

## Genes Involved in Copper Homeostasis in *Escherichia coli*

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Received 4 October 2000/Accepted 19 December 2000

**Recently, genes for two copper-responsive regulatory systems were identified in the *Escherichia coli* chromosome. In this report, data are presented that support a hypothesis that the putative multicopper oxidase CueO and the transenvelope transporter CusCFBA are involved in copper tolerance in *E. coli*.**

Copper is required for aerobic life and yet, paradoxically, is highly toxic even at low concentrations. Intracellular copper concentrations therefore need to be regulated within very narrow limits (14). Previous attempts to elucidate copper homeostasis in *Escherichia coli* have been incomplete. Genes such as *cutC*, *cutF*, and *ndh* have been suggested to be involved in copper homeostasis (9, 15, 17), but their exact roles have not been determined. Recently, two copper-responsive regulatory systems were identified. One is a two-component signal transduction system designated the Cu-sensing locus (*cus* locus). The *cusRS* genes form a sensor-regulator pair that activates the adjacent but divergently transcribed genes *cusCFBA* (10). The *cusCBA* genes are homologous to a family of proton-cation antiporter complexes involved in export of metal ions, xenobiotics, and drugs. CusF is a putative periplasmic copper-binding protein (5). The other system is regulated by CueR, a copper-activated homologue of MerR. CueR has been shown to regulate two genes, *copA* and *cueO* (formerly *yacK*) (13). CopA is a Cu(I)-translocating P-type ATPase, while CueO is a putative multicopper oxidase (6, 13, 16).

**CueO is involved in copper tolerance.** CueO and CopA are both regulated by CueR (13). To determine the role of CueO in copper tolerance, the *cueO* gene was disrupted. Chromosomal deletions were performed as described by Datsenko and Wanner (4), and the gene of interest was replaced by a chloramphenicol cassette. The  $\Delta cueO::cm$  cassette was transduced into *E. coli* W3110 by P1 transduction. The resulting strain, *E. coli* GR1 ( $\Delta cueO::cm$ ), was slightly more copper sensitive on complex medium than wild-type strain *E. coli* W3110 (Table 1; Fig. 1). At high copper concentrations the *cueO*-disrupted strain exhibited a distinctive colony morphology: the colonies were small, colorless, and often mucoid. The copper sensitivity of a *cueO* deletion-containing strain could be complemented by the presence of the *cueO* gene on plasmid pTYB2::*cueO* in *trans* (Table 1). Other metals tested did not have any effect on a *cueO*-disrupted strain (data not shown).

**The *cus* determinant encodes a copper efflux system.** There are at least two chromosomal copper-responsive determinants responsible for copper homeostasis. One is the *cus* determinant, regulated by a two-component signal transduction system

encoded by the *cusRS* genes (10). However, deletion of the *cus* determinant failed to result in decreased copper tolerance (Table 1; Fig. 1). Disruption of both *cueO* and *cusCFBA::cm* in the mutant *E. coli* GR10 ( $\Delta cueO \Delta cusCFBA::cm$ ) resulted in a substantial decrease in copper tolerance. *E. coli* GR10 was mucoid even at very low copper concentrations, indicating a stress response, and ceased growth at 1.3 mM CuCl<sub>2</sub>, compared with 2.75 mM for GR1 ( $\Delta cueO::cm$ ) and 3.5 mM for the wild-type *E. coli* W3110, *E. coli* GR5 ( $\Delta cusA::cm$ ), and *E. coli* GR6 ( $\Delta cusCFBA::cm$ ) (Table 1). This effect was observed in the presence of copper but not with cadmium, zinc, or cobalt (data not shown). The structural genes *cusCFBA* resemble genes of proton-cation antiporter complexes such as CzcCBA or SilCFBA involved in heavy metal resistance (8, 11). CusA is a member of the RND superfamily of proteins (18) and the central component of the multicomponent efflux pump. No difference was observed whether *cusA* or *cusCFBA* was deleted (Table 1), indicating that *cusA* is essential for function. These results strongly suggest that the *cus* determinant encodes a copper efflux system. The copper sensitivity conferred by a *copA* disruption is not additive when transferred into a  $\Delta cueO \Delta cusCFBA::cm$  double mutant, since the triple mutant *E. coli* GR16 ( $\Delta cueO \Delta cusCFBA::cm copA::km$ ) did not exhibit a further decrease in copper tolerance.

**CusCFBA may not transport Cu(I) from the cytoplasm.** The CzcCBA transenvelope transporter from “*Ralstonia metallidurans*” CH34 is thought to function as a proton-cation antiporter expelling cations from the cytoplasm across the inner and outer membranes. Two channels in CzcA are proposed to form a charge-relay system, where proton transport generates an electrical field that drives the transport of cations into the periplasm (7). However, the *cus* determinant probably does not transport cytoplasmic Cu(I), since *E. coli* GR13 (*copA::km*  $\Delta cusA::cm$ ) was no more sensitive than strain DW3110 ( $\Delta copA$ ) (Table 1). If CusCFBA extrudes copper from the cytosol, deletion of the *cus* determinant would be expected to have an additive effect on the phenotype of a *copA::km* mutant. Other transporters of the RND superfamily, such as AcrB and MexB, can efflux substrates that do not cross the cytoplasmic membrane (12). It was therefore suggested that binding of the substrate might occur on the periplasmic side of the transporter (19). CusA contains multiple methionine residues in the second large periplasmic domain. These residues, which are not present in CzcA, could be involved in copper binding. Furthermore, CusF is a putative periplasmic protein with potential

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TABLE 1. MICs<sup>a</sup> of copper for different genetic constructs of *E. coli* W3110

Strain	Genotype	MIC of CuCl <sub>2</sub> (mM)	Source
W3110	Wild type	3.5	Lab stock
GR1	W3110 $\Delta$ <i>cueO</i> :: <i>cm</i>	2.75 <sup>b</sup>	This work
GR5	W3110 $\Delta$ <i>cusA</i> :: <i>cm</i>	3.5	This work
GR6	W3110 $\Delta$ <i>cusCFBA</i> :: <i>cm</i>	3.5	This work
GR7	W3110 $\Delta$ <i>cueO</i> :: <i>cm</i> /pTYB2:: <i>cueO</i>	3.0	This work
DW3110	W3110 <i>copA</i> :: <i>km</i>	2.5 <sup>b</sup>	Rensing et al. (16)
GR8	W3110 <i>copA</i> :: <i>km</i> $\Delta$ <i>cueO</i> :: <i>cm</i>	2.25 <sup>b</sup>	This work
GR10	W3110 $\Delta$ <i>cueO</i> $\Delta$ <i>cusCFBA</i> :: <i>cm</i>	1.3 <sup>c</sup>	This work
GR12	W3110 <i>copA</i> :: <i>km</i> $\Delta$ <i>cusCFBA</i> :: <i>cm</i>	2.5 <sup>b</sup>	This work
GR13	W3110 <i>copA</i> :: <i>km</i> $\Delta$ <i>cusA</i> :: <i>cm</i>	2.5 <sup>b</sup>	This work
GR15	W3110 $\Delta$ <i>cueO</i> $\Delta$ <i>cusA</i> :: <i>cm</i>	1.3 <sup>c</sup>	This work
GR16	W3110 <i>copA</i> :: <i>km</i> $\Delta$ <i>cueO</i> <i>cusA</i> :: <i>cm</i>	1.3 <sup>c</sup>	This work
GR17	W3110 <i>copA</i> :: <i>km</i> $\Delta$ <i>cueO</i> <i>cusCFBA</i> :: <i>cm</i>	1.3 <sup>c</sup>	This work
GR18	W3110 $\Delta$ <i>cueO</i> $\Delta$ <i>cusCFBA</i> :: <i>cm</i> /pTYB2:: <i>copA</i> ( <i>R. metallidurans</i> CH34)	3.0	This work
GR19	W3110 $\Delta$ <i>cueO</i> $\Delta$ <i>cusCFBA</i> :: <i>cm</i> /pACYC:: <i>cueO</i>	3.0	This work
GR20	W3110 $\Delta$ <i>cueO</i> :: <i>cm</i> /pACYC:: <i>cueO</i>	3.5	This work
GR21	W3110 $\Delta$ <i>cueO</i> :: <i>cm</i> /pTYB2:: <i>copA</i> ( <i>R. metallidurans</i> CH34)	3.5	This work

<sup>a</sup> The MIC is defined as the concentration at which no growth was observed. Overnight cultures were diluted 1:500 into fresh Luria-Bertani medium, and after 2 h of growth at 37°C, they were streaked out on Luria-Bertani plates with different concentrations of CuCl<sub>2</sub>. Growth was monitored after 16 h at 37°C.

<sup>b</sup> Colonies were small, and colorless, and sometimes mucoid at concentrations well below the MIC.

<sup>c</sup> Colonies were small and mucoid at 0.5 mM CuCl<sub>2</sub>.

copper binding sites. Thus, it is possible that the CusCFBA transenvelope transporter binds and transports periplasmic copper. Since the *cus* determinant is also responsible for a small increase in Ag(I) resistance (5), it is likely that the transported copper species is Cu(I).

**CopA from “*R. metallidurans*” can functionally substitute for CueO.** CueO is homologous to the putative multicopper oxidases PcoA (*E. coli*), CopA (“*R. metallidurans*”; accession no. CAC07979) and CopA (*Pseudomonas syringae*), which are encoded by genes present in the plasmid-borne copper resistance operon *pco* in *E. coli* and the *cop* operons of “*R. metallidurans*” and *P. syringae*, respectively (3, 1). It should be pointed out that the “*R. metallidurans*” and *P. syringae* CopA proteins are not P-type ATPases, although PcoA, *P. syringae* CopA, and probably “*R. metallidurans*” CopA are essential components of their plasmid-encoded copper resistance determinants. PcoA, CopA (*P. syringae*), and CopA (“*R. metallidurans*”) are largely identical to each other and probably have similar functions. The degree of similarity between CueO and PcoA, CopA (*P. syringae*), and CopA (“*R. metallidurans*”) is much smaller, suggesting that they are distantly related. However, all four putative multicopper oxidases have leader sequences including a twin-arginine motif for export into the periplasm by the Tat pathway (13). CueO has a methionine-rich region that is also observed in PcoA and the CopA proteins from “*R. metallidurans*” and *P. syringae*. CopA (*P. syringae*) has been shown to be a periplasmic protein (2). CopA from *R. metallidurans* is functionally similar to CueO, since *copA* on plasmid pTYB2::*copA* can complement the single mutant *E. coli* GR1 ( $\Delta$ *cueO*::*cm*) (Table 1) and the double mutant *E. coli* GR10 ( $\Delta$ *cueO*  $\Delta$ *cusCFBA*::*cm*) (Table 1). Likewise, the double mutant *E. coli* GR10 ( $\Delta$ *cueO*  $\Delta$ *cusCFBA*::*cm*) can also be complemented by *cueO* on a plasmid (Table 1). By analogy, we suggest that CueO and CopA (“*R. metallidurans*”) are periplasmic proteins. Possibly, CueO prevents uptake of Cu(I) into the cytoplasm by oxidizing it to Cu(II). Additionally, by oxidizing Cu(I) to Cu(II), CueO might confer copper tolerance by preventing oxidative damage in the periplasm. The functions of PcoA,

CopA (*P. syringae*), and CopA (“*R. metallidurans*”) might be similar.

Another possibility is that the putative multicopper oxidases are secreted into the periplasm with their copper atoms already incorporated, consistent with the presence of the twin-arginine motifs. The synthesis and secretion of these proteins could result in a net efflux of copper from the cytoplasm, as was measured for the *pco* system (1). However, *E. coli* strain GR8, where both *copA* and *cueO* were disrupted, exhibited only a slight decrease in copper tolerance compared to strain DW3110, which has only *copA* disrupted (Table 1). One molecule of the homologous CopA protein (*P. syringae*) was shown

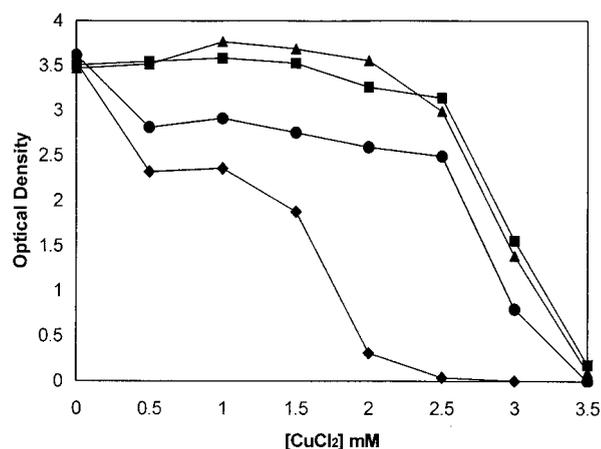


FIG. 1. Effect of several mutations on copper tolerance. Growth curves with different CuCl<sub>2</sub> concentrations are shown. Overnight cultures were diluted 1:500 into fresh Luria-Bertani medium, and after 2 h of growth at 37°C, cells were diluted 1:500 into fresh Luria-Bertani medium with the indicated concentrations of CuCl<sub>2</sub>. Cell growth was monitored as the optical density at 600 nm after 15 h of incubation at 37°C with shaking. Strains tested include *E. coli* GR1  $\Delta$ *cueO*::*cm* (●), *E. coli* GR6  $\Delta$ *cusCFBA*::*cm* (▲), *E. coli* GR10  $\Delta$ *cueO*  $\Delta$ *cusCFBA*::*cm*; (◆), and *E. coli* W3110 (■).

to bind approximately 11 atoms of copper (2). It seems unlikely that the amounts of copper transported by CopA (*P. syringae*) and possibly by CueO would be sufficient to confer copper resistance.

**Conclusions.** In this report we show that CueO and CusCFBA are involved in copper tolerance. CueO is homologous to multicopper oxidases such as ceruloplasmin, ascorbate oxidase, PcoA, and CopA (*P. syringae*) and is probably a periplasmic protein. CueO might oxidize Cu(I) to Cu(II) and can be functionally replaced by CopA from "*R. metallidurans*". The *cus* determinant encodes a copper efflux system that might transport periplasmic Cu(I) [and Ag(I)] across the outer membrane. This suggests that the two determinants provide alternate fates for periplasmic Cu(I): either oxidation by CueO or transport into the extracellular medium by CusCFBA. These studies are a starting point to further elucidate the molecular mechanisms of copper homeostasis in prokaryotes. Further biochemical studies are necessary to understand the function of CusCFBA, CueO, and other, yet-unidentified components.

This work was supported by hatch project 136713 to C.R.

We thank Barry Rosen for providing P1 lysate and for helpful suggestions and Dietrich Nies and Barry Wanner for the generous gift of strains.

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