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# Anaerobic Copper Toxicity and Iron-Sulfur Cluster Biogenesis in *Escherichia coli*

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**ABSTRACT** While copper is an essential trace element in biology, pollution of groundwater from copper has become a threat to all living organisms. Cellular mechanisms underlying copper toxicity, however, are still not fully understood. Previous studies have shown that iron-sulfur proteins are among the primary targets of copper toxicity in *Escherichia coli* under aerobic conditions. Here, we report that, under anaerobic conditions, iron-sulfur proteins in *E. coli* cells are even more susceptible to copper in medium. Whereas addition of 0.2 mM copper(II) chloride to LB (Luria-Bertani) medium has very little or no effect on iron-sulfur proteins in wild-type *E. coli* cells under aerobic conditions, the same copper treatment largely inactivates iron-sulfur proteins by blocking iron-sulfur cluster biogenesis in the cells under anaerobic conditions. Importantly, proteins that do not have iron-sulfur clusters (e.g., fumarase C and cysteine desulfurase) in *E. coli* cells are not significantly affected by copper treatment under aerobic or anaerobic conditions, indicating that copper may specifically target iron-sulfur proteins in cells. Additional studies revealed that *E. coli* cells accumulate more intracellular copper under anaerobic conditions than under aerobic conditions and that the elevated copper content binds to the iron-sulfur cluster assembly proteins IscU and IscA, which effectively inhibits iron-sulfur cluster biogenesis. The results suggest that the copper-mediated inhibition of iron-sulfur proteins does not require oxygen and that iron-sulfur cluster biogenesis is the primary target of anaerobic copper toxicity in cells.

**IMPORTANCE** Copper contamination in groundwater has become a threat to all living organisms. However, cellular mechanisms underlying copper toxicity have not been fully understood up to now. The work described here reveals that iron-sulfur proteins in *Escherichia coli* cells are much more susceptible to copper in medium under anaerobic conditions than they are under aerobic conditions. Under anaerobic conditions, *E. coli* cells accumulate excess intracellular copper, which specifically targets iron-sulfur proteins by blocking iron-sulfur cluster biogenesis. Since iron-sulfur proteins are involved in diverse and vital physiological processes, inhibition of iron-sulfur cluster biogenesis by copper disrupts multiple cellular functions and ultimately inhibits cell growth. The results from this study illustrate a new interplay between intracellular copper toxicity and iron-sulfur cluster biogenesis in bacterial cells under anaerobic conditions.

**KEYWORDS** copper toxicity, iron-sulfur proteins, iron-sulfur cluster biogenesis, cell growth inhibition, anaerobic conditions

Copper is an essential element required by at least 30 enzymes in living organisms (1). However, excess copper is highly toxic (2). In U.S. groundwater, copper concentrations range from undetectable to approximately 1 mM, with most samples in the nanomolar range (3). In industrial and mining waste streams, micromolar to millimolar

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concentrations of copper have also been found (4). Copper at high concentrations in water has become a threat to all living organisms (5). As a transition metal, copper was believed to produce reactive oxygen species via the Fenton reaction under aerobic conditions (6, 7). However, an elevated intracellular copper content did not damage DNA in *Escherichia coli* cells under aerobic conditions (8). In fact, copper is more toxic to *E. coli* cells under anaerobic conditions than under aerobic conditions (9, 10), indicating that other cellular mechanisms could be responsible for copper toxicity. Because copper needs only two thiol groups to form a linear biscysteinate coordination in proteins (11), excess copper in cells may directly compete with iron or iron-sulfur cluster binding sites in proteins to disrupt iron-sulfur clusters (12, 13) or block iron-sulfur cluster biogenesis (14–16). Since iron-sulfur proteins are involved in diverse and vital physiological processes ranging from energy metabolism to DNA replication and repair (17, 18), inactivation of iron-sulfur proteins by copper will have a broad impact on multiple physiological functions in cells.

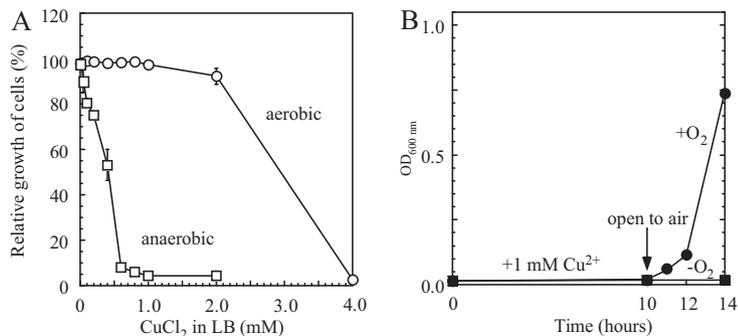
Recent studies have also established that formation of iron-sulfur clusters in proteins is not a spontaneous process in cells. A group of dedicated proteins are responsible for iron-sulfur cluster biogenesis (19). In *E. coli*, there are two iron-sulfur cluster assembly systems, the housekeeping *iscSUA-hscBA-fdx* gene cluster (20), which is conserved from prokaryotes (bacteria) to eukaryotes (18, 21), and the inducible *sufABCDE* gene cluster (22), which represents a redundant activity of *iscSUA-hscBA-fdx* and is found only in plastids in plants, archaea, and some bacteria (23). Among the proteins encoded by *iscSUA-hscBA-fdx*, IscS is a pyridoxal 5' phosphate-dependent cysteine desulfurase that provides sulfide for iron-sulfur cluster assembly (24). IscU is a scaffold protein that hosts transient iron-sulfur clusters (25) and transfers the assembled clusters to target proteins (26, 27). IscA is characterized as an alternative scaffold (28, 29). However, unlike the scaffold IscU, IscA has strong iron binding activity (30, 31) and is able to transfer the bound iron for iron-sulfur cluster assembly in target proteins *in vitro* (31–33). Based on these and other studies, we have postulated that IscA is an iron chaperone that recruits intracellular iron and delivers it for iron-sulfur cluster biogenesis (32, 33) and that IscS, IscU, and IscA may constitute the core members of the iron-sulfur cluster assembly machinery in cells (32, 34).

In previous studies, we reported that excess intracellular copper competes with ferrous iron for binding in the iron-sulfur cluster assembly protein IscA and effectively blocks iron-sulfur cluster biogenesis in *E. coli* cells under aerobic conditions (15). Here, we find that under anaerobic conditions, iron-sulfur proteins in *E. coli* cells are even more susceptible to copper in growth medium. It appears that, under anaerobic conditions, as reported by Outten et al. (9), *E. coli* cells accumulate more intracellular copper, which effectively inhibits iron-sulfur cluster biogenesis by binding to the iron-sulfur cluster assembly proteins IscA and IscU and the IscA paralog SufA. The results from this study suggest that the copper-mediated inactivation of iron-sulfur proteins does not require oxygen and that iron-sulfur cluster biogenesis is the primary target of anaerobic copper toxicity in cells.

## RESULTS

**Iron-sulfur proteins in *E. coli* cells are highly susceptible to copper under anaerobic conditions.** Figure 1A shows that the addition of 1.0 mM  $\text{CuCl}_2$  to LB medium had very little or no effect on cell growth of wild-type *E. coli* under aerobic conditions. However, the same copper treatment completely inhibited cell growth under anaerobic conditions. The results are consistent with those in a previous report by Outten et al. (9) showing that *E. coli* cells are more sensitive to copper in medium under anaerobic conditions than they are under aerobic conditions. Nevertheless, to date, the cellular mechanism underlying anaerobic copper toxicity is not understood.

When the *E. coli* cells grown in LB medium supplemented with 1.0 mM  $\text{CuCl}_2$  were shifted from anaerobic to aerobic conditions, the cell growth was immediately restored (Fig. 1B), indicating that the copper-mediated inhibition of cell growth under anaerobic conditions was reversible. Since iron-sulfur proteins are the primary targets of copper



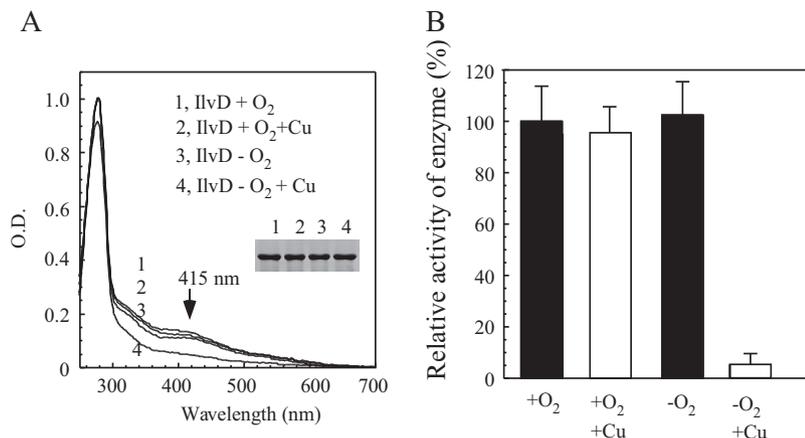
**FIG 1** Cell growth inhibition of *E. coli* by copper under aerobic and anaerobic conditions. (A) Cell growth of *E. coli* in LB medium supplemented with the indicated concentrations of copper under aerobic and anaerobic conditions. Wild-type *E. coli* cells (MC4100) were grown in LB medium supplemented with the indicated concentrations of CuCl<sub>2</sub> at 37°C for 4 h under aerobic (open circles) or anaerobic (open squares) conditions. Cell growth was determined by measuring turbidity at 600 nm. The data represent the averages  $\pm$  standard deviations from three independent experiments. (B) Reversal of copper-mediated cell growth inhibition. *E. coli* cells grown in LB medium supplemented with 1.0 mM CuCl<sub>2</sub> were incubated at 37°C for 10 h. Half of the cell culture (closed circles) was returned to aerobic conditions, while the other half (closed squares) remained under anaerobic conditions. Cell growth was monitored by OD determination at 600 nm.

toxicity in *E. coli* cells under aerobic conditions (12–16) and iron-sulfur clusters can be reversibly assembled in proteins without *de novo* protein synthesis (35), we sought to explore the effect of copper on iron-sulfur proteins in *E. coli* cells under anaerobic conditions. In our experiments, recombinant iron-sulfur protein was expressed in wild-type *E. coli* cells grown in LB medium supplemented with 0.2 mM CuCl<sub>2</sub> under aerobic and anaerobic conditions. The concentration of 0.2 mM CuCl<sub>2</sub> was used because it significantly inhibited the cell growth under anaerobic conditions but not under aerobic conditions (Fig. 1A). We chose dihydroxyacid dehydratase (IlvD), a key enzyme in the branched-chain amino acid biosynthesis pathway (36), as an example of a recombinant iron-sulfur protein expressed in *E. coli* cells. IlvD contains a [4Fe-4S] cluster which is essential for its enzyme activity (36). We then isolated the recombinant iron-sulfur protein from the *E. coli* cells, purified it, and subjected it to the UV-visible absorption spectrum measurements and enzyme activity analyses.

Figure 2A shows that addition of 0.2 mM CuCl<sub>2</sub> to LB medium had very little or no effect on the UV-visible absorption spectrum of recombinant IlvD expressed in the *E. coli* cells grown under aerobic conditions. While the yield of recombinant IlvD expressed in *E. coli* cells under anaerobic conditions was about one-third of that under aerobic conditions, the 0.2 mM CuCl<sub>2</sub> treatment did not significantly affect the protein expression in the cells under aerobic or anaerobic conditions. Nevertheless, the copper treatment significantly decreased the absorption peak at 415 nm of the [4Fe-4S] cluster (Fig. 2A) and the specific enzyme activity (Fig. 2B) of recombinant IlvD expressed in *E. coli* cells under anaerobic conditions. The iron and sulfide content analyses further showed that the anaerobic copper treatment had largely eliminated iron-sulfur clusters in IlvD in *E. coli* cells (data not shown). The enzyme activity of the purified IlvD was fully restored when iron-sulfur clusters were reassembled *in vitro* (15), further suggesting that the anaerobic copper treatment eliminated iron-sulfur clusters in IlvD and inactivated the enzyme activity of the protein in *E. coli* cells.

Similar results were also obtained when the recombinant iron-sulfur proteins acornitase B (37), endonuclease III (38), and phosphogluconate dehydratase (39) were expressed in *E. coli* cells grown in LB medium supplemented with 0.2 mM CuCl<sub>2</sub> under aerobic and anaerobic conditions (data not shown). Thus, copper is able to inactivate multiple iron-sulfur proteins by eliminating iron-sulfur clusters in the proteins in *E. coli* cells under anaerobic conditions.

**Copper inhibits the activities of iron-sulfur proteins in *E. coli* cells without affecting the proteins that do not have iron-sulfur clusters under anaerobic conditions.** The *E.*



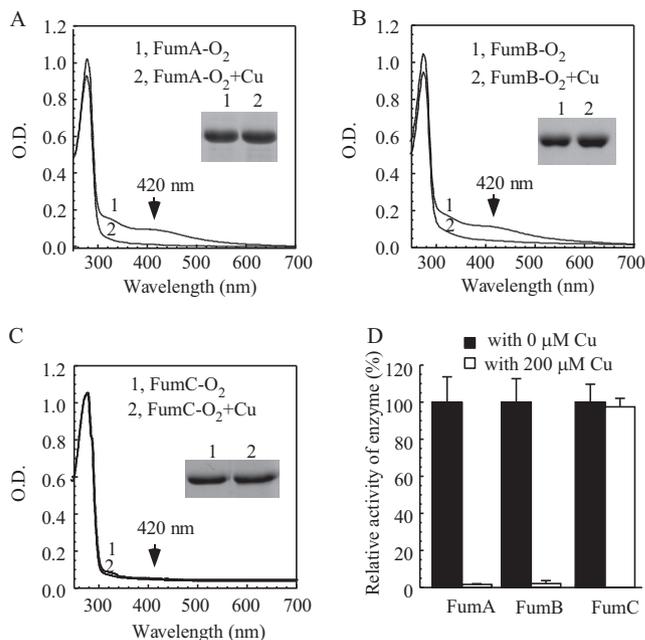
**FIG 2** Effect of copper on the iron-sulfur protein IlvD in *E. coli* cells under aerobic and anaerobic conditions. (A) UV-visible absorption spectra of recombinant IlvD purified from the *E. coli* cells grown in LB medium supplemented with or without 0.2 mM CuCl<sub>2</sub> under aerobic or anaerobic conditions. Purified IlvD was dissolved in buffer containing 500 mM NaCl and 20 mM Tris (pH 8.0). The concentration of IlvD was 28  $\mu$ M. (Inset) Photograph of SDS-PAGE gel of purified proteins. Samples: 1, IlvD under aerobic conditions; 2, IlvD under aerobic conditions with 0.2 mM CuCl<sub>2</sub>; 3, IlvD under anaerobic conditions; 4, IlvD under anaerobic conditions with 0.2 mM CuCl<sub>2</sub>. (B) Enzyme activity of IlvD. Recombinant IlvD was purified as described in panel A. For the enzyme activity assay, 1  $\mu$ M IlvD was used. The highest enzyme activity (100%) for purified IlvD was  $1.2 \pm 0.1$  mM/ $\mu$ M/min.

*coli* genome encodes three fumarases, fumarase A and fumarase B, each of which contains a [4Fe-4S] cluster (40), and fumarase C, which does not have iron-sulfur clusters (41). Each fumarase was expressed in the *E. coli* cells grown in LB medium supplemented with 0.2 mM CuCl<sub>2</sub> under aerobic or anaerobic conditions. While addition of CuCl<sub>2</sub> to LB medium had very little or no effect on iron-sulfur clusters in fumarase A and fumarase B expressed in *E. coli* cells under aerobic conditions (data not shown), the same copper treatment largely eliminated the iron-sulfur cluster content (Fig. 3A and B) and decreased the specific enzyme activity (Fig. 3D) of recombinant fumarase A and fumarase B expressed in the *E. coli* cells under anaerobic conditions. In contrast, the copper treatment did not affect the enzyme activity of recombinant fumarase C in *E. coli* cells under anaerobic conditions (Fig. 3D), suggesting that CuCl<sub>2</sub> at a concentration of 0.2 mM may specifically target iron-sulfur proteins in cells under anaerobic conditions.

**Copper inhibits iron-sulfur cluster biogenesis in *E. coli* cells under anaerobic conditions.** One possible explanation for the copper-mediated elimination of iron-sulfur clusters in proteins is that copper may directly disrupt iron-sulfur clusters in proteins in *E. coli* cells, as proposed by Macomber et al. (12). Alternatively, copper may block iron-sulfur cluster biogenesis (14–16), resulting in inactive apo-proteins in *E. coli* cells. To elucidate the possible effects, 0.2 mM CuCl<sub>2</sub> was added to LB medium before and after recombinant iron-sulfur protein was expressed in the *E. coli* cells under anaerobic conditions.

Figure 4A shows that, while addition of copper to LB medium before recombinant fumarase B was expressed largely eliminated iron-sulfur clusters in the protein, addition of copper to LB medium after fumarase B was expressed did not significantly affect iron-sulfur clusters in the protein under anaerobic conditions. The enzyme activity measurements further showed that copper blocked iron-sulfur cluster assembly without significantly affecting the assembled iron-sulfur clusters in fumarase B in the *E. coli* cells under anaerobic conditions (Fig. 4B).

The DNA repair enzyme endonuclease III hosts a stable [4Fe-4S] cluster (38). Unlike the solvent-exposed [4Fe-4S] clusters in IlvD, fumarase A, and fumarase B, the [4Fe-4S] cluster in endonuclease III is buried within the protein structure (38). Here, we used endonuclease III as another example of the effect of copper on iron-sulfur cluster biogenesis in *E. coli* cells under anaerobic conditions. Figure 4C shows that while addition of

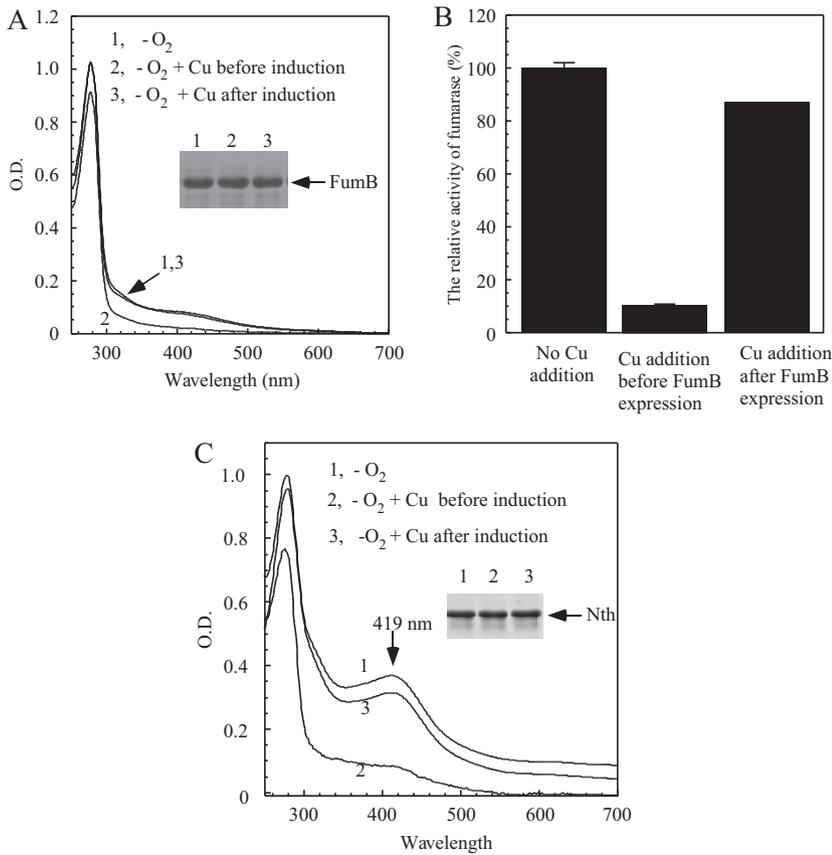


**FIG 3** Effect of copper on fumarase A, B, and C in *E. coli* cells under anaerobic conditions. (A) UV-visible absorption spectra of recombinant fumarase A (20  $\mu$ M) purified from *E. coli* cells grown in LB medium supplemented with 0 mM (spectrum 1) or 0.2 mM (spectrum 2)  $\text{CuCl}_2$  under anaerobic conditions. (B) UV-visible absorption spectra of recombinant fumarase B (20  $\mu$ M) purified from *E. coli* cells grown in LB medium supplemented with 0 mM (spectrum 1) or 0.2 mM (spectrum 2)  $\text{CuCl}_2$  under anaerobic conditions. (C) UV-visible absorption spectra of recombinant fumarase C (30  $\mu$ M) purified from *E. coli* cells grown in LB medium supplemented with 0 mM (spectrum 1) or 0.2 mM (spectrum 2)  $\text{CuCl}_2$  under anaerobic conditions. (Insets) Photograph of SDS-PAGE gels of purified proteins. (D) The enzyme activities of fumarases A, B, and C purified from *E. coli* cells grown in LB medium supplemented with 0 or 0.2 mM  $\text{CuCl}_2$  under anaerobic conditions. For the enzyme activity assay, 10 nM each fumarase was used. The highest enzyme activities (100%) for purified fumarase A, fumarase B, and fumarase C were  $19.2 \pm 0.3$ ,  $21.1 \pm 0.1$ , and  $16.6 \pm 0.3$  mM/ $\mu$ M/min, respectively. The results represent averages  $\pm$  standard deviations from three independent experiments.

0.2 mM  $\text{CuCl}_2$  to LB medium before endonuclease III was expressed eliminated the iron-sulfur cluster in the recombinant protein, addition of copper after endonuclease III was expressed did not significantly affect the assembled iron-sulfur clusters in the protein under anaerobic conditions. Altogether, these results show that copper appears to inhibit iron-sulfur cluster biogenesis without disrupting the assembled iron-sulfur clusters in proteins in *E. coli* cells under anaerobic conditions.

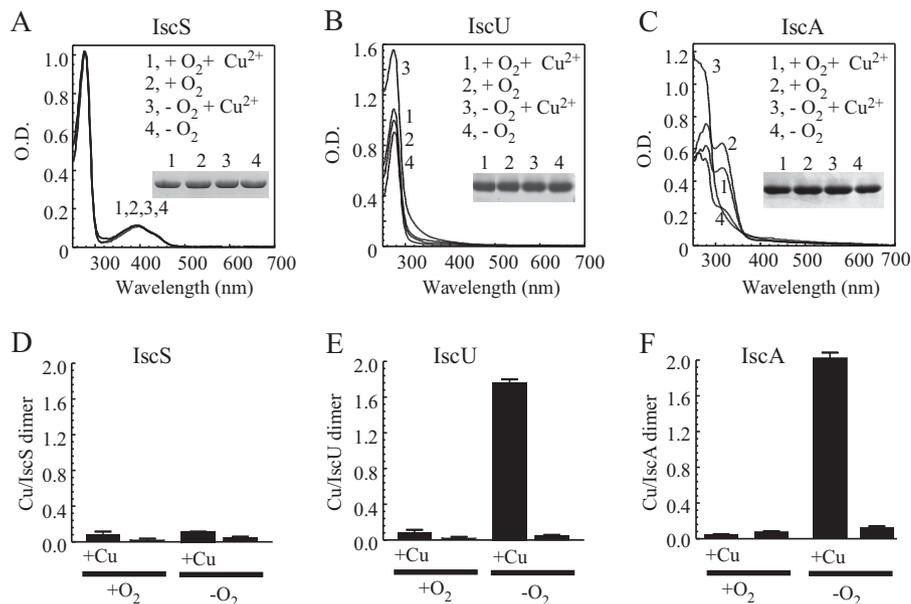
**Copper binding in the iron-sulfur cluster assembly proteins IscA and IscU in *E. coli* cells under aerobic and anaerobic conditions.** In *E. coli* cells, the proteins encoded by *iscSUA-hscBA-fdx* are the major source of the activity responsible for iron-sulfur cluster biogenesis (20). If copper inhibits iron-sulfur cluster biogenesis, we would expect that copper may directly bind to the iron-sulfur cluster assembly proteins. To test this idea, we expressed the core iron-sulfur cluster assembly proteins, cysteine desulfurase IscS (24, 34), scaffold protein IscU (27), and iron chaperone IscA (15), in *E. coli* cells grown in LB medium supplemented with 0.2 mM  $\text{CuCl}_2$  under aerobic and anaerobic conditions.

Figure 5A shows that addition of 0.2 mM  $\text{CuCl}_2$  to LB medium under aerobic or anaerobic conditions had very little or no effect on the UV-visible absorption spectrum of cysteine desulfurase IscS expressed in *E. coli* cells. The specific enzyme activity of purified IscS was also not affected by the copper treatment under aerobic or anaerobic conditions (data not shown). In contrast, when the *E. coli* cells were grown in LB medium supplemented with 0.2 mM  $\text{CuCl}_2$  under anaerobic conditions, purified IscU and IscA had an increased absorption peak of 260 nm (Fig. 5B and C), indicative of copper binding in the proteins (15). A copper content analysis further confirmed that IscU and IscA bind about 1.8 to 2.0 copper atoms per protein dimer (Fig. 5E and F).



**FIG 4** Copper inhibits iron-sulfur cluster assembly in *E. coli* cells under anaerobic conditions. (A) UV-visible absorption spectra of recombinant fumarase B purified from *E. coli* cells. Spectrum 1, fumarase B was expressed in the exponentially growing *E. coli* cells in LB medium without copper addition under anaerobic conditions for 2 h; spectrum 2, fumarase B was expressed in the exponentially growing *E. coli* cells in LB medium supplemented with 0.2 mM  $\text{CuCl}_2$  under anaerobic conditions for 2 h; spectrum 3, fumarase B was expressed in the exponentially growing *E. coli* cells in LB medium under anaerobic conditions for 2 h, followed by addition of 0.2 mM  $\text{CuCl}_2$  and an additional 2 h of growth under anaerobic conditions. Without addition of copper, fumarase B expressed in *E. coli* cells for 4 h had an absorption spectrum very similar to that of spectrum 3. The protein concentration of fumarase B was 20  $\mu\text{M}$ . (Inset) Photograph of SDS-PAGE gel of purified proteins. (B) Relative activities of purified fumarase B from panel A. For enzyme activity assay, 10 nM fumarase B was used. The highest enzyme activity (100%) for fumarase B was  $22.7 \pm 0.8 \mu\text{M}/\mu\text{M}/\text{min}$ . (C) UV-visible absorption spectra of recombinant endonuclease III (Nth) purified from *E. coli* cells. Spectrum 1, endonuclease III was expressed in the exponentially growing *E. coli* cells in LB medium without copper addition under anaerobic conditions for 2 h; spectrum 2, endonuclease III was induced in the exponentially growing *E. coli* cells in LB medium supplemented with 0.2 mM  $\text{CuCl}_2$  under anaerobic conditions for 2 h; spectrum 3, endonuclease III was expressed in the exponentially growing *E. coli* cells in LB medium under anaerobic conditions for 2 h, followed by addition of 0.2 mM  $\text{CuCl}_2$  and an additional 2 h of growth under anaerobic conditions. Without addition of copper, endonuclease III expressed in *E. coli* cells for 4 h had an absorption spectrum very similar to that of spectrum 3. The protein concentration of endonuclease III was 40  $\mu\text{M}$ . (Inset) Photograph of the SDS-PAGE gel of purified proteins.

SufA is a member of the inducible *sufABCDSE* gene cluster for iron-sulfur cluster biogenesis (22) and a paralog of IscA (42). In this context, we also assessed the copper binding activity of SufA in *E. coli* cells grown in LB medium supplemented with 0.2 mM  $\text{CuCl}_2$  under aerobic or anaerobic conditions. As shown in Fig. S1 in the supplemental material, SufA, like IscA, was able to bind copper in *E. coli* cells under anaerobic conditions but not under aerobic conditions. Since IscA, IscU, and SufA, each with bound copper, fail to assemble iron-sulfur clusters in target proteins (15), the bound copper in IscA, IscU, and SufA effectively blocks iron-sulfur cluster biogenesis in *E. coli* cells under anaerobic conditions. Interestingly, under anaerobic conditions, the gene cluster *iscSUA-hscBA-fdx* is significantly repressed in *E. coli* cells (43), which makes the



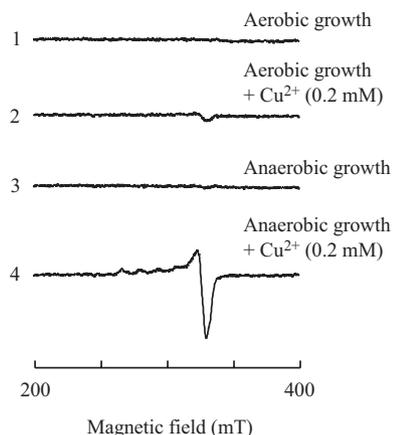
**FIG 5** Copper binding in IscS, IscU, and IscA in *E. coli* cells under anaerobic conditions. Iron-sulfur cluster assembly proteins IscS, IscU, and IscA were expressed in *E. coli* cells grown in LB medium supplemented with 0 or 0.2 mM CuCl<sub>2</sub> under aerobic or anaerobic conditions. UV-visible absorption spectra of purified IscS (25 μM) (A), IscU (100 μM) (B), and IscA (300 μM) (C) are shown. Spectrum 1, with 0.2 mM CuCl<sub>2</sub> under aerobic conditions; spectrum 2, with no CuCl<sub>2</sub> under aerobic conditions; spectrum 3, with 0.2 mM CuCl<sub>2</sub> under anaerobic conditions; spectrum 4, with no CuCl<sub>2</sub> under anaerobic conditions. (Insets) Photographs of SDS-PAGE gels of purified proteins. (D, E, and F) Copper contents of purified IscS, IscU, and IscA, respectively. The samples are the same as those in panels A through C. Copper contents in protein samples were determined as described in Materials and Methods and are presented as ratios of copper per protein dimer. The results are averages ± standard deviations from three independent experiments.

iron-sulfur cluster assembly proteins more sensitive to copper toxicity in the cells under anaerobic conditions.

Since the copper binding in IscA or IscU is not affected by oxygen *in vitro* (15), the observed copper binding in IscA and IscU in *E. coli* cells under anaerobic conditions (Fig. 5) could be due to the increased intracellular copper concentration, as reported by Outten et al. (9). Because different *E. coli* strains and growth media were used in the experiments by Outten et al. (9) and in the experiments described in this study, we reevaluated the intracellular copper content in *E. coli* cells grown in LB medium supplemented with 0.2 mM CuCl<sub>2</sub> under aerobic and anaerobic conditions using whole-cell electron paramagnetic resonance (EPR) measurements (15). As copper in *E. coli* cells exists as Cu(I) (9), which does not yield an EPR signal, the cells were washed twice and then treated with 2.5% (vol/vol) nitric acid to oxidize Cu(I) to Cu(II) for the EPR measurements (44). Quantification of the Cu(II) by EPR determination (Fig. 6) showed that *E. coli* cells grown in LB medium supplemented with 0.2 mM CuCl<sub>2</sub> contained about 10 to 15 times more intracellular copper content under anaerobic conditions than under aerobic conditions. We concluded that an elevated copper content in the *E. coli* cells (9) may effectively inhibit iron-sulfur cluster biogenesis by binding to the iron-sulfur cluster assembly proteins IscA, SufA, and IscU under anaerobic conditions.

**DISCUSSION**

Previous studies have shown that iron-sulfur proteins are the primary targets of copper toxicity in *E. coli* (12, 14, 15), *Bacillus subtilis* (13), and mitochondria (16) under aerobic conditions. Here, we report that iron-sulfur proteins in *E. coli* cells are much more susceptible to copper in medium under anaerobic conditions. While addition of 0.2 mM CuCl<sub>2</sub> to LB medium has very little or no effect on iron-sulfur proteins in *E. coli* cells under aerobic conditions, the same copper treatment largely inhibits the enzyme activity of iron-sulfur proteins by inhibiting iron-sulfur cluster biogenesis under anaer-



**FIG 6** Accumulation of intracellular copper content in *E. coli* cells under anaerobic conditions. *E. coli* cells were grown in LB medium supplemented with or without 0.2 mM  $\text{CuCl}_2$  for 4 h under anaerobic or aerobic conditions. The *E. coli* cells were then washed with buffer containing 20 mM Tris (pH 8.0) and 500 mM NaCl twice and treated with 2.5% (vol/vol) nitric acid. The samples were subjected to EPR measurements. Spectrum 1, *E. coli* cells grown in LB medium under aerobic conditions; spectrum 2, *E. coli* cells grown in LB medium supplemented with 0.2 mM  $\text{CuCl}_2$  under aerobic conditions; spectrum 3, *E. coli* cells grown in LB medium under anaerobic conditions; spectrum 4, *E. coli* cells grown in LB medium supplemented with 0.2 mM  $\text{CuCl}_2$  under anaerobic conditions.

obic conditions. It appears that under anaerobic conditions, *E. coli* cells accumulate extra intracellular copper, which blocks iron-sulfur cluster biogenesis by binding to the iron-sulfur cluster assembly proteins IscA and IscU and the IscA analog SufA. We also find that while copper inhibits iron-sulfur proteins, it does not affect the proteins that do not have iron-sulfur clusters in *E. coli* cells under anaerobic conditions. Since iron-sulfur proteins are involved in diverse and vital physiological processes (17, 18), inactivation of iron-sulfur cluster biogenesis by copper would have a broad effect on cellular functions under anaerobic conditions.

Cu(I) requires only two cysteine residues to form a linear biscysteinate coordination geometry, as seen in the Parkinsonism-associated DJ-1 protein (11). The thiol ligands in the iron-sulfur cluster assembly proteins IscA (45), SufA (42), and IscU (27) may facilitate strong copper binding. Previously, we found that copper can compete with iron for binding in IscA *in vitro* and in *E. coli* cells (15). While we did not observe significant copper binding in IscU in *E. coli* cells under aerobic conditions (15), we found copper binding in both IscA and IscU in *E. coli* cells grown in LB medium supplemented with 0.2 mM  $\text{CuCl}_2$  under anaerobic conditions (Fig. 5). Perhaps the accumulation of extra copper in *E. coli* cells under anaerobic conditions results in copper binding in IscU and IscA and its paralog SufA. Since IscA, SufA, and IscU bearing bound copper fail to assemble iron-sulfur clusters in proteins (15), copper may effectively block iron-sulfur cluster biogenesis in cells under anaerobic conditions. It should be pointed out that the repressed expression of the gene cluster *iscSUA-hscBA-fdx* in *E. coli* cells under anaerobic conditions (43) may further contribute to the increased susceptibility of iron-sulfur proteins to intracellular copper content. In this context, we propose that iron-sulfur cluster biogenesis is the primary target of copper toxicity in *E. coli* cells under anaerobic conditions.

Anaerobic copper toxicity in *E. coli* cells was first reported in 1976 (10). Accumulation of intracellular copper is likely responsible for increased copper toxicity under anaerobic conditions (9). In *E. coli*, intracellular copper content is regulated by three major copper homeostasis systems, CopA, a P-type ATPase that pumps copper ion out of the cytoplasm (46), copper oxidase (CueO), a periplasmic copper oxidase that oxidizes Cu(I) to Cu(II) in the periplasm to prevent adventitious entry into cytoplasm (47), and a copper pump (CusCBA) that directly transports copper ions from the cytoplasm to the extracellular environment (48). Under anaerobic conditions, CueO would be inactive because the enzyme requires oxygen to oxidize Cu(I), thus partially contributing to accumulation of intracellular Cu(I) (9). Expression of the genes *copA* and *cusCBA* also has

distinct responses to copper under aerobic and anaerobic conditions (9), which could further enhance copper accumulation in cells under anaerobic conditions. Nevertheless, the molecular mechanism underlying copper accumulation in *E. coli* cells under anaerobic conditions is not yet fully understood. Interestingly, *Saccharomyces cerevisiae* cells also accumulate intracellular copper under anaerobic conditions (49). Under anaerobic conditions, perhaps microorganisms have to promote the influx of copper or other metal ions for critical physiological functions, and accumulation of excess copper in cells will inadvertently inhibit iron-sulfur cluster biogenesis, as reported in this study. Nevertheless, the anaerobic copper toxicity appears to be reversible, as cell growth was almost immediately restored when the *E. coli* cells grown in LB medium supplemented with 1.0 mM  $\text{CuCl}_2$  were shifted from anaerobic to aerobic conditions (Fig. 1). Furthermore, when the *E. coli* cells grown in LB medium supplemented with 0.2 mM  $\text{CuCl}_2$  under anaerobic conditions were transferred to aerobic conditions, the iron-sulfur clusters and the enzyme activity of recombinant FumA expressed in *E. coli* cells were largely restored (see Fig. S2A and B in the supplemental material) with a concomitant decrease in the intracellular copper content (see Fig. S2C), suggesting a robust ability of bacteria to survive in response to copper toxicity.

While copper and iron are essential trace metals for living organisms, intracellular concentrations of both metals must be tightly controlled in order to balance their biological functions. In the presence of toxic concentrations of copper, bacteria may have to slow down or inactivate multiple physiological processes by blocking iron-sulfur cluster biogenesis in order to avoid potential damage to cells. On the other hand, excess iron may also interfere with components of the copper homeostasis and sensitize the bacteria to copper toxicity under anaerobic conditions (5). Since polluted water often contains different toxic metals (3, 4), the interplay between copper and iron/iron-sulfur clusters in microorganisms will require further investigation.

## MATERIALS AND METHODS

**Cell growth and protein purification.** The DNA fragments encoding fumarases A, B, and C were amplified from wild-type *E. coli* genomic DNA using PCR. The PCR products were ligated to an expression vector, pBAD (Novagen). The identity of the cloned DNA fragment was confirmed by direct sequencing. Each plasmid was then introduced into the wild-type *E. coli* strain (MC4100). For protein preparation, overnight cultures of *E. coli* containing expression plasmids were diluted (1:100) in freshly prepared LB medium. Cells were grown at 37°C with aeration to an optical density at 600 nm ( $\text{OD}_{600}$ ) of 0.6. The protein expression was induced by adding 0.2% (wt/vol) L-arabinose for 2 h. To express proteins under anaerobic conditions, the *E. coli* cells were purged with pure argon gas for 40 min before L-arabinose was added to the sealed flasks. The cells were grown for an additional 2 h before being harvested and washed twice with the protein purification buffer (20 mM Tris-HCl [pH 8.0] and 500 mM NaCl). Recombinant *E. coli* fumarase A, fumarase B, fumarase C, dihydroxyacid dehydratase (50), endonuclease III, IscS, IscU, and IscA were purified as described (15), and proteins were purified to a single band on SDS-PAGE gel. The UV-visible absorption spectra of purified proteins were recorded in a Beckman DU640 UV-visible absorption spectrometer equipped with a temperature controller. The extinction coefficients for purified *E. coli* IIVD, endonuclease III, fumarase A, fumarase B, fumarase C, IscS, IscU, and IscA are 35.2, 19.3, 51.4, 51.4, 34.3, 39.8, 11.2, and 1.6  $\text{mM}^{-1}\text{cm}^{-1}$ , respectively.

**Copper content analyses.** The copper contents of protein samples were determined by following the procedure described in reference 15, with slight modifications. Briefly, protein samples (160  $\mu\text{l}$ ) were incubated with 10  $\mu\text{l}$  neocuproine (5 mM in  $\text{H}_2\text{O}$ -ethanol [1:1]), 20  $\mu\text{l}$  SDS (20%), and 10  $\mu\text{l}$  sodium ascorbate (5 mM). Mixtures were incubated at room temperature for 20 min, followed by centrifugation. The amplitude of the absorption peak at 450 nm of the supernatant was used for quantification of copper content. Freshly prepared  $\text{CuCl}_2$  (10  $\mu\text{M}$ ) was used as a standard. The copper contents in protein samples were also analyzed by inductively coupled plasma mass spectrometry (ICP-MS) (Agilent 8800). The two metal content analyses produced similar results.

**Enzyme activity measurements.** The enzyme activity of dihydroxyacid dehydratase was measured using the substrate DL-2,3-dihydroxy-isovalerate, as described previously (15). Briefly, the enzyme activity was measured using the substrate DL-2,3-dihydroxy-isovalerate. The reaction solution contained 1  $\mu\text{M}$  dihydroxyacid dehydratase (IIVD), 50 mM Tris (pH 8.0), 10 mM  $\text{MgCl}_2$ , and 10 mM DL-2,3-dihydroxy-isovalerate. The reaction product (keto acid) was monitored at 240 nm using an extinction coefficient of 0.19  $\text{mM}^{-1}\text{cm}^{-1}$ . Fumarase activity was measured using malic acid as the substrate (51). The reaction solution contained 10 nM fumarase, 50 mM sodium phosphate (pH 7.4), and 50 mM malic acid (pH 7.4). The reaction product (fumaric acid) was monitored at 250 nm using an extinction coefficient of 1.48  $\text{mM}^{-1}\text{cm}^{-1}$ . The cysteine desulfurase activity of IscS was measured as described previously (34). The reaction solution contained 1  $\mu\text{M}$  IscS, 3 mM dithiothreitol, 20 mM Tris (pH 8.0), and 200 mM NaCl. The reaction solution was preincubated at 37°C for 5 min before 2 mM L-cysteine was added to

initiate the reaction. The product sulfide in the reaction solution was determined by adding 20 mM *N,N*-dimethyl-*p*-phenylene-diamine sulfate (in 7.2 M HCl) and 30 mM FeCl<sub>3</sub> (in 1.2 M HCl), as described previously (52). The sulfur production in the reaction solution was used to represent the relative enzyme activity of IscS.

**EPR measurements.** For the EPR (electron paramagnetic resonance) measurements, the *E. coli* cells were harvested, washed twice with buffer containing 500 mM NaCl and 20 mM Tris (pH 8.0), and treated with 2.5% (vol/vol) nitric acid to oxidize Cu(I) in cells as described previously (15, 44). The EPR spectra were recorded at X-band on a Bruker ESP-300 spectrometer equipped with an Oxford Instruments 910 continuous flow cryostat. The routine EPR conditions were a microwave frequency of 9.45 GHz, microwave power of 10 mW, modulation frequency of 100 kHz, modulation amplitude of 2.0 mT, sample temperature of 30 K, and receive gain of  $1.0 \times 10^5$ . A solution containing Cu(II)-EDTA (10 μM) was used as a standard for quantification of Cu(II) in the protein.

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.00867-17>.

**SUPPLEMENTAL FILE 1**, PDF file, 0.3 MB.

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