Detailed structures of the calcium ATPase of the sarcoplasmatic reticulum have given considerable insight into the working of such ATP-driven ion pumps (Toyoshima et al., 2003; Toyoshima and Mizutani, 2004). Copper-transporting ATPases are a subgroup of the of P-type ATPases. They have been termed heavy metal ATPases, CPx-type ATPases due to a conserved intramembranous CPC or CPH motif (Lutsenko and Kaplan, 1995; Solioz and Vulpe, 1996), or P1B-type ATPases, based on more systematic phylogeny (Lutsenko and Kaplan, 1995). CPx-type ATPases are widespread in nature and have been found to catalyze the transport of a range of transition and heavy metal ions, including Cu^+, Ag^+, Co^{2+}, Zn^{2+}, Cd^{2+}, Hg^{2+}, and Pb^{2+} (Axelsen and Palmgren, 1998).
Another typical feature of CPx-type ATPases is the presence of N-terminal metal binding domains. These domains can be of two kinds. In most CPx-type ATPases, including CopA of *E. hirae*, these domains consist of a CopZ-like module with a conserved CxxC motif for copper binding and the same βαββαβ fold exhibited by CopZ-like chaperones. Some prokaryotic ATPases possess two such CopZ-like domains and eukaryotic copper ATPases have two (yeast) or six (humans) such domains (Hanson et al., 2001; Solioz et al., 1994). The second type of N-terminal metal binding domain is found in CopB of *E. hirae* and a few poorly characterized bacterial CPx-type ATPases. It consists of a histidine- and methionine-rich region. Similar repeat structures were also found in two *Pseudomonas syringae* proteins which were

demonstrated to be periplasmic copper binding proteins (Cha and Cooksey, 1991).
The function of the N-terminal metal binding domains of heavy-metal ATPases remains unclear. Copper-transfer from chaperones to the N-terminal metal binding domains of CPx-type ATPases is now well documented, but it has never been shown that this copper can actually be transported across the membrane. Rather, it has been suggested that the N-terminus regulates the activity of the ATPase by domain-domain interaction (Arguello and Gonzalez-Guerrero, 2008). Copper transport may thus require a separate copper-donation event by the chaperone to the membrane region of the ATPases (Gonzalez-Guerrero and Arguello, 2008).
In *B. subtilis*, the copper export pump CopA features two N-terminal CopZ-like copper binding domains. It was shown that these motifs play a role in dimerization of CopA, which could constitute a regulatory mechanism of the ATPase (Singleton et al., 2008; Singleton and Le Brun, 2009).
ATP-driven copper transport from the cytoplasm to the extra-cytoplasmic space, catalyzed by copper ATPases, has been extensively studied and appears to take place in all bacterial species. Cu^{+} and Ag^{+} export by CopB of *E. hirae* have been directly demonstrated with radioisotopes in membrane vesicle and in whole cells loaded with silver (Odermatt et al., 1994; Solioz and Odermatt, 1995). Copper importing ATPases, on the other hand, have only been described in *E. hirae* (CopA), *Synechocystis* sp. (CtaA), and *B. subtilis* (YcnJ) (Chillappagari et al., 2009; Odermatt et al., 1994; Tottey et al., 2001). While the role of *E. hirae* CopA in cell physiology is still unclear, CtaA of *Synechocystis* sp. imports copper for plastocyanin, a copper-containing thylakoid protein which functions in the photosynthetic electron transport chain.
Cyanobacteria (e.g. *Synechocystis* sp.) are the one bacterial group that has a known demand for cytoplasmic copper for the synthesis of copper-containing, thylakoid-localized plastocyanin and cytochrome oxidase (Tottey et al., 2005). In other organisms, the cuproenzymes are localized at the cytoplasmic membrane or in the periplasm and copper loading of these proteins could take place in the periplasmic space. In many bacteria, including LAB, no intracellular copper requirements are known at all. The copper homeostatic machinery of these organisms may thus have the sole purpose of keeping copper out. Nevertheless, specific copper importers that are expressed under copper-limiting conditions have been described in *E. hirae* and *B. subtilis* (Chillappagari et al., 2009; Wunderli-Ye and Solioz, 2001). Energy-dependent copper uptake has, however, not been directly demonstrated, either by copper ATPases or alternative mechanisms such as with chalkophores (copper 'siderophores') (Balasubramanian and Rosenzweig, 2008; Kim et al., 2004) or as copper-substrate complexes through substrate transporters. In this light, copper import into the cytoplasm of LAB still needs rigorous experimental confirmation.

8.3.2.3 Regulation of copper homeostatic genes

CopY of *E. hirae* is a copper-responsive transcriptional regulator. It responds to excessive copper in the cytoplasm by derepressing the *cop* operon. In LAB, two types of copper responsive transcriptional regulators have been identified by bioinformatics analysis of sequenced genomes, namely CopY-type and CsoR-type regulators (Table 8.3). CopY-like repressors are the principal copper-responsive regulators of LAB and have experimentally been associated with gene regulation in *E. hirae* (Strausak and Solioz, 1997), *Enterococcus faecium* (Hasman et al., 2006), *L. lactis* IL1403 (Magnani et al., 2008), *Streptococcus mutans* (Portmann et al., 2006; Vats and Lee, 2001) and *Streptococcus gordonii* (Mitrakul et al., 2004).
CsoR-type regulators have only recently been described (Liu et al., 2007), although their occurrence is more widespread in the prokaryotic world than that of CopY-type repressors. In LAB, CsoR-related proteins occur only in a minority of the sequenced species and no biochemical studies are as yet available. Finally, CueR-type regulators which regulate copper homeostatic genes in *E. coli* (Outten et al., 2000), only occur in a few species of the *Ba-*

cillales, but not in LAB. CueR-type regulators are thus the primary copper-responsive regulators of Gram-negative bacteria.
CopY has a bi-partite structure: the N-terminus interacts with DNA, while the C-terminus interacts with zinc or copper. The N-terminus shows extensive sequence similarity to BlaI, MecI, and PenI, repressors which are involved in the regulation of β-lactamase in Gram-positive bacteria (Fig. 8.4A) (Garcia-Castellanos et al., 2004; Himeno et al., 1986; Van Melckebeke et al., 2003; Wittman and Wong, 1988). The structure of the N-terminus of CopR of *L. lactis*, a CopY-homolog, has been solved by solution NMR (Cantini et al., 2009) and is in fact nearly superimposable on the structure of BlaI of *Bacillus licheniformis* (Fig. 8.4B). The C-terminus of CopY exhibits sequence similarity to the yeast copper-inducible repressors AMT1, ACE1, and Mac, and to the β-domain of metallothioneins (Bird, 2008). All these proteins feature the consensus motif $CxCx_{4\text{-}5}CxC$. In newly synthesized CopY, this site is occupied by a single Zn^{2+}, which is coordinated by four sulfur atoms in a tetrahedral fashion (Cobine et al., 2002b).
At low ambient copper concentrations, CopY is present as a Zn(II)-containing homodimer and is bound to the operator-promoter region of the operon (Strausak and Solioz, 1997). The CopY-dimer binding sites feature the so called '*cop*-box' of consensus TACAnnTGTA, a motif which is widely conserved in the *Lactobacillales*. The DNA – CopY interaction has been assessed in quantitative terms by surface plasmon resonance analysis (Portmann et al., 2006). It was found that the CopY-type repressors of *L. lactis, E. hirae*, or *S. mutans* had very similar affinities for *cop*-boxes (Portmann et al., 2004). Interestingly, the β-lactamase regulators BlaI, MecI, and PenI, which feature an N-terminal DNA binding domain essentially identical to that of CopY-like repressors, also recognize a 'cop-box' (Sharma et al., 1998); the possible consequences of this have not been investigated.
Under low copper conditions, a CopY dimer is bound to the *cop*-box and prevents transcription. When media copper is raised, two Cu^{+}-CopZ donate the copper ion to one CopY monomer. This displaces the bound Zn(II) and CopY is released from the DNA as Cu^{+}_{2}CopY, allowing transcription to proceed (Fig. 8.2). Protein-protein interaction between CopZ and CopY could be demonstrated by surface plasmon resonance spectroscopy (Multhaup et al., 2001) and the overall induction mechanism of CopY by copper and CopZ is experimentally well supported (Cobine et al., 1999; Cobine et al., 2002a; Cobine et al., 2002b; Cobine et al., 2002c). At high intracellular copper levels, CopZ is degraded through a proteolytic pathway, conceivably because high levels of Cu^{+}-CopZ may be toxic to the cell (Lu and Solioz, 2001). Following release from the DNA, Cu^{+}_{2}-CopY is probably also proteolytically degraded (unpublished observation).
CsoR-type repressors have so far only been studied in *Mycobacterium tuberculosis* and *B. subtilis*. However, it can be assumed that CsoR-type repressors work similarly in LAB and will thus be briefly discussed. CsoR from *M. tuberculosis* represents the founding member of this new class of prokaryotic Cu(I) regulators and its structure has recently been solved (Liu et al., 2007). CsoR is tetrameric, with two monomers each forming a stable homodimer that adopts an antiparallel four-helix bundle architecture. This represents a novel DNA-binding fold because it lacks the obvious candidate DNA binding domains present in winged-helix type metalloregulators like CopY and CueR. Each CsoR homodimer binds two Cu^{+} such that they bridge the two subunits. By X-ray absorption spectroscopy, it was shown that Cu^{+} adopts a planar trigonal coordination involving two cysteines and a histidine residue (Liu et al., 2007).
CsoR has been shown to regulate the *copZA* operon of *B. subtilis* by copper-dependent derepression (Ma et al., 2009). The operon encodes a CopZ-type copper chaperone and a copper efflux ATPase. Two tetramers of apo-CsoR were shown to bind to a 30 bp DNA region overlapping the promoter of the *copAZ* operon. Cu^{+} weakened the CsoR-DNA interaction, thereby inducing the operon (Liu et al., 2007). CopY-type and CsoR-type repressors feature very dif-

ferent structures and activation mechanism to fulfill essential the same role. Why such diverse mechanisms for gene regulation by copper evolved remains an interesting open question.
In the study of acid-adaptation of *Lactobacillus bulgaricus*, it was found that, among a range of three dozens of other genes, three CPx-type ATPases were induced by low pH stress (Penaud et al., 2006). One of these ATPases resembles CopB of *E. hirae* and it appears likely that it serves in copper extrusion. Acidic conditions can lead to an increase in ambient copper concentrations through the release of bound copper and induction of copper export ATPases by acid stress makes physiological sense. No *cop* boxes were present in any of the promoters of the *L. bulgaricus* CPx-type ATPases and the induction mechanism by low pH remains unknown. Acid-sensitive mutants in the unrelated microorganisms *Rhizobium leguminosarum* bv. *viciae* and *Sinorhizobim meliloti* were similarly found to have disrupted *actP* genes which encode CPx-type ATPases (Reeve et al., 2002). These mutant strains were also more sensitive to ambient copper. Copper induced expression of the wild-type *actP* genes and low pH enhanced induction two to three-fold. Downstream of the ATPase genes of both organisms are genes encoding MerR-type transcriptional regulators, termed HmrR, which apparently regulate the expression of the ActP ATPases. In *E. coli*, a MerR-type transcriptional regulator, CueR, is responsible for copper-induced transcription of the CopA copper export ATPase and the periplasmic CueO copper oxidase (Outten et al., 2000; Outten et al., 2001). Acid-induction of copper ATPases may be a more general phenomenon. Unfortunately, acid induction of the copper homeostatic genes of neither *E. coli*, nor *E. hirae* or *L. lactis* has so far been addressed.

8.3.3 Copper homeostasis in *Lactococcus lactis*

In *L. lactis* IL1403, the copper-inducible *copRZA* operon encodes the CopR repressor, a CopY-type repressor, the CopZ copper chaperone, and the CopA copper ATPase (Fig. 8.5). The latter exhibits 45 % sequence identity to CopA of *E. hirae*. In contrast to *E. hirae* CopA, *L. lactis* CopA has been shown to be a copper export ATPase (Magnani et al., 2008). The CopR repressor of *L. lactis* regulates the CopR regulon in a fashion analogous to CopY of *E. hirae*. The CopZ-like copper chaperone, finally, can be assumed to function in intracellular copper routing (Arnesano et al., 2002; Cobine et al., 2002b).
A second putative copper ATPase in *L. lactis* is encoded by the unlinked, monocistronic *copB* gene, which is also under the control of CopR. CopB features a histidine-rich N-terminus and shares 55 % sequence identity with *E. hirae* CopB. However, no function could so far be assigned to this enzyme. It is notable that *E. hirae* CopB is encoded by the *copYZAB* operon, while CopB of *L. lactis* is encoded by a monocistronic gene. Whether these different gene organizations in *L. lactis* and *E. hirae* are a consequence of functional differences remains an open question.

8.3.3.1 Global responses to copper stress by *Lactococcus lactis*

The CopR repressors of *L. lactis* also recognizes the TACAnnTGTA consensus motif, the *cop*-box (Portmann et al., 2006). By performing a genome wide search for *cop* boxes in *L. lactis* IL1403, Barré and co-workers found 28 genes whose operator regions harbor the *cop*-box. Seven of these *cop*-boxes were shown to interact with CopR in a copper-responsive manner *in vitro*. The genes and operons associated with these *cop*-boxes were collectively termed 'CopR regulon'. It encompasses a total of 14 genes, organized into four operons and two monocistronic genes (Fig. 8.6) (Magnani et al., 2008). What is the function of these additional copper-regulated genes in copper homeostasis? Only for *lctO,* a NAD-independent, flavin-containing lactate oxidase, a function has been proposed (Barré et al., 2007). Since LctO requires oxygen to convert lactate to pyruvate, it could serve in the elimination of molecular oxygen under copper stress, thereby attenuating formation of reactive oxygen radicals (Barré et al., 2007). Similarly, an oxygen-consuming NADH oxidase has been proposed to be in-

volved in the defense against oxidative stress in *Lactobacillus delbrueckii* subsp. *bulgaricus* by removing oxygen and thereby preventing the generation of H_2O_2 and its reaction products (Marty-Teysset et al., 2000).

8.3.4 Response to silver

Silver has no known biological role and is highly toxic to microorganisms. In fact, silver-impregnated materials are starting to be employed to create aseptic surfaces or oderless clothing (Sondi and Salopek-Sondi, 2004). Silver is not redox active like other toxic metals, but remains in the Ag^+ form. Silver has a very high affinity to thiolates and binds avidly to sites normally occupied by Cu(I). Intracellular copper is always in the form of Cu(I), due to the reducing ambient of the cytoplasm, and any site normally occupied by copper can be taken over by silver. In this way, silver can activate the copper-responsive repressors like CopY of *E. hirae* or CopR of *L. lactis*, which has experimentally been verified (Odermatt and Solioz, 1995). It has also been shown that silver can be a substrate for copper transporting ATPases of bacteria, fish, and mammals (Bury et al., 1999; Hanson et al., 2001; Kanamaru et al., 1994; Stoyanov et al., 2003). The copper efflux ATPase of *E. hirae*, CopB, was shown to pump Ag^+ with the same affinity and velocity as Cu^+ (Solioz and Odermatt, 1995). Ag^+ also binds to copper chaperones in a fashion analogous to that of copper (Kihlken et al., 2008; Narindrasorasak et al., 2004). Silver is thus a Cu(I) mimetic and it can be assumed that all copper resistance systems can also handle silver. However, due to the higher toxicity of silver, organisms can generally tolerate much less silver than copper.

A plasmid-born silver resistance system has been isolated from silver-resistant *Salmonella* sp. (Gupta et al., 1999). The resistance determinant encodes a periplasmic silver-specific binding protein plus two apparently parallel efflux pumps: a CPx-type ATPase, SilP, and a membrane potential-dependent cation/proton antiporter. The *sil* determinants are regulated by a two-component sensor kinase-response regulator system. Due to the similarity of Ag^+ and Cu^+, it would be expected that the Sil-system can also handle copper, but this was apparently not tested.

8.4 Response to other heavy metals

Relatively few studies have been conducted on the response of LAB to heavy metals other than copper. We will therefore also discuss some of the key findings made in other bacterial species to the extent that they could be relevant to LAB. Mercury resistance, which has received considerable attention in many bacterial species, has not been addressed to any significant extent in LAB. The interested reader is referred to the excellent review on bacterial mercury resistance by Barkay et al. (Barkay et al., 2003).

4.1 Response to iron

In air, Fe^{2+} is rapidly oxidized to Fe^{3+}, which forms hydroxides which are barely soluble at neutral pH. For this reason, bacteria generally have to deal with iron limitation rather than with iron excess. Hence, bacteria have elaborated a range of strategies to acquire iron from the environment. For one, they produce high affinity chelators (siderophores) which can solubilize Fe^{3+}. In turn, corresponding ferrisiderophores uptake systems take up the iron-siderophore complexes to cover the cellular demand for iron (Neilands, 1995). It has been proposed that *Lactobacilli* do not require iron for growth, based on the growth in iron-deficient media and other observations (Imbert and Blondeau, 1998; Weinberg, 1997). However, the genomes of LAB do contain genes which are predicted to have roles in iron acquisition. Also, recent work in this laboratory has identified HemN as an iron-requiring protein involved in heme biosynthesis. Conceivably, the iron requirement of LAB is conditional: iron may only be required for aerobic growth, which also requires a supply of exogenous heme (Brooijmans et al., 2007).

LAB grown in the presence of oxygen produce damaging ROS, such as H_2O_2, $OH^{\cdot}$, or O_2^{-}. The hydroxyl and superoxide radicals, rather than H_2O_2, represent the ROS causing toxicity for LAB. H_2O_2 is membrane permeable and can be accumulated in significant amounts by LAB. Many species, including *L. lactis* IL1403, do not possess catalase for the removal of H_2O_2 (Marty-Teysset et al., 2000; Rochat et al., 2006). However, in the presence of iron, H_2O_2 can be converted to highly reactive $OH^{\cdot}$ by a Fenton-type reaction. Therefore, intracellular iron levels may contribute significantly to the impact of high H_2O_2 levels on cell survival.
Since the importance of iron for the growth of LAB has been discounted, little work has been performed on iron homeostasis. Here, findings from related Gram-positive bacteria, e.g. *B. subtilis* or *Lactobacillus plantarum,* will also be discussed since they may be extrapolated to LAB and may serve as starting point for further investigations. In *B. subtilis*, iron homeostasis has been investigated in some detail. In these cells, the ferric uptake regulator (Fur) represses genes involved in iron uptake. Fur is a dimeric DNA binding protein with one structural Zn^{2+}-ion per monomer and possesses a regulatory Fe^{2+} binding site (Bsat and Helmann, 1999; Kehres et al., 2000). Iron starvation induced by treatment of cultures with the iron-chelator 2,2'-dipyridyl induces the Fur regulon, encompassing 20 operons with 39 genes. The same set of genes is also induced in *fur* deletion mutants, supporting the nature of the Fur regulon (Baichoo et al., 2002). *L. lactis* IL1403 possesses a Fur-like protein of similar size to *B. subtilis* Fur (128 *versus* 132 amino acids) and with 28 % sequence identity, but experimental evidence for a function of this protein in iron homeostasis is not available.
The analysis of Fur-regulated genes in *B. subtilis* has led to the identification of various iron uptake pathways which may also be present in *L. lactis*, e.g. FeuB (Accession: ABX75613, 328 amino acids, 38 % sequence identity to *B. subtilis* FeuB, 334 amino acids). In *B. subtilis*, there is a range of iron uptake systems: FeuBC is believed to take up the siderophores enterobactin and corynebactin, the latter being the siderophore produced by *B. subtilis*. Except for the YebLMN elemental iron uptake system (related to the yeast FTS3 system), the iron transporters identified in *B. subtilis* belong to the ABC transporter family (Andrews et al., 2003; Moore and Helmann, 2005). Four ABC transporters for the uptake of ferric citrate, corynebactin and hydroxamate-type siderophores appear to be present in *B. subtilis* (FeuBC, YfiZ/YfhA, FhuBG and YfmDE). Of all these iron acquisition proteins, *L. lactis* appears to only possess FeuB. This suggests on one hand that there is a need for iron uptake by this LAB, but on the other hand, indicates a very low, maybe even non-essential demand for iron. An ABC transporter, MtsABC, involved in iron and zinc uptake has also been described for *Streptococcus pyogenes* (Janulczyk et al., 1999). The isolated protein exhibited high-affinity binding of Zn(II), Fe(III), and Cu(II) *in vitro*. An *mtsABC* mutant showed lower iron and zinc uptake, but was not affected in its growth. In the light of these observations and of what is known about copper homeostasis, it appears unlikely that MtsABC acts as a copper importer in *S. pyogenes*. Convincing evidence of an ABC-type copper importer in any prokaryote has yet to be produced. All sequenced LAB genomes encode two or more ABC-type transporters, but the function of most of these have not yet been experimentally addressed.
Recently, an iron homeostatic gene, *mntH*, was identified in *Lactococcus lactis* MG1363, based on resistance of tellurite (TeO_3^{2-}) and oxidative stress (Turner et al., 2007). Tellurite exerts oxidative stress by superoxide formation which accompanies its reduction in the cytoplasm (Perez et al., 2007). The tellurite-resistant strain with a non-functional *mntH* gene exhibited greatly increased survival after 24 h of aerated growth, compared to the wild-type. MntH is a member of the family of natural resistance-associated macrophage proteins (Nramp) (Richer et al., 2003). Members of this family have been shown to serve in Mn^{2+} and Fe^{2+} uptake (Kehres et al., 2000; Makui et al., 2000). The *mntH* mutant strain exhibited reduced iron uptake, suggesting that MntH serves in Fe^{2+} uptake. This observation does, however, not rule out that MntH also has a role in manganese acquisition (Turner et al., 2007). A

strain deleted in MntH was still respiration competent when supplied with heme or protoporphyrin IX, indicating that iron is still taken up. However, excess iron may be taken up by the wild-type *via* MntH and this iron could participate in oxygen dependent toxicity in *L. lactis.*

8.4.2 Response to zinc

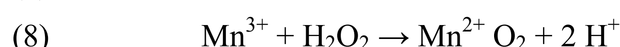
Zinc is an essential metal ion, but can be toxic if in excess. It plays a vital role as cofactor for more than 300 enzymes such as superoxide dismutase, alcohol dehydrogenase and DNA binding proteins. It also functions as structural scaffold for RNA polymerase, tRNA synthases and approximately 40 additional proteins (Coleman, 1998; Dunn et al., 2003; Outten and O'Halloran, 2001; Sun and Plapp, 1992; Vallee and Falchuk, 1993). Additionally, zinc can also function as an antioxidant by protecting sulfhydryl groups of proteins from the attack of reactive free radical species and by antagonizing free radical formation by competing with redox-active transition metals like copper and iron (Powell, 2000). In line with this, a mutant of *L. lactis* deficient in the low and high-affinity zinc uptake system was found to be more sensitive to H_2O_2 (Scott et al., 2000). In *B. subtilis*, H_2O_2 induces the Fur-like PerR repressor, which controls the expression of a dedicated zinc uptake system, ZosA, in addition to catalase and some other genes (Gaballa and Helmann, 2002). A *zosA* mutant exhibited significantly lower resistance to the diamine, a thiol-specific oxidizing agent. A similar regulatory system involved in zinc uptake and resistance to H_2O_2 was described in *L. lactis*. The two FNR-like (fumarate/nitrate reduction regulator) proteins, FlpA and FlpB, control the expression of a zinc uptake system which increases cellular zinc and enhance resistance to H_2O_2 (Gostick et al., 1999).
On the other hand, excess zinc can inhibit protein function by blocking pivotal thiols or by competing with other metal ions for binding to the active sites of proteins. Zinc at high concentrations can also bind to negatively charged domains of proteins which are crucial for function. It was for example shown that zinc inhibits cytochrome *c* oxidase, presumably by binding to the negatively charged proton entry site of the enzyme (Aagaard and Brzezinski, 2001). Clearly, zinc levels in the cell must be tightly regulated.
The first zinc resistance protein was identified in the extremely metal resistant bacterium *Ralstonia metallidurans*, followed by the CnrA protein from the same bacterium (see (Nies, 2003) for review). They are members of the RND protein family, which was first described as a related group of bacterial transport proteins involved in heavy metal resistance (*R. metallidurans*), nodulation (*Mesorhizobium loti*) and cell division (*E. coli*) (Saier, Jr. et al., 1994). This family has grown into a huge superfamily that includes seven protein families that can be found in all major kingdoms of life. In *R. metallidurans*, three genes are organized into the *czcCBA* operon. CzcCBA mediates resistance to Co^{2+}, Zn^{2+} and Cd^{2+}, driven by the proton motive force. Similar systems are also involved in nickel and manganese efflux (Claverys, 2001). These metal transporters belong to the cluster 9 family of ABC transporters, or ATP-binding cassette permeases. ABC transporters typically consist of a cytosolic metal binding protein, a membrane permease, and an ATPase, and can serve in the uptake as well as in the secretion of metal ions. ABC-transporters can be found in the genomes of all bacterial species, but the function has only been characterized in a few cases.
Zinc homeostasis has so far received little attention in LAB, but transport systems for zinc uptake as well as zinc efflux have been described for other Gram-positive bacteria (Hantke, 2005). In *Streptococcus pneumoniae*, it has been proposed that the *adcCBA* operon encodes an ATP-binding cassette transporter for zinc uptake, and *psa* one for manganese uptake (Dintilhac et al., 1997). A similar ABC-type manganese uptake system which is important for virulence has been described in *S. gordonii* (Dintilhac et al., 1997; Hantke, 2005; Hazlett et al., 2003; Jakubovics et al., 2000; Janulczyk et al., 1999) and an ABC transporter of *Streptococcus pyogenes* has been shown to bind copper, iron, and zinc, but no transport studies were performed (Janulczyk et al., 1999). In *L. lactis* IL1403, ZitSQP is an ABC transporter puta-

tively involved in high affinity Zn^{2+} uptake (Bolotin et al., 2001). The ABC transporter–encoding genes, *zitSQP*, are organized into the putative *zitRSQP* operon, also encoding the *zitR* repressor. Sequence similarities of the putative *zitR* metalloregulator suggest that *zit* expression could be regulated by zinc present in the environment, as already shown for other zinc transport operons in Gram-positive bacteria (Dintilhac et al., 1997; Gaballa and Helmann, 1998; Hantke, 2005). Several zinc uptake systems in bacteria have been shown to be under the control of a similar zinc–sensing Fur homolog, the zinc uptake repressor Zur (Dalet et al., 1999; Gaballa and Helmann, 2002; Lindsay and Foster, 2001; Patzer and Hantke, 2000).
An expression system P_{Zn}*zitR*, based on the regulatory signals (P_{Zn} promoter and *zitR* putative zinc repressor gene) of *L. lactis* IL1403 *zit* operon has been developed and shown to be highly inducible upon divalent cation starvation and strongly repressed in the presence of excess Zn^{2+}, thereby reinforcing the hypothesis of the involvement of the *zit* operon in Zn^{2+} high-affinity uptake and regulation in *L. lactis* IL1403 (Llull and Poquet, 2004).
Zinc efflux in Gram-positive bacteria has received even less attention than zinc uptake. In *Streptococcus thermophilus* 4134, the chromosomal *cadC* and *cadA* genes were shown to constitute a cadmium/zinc resistance system (Schirawski et al., 2002). They are organized in an operon, and their transcription is cadmium dependent *in vivo*. The predicted gene product of *cadA* is a CPx-type cadmium efflux ATPase and that of *cadC* an ArsR-type regulatory protein. The two *cad*-genes could also confer cadmium and zinc resistance to *L. lactis.* Clearly, the understanding of zinc homeostasis in LAB is still in its infancy and considerable more work is required to understand uptake, regulation, and secretion of zinc in these bacteria.

8.4.3. Response to manganese

Cytoplasmic manganese can help to protect bacteria against oxidative stress and the induction of manganese uptake by H_2O_2 has been observed in many bacteria (Horsburgh et al., 2002b). *L. plantarum* can accumulate manganese to over 30 mM (Archibald and Duong, 1984); uptake is accomplished by *mntA*, encoding a Mn^{2+}- and Cd^{2+}-transporting P-type ATPase (Hao et al., 1999). In *S. gordonii*, the scaCBA operon encodes an ABC-type manganese permease which is induced by low ambient manganese *via* the ScaR repressor (Jakubovics et al., 2000). ABC-type manganese permeases as described in *S. gordonii* are also widespread in LAB (Claverys, 2001). In addition to the ABC-type manganese permease MntABC, *Staphylococcus aureus* also possesses a Nramp-type manganese uptake system, MntH (Horsburgh et al., 2002a). These systems are regulated by the manganese-dependent MntR repressor and the PerR oxidative stress regulator in a concerted fashion. Similar transporters and regulators have also been described in other bacteria (see (Horsburgh et al., 2002b) for review), but studies in LAB have remained scarce.
Manganese homeostasis plays a key role in many organisms, chiefly to defy oxidative stress and/or during infection of a human host (Jakubovics et al., 2000). In many Gram-positive bacteria, the major superoxide dismutase which protects against oxidative stress is a manganese-containing enzyme (Gibson and Caparon, 1996; Poyart et al., 1998). Aside of its role as a cofactor of superoxide dismutase, manganese is able to directly protect against oxidative stress. *In vitro*, it has been shown that complexes of Mn(II) with bicarbonate can rapidly dismutate H_2O_2 (Stadtman et al., 1990). The most recently proposed scheme for this reaction involves cycling between reactions (7) and (8).

(7) $Mn^{2+} + H_2O_2 \rightarrow Mn^{3+} + 2\ OH^-$


(8) $Mn^{3+} + H_2O_2 \rightarrow Mn^{2+}\ O_2 + 2\ H^+$

The active species of manganese is a $Mn^{2+}(HCO_3^-)_2$ complex in which HCO_3^- acts as an acceptor for protons (Tikhonov et al., 2006). HCO_3^- also lowers the redox potential of the

Mn(II)-Mn(III) couple, which makes the reaction with H_2O_2 more favorable. Nevertheless, the rate constants of these reactions are still far lower than those of enzymic manganese-dependent SODs and Mn-catalases and it was recently shown that imported Mn does not significantly scavenge H_2O_2 in *E. coli* (Anjem et al., 2009). Rather, the beneficial effects of manganese appears to lies in its ability to metallate mononuclear enzymes in lieu of iron. When the iron is not deeply buried in iron-loaded enzymes, it can engage in the Fenton reaction and cause oxidative stress. The substitution of such iron by manganese under oxidative stress conditions could thus prevent protein damage. In line with this concept, *E. coli* mutants that could not import manganese were found to suffer high rates of protein oxidation (Anjem et al., 2009). Clearly, the protection of bacteria against oxidative stress by manganese remains an interesting area of investigation for the future.

8.4.4 Response to nickel

Nickel is an essential trace nutrient for some bacteria, required at nanomolar concentrations. To date, nine nickel-containing enzymes are known: urease, NiFe-hydrogenase, carbon monoxide dehydrogenase, acetyl-CoA decarboxylase/synthase, methyl coenzyme M reductase, certain superoxide dismutases, some glyoxylases, aci-reductone dioxygenase, and methylenediurease (Mulrooney and Hausinger, 2003; Ragsdale, 2009). None of these enzymes appears to play a role in LAB. Consequently, nickel is probably not an essential trace nutrient of these bacteria. Nickel in excess can induce oxidative stress in cells by cycling through the three redox states Ni^{+}, Ni^{2+}, and Ni^{3+} (Costa et al., 2002). In organisms requiring nickel, it is taken up by dedicated nickel uptake transporters, such as the NikABCDE import pump in *E. coli* (De Pina et al., 1999), or by high-affinity nickel/cobalt permeases (Eitinger et al., 2005). Members of these HoxN type permeases have been identified in Gram-negative and Gram-positive bacteria (Eitinger and Mandrand-Berthelot, 2000). Different Ni(II)-responsive metalloregulators that maintain nickel homeostasis in Gram-positive bacteria have been characterized. In *M. tuberculosis* the transcription factor NmtR of the Ars/SmtB family inhibits the expression of the gene for NmtA, an ATP-dependent transporter responsible for the efflux of nickel and cobalt. NmtR tightly binds to the promoter region of the *nmtR* and *nmtA* genes and releases the DNA when nickel, or to certain extent cobalt, is abundant (Cavet et al., 2002). A second nickel and cobalt sensor in *M. tuberculosis*, KmtA, represses the expression of a putative cation diffusion facilitator (CDF) metal exporter. NmtR and KmtA differ in their nickel/cobalt sensing affinity. It appears that first KmtR detects basal levels of cytosolic nickel or cobalt, which are then exported following expression of the CDF transporter. Only when a higher threshold of these metals accumulates does NmtR sense them and allows expression of the P-type ATPase (Campbell et al., 2007). In *Streptomyces coelicolor* the nickel-responsive regulator Nur, belonging to the Fur family, was characterized. This regulator represses the transcription of Fe-superoxide dismutase and simultaneously induces the transcription of Ni-superoxide dismutase under nickel stress (Ahn et al., 2006). It is not known how LAB respond to nickel stress. Natural environments are generally low in nickel and there may not have been a need for these organisms to evolve nickel detoxification systems. Indeed, genome analysis of sequenced LAB does not reveal any genes which are obviously connected to nickel.

8.4.5 Response to cobalt

Cobalt is a transition metal with the two naturally occurring oxidation states Co^{2+} and Co^{3+} and is primarily found in the corrin ring of coenzyme B12. To date, several also noncorrin-cobalt-containing enzymes have been isolated and characterized (Kobayashi and Shimizu, 1999). Cobalt undergoes redox chemistry and can thus participate in Fenton-type reactions, a fact making it potentially toxic at higher concentrations (Valko et al., 2005). Cobalt homeostasis is closely related to the homeostasis of nickel and other divalent ions. Both, cobalt and

nickel, are taken up by the cell via secondary metal transporters with different ion preferences, ranging from strict selectivity for nickel through unbiased transport of both ions to a strong preference for cobalt (Eitinger et al., 2005; Komeda et al., 1997). In *S. aureus*, the zinc/cobalt-responsive transcriptional repressor CzrA, which belongs to the ArsR/SmtB family, regulates the expression of the *czr* operon encoding a cobalt/zinc pump (Pennella et al., 2003). Transcriptional repressors with high sequence identities to CzrA can be found in the genomes of many LAB, but no characterization of their function has been performed to date.

8.4.6 Response to chromium

The widespread industrial use of the heavy metal chromium has caused it to be considered a serious environmental pollutant. It is mostly found in its trivalent or hexavalent forms in nature. Cr^{6+} is highly toxic to all forms of life, whereas Cr^{3+} is an essential micronutrient for many higher organisms (De Flora et al., 1990; Megharaj et al., 2003). However, for microorganisms and plants, chromium is non-essential. Chromate (CrO_4^{2-}) crosses biological membranes by means of the sulfate uptake pathway (Ramirez-Diaz et al., 2008). Inside the cell, Cr^{6+} is reduced to Cr^{3+}, a process in which free radicals may be formed (Liu and Shi, 2001). Bacterial chromium resistance systems related to plasmid genes usually encode membrane transporters which catalyze the efflux of chromate ions from the cytoplasm. The best-studied example is the *Pseudomonas aeruginosa* ChrA protein, which functions as a chemiosmotic pump that extrudes chromate from the cytoplasm using the proton motive force (Alvarez et al., 1999). A broad phylogenic analysis for *chrA* transporter genes revealed homologous genes in bacteria, archaea and fungi (Diaz-Perez et al., 2007). Several *Bacilli* possess homologous gene sequences, but none of the known chromium-defense genes are present in the sequenced LAB genomes.

8.4.7 Response to cadmium

Cadmium is a heavy metal with an oxidation state of +2. It is chemically similar to zinc and occurs naturally with zinc and lead in sulfide ores. Cadmium is not generally believed to have a biological function, however, one enzyme (cadmium-carbonic anhydrase) incorporating cadmium under low zinc conditions has been found in the marine diatom *Thalassiosira weissflogii* (Lane and Morel, 2000). In spite of not being a Fenton metal, cadmium is capable of inducing oxidative stress in cell culture models and in experimental animals (Joseph et al., 2001; Nigam et al., 1999) and may exhibit its toxicity in microorganisms in a similar way. Cadmium is accumulated by cells *via* uptake systems responsible for essential cations. In Gram-positive bacteria, such as *B. subtilis*, *S. aureus*, or *L. plantarum*, Cd^{2+} competes for transport with Mn^{2+} (Archibald and Duong, 1984; Burke and Pfister, 1986; Tynecka et al., 1981). To prevent toxic effects by cadmium, active efflux mechanisms have evolved in prokaryotes. The best characterized cadmium efflux system is that in the Gram-positive bacterium *S. aureus*, which consists of two plasmid-encoded genes, *cadA* and *cadC*. CadA, a CPx-type ATPase, catalyzes the efflux of Cd^{2+} (and probably also Zn^{2+}) and CadC is a transcriptional repressor (Nucifora et al., 1989). CadC binds specifically to the *cad* operator DNA and is released by the addition of Cd^{2+}, Pb^{2+}, and Bi^{3+} (Endo and Silver, 1995). Genetic analyses in *L. lactis* and *Oenococcus oeni* have shown the occurrence of similar plasmid-encoded cadmium resistance systems in LAB (Bon et al., 2009; Liu et al., 1997). Additionally, cadmium may be pumped out of the cell by multidrug transporters. The ATP-binding cassette (ABC) type multidrug transporters LmrA (*L. lactis*) and OmrA (*O. oeni*) could confer cadmium resistance to an *E. coli* mutant strain, which was hypersensitive to this heavy metal (Achard-Joris et al., 2005; Bourdineaud et al., 2004; Van Veen et al., 1996).

8.5 The phosphate-metal connection

Most bacteria store phosphate in phosphate polymers of up to hundreds of residues called

polyphosphates. It has been shown in a number of cases that polyphosphates are degraded under metal stress, such as by growth in the presence of lead or cadmium (Keasling, 1997). Presumably, phosphate derived from the degradation of polyphosphate is exported as complexes with toxic metal ions, thereby detoxifying the cytoplasm. For example, an *E. coli* mutant defective in both, polyphosphate kinase and polyphosphatase exhibited greatly increased cadmium sensitivity (Keasling and Hupf, 1996). The extrusion of neutral metal phosphate complexes of the form $MeHPO_4$ has in fact been directly demonstrated in *Acinetobacter johnsonii* and been shown to generate electron-motif force (Van Veen et al., 1994b). Species of *Sulfolobus* have also been shown to accomplish high copper-tolerance by induction of polyphosphatase and secretion of copper-phosphate (Remonsellez et al., 2006). The extrusion of metal-phosphate complexes takes place *via* the same Pit systems which also work in phosphate uptake (see below). Pit-systems have been shown to catalyze the translocation of phosphate complexed to Mg^{2+}, Ca^{2+}, Co^{2+}, Cu^{2+}, or Mn^{2+} (Van Veen et al., 1994a). Conceivably, complexes of phosphate with other divalent metal ions may also be translocated. LAB are generally not considered to produce polyphosphate, but there are findings which suggest that at least some of them do (Benthin et al., 1994).
The phosphate transport systems which can participate in $MeHPO_4$ extrusion can also serve in phosphate uptake under phosphate-limiting conditions. In *E. coli*, the low-affinity PitA and PitB phosphate transport systems were shown to catalyze the uptake of neutral metal-phosphate complexes (interestingly, PitA is non-functional in *E. coli* K-12 lab strains due to a mutation; (Harris et al., 2001)). Mutation of *E. coli pitA* conferred increased zinc resistance (Beard et al., 2000), and growth in the presence of zinc reduced the intracellular magnesium concentration and increased intracellular zinc, presumably due to competition between the two ions (Jackson et al., 2008). High-affinity phosphate uptake by *L. lactis* and related organisms is accomplished by an ATP-driven ABC-type transporter encoded by the *pstFEDCBA* operon. Mutations in *pstFEDCBA* were found to increase resistance to copper and zinc by lowering the intracellular reactivity of these metals, which in turn also reduced the sensitivity of the cells to oxygen (Cesselin et al., 2009). This suggests that the *pst*-system can (or must) also transport metal-phosphate complexes.
The observation of Pit- and Pst-catalyzed metal-phosphate co-transport is a surprising aspect of these transporters, which was not taken into consideration in most studies of either phosphate or metal transport. This masquerade may have disguised the true function of many transporters. The magnesium transporter, CorA, which is ubiquitous in Gram-negative bacteria, may in fact be a metal-phosphate transporter, and the magnesium transporter, MgtE, which also occurs in Gram-positive bacteria, may similarly be a metal-phosphate transporter. Clearly, much more work is required for a detailed understanding of bacterial metal transport.

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FIGURE CAPTIONS

Figure 8.1

Major oxidative damage mechanisms and their coupling to redox-active metals. Superoxide ($O_2^{\cdot -}$) produced by physiological reactions can attack iron-sulfur centers of enzymes and cause loss of the iron from the reactive center. For detoxification superoxide is converted by superoxide dismutase (SOD) to hydrogen peroxide (H_2O_2). Hydrogen peroxide can be dismutated to water and oxygen either in a non-enzymatic reaction with Mn^{2+} or by catalase, but can also undergo Fenton chemistry catalyzed by Fe^{2+} or Cu^{+}, resulting in highly toxic hydroxyl radicals ($OH^{\cdot}$). These can lead to thiol oxidation and hydroxylation of cellular constituents. Superoxide also can react with nitrous oxide radicals ($NO^{\cdot}$) to form reactive peroxynitrite ($ONOOH^{-}$) which can nitrate or hydroxylate cellular components.

Figure 8.2

Cartoon of copper homeostasis in *E. hirae*. Copper enters the cell via *CopA* or by non-specific leakage. Excess cytoplasmic copper binds to CopZ, which can then donate Cu^{+} to CopB for export and to the CopY repressor to induce the *cop* operon. In low copper conditions, CopY-dimers in the zinc form are bound to the *cop*-boxes in front of the *cop* operon. When CopZ donates Cu^{+} to CopY, one Zn^{2+} per CopY monomer is replaced by two Cu^{+} with concomitant release of CopY from the promoter and induction of transcription of the downstream genes.

Figure 8.3

Model of Cu^{+}-CopZ dimer formation. Each Cu^{+} ion is coordinated by three sulfur atoms of the cysteine ligands of two CopZ molecules.

Figure 8.4

A. Alignment of the protein sequence of CopY of *E. hirae* with those of β-lactamase regulators in the N-terminal region and fungal transcriptional regulators and metallothionein in the C-terminal region. B. Overlay of the N-terminal DNA binding domain of *L. lactis* CopR (blue) and the BlaI β-lactamase regulator of *B. licheniformis* (gold).

Figure 8.5

Cartoon of copper homeostasis in *L. lactis*. How copper enters the cell is unknown. Excess cytoplasmic copper binds to CopZ, which can then donate Cu^{+} to either the copper ATPases for export or the CopR repressor to induce transcription. In low copper conditions, a CopR-dimer in the zinc form is bound to the *cop*-box in front of the *copRZA* operon and the *copB* gene. When CopZ donates Cu^{+} to CopR, one Zn^{2+} per CopR monomer is replaced by two Cu^{+} with concomitant release of CopR from the promoters and induction of transcription of the downstream genes. CopA then accomplishes copper export from the cytoplasm. The function of CopB is unknown.

Figure 8.6

CopR regulon of *L. lactis* IL1403. The genes in full color are regulated by CopR. *Vertical lines* indicate the location of *cop*-boxes and the *lariats* those of ρ-independent transcriptional terminators. The predicted functions of the genes are indicated on the right. Genes are drawn to the scale indicated in basepairs (bp) on the top of the Figure.

TABLE CAPTIONS

Table 8.1

Use of selected metals by lactic acid bacteria

Table 8.2

Known bacterial copper-containing enzymes

Table 8.3

Copper-responsive regulators of Actinobacteria, Firmicutes, and Proteobacteria

Table 8.1

Species	Genomes	Cu users[1]	Ni users[2]	Co users[2]	Mo users[3]	Se users[4]
Enterococci	2	0	0	1	1	0
Lactobacilli	10	0	0	7	2	0
Lactococci	1	0	0	0	0	0
Leuconostoc	1	0	0	0	0	0
Oenococcus	1	0	0	0	0	0
Pediococcus	1	0	0	0	0	0
Streptococcus	6	0	1	2	0	0

[1] (Ridge et al., 2008)
[2] (Zhang et al., 2009)
[3] (Zhang and Gladyshev, 2008)
[4] (Zhang et al., 2008)

Table 8.2

Enzyme	Function	References
Cytochrome *c* oxidase	Terminal oxidase	(Cavet et al., 2003)
NADH dehydrogenase-2	Electron transport, Cu reduction	(Rapisarda et al., 2002; Rodriguez-Montelongo et al., 2006)
Nitrosocyanin, cuproredoxin-like	Electron transfer, other?	(Arciero et al., 2002)
Plastocyanins	Electron transfer	(Cavet et al., 2003)
Cu-containing nitrite reductases	Nitrous oxide reduction	(Ellis et al., 2007)
Tyrosinase	Phenol oxidation, melanin synthesis	(Lopez-Serrano et al., 2004; Tsai and Lee, 1998)
Cu amine oxidases	Oxidation of primary amines	(Brazeau et al., 2004)
Particulate methane monooxygenase	Methane oxidation	(Chan et al., 2004)
Cu,Zn-superoxide dismutase (cuprein)	Defense during infection?	(Battistoni, 2003)
Cu-containing laccase	Polyphenol oxidase	(Hullo et al., 2001)

Table 8.3

Organisms	CopY-type	CsoR-type	CueR-type
Actinobacteria	0	43	0
Firmicutes			
Bacillales	3	30	7
Clostridia	0	25	0
Lactobacillales	39	3	0
Proteobacteria	0	54	215

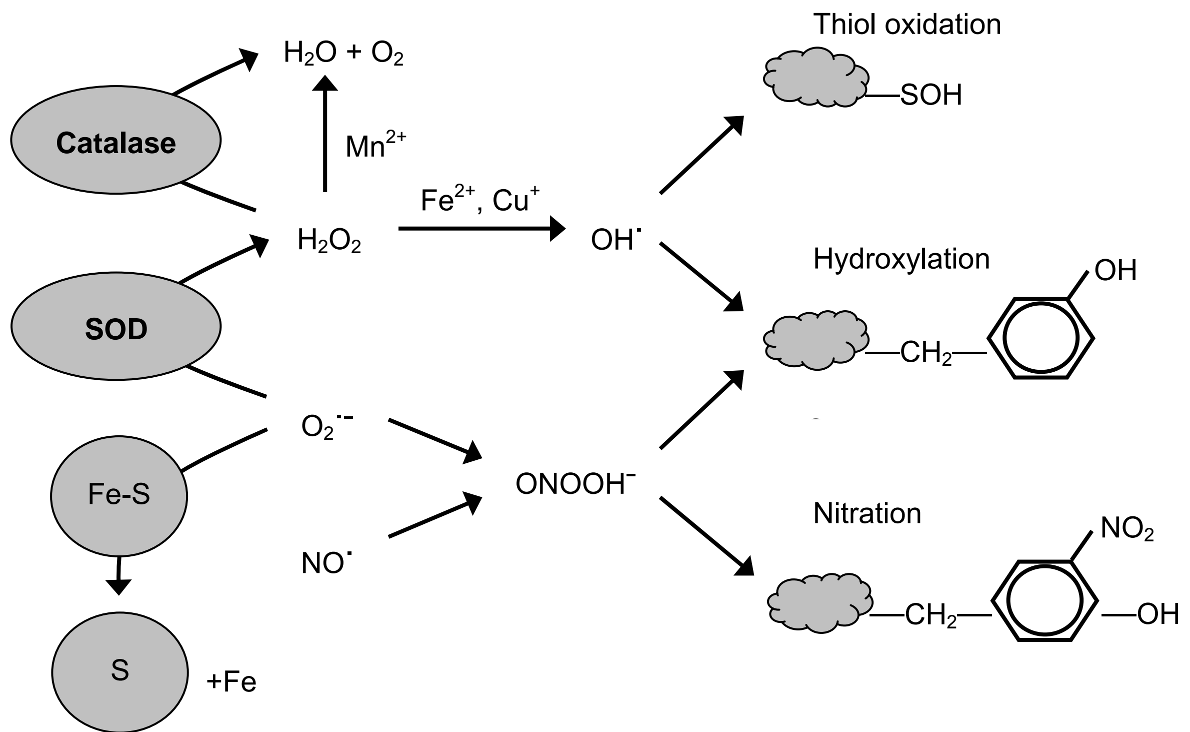


Fig. 1

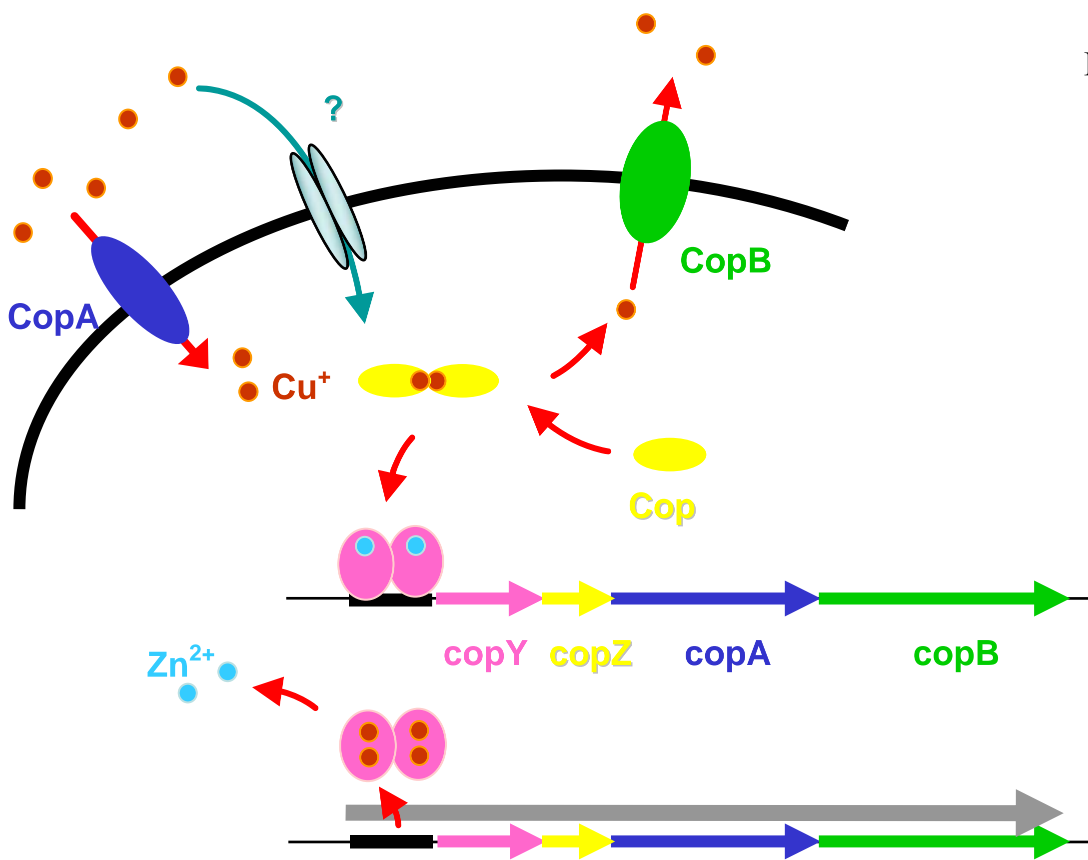


Fig. 2

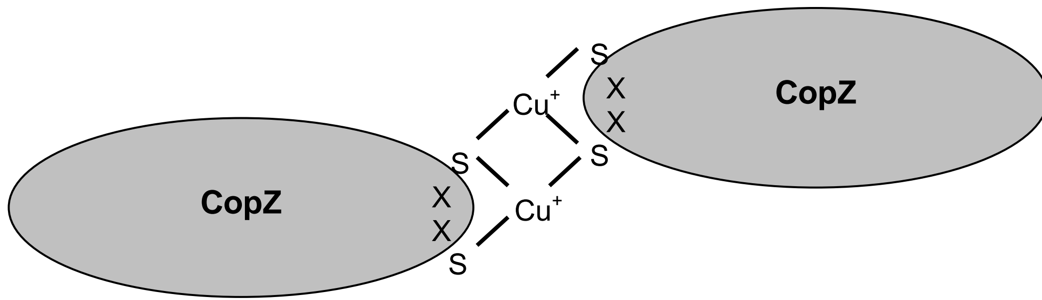


Fig. 3

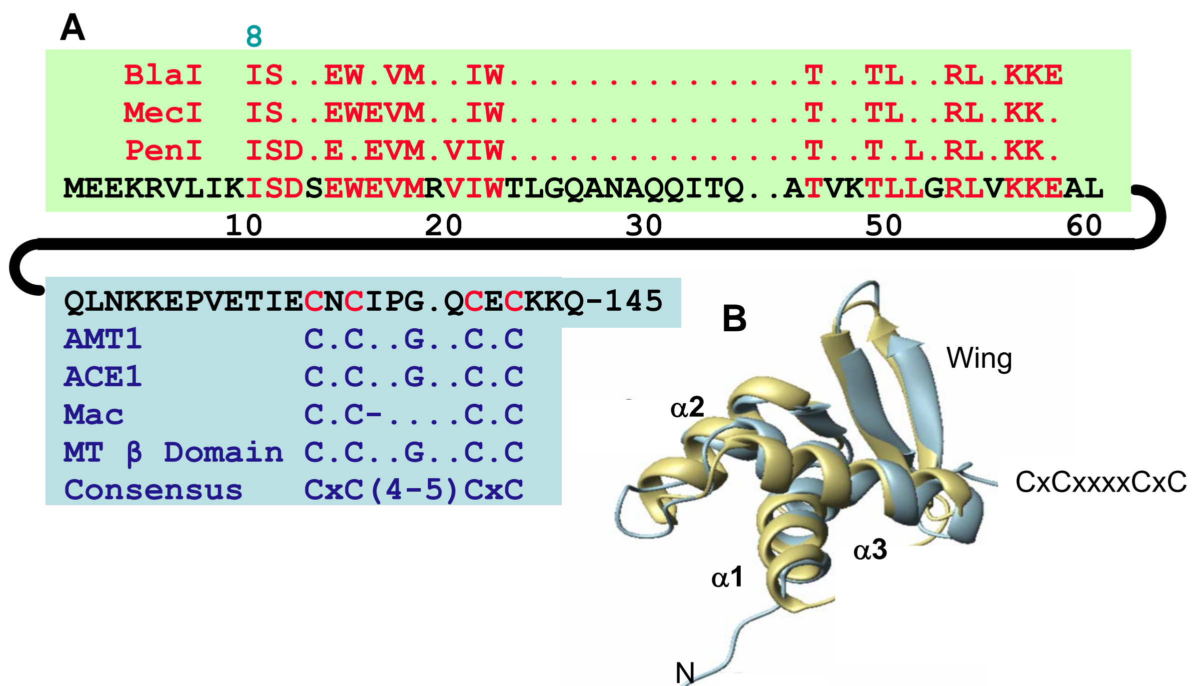


Fig. 4

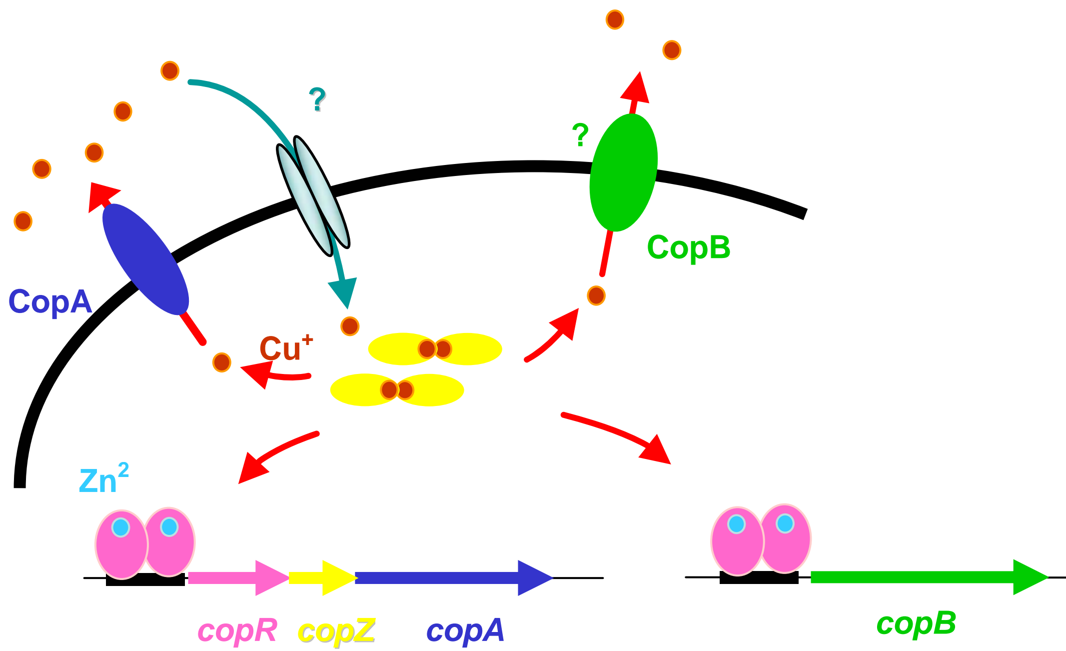
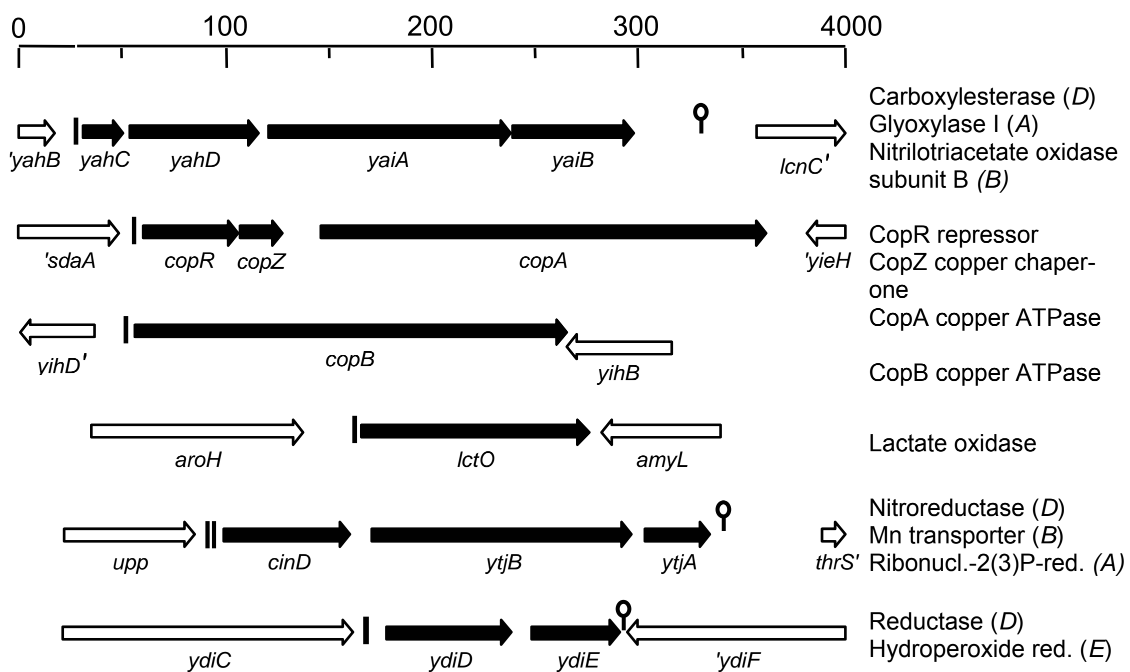


Fig. 5



Chapter III: Discussion and Outlook

Copper plays a pivotal role in cell physiology and has been regarded as important for life since pre Christian times. 1500 years before Christ, Iphyclus, prince of Thessalia was cured of his sexual impotence by Melampus, a seer. He scraped the rust off of an old knife into wine and administered the beverage to Iphyclus (6). Decades have past in which mankind has taken benefits from this metal without knowing anything about copper proteins or the molecular mechanisms of copper homeostasis. In 1962, John H. Menkes, an Austrian physician, first described an X-linked chromosomal disorder in the male offspring of an Irish family. All five boys, normal at birth, developed neurological symptoms and eventually died between the age of seven month to three years (88). It was the characteristic kinky hair of these boys that ten years later prompted the Australian pediatrician David M. Danks to draw the right conclusion (89). It caught his eye that this special hair resembled the brittle and fragile wool of sheep that had been grazing on copper poor soils and was thus connected to copper deficiency. When in 1992 the first copper pump was identified in *E. hirae* (55) it became clear that the defective Menkes gene also encoded a similar pump and that Menkes patients died of the inability to pump copper from the intestine into the body. There has been major progress in understanding copper homeostasis since then, and the high degree of conservation of the different components of copper homeostasis between bacteria and eukaryotes has allowed expanding knowledge from one cellular system to others. Nevertheless, even in bacteria, some major questions in copper homeostasis still remain open.

One unanswered question is that of how copper enters bacterial cells. It is not yet known by which pathways copper crosses the outer and inner membranes. Porins such as OmpC or OmpF have been suggested as way of entry, but porin deficient mutants of *E. coli* did not show significant increase in metal tolerance (90). In higher cells, copper is actively taken up by high-affinity copper transporters of the Ctr family in the cytoplasmic membrane (91), but in bacteria no similar system has so far been characterized. In *E. hirae*, the ATPase CopA has been proposed to serve in copper uptake (64). Direct proof of this hypothesis is, however, still missing. More recent experiments conducted with the homologous ATPase in *L. lactis*, which clearly support a role for LlCopA in copper export, cast doubt on the role of EhCopA as copper importer (56). The fact that members of the order Lactobacillales, to which *E. hirae* and *L. lactis* belong, appear to be copper "non-users" in general (12) raises the question if here specific copper importers are at all necessary. Copper import could occur via substrate-copper complexes by substrate transporters such as sugar or amino acid transporters. Alternatively, transporters for other metal ions may be leak pathways for copper (3). Neverthless, bacteria that have a clear need for copper must have an effective mechanism to acquire copper from the environment. Methanogenic bacteria have been shown to synthesize mole-

cules (methanobactin) for extracellular copper binding, analogous to the widely used iron-siderophores (92, 93). Siderophores are first secreted to the environment where they chelate iron and are subsequently taken up again by specialized transport systems as siderophore-iron complexes.

E. coli, the prokaryotic system used in the present study, expresses several cuproenzymes, and a mechanism to actively acquire copper is somehow assumed. The identification of the repressor protein YcfQ and its interaction with *ycfR* might be a small step in the direction of unraveling the question of copper entry. YcfR is a small outer membrane protein and seems to lower the permeability of the membrane under copper stress. Several studies have reported an overexpression of YcfR under stress conditions, such as acid, heat, hydrogen peroxide, or heavy metals (cadmium) and to be involved in biofilm formation (94-99). It was proposed that the expression of YcfR might be a general response to environmental stress situations. It would be interesting to know if the repressor YcfQ, which was newly identified in this study, really binds copper and as a result allows transcription of *ycfR*. (Binding studies with the purified repressor protein are currently in progress in our laboratory.) An induction directly through the metal would point towards a specific defense mechanism against copper stress. Microarray studies by *Zhang et al*. showed that a deletion of the *ycfR* gene has far-ranging effects on the expression of genes encoding cell-surface proteins, including membrane-associated transporters such as the high affinity D-Ribose transport system or transport systems for carbohydrates, organic acids, and alcohols (99). A closer look at the genomic context of *ycfR* and *ycfQ* also reveals the presence of *ndh*, the gene coding for the NADH dehydrogenase 2, just two genes downstream of *ycfQ*. The membrane bound NADH dehydrogenase 2 of *E. coli* has been shown to exhibit cupric reductase activity and might contribute to Cu^{+} uptake into cells (100). A similar copper reduction process prior to uptake has already been reported in yeast (101). Further work on the YcfRQ system will have to show the connection between this copper-induced repressor, the controlled outer membrane protein, the cupric reductase and any further interaction partner and might not only resolve the question how copper enters bacterial cells, but also in which form (Cu^{+} or Cu^{2+}).

Another open, but also far-reaching question was raised by the genome wide search for *cop* boxes by *Barré et al* (56). A *cop* box is the binding site of the copper-induced regulator CopR in *L. lactis*. The search revealed 14 candidates, arranged in four operons and two monocistronic genes, under the control of CopR (Fig. 4 on p. 10). Whereas the function for the *copRZA* operon and the *copB* gene were quickly assigned, the functions, physiological roles, and the connection to copper homeostasis of all the other copper-induced genes remained less obvious. The lactate oxidase LctO was proposed to serve in the elimination of hydrogen peroxide by nonenzymatic reaction with pyruvate (102). The role of CinD, a predicted nitroreductase, was more difficult to assign. Despite the clear gene induction by cop-

per, a gene knockout did not show any copper related phenotype. It was found instead that CinD could protect cells against the oxidative agent 4-nitroquinoline-*N*-oxide (NQO), which in turn showed only marginal induction of the gene. NQO is a known mutagenic agent and exerts its toxicity by forming DNA-adducts (103). It is possible that CinD serves in the detoxication of some specific mutagenic compounds in *L. lactis*, an action which may not only be beneficial for the bacterium itself, but also for its host (104). Different *Bacillus* strains have been shown to be able to reduce NQO and to inhibit further genotoxic effects on other organisms (105). However, the induction of the gene by copper still remains a bit puzzling and cannot be explained at this point. Copper stress might also trigger the formation of an *in vivo* substrate with similar structure, but to elucidate this, extensive metabolomic studies will be necessary. CinD was also found to exhibit catalase activity and to contribute to the general oxidative stress defense system together with LctO and the putative Manganese transporter encoded by *ytjB* (downstream of *cinD*). The functions of the other genes encompassing the CopR regulon have not yet been resolved, but many of them are likely to contribute to the same defense system. The global response system of *L. lactis* via the CopR regulon thus appears to serve in the control of oxidative stress in addition to the direct control of cytoplasmic copper levels.

The identification of CinD, YcfQ and YcfR as new components of bacterial copper homeostasis adds a new level of complexity to the process of cellular stress response to copper. Although the primary mechanisms involved in avoiding too high intracellular copper concentrations have been investigated quite in detail in several organisms, the genome wide effects and secondary interactions caused by the unusual stress situation are still mostly unknown. Insight gained into the mechanisms of bacterial copper homeostasis proved to be widely applicable, and a better understanding of these processes is fundamental to understand the role of copper in health and disease. The aim of this work was to contribute a small piece to the puzzle of copper homeostasis in bacteria, and only further investigations will show if one day, we will really be able to marvel at the whole picture.

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Manuscript in preparation

Stoyanov, J. V., Mermod, M., Magnani, D., and Solioz, M. The copper-inducible YcfQ repressor regulates YcfR expression and affects copper entry into *Escherichia coli*. J. Biol. Chem.

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Gone too soon

You took chances
Once too many times.
As a child you thought -
"Oh no, never me."
Life is a gift,
Given and taken at some
Unknown time.
Your time came too soon -
Your life was over in a flash.
The fun you shared,
The joy you brought,
All just a memory -
Behind us.

Joëlle, 1983 – 2009

Declaration of Originality

Last name, first name: **Mermod, Mélanie Jeanne**

Matriculation number: **98-909-278**

I hereby declare that this thesis represents my original work and that I have used no other sources except as noted by citations.
All data, tables, figures and text citations which have been reproduced from any other source, including the internet, have been explicitly acknowledged as such.
I am aware that in case of non-compliance, the Senate is entitled to divest me of the doctorate degree awarded to me on the basis of the present thesis, in accordance with the "Statut der Universität Bern (Universitätsstatut; UniSt)", Art. 20, of 17 December 1997.

Place, date   Signature

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