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The Independent *cue* and *cus* Systems Confer Copper Tolerance during Aerobic and Anaerobic Growth in *Escherichia coli**

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Copper is essential but can be toxic even at low concentrations. Coping with this duality requires multiple pathways to control intracellular copper availability. Three copper-inducible promoters, controlling expression of six copper tolerance genes, were recently identified in *Escherichia coli*. The *cue* system employs an inner membrane copper transporter, whereas the *cus* system includes a tripartite transporter spanning the entire cell envelope. Although *cus* is not essential for aerobic copper tolerance, we show here that a copper-sensitive phenotype can be observed when *cus* is inactivated in a *cueR* background. Furthermore, a clear copper-sensitive phenotype for the *cus* system is revealed in the absence of O_2. These results indicate that the *cue* pathway, which includes a copper exporter, CopA, and a periplasmic oxidase, CueO, is the primary aerobic system for copper tolerance. During anaerobic growth, however, copper toxicity increases, and the independent *cus* copper exporter is also necessary for full copper tolerance. We conclude that the cytosolic (CueR) and periplasmic (CusRS) sensor systems differentially regulate copper export systems in response to changes in copper and oxygen availability. These results underscore the increased toxicity of copper under anaerobic conditions and the complex adaptation of copper export in *E. coli*.

Cellular responses to starvation are complicated if the nutrient in question is essential but also toxic. The transition metal copper is one such nutrient. The unique redox properties of copper lead to a central role in numerous respiratory and metabolic enzymes, especially those that utilize dioxygen or reactive oxygen species as substrates. Copper can also be quite toxic. This toxicity led to widespread use of copper compounds in anti-fungal and anti-microbial agricultural applications (1) or as in anti-fouling paint additives. This cytotoxicity is further manifest in a number of fatal diseases in humans, such as Menkes and Wilson disease, which involve significant disruption of intracellular copper trafficking (2). Although the mechanisms of cytotoxicity are not yet established, excess copper can apparently lead to deleterious side reactions, including competition with other essential transition metals for binding to key metalloprotein metal centers.

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Given the dual nature of copper, elaborate mechanisms for copper homeostasis can be found in all kingdoms. In eukaryotes, ranging from yeast to humans, highly conserved systems are used to acquire copper, to direct it to appropriate enzyme metal centers and intracellular compartments, or to eject copper from the cell (3, 4). The ensemble of these activities maintains the cellular quota for this metal within an acceptable range, depending upon the growth and stress conditions. The minimal copper quota is that amount necessary for growth and metabolism per cell, but this value is influenced by the specific growth conditions and by the genetic and biochemical background of the organism.

Recent studies in the Gram-negative bacterium *Escherichia coli* have identified two chromosomal systems involved in copper tolerance, both of which catalyze removal of excess copper from the cell. The *cue* system (for Cu efflux) consists of a copper-responsive metalloregulatory protein, CueR, that upregulates expression of two genes, *copA* and *cueO* (5). CopA is a copper efflux P-type ATPase with significant homology to eukaryotic copper transporters in yeast and humans (6). CueO is a putative oxidase that bears similarity to a large family of multicopper oxidases, which includes ascorbate oxidase and the ferroxidases Fet3 and ceruloplasmin (5).

An independent copper efflux system on the *E. coli* chromosome, the *cus* system (for Cu-sensing) is regulated by a two-component signal transduction system encoded by *cusR* and *cusS* (7). CusRS activate expression of the *cusCFBA* operon in response to elevated concentrations of copper (7, 8). The *cus* system can also be activated by silver, although at higher concentrations than observed for copper (8). CusC is an outer membrane protein with homology to the TolC stress-response protein (9). CusB belongs to the membrane fusion protein family (10). Membrane fusion protein family members typically are anchored in the inner membrane with a long periplasm-spanning domain (11). CusA is part of the RND family of H^+ antiporters, which export numerous substrates ranging from heavy metals to proteins (12). Together these three proteins are envisioned to form a multiunit transport complex that spans both membranes and the periplasmic space.

Based on the precedent of homologous systems, the CusCBA transport complex is proposed to transport copper from the cytoplasm across the cell envelope and into the extracellular space utilizing the proton motive force as an energy source. The proposed copper export function of the *cus* locus has been challenged (8). In an alternative model, it is argued that the absence of a copper-sensitive phenotype in the *cusA* strain is not consistent with a role in copper tolerance and that the *cus* genes mediate resistance to silver (8). The *cus* and *cue* systems, however, are induced at similar concentrations of copper sulfate in the medium (5, 7), which implies that there may be significant overlap in function. Such overlap would make the copper phenotype difficult to discern. A role for the *cus* deter-

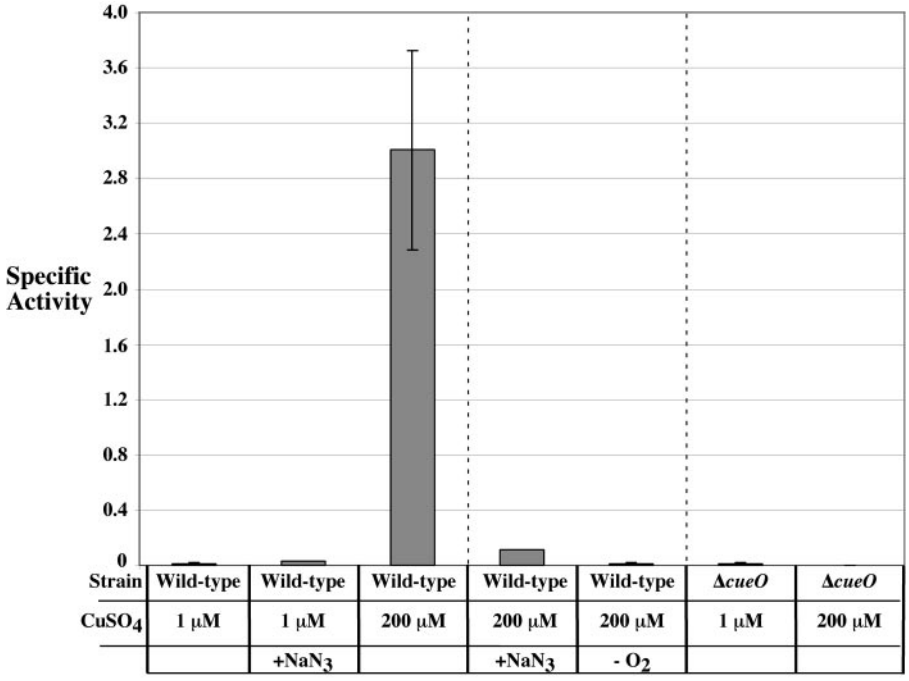

FIG. 1. **Oxidase activity assay of periplasmic extracts from wild-type and *cueO* strains.** Activity was determined as described under "Experimental Procedures." Wild-type and *cueO* strains were exposed to 1 or 200 μM copper sulfate prior to isolation of periplasmic extracts. Sodium azide (NaN_3) was added as described under "Experimental Procedures." Some assays were conducted in an anaerobic glove box ($-O_2$). Shown is the average of at least three replicates.

minant in copper tolerance is further supported by the recent observation of a copper-sensitive phenotype in a *cueO*/*cusCFBA* strain (13).

To test these alternatives, we have deconvoluted the regulation of three loci within these two systems and examined how anaerobic growth alters the copper phenotypes. We show that CueO has multicopper oxidase activity in the periplasm as initially proposed. This activity and several of the Cu^s phenotypes are dependent upon oxygen, suggesting differential roles for the *cus* and *cue* systems under aerobic and anaerobic conditions. In fact the systems are partially redundant, but each operon functions optimally in different environments providing protection for both cytosolic and periplasmic factors from adverse effects of copper.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Media—The *E. coli* strains used in this work (Table I) are all derivatives of BW25113 except for LMG194/*copA*. Construction of the strains used in this study is described below. All growth and gene reporter studies were conducted in a metal-limited, chemically defined media (CDM)[1] described previously (5). Where required, antibiotics were used at the following concentrations: chloramphenicol at 30 μg/ml, kanamycin at 40 μg/ml, and carbenicillin at 100 μg/ml. Media and other solutions used anaerobically were purged of oxygen through three cycles of vacuum evacuation/nitrogen replacement. All anaerobic experiments were performed in a Vacuum Atmospheres™ glove box under a N_2 atmosphere.

Construction of Mutant and Gene Reporter Strains—Insertional inactivation of *cueO* was accomplished using the mutagenesis system described previously (14) to create JHI41. Strain BW27102[2] containing the *cusR*::*kan* mutation was acquired from B. Wanner, and the mutation was transduced via P1 phage into BW25113 to create strain WOII301A and into WOII248B to create WOII301C. Strain LMG194/*copA* (6) containing the *copA*::*kan* mutation was obtained from B. Rosen, and the mutation was transduced via P1 phage into BW25113 to create JHI42A. Single copy gene reporter strains containing *cusC-lacZ*, *cueO-lacZ,* or *copA-lacZ* were constructed using a λ phage vector as described elsewhere (16).

Gene Reporter Studies—Lysogens containing the various promoter *lacZ* fusions were grown aerobically at 37 °C until exponential phase

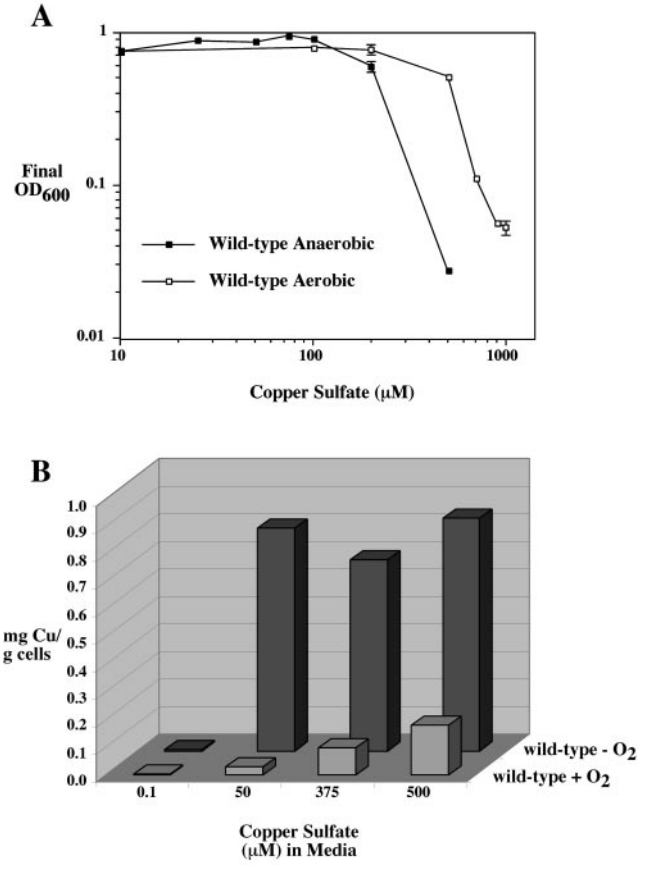

FIG. 2. **Analysis of copper sensitivity and copper accumulation during anaerobic growth.** *A,* copper sensitivity of BW25113 wild-type *E. coli* grown in the presence and absence of oxygen. Shown is the final A_{600} measured after growth in varying concentrations of copper sulfate. All points are the average of three replicates. *B,* copper accumulation of BW25113 wild-type *E. coli* grown in the presence and absence of oxygen as measured by ICP-MS. Cells were exposed to varying amounts of copper sulfate in the media for 80 min prior to harvesting. Data are shown as milligrams of copper per g of cell dry weight. Each measurement is the average of three replicates.

[1] The abbreviations used are: CDM, chemically defined media; ICP-MS, inductively coupled plasma-mass spectrometry.

[2] J. Masella and B. L. Wanner, personal communication.

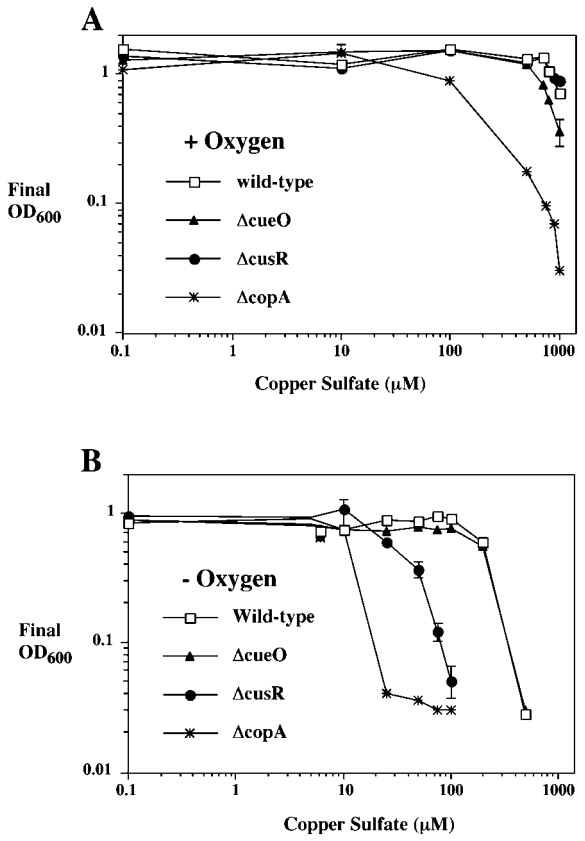

FIG. 3. **Aerobic and anaerobic copper sensitivity phenotypes of wild-type, *cueO*, *copA*, and *cusR* strains.** *A,* final A_{600} of wild-type (*squares*), *cueO* (*triangles*), *cueR* (*circles*), and *copA* (*asterisks*) grown in various amounts of copper sulfate under aerobic conditions. *B,* final A_{600} of wild-type (*squares*), *cueO* (*triangles*), *cueR* (*circles*), and *copA* (*asterisks*) strains grown in various amounts of copper sulfate under anaerobic conditions.

(A_{600} ≈0.5–0.7) at which time they were exposed to varying amounts of copper sulfate for 1 h. After copper exposure promoter activity was measured using the standard Miller assay for β-galactosidase. For anaerobic copper induction profiles, cells were grown as described for the copper sensitivity experiments except that no copper was added to cultures until cells were growing exponentially (A_{600} ≈0.5–0.7). Cells were exposed to various copper concentrations for 1 h at which time cells were brought out of the anaerobic glove box. An aliquot of cells was immediately transferred into Z buffer for the Miller assay, whereas the remaining cells were used for A_{600} determination. The measurements shown are the average of triplicate experiments.

Preparation of Periplasmic Extracts—Overnight cultures of the various strains were diluted 1:100 into 5 ml of CDM. Cells were grown at 37 °C to an A_{600} of 0.6–0.8, followed by addition of various concentrations of copper sulfate. After 1 h, the cells were harvested, and a periplasmic extract was prepared by the chloroform method (17). Briefly, the media were drained, and the cells were resuspended into ~100 μl of remaining media, followed by the addition of 50 μl of $CHCl_3$. After brief mixing, the cell suspension was incubated for 15 min at room temperature. Then 500 μl of 10 mM Tris-Cl, pH 8, was combined with the cell suspension to extract the released components of the periplasm. After centrifugation, the top layer, containing the periplasmic extract, was carefully removed and stored on ice, prior to the oxidase assays. Total protein concentration of the extract was determined by the method of Bradford with IgG (Biorad) used as a standard (18).

Multicopper Oxidase Activity Assays—The activity assays were performed utilizing 3,3′-dimethoxybenzidine (Sigma) as the colorimetric indicator for oxidase activity (19). Reactants were pre-equilibrated at 37 °C, and the reaction was performed at 37 °C. In the assay 15 μl of periplasmic extract was combined was 225 μl of 0.1 M sodium acetate, pH 5.5. The reaction was initiated by the addition of 60 μl of 7.88 mM 3,3′-dimethoxybenzidine. The reaction was performed in duplicate for each assay, and the reactions were stopped at 5 and 120 min, respectively, by the addition of 600 μl of 18 N H_2SO_4 to each reaction. The difference in absorbance at 540 nm between the 120- and 5-min reactions was used to calculate the amount of oxidized 3,3′-dimethoxybenzidine (ϵ = 9600 M^{-1} cm^{-1}). A unit of activity is defined as micromole of substrate oxidized per min per ml of extract. The specific activity is defined as the micromole of substrate oxidized per min per mg of total protein in extract. Measurements shown are the average of triplicate experiments.

TABLE I
Strains

Strain	Description	Ref.
BW25113	Wild-type ($lacI^q rrnB_{T14}$ $lacZ_{WJ16}$ *hsdR514* $araBAD_{AH33}$ $rhaBAD_{LD78}$)	14
BW27102	*cusR::kan, creABCD, phoR*	Footnote 2
LMG194/copA	$copA::kan^R$	6
WOII248B	*cueR*	5
WOII301A	$cusR::kan^R$	This work
WOII301C	$cueR/cusR::kan^R$	This work
JHI41	*cueO*	This work
JHI42A	$copA::kan^R$	This work
WOII260B	Wild-typeΦ(cueO-lacZ)	5
WOII260E	Wild-typeΦ(*copA-lacZ*)	This work
WOIII1A	Wild-typeΦ(*cusC-lacZ*)	This work
WOIII10	*cueR*Φ*(cusC-lacZ)*	This work

Copper Sensitivity Determination—For aerobic copper sensitivity, overnight cultures of the various strains were diluted 1:100 into 5 ml of CDM containing various concentrations of copper sulfate. Cells were grown at 37 °C with shaking, and a final A_{600} measurement was taken after 9 h of growth. For anaerobic copper sensitivity, overnight cultures of the various strains were sealed into a 1.5-ml tube, and oxygen was replaced with nitrogen gas. These tubes were then transferred into an anaerobic glove box, and the cells were diluted 1:500 into 20 ml of CDM containing various concentrations of copper sulfate. These cells were well dispersed by vigorous pipetting and grown in Petri dishes at 30 °C. A final A_{600} measurement was taken after 24 h of growth. Measurements shown are the average of triplicate experiments. Copper gradient plates were made using equal portions of CDM agar with no added copper and with 1 mM copper sulfate. Plates were allowed to equilibrate overnight prior to use. Equal numbers of cells from each strain were dotted in 10-μl portions across the copper gradient. Growth was examined after 24 h at 37 °C.

Copper Accumulation Measurements—For aerobic copper accumulation, cells were grown to mid-log phase and then exposed to varying amounts of copper sulfate for 80 min. Following copper treatment, 1 ml of cells was removed for determination of final A_{600}, which was used to calculate the total milligrams of cells based on a calibration curve. Cells were then pelleted and washed three times with media containing 1 mM EDTA. The cell pellets were dried overnight at 80 °C in 10-ml Teflon tubes. The dried pellets were then dissolved in 500 mg of Trace Metal Grade (Fisher) nitric acid by heating at 80 °C for 30 min. After cooling to room temperature the samples were transferred to a 50-ml sterile polypropylene centrifuge tube (Fisher). Teflon tubes were rinsed 3 times with Milli-Q H_2O, and the rinse was also transferred to the 50-ml tube. These samples were mixed with an internal standard consisting of ^{45}Sc, ^{89}Y, ^{115}In, ^{159}Tb, and ^{209}Bi (CPI International). The volume was adjusted to 25.0 g with Milli-Q H_2O giving final concentrations of 5 ppb for the internal standard and 2% for nitric acid. The metal content of the samples was measured by inductively coupled plasma mass spectrometry (ICP-MS) (PQ ExCell, VG Elemental). Control experiments without cells were run in parallel to determine the background metal contamination in the materials used. A 10-ppm stock of the metal standards used for calibration of ICP-MS was custom-ordered from CPI International and contained ^{25}Mg, ^{51}V, ^{53}Cr, ^{55} Mn, ^{59}Co, ^{60}Ni, ^{65}Cu, ^{66}Zn, ^{75}As, ^{82}Se, and ^{100}Mo. This 10-ppm stock was diluted to make standards ranging from 0 to 20 ppb. All glassware and plasticware was acid-washed overnight with 10% nitric acid prior to use. For anaerobic copper accumulation, media were measured into flasks and sealed with rubber septa in the nitrogen atmosphere of an anaerobic glove box. Cells were added to the anaerobic flask using a sterile syringe and were grown at 37 °C without shaking. Addition of copper, copper exposure, and ICP-MS analysis were conducted as described for the aerobic copper accumulation experiments. Measurements shown are the average of triplicate experiments.

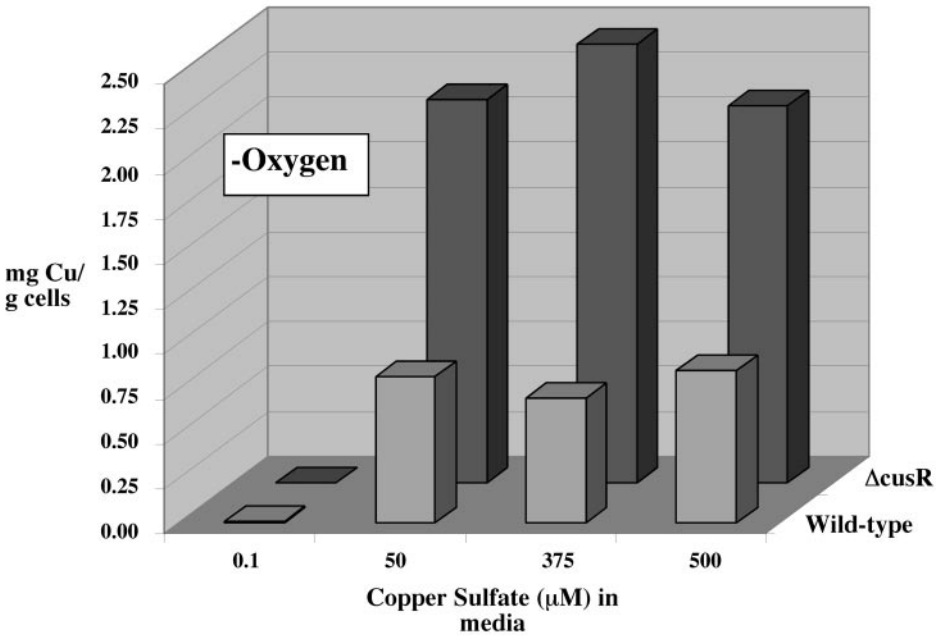

FIG. 4. **Copper accumulation of *cusR* and wild-type strains during anaerobic growth.** The strains were exposed to varying amounts of copper sulfate in the media for 80 min prior to harvesting and copper measurement with ICP-MS. Measurements are the average of three replicates.

RESULTS

CueO Is a Periplasmic Multicopper Oxidase—CueO (encoded by the *yacK* gene) contains a twin arginine translocation motif at its amino terminus, which targets it for export to the periplasm via the "folded protein" export pathway (20, 21). To determine if CueO may function in the periplasm as a multicopper oxidase, periplasmic extracts made from wild-type and *cueO* strains were tested for oxidase activity. Oxidase activity from wild-type extracts is clearly present, and this activity is inducible by copper (Fig. 1). This oxidase activity was abolished in the *cueO* strain regardless of the presence of added copper. The copper-inducible oxidase activity was also sensitive to treatment with sodium azide (Fig. 1), an established inhibitor of multicopper oxidases, such as ascorbate oxidase, ceruloplasmin, and Fet3 (22–24). These results demonstrate that *cueO* encodes for a copper-inducible, periplasmic multicopper oxidase.

The catalytic cycle of multicopper oxidases involves oxidation of a substrate and transfer of the liberated electrons to a copper cluster, where the four-electron reduction of O_2 to form H_2O occurs (25, 26). Multicopper oxidases require dioxygen for activity and are thus sensitive to oxygen deprivation. In fact, we observe no CueO oxidase activity when the assay is conducted anaerobically (Fig. 1). One would therefore predict that CueO is unable to function during growth in the absence of oxygen.

Copper Toxicity and Accumulation Increases Under Anaerobic Growth Conditions—The requirement for molecular oxygen for the *cue* system led us to hypothesize that this system may play a significant role in copper efflux under aerobic conditions, whereas the *cus* system could be important for copper export under anaerobic conditions. To determine a base line for anaerobic copper toxicity, we measured the copper tolerance of wild-type *E. coli* strain BW25113 grown under aerobic and anaerobic conditions (Fig. 2*A*). The toxicity of copper increases dramatically under anaerobic growth conditions. This trend supports an early report of increased copper toxicity in *E. coli* under anaerobic conditions (27). This is interesting because the toxic effects of copper are often attributed to its ability to catalyze adventitious formation of reactive oxygen species from O_2. Given that copper is more toxic under oxygen limited conditions, copper must be able to alter physiological processes in other ways.

Previous work (28) has shown that copper accumulation increases in anaerobic cultures of *Saccharomyces cerevisiae* as compared with aerobic cultures. To determine if a similar change in copper accumulation occurs in *E. coli* under anaerobic conditions, we measured total copper content of wild-type aerobic and anaerobic cultures (Fig. 2*B*). Strikingly, cells grown under anaerobic conditions reach a point of maximum copper accumulation at a media copper concentration of 50 μM, whereas aerobic copper accumulation fails to reach similar levels even out to 500 μM copper. Furthermore, anaerobic cells accumulated up to 20-fold more copper than aerobic cells. These results suggest that the essential copper quota may increase during anaerobic growth. Alternatively, copper homeostasis may be perturbed in the absence of oxygen, either through uncontrolled uptake or through disruption of copper export.

cusR and copA Are Necessary for Anaerobic Copper Tolerance but cueO Is Not—To test whether the roles of *cue* and *cus* in copper tolerance shift in response to oxygen, the *cue* and *cus* mutant strains were assayed in the presence and absence of air. Disruption of *copA* leads to a significant copper sensitivity under aerobic growth conditions (Fig. 3*A*). The *cueO* strain shows only a mild aerobic copper sensitivity, whereas the copper sensitivity of the *cusR* strain is not different from wild type in the presence of air (Fig. 3*A*). These results are corroborated by the recent findings of Grass and Rensing (13).

The sensitivity of these strains to copper changes significantly under anaerobic growth conditions. As anticipated by the biochemical analysis above, deletion of *cueO* does not affect copper sensitivity relative to that of wild-type strains. The *copA* strain is more sensitive to copper than wild-type (Fig. 3*B*), indicating that it continues to play a role in copper tolerance during anaerobic growth. The most striking difference between the anaerobic and aerobic conditions is the appearance of copper sensitivity in the *cusR* strain in the absence of air (Fig. 3*B*).

These results imply that disruption of copper export machinery leads to increased copper toxicity, perhaps through increased accumulation. It has been shown previously that disruption of *copA* leads to a decrease of copper efflux and accumulation of cellular copper (6, 29). To determine if deletion of *cusR* and inactivation of the CusCFBA transporter lead to a similar disruption in copper export under anaerobic conditions,

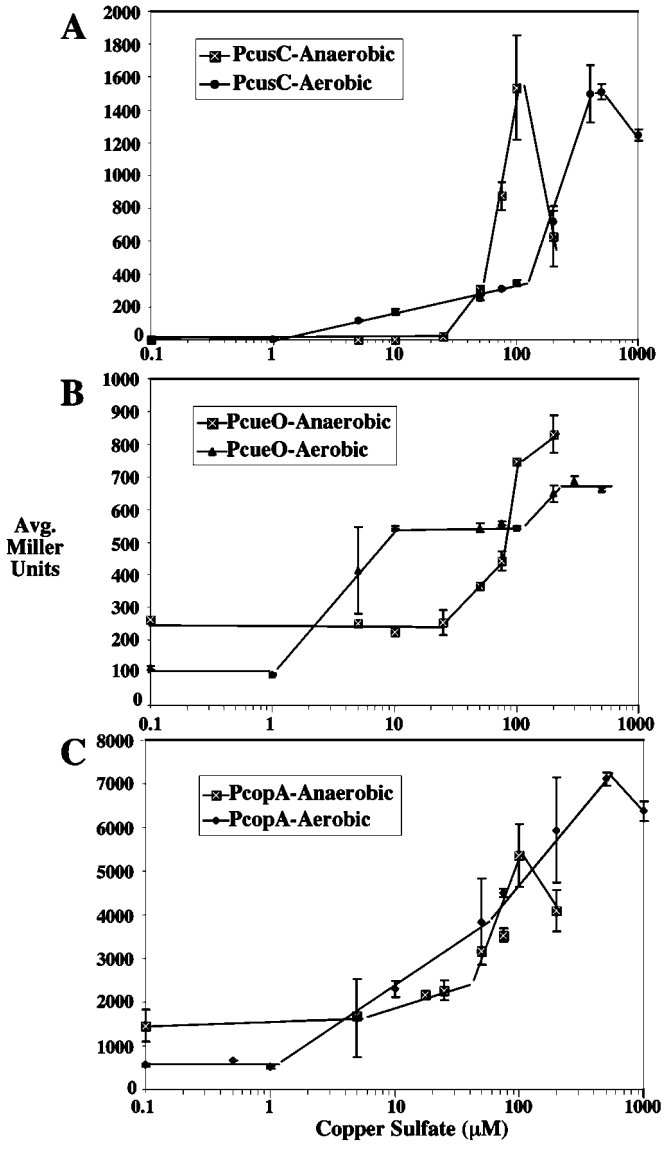

FIG. 5. **Changes in copper regulation of *cue* and *cus* promoters under anaerobic conditions.** *A, cusC-lacZ* lysogens were exposed to varying concentrations of copper sulfate during exponential growth under anaerobic (*x in squares*) or aerobic (*circles*) conditions. *B*, *cueO-lacZ* lysogens were exposed to varying concentrations of copper sulfate during exponential growth under anaerobic (*x in squares*) or aerobic (*triangles*) conditions. *C, copA-lacZ* lysogens were exposed to varying concentrations of copper sulfate during exponential growth under anaerobic (*x in squares*) or aerobic (*diamonds*) conditions. β-Galactosidase activity was measured by Miller assay.

the copper content of the *cusR* strain grown anaerobically was measured. Fig. 4 indicates that copper accumulation increases markedly in the *cusR* strain compared with the wild-type strain, suggesting that anaerobic copper export is disrupted in the absence of the *cus* system. This accumulation implies that the *cus* system may exhibit different patterns of gene expression in response to oxygen.

The Cus and Cue Systems Are Differentially Regulated under Aerobic and Anaerobic Conditions—A number of metabolic and stress-responsive pathways include metalloproteins that are specifically regulated in response to molecular oxygen (30, 31). Although none of the *cue* or *cus* gene promoters contain obvious binding sites for oxygen-dependent regulators, such as FNR or ArcB, copper induction profiles for the *cusC* and *cueO* promoters reveal a strong oxygen-dependent effect (Fig. 5).

First, we consider the relative effects of copper on transcription under aerobic conditions for each promoter (Fig. 5). The half-maximal induction of the *cueO* promoter occurs at ~3 μM, which is a lower copper concentration than observed for the *cusC* promoter (~200 μM) (compare Fig. 5, *A* and *B*). Induction of the *copA* promoter directly parallels that of *cueO* in the initial response (*i.e.* up to 10 μM) as expected (Fig. 5, *B* and *C*). The base-line expression of both *copA* and *cueO* in the absence of any copper stress is significantly higher than that observed with the *cus* system (Fig. 5). Thus at these copper levels we observe the earliest indications of copper stress, up-regulation of *cueO* and *copA*. The early induction of the *cue* system suggests that *copA* and *cueO* together form the primary export pathway under these moderate copper concentrations. Further induction of the *copA* promoter is observed at higher copper concentrations, and as suggested previously (5) this may arise from additional activation by other regulatory factors, such as *cpxR*, that respond to cell envelope stress.

A very different pattern of copper-responsive gene expression is observed under anaerobic conditions. First, the half-maximal induction of the *cusC* promoter now occurs at a lower copper concentration (70 μM) under anaerobic conditions than that observed aerobically (200 μM) (Fig. 5*A*). This is consistent with the hypothesis that the *cus* system plays a more important role under anaerobic conditions. In contrast, the half-maximal induction of the *cueO* promoter occurs at a higher copper concentration compared with the aerobic condition (60 *versus* 3 μM) (Fig. 5*B*). Activity at the *copA* promoter changes little under anaerobic conditions (Fig. 5*C*), but the higher basal level under anaerobic conditions suggests an elevation of cytoplasmic copper concentration relative to aerobic growth. Despite the lack of any clear connection to known oxygen-responsive regulatory systems, the copper responses of the *cueO* and *cusC* promoters are shifted in opposite directions by oxygen deprivation. This shift in the regulation pattern may allow the cell to adapt to changes in the periplasmic Cu^{1+}/Cu^{2+} ratio under anaerobic conditions.

The Cus System Also Functions in Aerobic Copper Tolerance—Initial characterization of a *cusA* strain showed no measurable loss of aerobic copper tolerance (8). Our results here demonstrate that a *cusR* mutant strain also has no measurable copper sensitivity phenotype under aerobic conditions. This led to the suggestion that the *cus* operon is not important for aerobic copper tolerance despite the fact that copper is the most potent inducer, giving a greater induction ratio than silver (data not shown) (8). To evaluate this hypothesis further, we deleted *cusR* in a *cueR* background to create a *cueR*/*cusR* strain. In this double mutant strain, the *cue* system (*copA* and *cueO*) is expressed at basal levels but is not copper-inducible due to the absence of *cueR* (5). Furthermore, the *cus* system is not present; even a low basal expression of the *cusC* promoter cannot be detected in the absence of CusR-dependent activation (7) (Fig. 5).

Fig. 6*A* shows that, whereas the copper tolerance of the wild-type and single mutant strains is very similar, a *cueR*/*cusR* double mutant is markedly sensitive to copper stress. This establishes that the *cusCBA* transporter is necessary for aerobic copper tolerance when the *cue* system cannot be induced. An aerobic function for *cus* is further supported by the observation that the *cusC* promoter is induced at a lower copper concentration in a *cueR* background relative to wild type, as shown in Fig. 6*B*. Here the *cus* expression profile is seen to shift to significantly lower copper concentrations in the absence of *cue* induction. This early activation of the *cus* system in a *cueR* background parallels the early activation of the *cus* system under anaerobic conditions (Fig. 5*A*). Together these results show that the *cus* and *cue* systems play distinct but somewhat

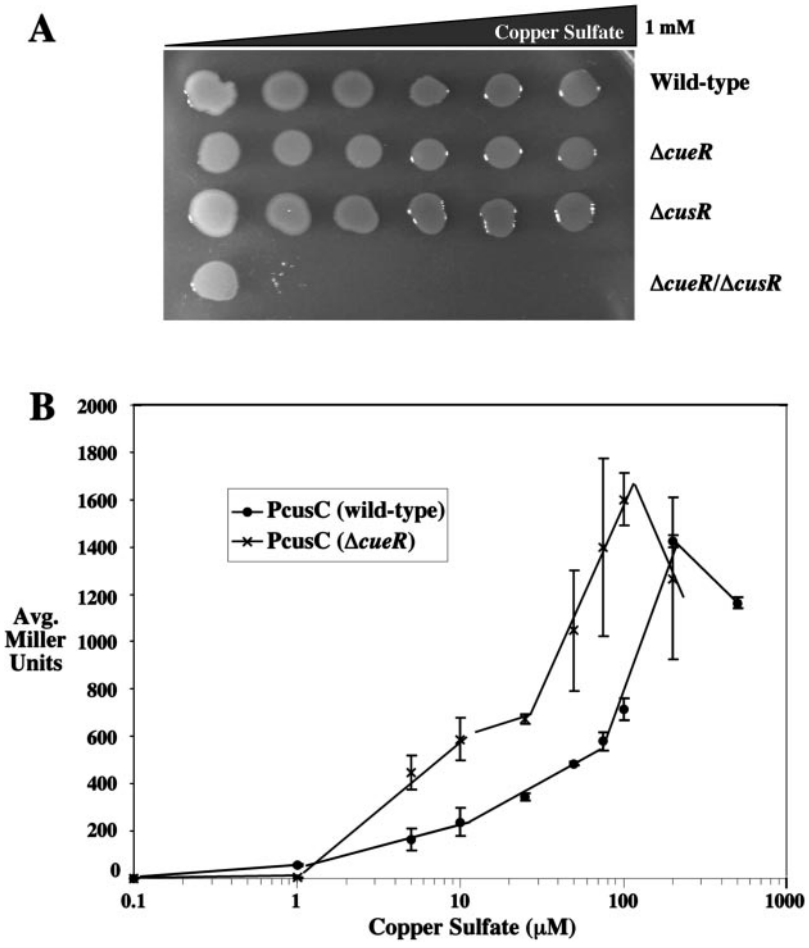

FIG. 6. **Role of the *cus* system in aerobic copper tolerance.** *A*, growth of wild-type, *cueR*, *cusR*, and *cueR*/*cusR* mutant strains across a gradient of copper concentrations. *B*, copper expression profile of a *cusC-lacZ* reporter in wild-type (*circles*) and *cueR* (×) strains. β-Galactosidase activity was measured by standard Miller assay.

reciprocal roles in copper tolerance under aerobic growth conditions and that these roles diverge further during anaerobic growth.

DISCUSSION

The Cue System Is the Primary Copper Homeostasis System—Under both aerobic and anaerobic conditions, the *cue* regulon is expressed at moderate basal levels. In contrast, the *cus* system has extremely low basal levels in both cases. As copper stress increases, the *cue* system activates to a maximal induction of ~12-fold. The *cus* system, on the other hand, is not transcribed until a higher threshold of copper is reached (~200 μM $CuSO_4$ for half-maximal transcription) and shows an 800-fold induction. These observations suggest that the *cue* system is a primary copper efflux system and that *cus* is only activated when the housekeeping systems are overwhelmed. In this sense *cue* confers copper tolerance under moderate and high copper concentration, whereas *cus* augments tolerance under conditions of extreme copper stress.

At low (0.1 μM) to intermediate (50 μM) copper concentrations under aerobic conditions, the periplasm may actually function as a copper storage compartment for *E. coli*. When environmental copper levels increase beyond the essential cellular copper quota, the *cue* system is the first to be induced (Fig. 7). It is mildly up-regulated and apparently clears the cytoplasm of excess copper without depleting the cell of a locally available periplasmic copper pool. If the copper detoxification capacity of the *cue* system begins to saturate, the *cus* system activates and transports copper beyond the periplasm to the extracellular space. There may also be additional gene products that interact with the *cue* system in the periplasm and outer membrane.

The fact that deletion of *cusA* or *cusR* does not lead to measurable copper sensitivity during aerobic growth leads Nies and co-workers (8) to propose that *cus* is not involved in copper tolerance. Since the *cusA* strain was sensitive to silver, it was argued that *cus* is a silver resistance locus; however, many genes involved in copper export also protect against silver toxicity (32, 33). Furthermore, the regulatory and phenotypic data indicate that during aerobic growth the *cus* system provides significant protection against copper only when concentrations overwhelm the primary *cue* system or if the *cue* system is not fully functional (as in the *cueR* strain). Under aerobic growth conditions in wild-type strains, the role of the *cus* system as an auxiliary copper efflux system is minimal because the elements of the housekeeping system, namely CopA and CueO, are fully functional.

The Cus System Plays an Important Role in Copper Tolerance under Anaerobic Growth and, under Extreme Copper Stress, in Aerobic Growth—During anaerobic growth, the cell can tolerate low to moderate copper stress levels without inducing either system (Fig. 7) even though a significant increase in anaerobic copper accumulation is observed. As copper concentrations further increase to the moderate stress levels, the cellular copper export systems seem to be overwhelmed at a lower copper concentration under anaerobic, as compared to aerobic, conditions.

The increased anaerobic toxicity of copper to *E. coli* was previously purposed to result from a shift in total copper from the Cu^{2+} to the Cu^{1+} oxidation state (27). In fact a loss of EPR signal was observed in samples of *E. coli* that were shifted to anaerobic growth conditions, suggesting a loss of Cu^{2+} and an increase in Cu^{1+} as oxygen is depleted (27). This shift could be due to reduction of Cu^{2+} to Cu^{1+} by biological reduction mech-

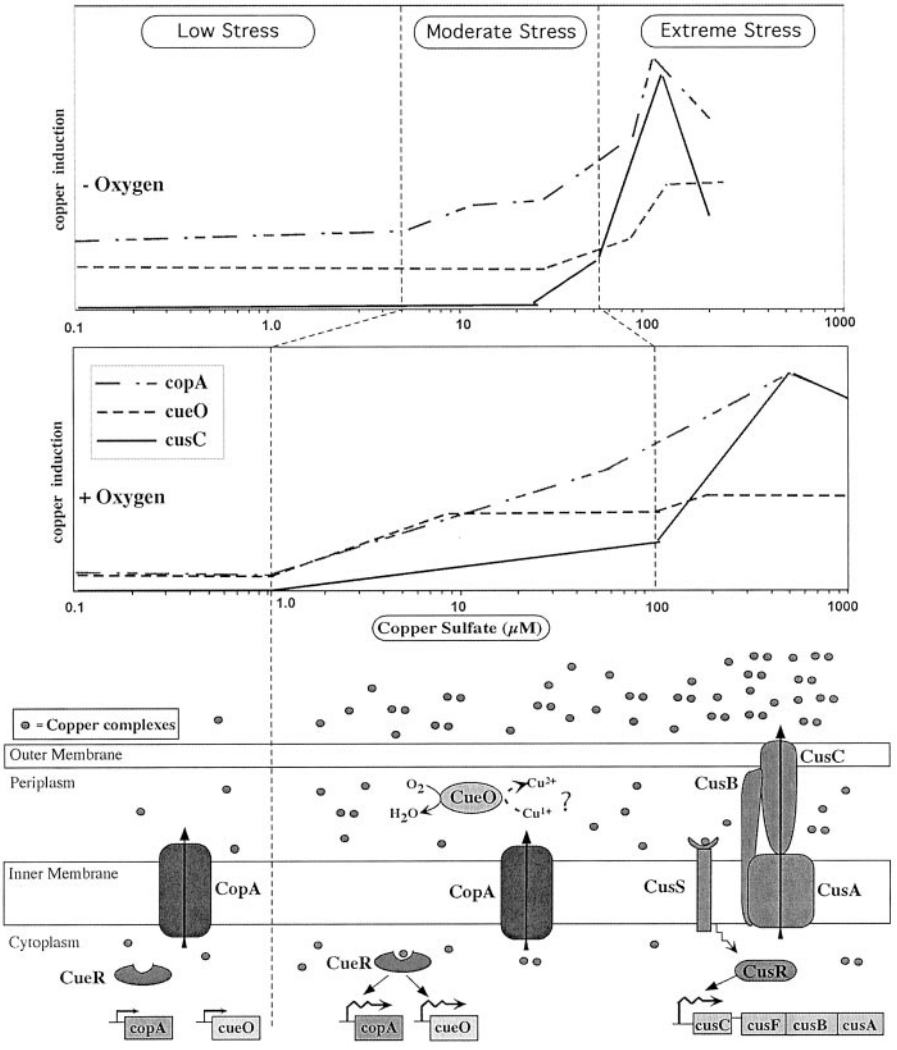

FIG. 7. **Summary of *cue* and *cus* regulation in response to copper and oxygen availability.** *Top panels, in vivo* transcription responses over a continuum of copper concentrations for both aerobic and anaerobic conditions. Three domains of the copper continuum are depicted: low, moderate, and extreme copper stress. Note that the region of moderate stress is much smaller for the anaerobic condition. The gene expression profiles are from Fig. 5, with the *left axes* scaled as shown in Fig. 5, *A* and *B,* for *cusC* and *cueO*, and right axes scaled as shown in Fig. 5*C* for *copA*. *Lower panel,* a model for the localization and regulation of the three loci.

anisms such as cell-surface reductases, small molecule reductants, or other components of the electron transport machinery.

The observation that Cu^{1+} is more toxic than Cu^{2+} suggests that the role of CueO during aerobic growth may be to convert periplasmic Cu^{1+} to a less toxic form, namely Cu^{2+}. Thus, one factor that may contribute to these changes in copper toxicity and accumulation is the loss of CueO function in the absence of oxygen. Disruption of CueO function may lead to an increase in the Cu^{1+} concentration gradient on the periplasmic side of the inner membrane. Cu^{1+} accumulation could then occur via passive or active uptake of Cu^{1+} through yet unidentified inner membrane factors, possibly involving nonspecific uptake systems for other metals such as sodium or potassium. Alternatively, CueO may not use Cu^{1+} as an electron donating substrate *in vivo*. It may have another biochemical function, such as that of a terminal oxidase that helps energize a copper transport pathway.

Regardless of the exact function of CueO, it is clear that one facet of the biochemical mechanism of the *cue* system is crippled in the absence of molecular oxygen. We have shown here that under anaerobic growth conditions, *cusR*-dependent activation of the *cus* system is absolutely required for tolerance to high copper concentrations. We propose that the role of *cus* as an auxiliary copper efflux system is enhanced under anaerobic conditions due to the biochemical limitations of the *cue* primary copper export system.

Cytoplasmic and Periplasmic Sensors Balance Copper Efflux in Response to Multiple Stresses—During aerobic growth, both *cueO* and *copA* are induced at lower copper concentrations than *cusC*. Under anaerobic conditions, this situation reverses and *cusC* is activated earlier while the copper induction point for *cueO* shifts higher. The independent localization of the two sensor systems that control their expression may explain the differences between aerobic and anaerobic regulation. As cells grow aerobically, copper in the oxidizing environment of the periplasm can persist in the Cu^{2+} state, but membrane-based reductases and the electron transport chain most likely convert some fraction of the copper to the Cu^{1+} state. Since Cu^{1+} is, based on first principles, energetically easier to move across a lipid bilayer, it may well be the form taken up by the cell. If any Cu^{2+} is moved across the inner membrane, the reducing environment of the cytoplasm can lead to reduction (Reaction 1). Further stabilization of the Cu^{1+} form through binding to thiol moieties can be anticipated from copper chemistry.

$$\begin{array}{ll} & E_O \\ Cu^{2+}{}_{(aq)} + 1e^- \rightarrow Cu^{1+}{}_{(aq)} & +0.15\ V \\ GSH \rightarrow \frac{1}{2}\,GSSG + 1e^- & +0.22\ V \\ \hline Cu^{2+}{}_{(aq)} + GSH \rightarrow Cu^{1+} + \frac{1}{2}GSSG & +0.37\ V \end{array}$$

REACTION 1

The cytoplasmic sensor CueR, therefore, is most likely activated in some way by increasing levels of Cu^{1+} in the cytoplasm. Silver induction of the *copA*/*cueO* system also supports this idea since Ag^{1+} is chemically similar to Cu^{1+} but not Cu^{2+} (6). As a cytoplasmic Cu^{1+} sensor, CueR is ideally placed to up-regulate the primary copper efflux system, represented by *cueO* and *copA*, in order to clear the cytoplasm of excess copper.

The periplasmic CusS sensor is also thought to respond to Cu^{1+} even though the periplasm is an oxidizing environment (34). This hypothesis is based on the observation that monovalent cations such as Ag^{1+} activate the *cus* system but divalent cations like Zn^{2+} and Cd^{2+} do not (15). So long as CopA and CueO are able to export and detoxify Cu^{1+}, CusS is not activated, and the *cus* system remains off. As environmental copper levels increase, CueR stimulates transcription of the *cue* system by a maximum of 12-fold. Upon exposure to extremely high concentrations of copper, however, the CopA/CueO copper export/detoxification system is overwhelmed, leading to Cu^{1+} accumulation locally in the periplasm. Excess Cu^{1+} then triggers the periplasmic sensor, CusS. Once the *cus* system activates, copper is ejected from the cell and into the extracellular space. This additional efflux capacity coupled with the *cue* housekeeping system provides the full copper tolerance observed in wild-type strains under aerobic growth conditions.

Copper Induction under Anaerobic Conditions—Growth in the absence of oxygen clearly alters copper physiology. The activation of the CusS periplasmic sensor at lower copper concentrations is consistent with a shift of periplasmic copper ratio toward the Cu^{1+} state. If CueO oxidizes Cu^{1+} to Cu^{2+}, this shift in the Cu^{1+}/Cu^{2+} ratio may be exacerbated by the loss of CueO function in the absence of oxygen. Increased Cu^{1+} accumulation in the periplasm activates the CusS sensor at a lower total copper concentration in the medium. Furthermore, early activation of the *cus* system might explain the delayed induction of *cueO* if *cus* decreases cytoplasmic copper levels and delays CueR activation. Thus, the differential localization of two Cu^{1+}-sensing systems between the periplasm and cytoplasm explains the regulatory behavior observed in this study.

By compartmentalization of the CueR and CusRS copper sensors, *E. coli* can tailor expression of the *cue* and *cus* efflux systems to manage a range of copper exposure levels and a variety of growth conditions. The potential differences in metal affinity of the two primary sensors, CueR and CusS, may provide an additional mechanism for tuning copper efflux machinery in *E. coli*. The requirement for such elaborate mechanisms to control copper efflux stems from the essential yet potentially toxic nature of copper. Similar strategies are likely to be necessary for other essential but potentially toxic transition metals.

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