Escherichia coli mechanisms of copper homeostasis in a changing environment

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Abstract

Escherichia coli is equipped with multiple systems to ensure safe copper handling under varying environmental conditions. The Cu(I)-translocating P-type ATPase CopA, the central component in copper homeostasis, is responsible for removing excess Cu(I) from the cytoplasm. The multi-copper oxidase CueO and the multi-component copper transport system CusCFBA appear to safeguard the periplasmic space from copper-induced toxicity. Some strains of E. coli can survive in copper-rich environments that would normally overwhelm the chromosomally encoded copper homeostatic systems. Such strains possess additional plasmid-encoded genes that confer copper resistance. The pco determinant encodes genes that detoxify copper in the periplasm, although the mechanism is still unknown. Genes involved in copper homeostasis are regulated by MerR-like activators responsive to cytoplasmic Cu(I) or two-component systems sensing periplasmic Cu(I). Pathways of copper uptake and intracellular copper handling are still not identified in E. coli.

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1. Introduction

Copper ions undergo unique chemistry due to their ability to adopt distinct redox states, either oxidized as Cu(II) or in the reduced state, Cu(I). The stable Cu(II)–N bonds are often inert while the bonds with oxygen donor ligands are more labile. Cu(I) is considered a soft metal and preference bonds with ligands such as sulfhydryl groups. Biological systems did not utilize copper before the advent
of atmospheric oxygen. In the prevailing reducing conditions before this event, copper was in the water-insoluble Cu(I) state, in the form of highly insoluble sulﬁdes, and was not available for biological processes. Cyanobacteria are thought to be responsible for the beginning of dioxygen production about 10^9 years ago. The appearance of a signiﬁcant O2 concentration in the atmosphere required another 200–300 million years because the oxygen produced was initially consumed by the oxidation of ferrous iron in the oceans. This event irreversibly changed life on earth. While enzymes involved in anaerobic metabolism needed to work in the lower portion of the redox spectrum, the presence of dioxygen created the need for a redox-active metal with $E^0_{Mn^{1+/Mn}}$ from 0 to 0.8 V. The redox potential Cu(II)/Cu(I) of copper is usually higher than that of iron Fe(III)/Fe(II). Most copper enzymes work between 0.25 and 0.75 V. This high potential can be utilized for direct oxidation of easily oxidized substrates such as superoxide, ascorbate, catechol or phenolates [1].

Copper proteins are widely distributed in aerobic organisms and mainly have two functions, electron transfer and dioxygen transport and activation. Copper proteins are involved in vital processes such as respiration, iron transport, oxidative stress protection, blood clotting and pigmentation [2]. Almost all copper proteins, such as multicopper oxidases, amine oxidases or lysine oxidase, are periplasmic or extracellular. In bacteria, cytochrome c oxidase is localized in the cytoplasmic membrane whilst in the mitochondria of eukaryotes it is in the inner mitochondrial membrane. In bacteria, including Escherichia coli, Cu,Zn-superoxide dismutase (Cu,Zn-SOD) is a periplasmic enzyme. In contrast, Cu,Zn-SOD is a cytosolic enzyme in eukaryotes. These differences are probably the result of endosymbiosis. Mitochondria are thought to be descendants of an endosymbiotic α-proteobacterium [3]. Accordingly, the bacterial (mitochondrial) periplasmic Cu,Zn-SOD would be located outside, i.e. in the eukaryotic cytoplasm.

Copper requiring proteins are involved in a variety of biological processes and deﬁciency in these enzymes, or alteration in their activities, often cause disease states or pathophysiological conditions [4]. Copper homeostasis is a requirement for the evolution of aerobic metabolism since copper is highly toxic even at low concentrations in part because copper is a redox-active transition metal. Copper appears to be responsible for the intracellular generation of superoxide or other reactive oxygen species [5]. In addition, under anaerobic conditions copper appears to shift from the Cu(II) to the Cu(I) oxidation state and becomes much more toxic, possibly because Cu(I) can diffuse through the cytoplasmic membrane (Fig. 1) [6,7]. Therefore, intracellular copper concentrations need to be regulated within very narrow limits. Disturbed copper homeostasis has recently been implicated in diseases such as Alzheimer disease, cystic ﬁbrosis and Parkinson disease [8,9].

The mechanisms involved in copper transport and homeostasis in bacteria are only partially understood (Fig. 1). In this review we will attempt to summarize what is presently known with an emphasis on E. coli since copper homeostasis and trafﬁcking in bacteria is best understood in E. coli and in Enterococcus hirae [169]. Relevant ele-
ments of copper homeostasis and their function in E. coli are listed in Table 1. Known copper-dependent proteins in E. coli, apart from copper homeostasis factors such as CueO, include the periplasmic zinc, copper SOD (SodC), NADH dehydrogenase-2 (NDH-2), cytochrome oxidase (CytBO(3)), aromatic amine oxidase (MaoA) and 3-deoxy-darabino-heptulosonate-7-phosphate synthase (AroF) [10–13].

Copper uptake systems should be cation specific, so as to ensure an adequate supply of copper in the presence of varying levels of related cations such as nickel or zinc. Once inside the cells, additional proteins are needed for sequestration and trafficking of copper since intracellular free cations are toxic. In the case of copper, intracellular unbound Cu$^{2+}$ can result in oxygen-radical damage [14]. However, in yeast intracellular levels of free copper were not detectable, indicating that all copper under physiological, non-toxic conditions is bound to proteins, chelators and metallochaperones [15]. In addition, cellular efflux systems have been shown to remove excess levels of cations and to protect the intracellular environment [16].

E. coli is a facultative aerobic, enteric bacterium living in the digestive tract of warm-blooded animals. The concentration of copper can be high in the digestive tract, particularly in the stomach and duodenum, but even there it probably does not exceed 10 μM. However, it has been shown that under acidic anaerobic conditions, which prevail in that part of the digestive tract, copper becomes much more toxic. Thus, intestinal bacteria may have evolved elaborate mechanisms for copper homeostasis as an adaptation to their specific ecological niche, the animal gut. Recently, two copper-responsive regulatory systems were identified. One is a two-component signal transduction system designated the Cu-sensing or cut locus. The cutRS genes form a sensor/regulator pair that activates the adjacent but divergently transcribed genes cutCFBA [17,18]. The cutCBA gene products are homologous to a family of proton/cation antiporter complexes involved in export of metal ions, xenobiotics, and drugs. CutF is a periplasmic copper-binding protein (S. Franke et al., unpublished). The second system, termed cue (for Cu efflux), is regulated by CueR, a MerR-like transcriptional activator induced by copper [19–21]. CueR has been shown to regulate two genes, copA and cueO (formerly yacK) [19–21]. CopA is a Cu(II)-translocating P-type ATPase while CueO is a multi-copper oxidase [19,22,23].

Early attempts to elucidate copper homeostasis in E. coli have been inconclusive. On the basis of a preliminary characterization of copper-sensitive mutants, it was initially proposed that six genes (cutA, -B, -C, -D, -E, -F) are involved in the uptake, intracellular storage, delivery, and efflux of copper in E. coli [24]. However, few of these genes have been directly linked to copper metabolism, transport, or regulation. Of these six structural genes, the cutA locus [25] and the cutC [26], cutE [27] and cutF [26] genes have been cloned and a putative function has been assigned to them. These proteins could have an ancillary role such as the reduction of methionines in CueO, altering porins or ensuring proper protein folding in the periplasm. Whatever their role, they only have a slight influence on copper-resistance levels with the exception of CutE. The cutE (int) gene encodes apolipoprotein N-acyltransferase. CutE might function in acylation of CusC and other apolipoproteins required for copper tolerance [17,28]. However, most of the cut genes are probably only indirectly involved in copper homeostasis [19,28].

Copper appears to cross the outer membrane through porins since a copper-resistant, porin-deficient mutant E. coli could be isolated [29]. Similar results could be obtained in laboratory-selected silver-resistant mutants of E. coli [30]. However, various isogenic OmpF- and/or OmpC-deficient mutants of E. coli did not differ significantly in copper or silver resistance [30]. This might indicate that in addition to lowering the permeability of the outer membrane, cells need to increase their rate of copper or silver efflux. How copper crosses the cytoplasmic membrane has not been conclusively described in bacteria besides the possibility that Cu(I) may equilibrate across this barrier [7]. Since specific transporters are needed for Cu(I) uptake in eukaryotes it appears unlikely that this is a major route of copper entry.

The cupric reductase NDH-2 has been described in E. coli [13,31] suggesting that Cu(II) is reduced during uptake as was described in eukaryotes (Fig. 1). Copper

<table>
<thead>
<tr>
<th>Homeostatic mechanism</th>
<th>Regulated by</th>
<th>Function</th>
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<tbody>
<tr>
<td>CopA</td>
<td>CueR (sensing cytoplasmic Cu(I) and CpxR (sensing cell envelope stress)</td>
<td>Detoxification of cytoplasmic Cu(I)</td>
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<tr>
<td>CueCFBA</td>
<td>cutRS (two-component regulation system, sensing periplasmic Cu(I))</td>
<td>Detoxification of periplasmic (and possibly cytoplasmic) Cu(I)</td>
</tr>
<tr>
<td>CueO</td>
<td>CueR (sensing cytoplasmic Cu(I))</td>
<td>Protection of periplasmic proteins from copper-induced toxicity</td>
</tr>
<tr>
<td>PcoABCD</td>
<td>PcoRS, (two-component regulation system, sensing periplasmic Cu(I) (and CusRS)</td>
<td>Protection from extreme periplasmic copper stress</td>
</tr>
<tr>
<td>PcoE</td>
<td>CusRS (PcoRS)</td>
<td>Periplasmic copper chaperone, copper binding</td>
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uptake in *Saccharomyces cerevisiae* is mediated by separate high- and low-affinity systems. High-affinity copper uptake requires plasma membrane reductases to reduce Cu(II) to Cu(I). This reduction is mediated by the same plasma membrane reductases, Frel and Fer2, that are involved in iron uptake [32]. Once reduced to Cu(I), the ions are taken up by separate high-affinity transporter proteins encoded by the *CTR1* and *CTR3* genes. High-affinity copper uptake is energy dependent and specific for Cu(I) over other metals [2]. A third, lower-affinity system, Fet4, for copper uptake has also been detected in yeast [33–35].

This review will focus on the better-understood copper homeostatic mechanisms from *E. coli*. These encompass the P-type ATPase CopA, the four-part Cus copper efflux complex, the multi-copper oxidase CueO and the plasmid-borne Pco system. Finally, we will try to combine recent findings in enzymatic functions of copper detoxification with regulatory networks, to form a complex picture of copper homeostasis in the model organism *E. coli* within its changing environment.

2. CopA is responsible for cytoplasmic copper homeostasis

CopA is the central component of copper homeostasis in *E. coli* and is required for intrinsic copper resistance under both aerobic and anaerobic conditions. The 834-amino acid (aa) CopA is a Cu(I)-translocating P-type ATPase that is regulated by CueR. CueR is activated by intracellular Cu(I) and Ag(I) levels [19–21]. In addition, the expression of copA may be influenced by CpxR, in response to cell envelope stress [19]. The activity of the copA promoter is not very different under both aerobic and anaerobic conditions. However, the basal level of transcription is higher under anaerobic conditions indicating that cytoplasmic copper concentrations might be higher than under aerobic conditions [7].

The superfamily of P-type ATPases is a ubiquitous group of proteins involved in transport of charged substrates across biological membranes [36]. One branch contains the phylogenetically related subgroup of P-type ATPases that catalyze transport of transition- or heavy-metal ions. They have previously been described as CPx-type ATPases [37] or soft-metal-transporting P-type ATPases [16]. These ATPases have eight transmembrane domains [38], in contrast to the hard-metal P-type ATPases, which have 10 transmembrane segments. Soft-metal-transporting P-type ATPases can be further divided into subgroups that contain Cu(I)/Ag(I)-translocating ATPases and Zn(II)/Cd(II)/Pb(II)-translocating ATPases [16,39,40]. Well-studied prokaryotic copper transport systems are the copper ATPases of *E. hirae*, CopA and CopB [41], and CopA from *Helicobacter pylori* [42] and *E. coli* [22]. Eukaryotes possess similar copper ATPases, such as Ccc2 in *S. cerevisiae*, RESPONSIVE-TO-ANTAGO-NIST1 in *Arabidopsis thaliana* [43] and ATP7A [44] and ATP7B [45] in humans. The results of in vitro copper or silver transport experiments and copper-stimulated ATPase activity demonstrate that the transported species are Cu(I) and Ag(I). ATP7A (Menkes disease-related protein), *E. hirae* CopB and *E. coli* CopA were shown to transport copper only in the presence of dithiothreitol (DTT), consistent with transport of Cu(I). Recent results demonstrated that CopA formed an acylphosphate intermediate with [γ-32P]ATP only in the presence of Cu(I) or Ag(I) but not with Cu(II), Zn(II), or Co(II) [46]. ATPase activity of purified CopA was also only stimulated by Cu(I) and Ag(I). CopA from the thermophile *Archeoglobus fulgidus* required both DTT and cysteine for copper-dependent stimulation of ATPase activity. DTT was needed for reduction of copper to Cu(I) [47].

CopA possesses two putative CXXC motifs in the N-terminal domain that are potential metal-binding sites. However, aa substitutions by site-directed mutagenesis showed that these motifs are not required for function and do not confer metal specificity [48]. Similar results were also obtained with ATP7A, ATP7B, CadA from *Listeria monocytogenes* and ZntA from *E. coli* [49–52]. While these metal-binding motifs might not be required for function they might have a regulatory effect. The apparent *Kₘ* of Cu(I) in acylphosphate intermediate formation in wild-type CopA is 1.5 μM but in a quadruple mutant with all cysteines substituted by serines by serines the *Kₘ* is lowered to 0.45 μM in an ATPase assay with purified CopA. However, there could be an alternative explanation of these counterintuitive results. It is possible that the CXXC motifs bind copper in this in vitro assay and the apparent *Kₘ* is higher than in the absence of these motifs [46].

3. The proton-driven Cus system

In contrast to Gram-positive bacteria such as *Bacillus subtilis*, a Gram-negative bacterium not only needs to safeguard the cytoplasm but also has to translocate metals across the outer membrane to protect the vital periplasmic compartment from metal-induced damage. A family of related transport systems exclusively found in Gram-negative bacteria is that of the CBA-transport systems. These are involved in export of metal ions, xenobiotics, and drugs [53–55], and usually contain three different structural proteins encoded by a single operon. The central pump protein belongs to the resistance nodulation cell division family (RND). RND proteins are secondary transporters probably energized by proton–substrate antiport [54,56–58]. The two other components are a membrane fusion protein (MFP) [59] and an outer membrane factor (OMF). MFPs were also grouped into the family of periplasmic efflux proteins [60] or periplasmic adaptor proteins [61]. OMFs are associated with the outer membrane and span the periplasmic space [53,62]. Recently, crystalliza-
tion of the OMF TolC has revealed that TolC, as a trimer, assembles into a transperiplasmic channel-tunnel embedded in the outer membrane [63]. Therefore, according to a recent model RND, MFP and OMF interact to form an active channel spanning the periplasm and thus connect the cytoplasm to the outer membrane (Fig. 2). Prominent metal-transporting CBA-type systems have been characterized in Pseudomonas, Ralstonia, Synechococcus, Salmonella and E. coli. Thus, they seem to be widespread in Gram-negative bacteria and probably confer additional resistance to certain heavy metals. Currently, the czc determinant is the best-characterized metal CBA-like transporter. In the highly metal-resistant bacterium Ralstonia metallidurans strain CH34, resistance to zinc, cadmium, and cobalt is catalyzed by the gene products of the cobalt–zinc–cadmium (czc)-resistance operon [56,58]. The Czc system is an efflux pump that functions as a chemiosmotic divalent cation/proton antiporter [56,58,64]. The czc determinant encodes three structural proteins, CzcA, CzcB and CzcC. These proteins have become the prototype CBA-type family of three-component chemiosmotic exporters, including members that efflux toxic cations or organic compounds [65]. An in-depth look into CBA transporters is provided by a review by Nies [170].

The RND proteins confer substrate specificity [66,67] whereas the OMF proteins are often interchangeable since members of the OMF family could functionally substitute for each other. A R. metallidurans strain deleted in cnrC encoding the OMF protein of the cobalt–nickel-resistance determinant was functionally substituted in trans by the related czcC or nccC gene [68]. A similar result was obtained with antibiotic-resistance systems from Pseudomonas aeruginoso [69–74].

In E. coli the copper (and silver)-translocating Cus system is encoded on the chromosome as a determinant comprising two operons transcribed in opposite directions. One operon encodes the regulators CusRS that form a two-component regulatory system. CusS is a histidine kinase located at the cytoplasmic membrane probably sensing copper ions in the periplasm. CusR acts as a response regulator that activates transcription of cusCFBA. The cus determinant is induced by copper (and to a lesser extent by silver) and both genetic and phenotypical analysis strongly suggests that the products of cusCFBA form a copper-extruding complex ([7,17,75]; S. Franke et al., unpublished) (Fig. 2).

In our opinion the Cus system transports copper directly from the periplasm across the outer membrane. Initial evidence suggesting that cations are transported from the periplasm and not the cytoplasm came from genetic analysis in E. coli. Although transcription of the cus determinant was induced by copper, deletion of cusCFBA failed to show a copper-sensitive phenotype under aerobic conditions. Cells disrupted in copA (and no longer encoding the Cus(I)-translocating P-type ATPase) are more copper sensitive than wild-type cells. A double mutant, where both copA and cusCFBA are disrupted, is no more copper sensitive than a strain only disrupted in copA [75]. This indicates that cusCFBA is not an alternative pump for the removal of copper ions from the cytoplasm. However, a disruption of both cueO, encoding the multi-copper oxidase in E. coli (see Section 4), and cusCFBA showed a
dramatic increase in copper sensitivity even in the presence of CopA. These results suggest that CusCFBA transports periplasmic copper. We also assume that this is true for homologous systems such as CzcCBA. This hypothesis is supported by the observation that other transporters of the RND superfamily such as AcrB and MexB can also transport substrates that do not cross the cytoplasmic membrane [76]. These findings indicate that binding of the substrate can occur on the periplasmic side of the transporter [77].

A previous model of the well-studied RND pump, CzcA, suggested that cations are pumped out of the cytoplasm while pumping protons in, since CzcA possesses both a putative proton channel and a putative cation channel. However, we think this model is only partially correct because the $K_m$ measured in transport experiments is not physiological at 10 mM for zinc [57]. Rather, cations could also be transported from the periplasm or the periplasmic side of the cytoplasmic membrane across the outer membrane. The recent crystal structure of AcrB, a related RND transporter, suggests the presence of two possible pathways. Substrates could be transported from the cytoplasm or the periplasm [78].

Data obtained with CzcA suggested a protein topology encompassing 12 transmembrane domains and two large periplasmic loops that probably also applies to CusA (Figs. 2 and 3) [57]. The periplasmic loops potentially contain metal-binding sites and may prevent metal cations from entering the cell by pumping them across the outer membrane while they are at or near the cytoplasmic membrane. Recent mutational analysis of CusA indicated that some methionine residues located in the second periplasmic loop of CusA are important for function (Fig. 3; S. Franke et al., unpublished). Those methionines are not present in Zn(II)- or Ni(II)-transporting RND proteins. However, they are conserved in other homologous, putative copper-translocating CBA-like transporters. Therefore, it is tempting to speculate that some of those methionine residues are involved in copper binding or transport. Moreover, transmembranesegment four of monovalent metal cation (Cu[I], Ag[I])-translocating RND transporters distinctively differs from those transporting divalent metal cations or antibiotics (Table 2).

The CusA protein (1047 aa) is thought to be the central component, responsible for copper transport. Additionally, the putative channel-forming CusC protein (457 aa) and the clamping CusB (407 aa) are necessary for full Cus-mediated copper resistance (S. Franke et al., unpublished). An in-frame deletion of either $\text{cusB}$ or $\text{cusC}$ resulted in a dramatic decrease in copper resistance.

### Table 2

<table>
<thead>
<tr>
<th>Transport of Substrates</th>
<th>Amino acid sequence</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monovalent metals</td>
<td>$A_2D_3X_6E$</td>
<td>CusA (E. coli)</td>
</tr>
<tr>
<td></td>
<td>$S_1A_2$</td>
<td>SilA (S. enterica)</td>
</tr>
<tr>
<td>Divalent metals</td>
<td>$D_4X_2D_3X_6E$</td>
<td>NccA (A. xylosoxidans)</td>
</tr>
<tr>
<td></td>
<td>$X_2C_4E$</td>
<td>CzcA (R. metallidurans)</td>
</tr>
<tr>
<td>Antibiotics</td>
<td>$X_2D_4X_3E$</td>
<td>AcrB (E. coli)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MexB (P. aeruginosa)</td>
</tr>
</tbody>
</table>

*Binding motifs were derived from Goldberg et al. [57], Guan and Nakae [79] and Aires et al. [80].

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Fig. 3. Two-dimensional topological model of CusA with effects of aa substitutions. Topology of CusA was modeled by analogy to other RND transporters such as CzcA (from R. metallidurans), AcrB (from E. coli), MexB and MexD from P. aeruginosa. Changed aa residues are boxed and the effects of these mutations are shown on the right. Aa residues A399, D405 and E412 in transmembrane segment IV denote the signature motif of monovalent cation-translocating RND proteins. PP, periplasm; CPM, cytoplasmic membrane; CP, cytoplasm.
resulted in nearly complete loss of function of the Cus system. OMFs are thought to be associated with the outer membrane. Some OMFs such as OprM are predicted to be lipoproteins and possess a cysteine residue at their N-terminal end after signal peptidase processing. This lipoprotein box is responsible for thiol-ether linkage to diacylglyceride. Since these proteins do not contain an aspartate at position two of the mature protein they are localized to the outer membrane [81–83]. CusC contains both a signal sequence and a cysteine residue at aa residue position 18 [17] and is closely related to OprM and MtrE, both involved in drug efflux. Other OMFs of metal-resistance [17] and is closely related to OprM and MtrE, both in sequence and a cysteine residue at aa residue position two of the mature protein they are localized to the outer membrane [81–83]. CusC contains both a signal sequence and a cysteine residue at aa residue position 18 [17] and is closely related to OprM and MtrE, both involved in drug efflux. Other OMFs of metal-resistance determinants such as CzcC or NccC are only distantly related and do not have a cysteine residue at their N-terminus ([63]; G. Grass and D.H. Nies, unpublished). If attachment to the outer membrane is accomplished in both cases, the underlying mechanisms might be different. However, the activity and expression of OprM with a mutation of the highly conserved N-terminal cysteine residue was not affected indicating acylalation is not important for function [84].

A unique feature of copper/silver CBA-type transporters is the presence of a small (110-aa) periplasmic protein, CusF, in the Cus system. CusF was characterized recently (S. Franke et al., unpublished). It could be shown that the mature protein binds one copper per polypeptide and that the purified copper-containing protein is pink in color. A non-polar deletion of cusF led to a decrease in Cus-mediated copper resistance. Mutational analysis indicated that copper binding in CusF is accomplished in part by two conserved methionine residues. Other potential metal-binding residues such as five histidine residues, a conserved phenylalanine or a conserved aspartate residue seem not to be involved in copper binding. Possibly CusF is a periplasmic metallochaperone transporting copper to the CusCBA efflux complex and thus facilitating copper detoxification of the periplasm (Fig. 2). Since the cus determinant is also responsible for a small increase in Ag(I) resistance [85], it is likely that the transported copper species is Cu(I).

In contrast to the CueO multi-copper oxidase that requires oxygen for function, the CusCBA efflux complex works under both anaerobic and aerobic conditions. Recent results from Outten et al. [17] clearly showed Cus-mediated copper detoxification is especially important under anaerobic conditions. Under aerobic conditions copper sensitivity is only observed when both cueO and cusCFBA are deleted.

4. CueO is a multi-copper oxidase protecting the periplasm from copper-induced damage

The copA gene encoding the copper efflux P-type ATPase and cueO are both regulated by CueR. Together they constitute the Cue system. CueO (516 aa) is a multi-copper oxidase that possesses laccase-like activity. Recently, several other microbial protein sequences showing similarities to fungal laccases were identified [86]. These bacterial multi-copper oxidases have been implicated in antibiotic biosynthesis, sporulation, copper tolerance, morphogenesis and manganese oxidation [23,87–89]. Multi-copper oxidases couple the one-electron oxidation of substrate(s) to full reduction of molecular oxygen to water by employing a functional unit formed by three types of copper-binding sites with different spectroscopic and functional properties [90]. Type 1 blue copper (T1) is the primary electron acceptor for the substrate, while a trimuclear cluster formed by type 2 copper and binuclear type 3 copper (T2/T3) is the oxygen-binding and -reduction site. Prominent members of this group are mammalian ceruloplasmin, plant ascorbate oxidases and fungal laccases. By definition, laccases (p-diphenol-O2 oxidoreductase EC 1.10.3.2) catalyze the oxidation of p-diphenols with the concurrent reduction of dioxygen to water, although the actual substrate specificities of laccases are often quite broad and vary with the enzyme source. These multi-copper oxidases are widely found in fungi and plants and numerous laccases have been cloned from these sources [91–93]. In contrast to our understanding of the electron transfer reactions that occur in laccases, relatively little is known about their physiological function. In fungi, laccases have been implicated in processes such as pigmentation, fungal sporulation, lignin degradation and biosynthesis, and pathogenesis [94]. However, very few of these functions have been experimentally proven.

It has been shown that CueO protects periplasmic enzymes from copper-induced damage [23]. Sequence analysis of CueO revealed a twin-arginine motif within the leader sequence. In fact, CueO possesses a perfect signal peptide sequence recognized by the Tat translocation machinery. The Tat pathway transports folded proteins, with their cofactors already bound before translocation into the periplasm [95]. Therefore, loading of copper into CueO in the cytoplasm with subsequent transport into the periplasm could contribute to cytoplasmic copper detoxification. However, the impact of this CueO-dependent copper translocation on copper resistance would only be marginal. The P-type ATPase CopA can transport copper much more efficiently across the cytoplasmic membrane. CueO function is strictly oxygen dependent even though expression of the cueO gene also occurred under anaerobic conditions. However, the cueO promoter revealed a strong dependence on oxygen. Furthermore, expression of cueO is very sensitive to copper. Compared to other copper homeostasis mechanisms examined in E. coli, expression of cueO showed the lowest half maximum induction (3 μM) by copper [17].

The three-dimensional structure of CueO was recently solved at 1.4 Å resolution [96]. Four copper ions are bound in the CueO monomer, forming an active center typical for multi-copper oxidases such as ascorbate oxi-
A copper (II) ion is found to bind to the protein 7.5 Å from the T1 copper site in a region rich in methionine residues. Copper was bound to two methionine sulfur atoms, Met 441 and Met 359, and two aspartate carboxylic acid oxygens, Asp 439 and Asp 460. The other carboxylate oxygen of Asp439 is only 2.9 Å apart from and apparently hydrogen bonded to His 443. The ND1 atom of this histidine ligates the T1 copper. Thus, this bound copper is in a position to mediate electron transfer from substrates to the T1 copper (S.A. Roberts et al., unpublished).

At this point the mechanism by which CueO protects cells from copper-induced damage is still unknown. Characterization of purified CueO demonstrated that it is also able to oxidize ferrous iron [23,97]. Iron oxidation prior to uptake was experimentally determined to be the function of Fet3, a multi-copper oxidase from yeast S. cerevisiae [98]. However, it is not clear if this is one of the physiological roles of CueO. CueO is able to oxidize enterobactin and related catechols in vitro, producing a colored precipitate [97]. This suggested that the formation of the colored precipitate might be related to polymerization reactions involved in microbial melanization processes. Synthesis of a copper-binding compound would be an excellent way to decrease toxic copper concentrations and could be one of the physiological roles of CueO. Another hypothesis is that CueO oxidizes periplasmic Cu(I) to the less permeable and thus less toxic Cu(II) [7,75]. This activity could stop both membrane damage and Cu(II) reduction, thus protecting the cells from copper-induced damage by preventing the Fenton-like reaction of redox-active copper.

All multi-copper oxidases with a function in copper resistance possess an extensive methionine-rich region proximal to the copper centers (Fig. 4). The role of this domain in enzymatic function is yet unknown. It could be a regulatory domain that binds copper and then activates the enzyme or it could be involved in protein–protein interactions [99]. Another possibility is that this region has a protective role for the enzyme preventing fragmentation of the multi-copper oxidase by copper-generated oxygen radicals [100].

5. The plasmid-encoded pco determinant confers additional copper resistance

Copper can be introduced into soils via sewage sludge, mine effluents and industrial waste. For more than 100 years copper has been used as the active ingredient in bacteriocides and fungicides on fruits and vegetables. Since the mid-1980s, copper-resistant bacterial pathogens have been detected repeatedly [101]. Copper resistance was the most frequently found resistance in bacteria from pre-antibiotic era isolates [102]. Moreover, copper can inhibit soil organisms and has recently been implicated in induction of the viable-but-not-cultivable condition [103].

Many soils are deficient in essential metals such as copper or cobalt, resulting in mineral disorders that affect livestock at pasture. A well-documented example is copper deficiency termed ‘swayback’ in sheep from Australia. The condition appears to occur as a result of copper interacting with other dietary components, particularly molybdenum and sulfur, resulting in a decrease in copper absorption. This has led to supplementing the diet of sheep with copper. Paradoxically, sheep are very susceptible to copper toxicity and even moderate dietary supplements to prevent deficiency can result in elevated hepatic copper concentrations, liver failure and death of the animal [104]). All breeds of sheep have a reduced ability to excrete copper in the bile and will accumulate copper in the liver [105]. However, sheep display a variant copper susceptible phenotype when compared with other mammals. For example, copper and antibiotics have been used as growth promoters in pig diets for at least 45 years [106]. This practice has continued because pigs fed such supplemented diets exhibited an improved average daily weight gain and food conversion efficiency. It has been proposed that cop-
per elicits an antibacterial effect on the bacteria of the pig gut [107].

Some strains of *E. coli* can survive in copper-rich environments that would normally overwhelm the chromosomally encoded copper homeostatic systems. Such strains possess additional plasmid-encoded genes that confer copper resistance. Copper-resistance operons have been characterized from *E. coli*, *Pseudomonas syringae* pv. *tomato* and *Xanthomonas campestris* [108]. The genes from these copper-resistant determinants are largely homologous and probably have similar functions. The conjugative plasmid pRJ1004 confers copper resistance and was isolated from *E. coli* in the gut flora of pigs fed a diet supplemented with copper sulfate as a growth promoter [109]. The copper resistance specified by this plasmid involves the *pco* gene cluster, which contains seven genes, *pcoABCDRSE* [110]. Copper resistance in *P. syringae* pv. *tomato* is specified by the *cop* determinant, which contains six genes, *copABCDRS*, arranged in two operons, *copABCD* and *copRS*, respectively. This arrangement is also found in the *pco* determinant but with an additional gene, *pcoE*, further downstream [110–112]. In all cases copper resistance has been shown to be inducible [113,114]. Radioactive 64Cu uptake experiments suggested that an energy-dependent copper efflux mechanism is associated with the *pco* copper-resistance genes from plasmid pRJ1004 [110]. However, critical evaluation of these experiments shows that reduced uptake does not necessarily imply an additional efflux mechanism.

Our current model of the resistance mechanism of the *pco* determinant is illustrated in Fig. 5. PcoA (605 aa) is the central protein of the *pco* determinant. The *pcoA* gene encodes a 605-aa protein of the multi-copper oxidase family. Since periplasmic extract containing PcoA showed copper-inducible oxidase activity [115], an indication on function of the products of the *pco* determinant might be gained from the recent results with CueO. In vitro, catechols, especially enterobactin, were the substrate with the lowest *K_m* of all substances tested ([97]; G. Grass et al., unpublished). CueO-mediated oxidation of enterobactin (and its precursors) led to the formation of colored precipitates. Tetaz and Luke [109] already noticed that *E. coli* strains harboring the *Pco*-plasmid pRJ1004 produced brown colonies with dark brown centers when streaked on medium containing CuSO4. In addition, PcoA could functionally substitute for CueO indicating they have a similar function. Similar to CueO, PcoA also has a twin-arginine motif in its leader sequence and is probably translocated by the TAT pathway with copper bound to its active sites.

PcoB is a 296-aa protein predicted to be an outer membrane protein. In a copper-sensitive *E. coli* strain *pcoA* and *pcoB* could confer copper resistance at a much lower expression level compared to *pcoA* alone, indicating that they might interact. These findings are similar to results obtained in *P. syringae*, where expression of *copA* with *copB* alone could also confer copper resistance. The presence of CopC and CopD was only needed for maximal

![Fig. 5. Proposed mechanism of Pco-mediated copper detoxification. Copper enters the periplasm by an unknown mechanism, possibly through OmpC and OmpF. The function of the outer membrane protein PcoB has not been elucidated. Consequently, copper might be detoxified by sequestration on oxidized catechol siderophores or Cu(I) could be oxidized to the less toxic Cu(II) by the multi-copper oxidase PcoA. In order to load copper into catalytic sites within PcoA, PcoC and PcoD might transport copper across the cytoplasmic membrane with PcoC delivering copper to PcoD. PcoE binds copper in the periplasm and possibly shuttles copper to PcoA.](https://academic.oup.com/femsre/article-abstract/27/2-3/197/614329)
resistance. These observations are reflected when looking at genomic sequences. Often, only homologs of PcoA and PcoB are encoded on a genome but not PcoC and PcoD. However, in both the Cop and the Pco system PcoC and PcoD are required for full resistance. In P. syringae studies indicated that CopC and CopD may function together in copper uptake [116]. The homologous PcoC and PcoD might have a similar function. PcoC containing 126 aa was shown to bind one copper atom per molecule and might act as a dimer [115,117]. PcoC could regulate copper uptake by PcoD, a 309-aa integral membrane protein with eight putative membrane-spanning domains as predicted by topology modeling according to Klein et al. [118]. Interestingly, in B. subtilis homologs of pcoC and pcoD are not encoded as separate genes but as a fusion gene termed ycnJ. The YcnJ protein consists of 541 aa with the N-terminus being homologous to PcoC and the C-terminus to PcoD. This suggests that in E. coli PcoC and PcoD might also interact to form a functional unit (Fig. 5). In our current model PcoC would deliver periplasmic copper to PcoD for uptake into the cytoplasm, probably for loading into PcoA.

The pco determinant contains another gene, pcoE, required to confer full resistance. The pcoE gene is not part of the pcoABCD operon but is located further downstream on plasmid pRJ1004. Expression of pcoE is regulated by its own copper-regulated promoter and is under the control of the two-component systems CusRS [17,119]. A related protein, SilE, is encoded by the sil determinant [120,171]. Expression of pcoE alone led to copper accumulation in the periplasm indicating a role in binding of copper in the periplasm [121].

Copper resistance in P. syringae pv. tomato is specified by the cop determinant, which contains six genes, cop-ABCDRS, homologous to the equivalent pco genes [111]. The pco and the cop determinants are regulated by a plasmid-encoded and a chromosomally encoded two-component system. In P. syringae alginate synthesis is induced by copper [122]. Alginates were shown to protect cells from reactive oxygen stress. However, a similar system does not exist in E. coli. This difference might be one of the reasons why copper-resistant E. coli turn brown [110,111]. Another possibility is that copper bound to oxidized siderophores from Pseudomonas exhibits a blue color, and in E. coli, with different siderophores, this might look brown.

Interestingly, homologs of these plasmid-encoded gene products are also present on the E. coli chromosome. CueO is related to PcoA and probably has a similar function. A PcoC homolog, YobA (124 aa), does not contain any methionines and its function in copper homeostasis is unknown. In addition, a homolog of PcoD, YebZ (290 aa), with unknown function is present on the E. coli chromosome (Table 3). Like pcoCD, the adjacent yabA and yebZ genes are probably translated as a dicistronic transcript.

### 6. Regulation of genes involved in copper homeostasis reflects different demands in a changing environment

Copper homeostasis in E. coli is a multi-layered process not only regulating uptake and efflux but also periplasmic copper sequestration. Genes participating in these processes are regulated differently reflecting different environmental demands. Cells are continuously challenged to keep copper in a delicate balance. While a hypersensitive regulation of expression of genes involved in resistance of a solely toxic metal, such as mercury, would be beneficial [172], this mechanism would be only inappropriate for homeostasis of an important trace metal like copper [20].

Three copper-dependent transcriptional regulatory systems have been identified in E. coli, the DNA-binding CueR regulator, and the chromosomal (CusRS) and plasmid-encoded (PcoRS) two-component regulators [121]. CueR is a member of the MerR family of metal-responsive regulators and regulates copA and cueO [19]. Metal-responsive MerR-like regulators are broadly distributed in bacteria. Previously characterized members include the archetype mercury-responsive MerR [123,124], ZntR responsible for zinc-dependent expression of ZntA, the Zn(II) efflux ATPase from E. coli [125], and PbrR, regulating the R. metallidurans CH34 lead-resistance determinant [126]. MerR-like regulators are discussed more extensively in a review by Brown et al. [172]. Even though members of

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**Table 3**

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<tr>
<th>Homologs</th>
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<tr>
<td>PcoA</td>
<td>CueO</td>
<td>Periplasm, monomeric</td>
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<td>PcoE</td>
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<td>Outer membrane</td>
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<td>PcoC</td>
<td>YobA*</td>
<td>Periplasm, dimeric?</td>
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<tr>
<td>PcoD</td>
<td>YebZ*</td>
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<td>PcoS</td>
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*P*Plasmid pRJ1004 carries the pco determinant [110].

*Y*obA (P76279) and YebZ (P76278) are putative ORFs located on the E. coli chromosome.
the MerR family share crucial features such as DNA and metal binding they recognize distinct metal substrates resulting in activation of cognate promoters.

Initially an unusually long spacing in PcopA suggested a possible copper-responsive signal transduction system of a MerR-like regulator [19]. Consequently, an E. coli strain deleted in cueR lost copper-dependent PcopA induction. In contrast to the regular 17-bp spacer in RpoD-dependent promoters, a palindrome sequence in an extended 19-bp spacer between the −35 and −10 sites of P cueO and PcopA could be identified [20]. This sequence is reminiscent of those found within promoters regulated by other members of the MerR family of transcriptional regulators. The dyad sequence has 7 bp of perfect symmetry and is shared with the binding motif of MerR. However, there is no obvious similarity between the CueR- and MerR-binding 7-bp motifs [20].

Upstream of the −35 site a weak consensus UP element can be identified next to a CpxR-binding site [19]. CpxR is the cell envelope stress response regulator [127]. A deletion of this site led to reduced induction of PcopA under high copper concentrations, indicating PcopA is not only regulated by CueR but also by CpxR [19]. In Salmonella typhi and Salmonella typhimurium cueR and copA are transcribed divergently but in E. coli they are separated by two open reading frames (ORFs) [20]. It is not known if those ORFs are involved in copper homeostasis or, more likely, represent recent events of genomic rearrangement.

CueR senses cytoplasmic copper and silver, and transcription of PcopA is activated in response to elevated concentrations [20]. CueR-dependent induction of PcopA exhibits a hypersensitive response to silver ions, but a linear response to copper ions. Such a regulation mechanism makes biological sense considering that silver occurs as Ag(I) only but copper is in a steady state between Cu(I) and Cu(II) and that both Ag(I) and Cu(II) are toxic for cells. CueR shares a helix-turn-helix motif for DNA binding at the N-terminal end with other MerR-like regulators such as ZntR [128] or MerR [129] and a C-terminal metal-binding site. It could be demonstrated that purified CueR bound to PcopA between −35 and −10 in a DNase I footprint assay [19–21]. Interestingly, a conserved cysteine residue in ZntR and MerR is absent in CueR. Additionally, spacing between domains within the proteins is different which might be responsible for specificity towards copper [19].

Several promoters of copper (and silver)-inducible genes are preceded by a palindrome sequence, termed ‘cop-box’. Cop-boxes are the sites of DNA binding of a cognate response regulator. On the E. coli chromosome cop-boxes can be found upstream of cusC and on plasmid pAPI73 upstream of pcoA and pcoE [17,119]. In other organisms such as Salmonella enterica serovar Typhimurium or P. syringae similar cop-boxes can be identified preceding sitC and sitE or copA and copH, respectively [130,131]. Whereas the copA, cueO and cusCFBA regulation reflects copper handling under ‘daily life’ circumstances for E. coli, expression of the plasmid-borne pco determinant is only required when these housekeeping systems are overwhelmed. Accordingly, expression of the pco structural genes is not hypersensitive to external copper concentrations [119]. The highly effective pco determinant is only expressed under elevated copper concentrations.

The regulators PcoRS are required for copper-inducible expression of copper resistance mediated by the pco determinant [110]. PcoS possesses two transmembrane segments with peptide loops extending into the periplasm. With increasing copper levels, this kinase phosphorylates the cognate response regulator PcoR. It in turn can control transcription. Mutation disrupting pcoRS abolishes high-level copper resistance [110]. However, copper-dependent expression from PpcoA and PpcoE was not completely lost [119]. Yet, the regulators PcoRS are required for maximum copper-inducible expression from PpcoA. In contrast, the pcoRS genes are not required for copper-inducible expression of PpcoE located downstream of pcoABCD. PcoE reduces the time required for E. coli strains to recover from copper-mediated stress [17]. Rouch and Brown [119] recognized that the two promoters of pco remain regulated by a chromosomal determinant, now identified as CusRS, even when the pcoRS genes were deleted.

The similarity between CusR and PcoR is 61% and between CusS and PcoS 38%. In the absence of CusS, CusR might be activated by (an)other histidine kinase(s). In a ΔcussRS strain pcoRS were able to provide copper-inducible expression of a β-galactosidase reporter under the control of PpcoA, but not from PpcoE or PcusC. Thus, the regulators CusRS provide copper-inducible expression of PpcoE and PcusC and an increased basal level of PpcoA. On the other hand, the regulators PcoRS regulate PpcoA but not PpcoE and PcusC. Taken together the related two-component regulator pairs CusRS and PcoRS are able to function independently [17].

CutC is a cytosolic protein of 248 aa and a ΔcutC deletion strain showed copper sensitivity at high copper concentrations. Previous studies have implicated CutC in copper efflux, suggesting a role of CutC in intracellular trafficking of Cu(I), since an increase in cytosolic Cu(I) would undoubtedly lead to intracellular damage [5,132]. The cutC gene is probably regulated by the extracytoplasmic function sigma factor RpoE [133,134]. The RpoE-dependent extracytoplasmic stress response in E. coli is induced by excessive amounts of unfolded proteins in the envelope of the cell, particularly unfolded outer membrane porins [135–138].

7. Conclusions and perspective

Considerable progress in understanding the basic mechanisms of copper handling in E. coli has been made in
recent years. However, large pieces of the puzzle are still missing. We are only beginning to understand the metallochaperones involved in cytoplasmic and periplasmic copper trafficking, and not much is known about copper uptake systems in bacteria including *E. coli*. While copper uptake mechanisms in *E. coli* still await discovery, in the Gram-positive bacterium *E. hirae* copper homeostasis is accomplished by the *cop* operon, which consists of the four genes *copA*, *copB*, *copY* and *copZ* [41,169]. *CopA* and *CopB* are both copper-translocating P-type ATPases and earlier data suggested that *CopA* serves in copper uptake under conditions of copper limitation, whereas *CopB* serves in copper extrusion when intracellular copper reaches toxic levels [139]. However, this has not been shown for *CopA* through direct transport measurements. In contrast, in *E. coli* the chromosomally encoded P-type ATPase *CopA* exclusively functions as an efflux transporter.

Transport of copper across the outer membrane into the periplasm has been shown for *NosA* in *Pseudomonas stutzeri* and *OprC* in *P. aeruginosa* [140,141]. At this point, no protein in bacteria responsible for copper uptake across the cytoplasmic membrane has conclusively been identified and characterized.

No intracellular copper chaperone has yet been identified in *E. coli* but we believe that in the future, metallochaperones with specificity for copper will be identified in *E. coli* and other related bacteria. The *E. coli* genome contains many ORFs of unknown function containing a CXXC motif that could potentially be involved in copper binding. However, other proteins that do not possess a CXXC motif such as CutC might also be copper chaperones. Eukaryotes possess specific copper chaperones responsible for intracellular copper trafficking [142,143]. These proteins possess no obvious sequence similarities except for a conserved CXXC metal-binding site [15,144]. In yeast, the copper metallochaperone Atx1 delivers copper to the Cu(I)-translocating P-type ATPase Ccc2 which delivers copper into the Golgi apparatus for insertion into the multi-copper oxidase Fet3 [145]. In some prokaryotes, Atx1-related proteins have also been identified. Examples include Atx1 from the cyanobacterium *Synechocystis* PCC 6803 [146] and *CopZ* from *E. hirae* and *B. subtilis* [147,148]. *CopZ* from *E. hirae* and *B. subtilis* might interact with the N-terminus of the Cu(I)-translocating P-type ATPases in these organisms [149,150]. So far no copper-specific cytoplasmic chaperone has been identified in *E. coli*. It was speculated that the cystolic N-terminal part of the copper-ATPase *CopA* is cleaved off and acts as a copper chaperone [151]. However, the absence of a phenotype in a *CopA* mutant with all four cysteines substituted by serines suggests that this region is not required for function and is probably not a chaperone [48]. In several *Streptomyces* species the copper chaperone MelC1 delivers copper to a binuclear copper center (CuA and CuB) in tyrosinase that is required for melanin biosynthesis. The chaperone MelC1 maintains the state of apotyrosinase, which facilitates copper incorporation and secretion [152,153].

These results indicate copper chaperones in bacteria are widespread and this might also be true for zinc. Recently, it could be shown that several bacterial species harbor zinc-specific metallothioneins [154]. The bacterial metallothioneins are cysteine-rich small proteins that bind multiple metal ions. A deletion of the metallothionein gene smtA in *Synechococcus* PCC 7942 resulted in a five-fold decrease in zinc resistance [155]. GatA, a related protein, has recently been identified in *E. coli*. GatA binds one Zn(II) through its cysteine residues whereas SmtA from *Synechococcus* is able to sequester four zinc cations [154]. A role for GatA in the sequestration of excess zinc appears unlikely since GatA contains an SmtA-like zinc finger fold but a metallothionein cluster is not present. These results also illustrate that caution needs to be exercised in the prediction of specific roles for proteins by sequence comparison. A protein might bind copper or zinc but this ability to bind metals is without physiological consequence. We might also discover metal chaperones with previously unknown metal-binding motifs.

Copper is required in electron transport systems and as a cofactor in enzymes such as oxidases and hydrolases. However, it is toxic in its cuprous form for its ability to generate hydroxyl radicals in the presence of superoxide. This reaction is analogous to the Fenton reaction of ferrous iron [155,156]. The *soxRS* regulon of *E. coli* is expressed under oxidative stress. The SoxR protein is the superoxide-sensitive activator of the *soxS* gene. The constitutively expressed SoxR protein has homology to the MerR-type family of regulators and is a homodimer with two [2Fe–2S] centers per dimer. The oxidation of the reduced [2Fe–2S]1+ form of SoxR to a [2Fe–2S]1+ form leads to SoxR activation [157]. This active form of SoxR leads to enhanced *soxS* transcription and increased levels of SoxS activate expression of the *soxRS* regulon [158]. It was shown that in the presence of molecular oxygen, CuSO4 acted as an inducer of SoxR-dependent *soxS* expression. This indicates that copper toxicity in *E. coli* is caused by the generation of reactive oxygen species. Therefore, an *E. coli* strain lacking either oxidative DNA repair enzymes or SODs was hypersensitive to killing by copper [5]. Additionally, copper toxicity might be enhanced by enzymes such as NADH reductase-2, which was shown to be capable of reducing cupric to cuprous ions in *E. coli* [31]. Copper (CuSO4) might affect NADH dehydrogenase or other respiratory enzymes by causing a redox imbalance [5]. However, the same properties that make copper toxic help copper-containing enzymes achieve rapid rates of catalysis.

Pathogens often have to ward off toxicity due to the production of reactive oxygen species as part of the host defense. There has been clear evidence of copper-contain-
ing enzymes such as Cu,Zn-SOD or multi-copper oxidases as virulence factors. Expression of Cu,Zn-SOD enhances intracellular survival of *E. coli* and *S. enterica* Typhimurium [159,160]. The *E. coli* multi-copper oxidase CueO is an excellent model system to understand how related enzymes protect pathogenic organisms. In the fungus *Cryptococcus neoformans* the multi-copper oxidase laccase and its product melanin were identified as important virulence factors. Host cellular immunity might be compromised by melanin and its ability to act as an antioxidant or to protect the cell wall surface [161]. Melanized cells of *C. neoformans* were more resistant to antibody-mediated phagocytosis and the antifungal effects of murine macrophages than non-melanized cells [162]. Because melamins are efficient radical scavengers, melanized cells were less susceptible to killing by oxygen- or nitrogen-derived radicals [163,164]. Electron spin resonance spectra indicated electron transfer between melanin and chemically generated free radicals. Furthermore, melanin appears to contribute to virulence by protecting fungal cells against host defense attack [165].

Some of the copper-regulated genes can accomplish surprising additional tasks. One example is IbeB (CusC) from *E. coli* K1. *E. coli* K1 is the most common Gram-negative organism causing neonatal meningitis. Invasion of brain microvascular endothelial cells is a prerequisite for penetration into the human central nervous system. It was demonstrated that the IbeB protein of *E. coli* K1 is an important contributing determinant [166,167]. However, at this moment it is not apparent how IbeB functions in bacterial penetration.

Elucidation of copper homeostasis in the bacterial model organism *E. coli* will result in understanding of the delicate balance of this important yet toxic redox-active metal. We anticipate great progress in the near future, not only through genomics and proteomics, but also from an ambitious, global project to model *E. coli* called the International *E. coli* Alliance [168].

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