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Lability and Liability of Endogenous Copper Pools

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Although the genome of *Escherichia coli* K-12 encodes just a few copper-containing enzymes, this transition metal is critical for the bacterium to derive energy from oxygen reduction and to protect itself from injury (1). For example, the Cu_B site within cytochrome *bo* oxidase facilitates the flow of electrons from ubiquinol to oxygen during aerobic respiration. A putative copper-binding site with cupric reductase activity has also been identified within NADH dehydrogenase II, but this site may involve an adventitious activity rather than a bona fide role for the enzyme in copper homeostasis (2). More certain is the role of copper within TynA, a periplasmic monoamine oxidase that allows the cell to use phenylethylamine as its sole carbon and energy source (3). The homodimeric enzyme contains two copper atoms that facilitate autocatalytic generation of the enzyme's cofactor (4, 5). Damaging reactive oxygen intermediates, such as those generated by the oxidative burst of professional phagocytes, are detoxified in the periplasm by Cu,Zn superoxide dismutase (6). Periplasmic Cu(I) is oxidized to the less toxic Cu(II) by CueO, a multicopper oxidase (7).

Despite the utility of copper in enzymatic reaction centers, at least two factors may have selected against its usage. The first involves the limited bioavailability of copper necessitating competition between commensal *E. coli*, other microflora residing within the intestinal lumen, and the mammalian host, for which copper is an essential micronutrient. Indeed, agricultural settings are likely to be among the few ecological niches where the bacterium may encounter an abundance of copper, because copper salts have been used as both a feed additive and fungicide (8). Even within the laboratory, the trace amounts of copper present in culture media are suboptimal for growth, and the concentration of copper within the bacterium exceeds that of its environment (9, 10). Under such copper starvation conditions, this cellular copper is thought to be tightly sequestered within enzymatic reaction centers (11). However, that view may require revision in light of the accompanying article by Fung et al., which suggests that the cell maintains a labile pool of copper ions that flux in response to nutritional status and environmental conditions (12).

As demonstrated by Fung et al., the maintenance of an endogenous pool of copper is not without costs; like most transition metals (essential and nonessential), copper can be toxic *in vivo*. Thus, toxicity is the second factor that may limit copper's biological use. Despite early hypotheses about copper toxicity that focused on the ability of Cu(I) to generate reactive oxygen species (ROS), it has become clear that much of the copper toxicity in *E. coli* is actually due to disruption of iron metabolism via non-ROS mechanisms (13, 14). In fact, the toxicity of copper increases under anaerobic conditions, when there is no oxygen available for ROS generation (13). The most sensitive target of copper toxicity appears to be iron-sulfur (Fe-S) cluster metabolism, both at the stage of Fe-S cluster biogenesis as well as via disruption of mature Fe-S cluster-containing metalloenzymes (13, 15). Furthermore, the most toxic oxidation state of copper appears to be Cu(I), which likely predominates in the reducing environment of the cell, especially under anaerobic conditions (16). Much of the Cu(I) toxicity likely stems from its thiolphilic nature, which allows it to directly displace other metal ions, such as iron, that are bound less tightly to thiolate or sulfide ligands, as predicted by the Irving-Williams series.

To combat copper toxicity, *E. coli* utilizes an efflux strategy to remove excess copper from the cytoplasm via CopA, a P-type ATPase efflux pump. Full functionality of the CopA system depends on a periplasmic multicopper oxidase, CueO, that may oxidize Cu(I) as a substrate in addition to using copper ions as cofactors (7). The expression of both CopA and CueO is regulated by the Cu(I)-dependent activator CueR (17).

Supplementing CopA is CusCBA, a tripartite RND transport system that spans the cell envelope and clears Cu(I) from the cytoplasm or, with the assistance of CusF, from the periplasmic compartment (18). In contrast to CopA, the CusCFBA system relies on the proton motive force to drive copper efflux, and transcription of *cusCFBA* is dependent upon a two-component regulatory system consisting of CusR and CusS (19). The signal cascade is initiated by Cu(I), which is presumably detected by a periplasmic sensor domain of CusS (20). The signal is then transmitted across the cytoplasmic membrane via CusS-dependent phosphorylation of the response regulator CusR, followed by activation of the *cusC* promoter, P*cusC*. Although the addition of exogenous copper ions is known to activate the CusRS system, Fung et al. observed activation of P*cusC* in anaerobic cultures deprived of amino acids that was independent of exogenous copper. They attributed this effect to an endogenous pool of Cu(I), because it was suppressed by the membrane-permeable Cu(I) chelator neocuproine but not by bathocuproine, a membrane-impermeable derivative. In addition, the sulfur-containing amino acids methionine and cysteine were shown to suppress the anaerobic activation of P*cusC*, suggesting a potential role for these amino acids in the maintenance of endogenous pools of copper ions.

Intriguingly, Fung et al. also found that anaerobic, amino acid-deprived conditions compromised the growth of *copA* and/or *cusC* mutants on fumarate. Through a series of logical experiments, the authors were able to demonstrate that the growth defect was due to inactivation of fumarate reductase (Frd), a critical

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enzyme for anaerobic fumarate respiration that contains three Fe-S clusters. Fung et al. also demonstrated copper-specific auxotrophies for amino acids that require Fe-S cluster dehydratase enzymes under anaerobic amino acid-limited conditions. These startling phenotypes indicate that fluxes in endogenous copper pools are sufficient to poison Fe-S cluster metabolism under anaerobic conditions when amino acids are limiting. Such an effect was further supported by the observed induction of the *sufABCDSE* stress response Fe-S cluster biogenesis pathway under these conditions. The Suf Fe-S cluster biogenesis pathway is only utilized under conditions that perturb the normal housekeeping Fe-S cluster pathway, which is encoded by *iscRSUA-hscBA-fdx-iscX* (21).

These results further illustrated a complication stemming from the ecological niche of *E. coli* and its ability to grow as a facultative anaerobe in the absence of oxygen as a terminal electron acceptor. The majority of the copper-containing enzymes characterized in *E. coli* are utilized under aerobic conditions (cytochrome *bo*, Cu,Zn superoxide dismutase, and CueO) and are not expressed under anaerobic conditions (1). However, *E. coli* can grow quite productively in the absence of oxygen by fermenting or by utilizing alternate electron acceptors, such as fumarate, that contain their own specific terminal oxidases, none of which appear to utilize copper as a cofactor. Thus, one might expect that the cell's anaerobic copper quota would be diminished relative to that under aerobic conditions. Remarkably, Fung et al. found that anaerobically cultured bacteria accumulated the same amount of copper as those cultured aerobically. However, anaerobic copper accumulation was dependent on the presence of Casamino Acids. In the absence of Casamino Acids, anaerobically cultured cells accumulated significantly less copper than those cultured with Casamino Acids.

What explains the decreased cellular copper content of cells from media lacking amino acids and how do amino acids affect copper homeostasis? Several observations by Fung et al. provided answers, beginning with their observation that the *cusC* promoter is activated under anaerobic conditions. This suggests that CusS senses a flux in endogenous copper pools that occurs under amino acid limitation in the absence of oxygen. In response, CusS stimulates CusR and increases transcription from P*cusC*. An increase in CusCFBA-dependent efflux of Cu(I) could account for the lower level of intracellular copper observed under this growth condition compared to growth in Casamino Acids. To test this hypothesis, Fung et al. measured copper accumulation of *cusC* and *cusCFBA* deletion mutants. As expected, they found that the deletion mutants grown without Casamino Acids retained as much copper as wild-type cells grown in medium supplemented with Casamino Acids. Taken together, these observations suggest that the cell maintains a reservoir of copper ions that is dependent on free amino acids, specifically, the sulfur-containing amino acids methionine and cysteine.

The possibility of a labile pool of copper ions challenges the conventional wisdom that nearly all cellular copper is tightly sequestered within enzymatic reaction centers. However, the conclusions drawn by Fung et al. are bolstered by the transcriptomic study of Tagkopoulos et al., which revealed that an oxygen downshift induces *cusC* expression within 12 min (NCBI GEO Datasets accession number GSE10855) (22). An inverse trend, that of decreased expression of *cusC*, is observed within minutes of an oxygen upshift. Since it is highly improbable that copper is alternately released and sequestered by copper-containing enzymes on this time scale, these studies provided additional evidence for a labile pool of cellular copper.

However, a number of questions remain to be answered about this endogenous copper pool. First, where is it localized? Activation of P*cusC* suggests the periplasmic compartment, because CusS is thought to detect Cu(I) via a periplasmic sensor domain. As proposed by Fung et al., it is also possible that CusS detects Cu(I) after CopA-dependent efflux from a cytoplasmic pool. Indeed, damage of Fe-S clusters would not be possible in the absence of cytoplasmic Cu(I), since cytoplasmic Fe-S-dependent dehydratase enzymes are the most sensitive targets of copper toxicity. Unfortunately, this does not exclude the possibility that Cu(I) enters the cytoplasm after reduction of a periplasmic pool of Cu(II). Second, how does copper speciation change under anaerobic, amino acid-limited conditions? One would predict that Cu(II) pools would rapidly shift to Cu(I) in the absence of oxygen, and this was supported by the transcriptomic study described above. The lack of copper-chelating amino acids could further exacerbate the change in copper speciation or the bioavailability of copper under these conditions. The specific disruption of Fe-S cluster metabolism under anaerobic and amino acid-limited conditions also supports a change in copper speciation, since Cu(I) is more potent than Cu(II) with regard to toxic effects. Third, what is the ligand(s) for the endogenous pool of copper? The work by Fung and colleagues clearly implicated methionine and cysteine as candidate low-molecular-weight ligands for copper under anaerobic conditions, because either of these amino acids could inhibit Cu(I)-dependent activation of P*cusC*. This may involve Met and/or Cys sequestration of Cu(I), thus denying CusS its metal ligand. Alternatively, an indirect mechanism may be responsible for the effect. Despite these and other questions, the work of Fung et al. provides a new perspective for copper homeostasis that will no doubt generate considerable conversation within the field of metal ion metabolism.

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