Critical role of the periplasm in copper homeostasis in Gram-negative bacteria

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Abstract

Copper is essential for life, but is toxic in excess; that is, cells must keep an optimal internal copper concentration. Under aerobic conditions, less toxic Cu(II) taken up by bacterial cells is reduced to more toxic Cu(I) in the cytoplasm. Copper homeostasis is achieved in the cytoplasm and the periplasm as a unique feature of Gram-negative bacteria. The copper efflux pumps, CopA and CusCBA export Cu(I) from the cytoplasm or the periplasm to outside of the cells in *Escherichia coli*. In addition, the periplasmic proteins, such as a multi-copper oxidase CueO, play a role in the periplasmic detoxification. While the efflux pumps are highly conserved in Gram-negative bacteria, the periplasmic proteins are diversified, indicating that copper homeostasis in the periplasm could contribute to adaptation to various living environments. However, the role of the periplasm and periplasmic proteins in regard to whole-cell copper homeostasis remains unknown. In this study, we addressed the role of the periplasm and periplasmic proteins in copper homeostasis to adapt to various ecological niches. We have used a systems approach, alternating rounds of experiments and models, to further elucidate the dynamics of copper efflux system. We measured the response to copper of the main specific copper export systems in the wild type *E. coli* strain, and a series of deletion mutant

strains. We interpreted these data using a detailed mathematical model and Bayesian model fitting routines, and verified copper homeostasis. Compared with the simulation and the growth in response to copper, we found that the growth was associated with copper abundance in the periplasm. In particular, CueO unique to Gram-negative bacteria contributes both to protection against Cu(I) toxicity and to incorporating copper into the periplasmic components/proteins, resulting in maximizing the growth. These results suggest that Gram-negative bacteria have evolved to utilize the periplasm as a sensor and store for copper, in order to enable Gram-negative bacteria to adapt to a wide range of environmental copper concentrations.

Introduction

Metals such as copper and zinc are essential for life, but toxic in excess. Metal cofactors are a prerequisite for central bioenergetic processes such as respiration, photosynthesis and nitrogen fixation (Merchant and Helmann, 2012). Metals can be bioavailable but not synthesized, and therefore complex mechanisms are required to stably maintain the concentrations of essential metals in the cell. The homeostasis of essential metals, such as copper and zinc, is achieved in a metal-

specific manner in *Escherichia coli* (Chandrangsu et al., 2017; Pal et al., 2017). Zinc is imported by a high-affinity ABC-type zinc uptake system ZnuABC (Patzer and Hantke, 1998; Patzer and Hantke, 2000; Yatsunyk et al., 2008) and exported by a P-type ATPase ZntA (Rensing et al., 1997), respectively. In contrast with zinc homeostasis, copper homeostasis is achieved by the export system. In addition, copper adopts distinct redox states, either less toxic Cu(II) or toxic Cu(I). *E. coli* can survive in the digestive tract of warm-blooded animals such as the stomach and duodenum, where the concentration of Cu(I) can be high, probably less than 10 μ M (Rensing and Grass, 2003).

Copper can enter *E. coli* cell without a specific import system (Rensing and Grass, 2003), and can diffuse from the periplasm to the cytoplasm without any known specific transporters (Beswick et al., 1976; Outten et al., 2001). The reducing environment of the cytoplasm leads to reduction of Cu(II) (Outten et al. 2001), and Cu(II) reductase, such as NDH-2, could reduce Cu(II) during uptake as was described in eukaryotes (Rapisarda et al., 1999; Rapisarda et al., 2002). Subsequently, it is conceivable that less toxic Cu(II) is reduced to toxic Cu(I) in the cytoplasm under aerobic conditions (Rensing and Grass, 2003). Cu(I) in the

cytoplasm is regulated at most one atom per cell, because the MerR homologue protein CueR with zeptomolar sensitivity to Cu(I) functions as a Cu(I) sensor (Changela et al., 2003). The periplasm is a unique cellular compartment in Gram-negative bacteria, which is surrounded by the inner and outer membranes and has a distinctive function in substrate oxidation. The periplasm is involved in several functions such as protein oxidation and metal transport (Miller and Salama, 2018). Besides Cu(II), copper ions can be as the free Cu(I) or the binding form with proteins such as metallochaperone CusF in the periplasm (Rensing and Grass, 2003).

To achieve copper homeostasis in both the cytoplasm and the periplasm, *E. coli* carries two export systems: the Cu-efflux (*cue*) system, which transports copper from the cytoplasm to the periplasm; and the Cu-sensing (*cus*) system, which transports copper from the cytoplasm and the periplasm to the outside of cells (Rensing and Grass, 2003). In addition to the export systems, it is well known that a periplasmic multi-copper oxidase CueO comprised of the *cue* system, which oxidizes Cu(I) to Cu(II), safeguards against Cu(I) toxicity in the periplasm (Grass et al., 2001; Singh et al., 2004). The *cue* system is a primary mechanism

91 responsible for copper resistance under both aerobic and anaerobic conditions
 92 (Rensing and Grass, 2003; Bondarczuk and Piotrowska-Seget, 2013), and the *cus*
 93 system is induced under anaerobic or copper-replete conditions (Outten et al.,
 94 2001). The *cue* system is comprised of CopA, an inner membrane Cu(I)-
 95 translocating P-type ATPase, and CueO (Petersen and Møller, 2000; Rensing et al.,
 96 2000; Grass et al., 2001; Singh et al., 2004). Expression of *copA* and *cueO* regulated
 97 by CueR is induced by Cu(I) levels in the cytoplasm (Outten et al., 2000; Stoyanov
 98 et al., 2001; Petersen and Møller, 2000). Upon exposure to copper stress such as 10
 99 μ M copper, the *cue* system is induced in order not to accumulate an excess of Cu(I);
 100 Cu(I) in the cytoplasm is transported to the periplasm by CopA, followed by the
 101 oxidization of Cu(I) by CueO. Under anaerobic or copper-replete conditions such as
 102 500 μ M copper, the *cus* system is activated; a copper-responsive two-component
 103 system CusRS induces the expression of *cusCFBA* (Yamamoto and Ishihama,
 104 2005), encoding a multiunit copper efflux pump that spans from the outer
 105 membrane to the periplasm, which exports copper in the cytoplasm and the
 106 periplasm to the extracellular environment (Munson et al., 2000; Outten et al.,
 107 2001; Oshima et al., 2002; Franke et al., 2003). CusS senses Cu(I) in the periplasm
 108 (Outten et al., 2001), followed by the phosphorylation of CusR (Yamamoto et al.,

2005). Phosphorylated CusR induces the expression of *cusC* (Rensing and Grass, 2003; Loftin et al., 2005; Kittleson et al., 2006).

Although the biochemical activity of CueO was corroborated, the effect of Cu(I) detoxification by CueO on copper stress has remained elusive. Outten et al. (2001) reported that while the expression of *cueO* was induced at a copper concentration of 10 μ M, the disruption of *cueO* had little effect on the growth compared with the growth in the Δ *copA* strain even upon exposure to a higher copper concentration of 500 μ M (Outten et al., 2001). Hernández-Montes et al. (2012) reported that the periplasmic proteins, such as CueO, involved in copper homeostasis are extensively diversified among 268 gamma proteobacteria, while copper transport from the cytoplasm to the periplasm by CopA and from the cytoplasm and the periplasm to the outside of the cells by CusCBA is highly conserved (Hernandez-Montes et al., 2012). Although this finding indicates that periplasmic proteins function in a species-specific manner, the role of the periplasm in copper stress is also unknown. These raise the question of whether Cu(I) detoxification by CueO serves as protection against copper stress, and whether there are the common features of periplasmic components/proteins in copper homeostasis.

127

128 In this study, we addressed the role of CueO and the periplasm in sensing and
 129 storing of copper. We initially performed ChIP-chip analysis to confirm the regulons
 130 controlled directly by CueR and CusR in *E. coli*, and measured the transcription
 131 responses to copper of the copper efflux systems by Lux reporter analysis. We then
 132 derived a mathematical model, and integrated the model with both published data
 133 and our current experimental data using a Monte Carlo Markov chain approach to
 134 evaluate model fits and infer the plausible ranges of parameter values. Compared
 135 the stochastic simulations with the growth measurements, we suggested that CueO
 136 unique to Gram-negative bacteria contributes both to protection against Cu(I)
 137 toxicity and to incorporating copper into the periplasmic components/proteins.
 138 Taken together with comparative genome analysis, we propose that Gram-negative
 139 bacteria have evolved to utilize the periplasm as a sensor and store for copper,
 140 contributing to the expansion of living niche with a wide range of environmental
 141 copper concentrations.

142

143 **Results**

144 **Confirmation of the genes directly regulated by CueR and CusR**

In order to identify a set of genes directly regulated by CueR and CusR of the *cue* and *cus* systems, respectively, we performed ChIP-chip analysis. The *E. coli* TM01 and TM02 strains expressing 3x Flag-tagged CueR and CusR, respectively, were grown aerobically in Defined Medium A (DMA) supplemented with 0 μ M, 750 μ M and 2 mM CuSO₄. Seeking the binding peaks of CueR and CusR, we found that two CueR-binding peaks were located at the upstream regions of *copA* and *cueO* across the entire *E. coli* chromosome at 750 μ M and 2 mM CuSO₄ (Figure 1B). We found that one CusR-binding peak was located at an intergenic and upstream region of *cusCFBA* and *cusRS* operons at 2 mM CuSO₄ (Figure 1B). This result was consistent with a previous finding that the CusR binding sequence (CusR box) was located only at that region (Yamamoto et al., 2005).

Since auto-regulation of CusR remains unclear, we investigated the auto-regulation of CusR by the plate assay of *cusR-lacZ* fusion strains. The TM20 strain formed red colonies on MacConkey agar plates supplemented with 750 μ M CuSO₄, while the MC4100 and TM21 strains formed white colonies (Figure 1C). In contrast, we confirmed that all strains formed white colonies on MacConkey agar plates without CuSO₄. Our results clearly indicated that CusR directly and positively regulated

cusRS in the presence of CuSO₄. Taken together, we concluded that four promoters, that is, *copA*, *cueO*, *cusCFBA*, and *cusRS* are regulated by CueR and CusS, of the *cue* and *cus* systems in *E. coli*.

Time course of promoter activities following addition of copper

To elucidate the transcription responses of the *cue* and *cus* systems, the promoter activities of three genes, *copA* (henceforth called P_{*copA*}), *cueO* (P_{*cueO*}), and *cusCFBA* (P_{*cusC*}) in the wild-type MG1655 (WT) strain, and its derivatives Δ *copA*, Δ *cueO*, and Δ *cusC* strains, were measured using a Lux reporter system (Burton et al., 2010; Takahashi et al., 2015). Excess external copper conditions included the addition of either 100 μ M, 200 μ M, 300 μ M, or 400 μ M CuSO₄ to DMA (henceforce called DMA¹⁰⁰, DMA²⁰⁰, DMA³⁰⁰, or DMA⁴⁰⁰). We confirmed no severe growth defects in all strains (data not shown).

Figure 2J shows the maximum induction levels of promoters relative to the promoter signal at 0 min in the WT strain. The maximum induction levels of P_{*cusC*} were associated with the addition of CuSO₄ in a concentration-dependent manner. In contrast, we observed little change in maximum induction levels of P_{*copA*} above

DMA³⁰⁰, and P_{cueO} above DMA²⁰⁰, respectively. These results suggested that the *cue* system could sense the differences between lower external copper concentrations such as DMA¹⁰⁰ and DMA²⁰⁰, while the *cus* system could sense the differences between higher external copper concentrations such as DMA³⁰⁰ and DMA⁴⁰⁰, which could not be distinguishable by the *cue* system.

Next we investigated the effects of a CopA deletion on the inductions of P_{copA}, P_{cueO}, and P_{cusC}. All tested genes were affected by the CopA defect (Figure 2D–F). CopA is the only known chromosomally encoded Cu(I) efflux pump from the cytoplasm to the periplasm in *E. coli*. The inductions of P_{copA} and P_{cueO} continued for 60 min after addition of CuSO₄ in the Δ *copA* strain, although the inductions of these promoters in the WT strain plateaued after 20 min (Figures 2A, B, D and E). This could be because the deletion of CopA causes Cu(I) accumulation in the cytoplasm, resulting in the continued activation of P_{copA} and P_{cueO} in DMA¹⁰⁰ and DMA⁴⁰⁰. We also expected little change of P_{cusC} induction, because the expression of *cusC* was positively regulated by the two-component system CusRS which was activated through the increase of Cu(I) in the periplasm (Rensing and Grass, 2003). Interestingly, P_{cusC} was induced in the Δ *copA* strain following addition of CuSO₄

(Figure 2F), supporting the activity of alternative Cu(I) exporters such as AcrD and MdtABC from the cytoplasm to the periplasm in addition to CopA (Nishino et al., 2010; Hobman and Crossman, 2015). The induction levels of P_{cusC} were, however, drastically reduced compared to those in the WT strain (Figures 2C and F).

In addition, the effects of a CueO deletion on the induction of P_{copA} , P_{cueO} , and P_{cusC} were investigated (Figures 2G–I). We hypothesized that as CueO plays an important role in oxidation of Cu(I) to Cu(II) in the periplasm, the disruption of CueO had no impact on the inductions of P_{copA} and P_{cueO} . As expected, we observed similar expression profiles of P_{copA} and P_{cueO} in both the WT and $\Delta cueO$ strains. In contrast to the effects of the CueO defect on P_{copA} and P_{cueO} , it was thought that the induction of P_{cusC} could occur at lower concentration of $CuSO_4$ in the $\Delta cueO$ strain compared to the WT strain, because deletion of *cueO* causes a decrease in periplasmic Cu(I) oxidation activity, leading to higher Cu(I) levels in the periplasm. As expected, P_{cusC} in the $\Delta cueO$ strain exhibited 4.4-fold change relative to that in the WT strain in DMA¹⁰⁰.

Finally, we investigated the effects of a CusC deletion on the induction of P_{copA} ,

P_{cueO} , and P_{cusC} (Figures S1A–C). Copper concentrations we tested were lower than the concentration for optimum growth, that is, 750 μM (Kershaw et al., 2005). As expected, the disruption of CusC had no effect on the maximum induction levels of P_{cusC} relative to the promoter signal at 0 min (Figures 2J and S1D). Consistent with previous observation (Munson et al., 2000), the *cus* system was induced at relatively high concentrations of CuSO_4 , while the *cue* system responded to relatively low concentration of CuSO_4 under aerobic conditions.

A new mathematical model for *in vivo* copper homeostasis

In order to understand the dynamics of the *cue* and *cus* systems, in conjunction with published data, we developed a mathematical model that describes the molecular process of copper homeostasis including Cu(I) concentrations in the cytoplasm and the periplasm (Figure 1A). In the model, we mainly focused on the interactions between the *cue* and *cus* systems which dominate the dynamics of Cu(I) in the cell. We set three variables for expressing copper concentrations as Cu(I) in the cytoplasm, Cu(I) in the periplasm, and Cu(II) in the periplasm, leading us to incorporate the spatiotemporal dynamics of Cu(I) and Cu(II) in the cell. Furthermore, we use the model to test the role of CueO in copper homeostasis. In

the model, we assumed two features. First, that there is a Cu(I) reservoir in the periplasm that protects the cells against Cu(I) toxicity. This is because there exist proteins acting as the copper reservoir, such as CusF, which play a role in keeping vast majority of Cu(I) in the periplasm to transfer Cu(I) to other proteins (Loftin et al., 2005, Kittleson et al., 2006, Pal et al., 2017). Second, we hypothesize that CusS phosphorylates and dephosphorylates its two-component partner CusR in the same manner as the other sensor kinases (Miyashiro and Goulian, 2008, Ray and Igoshin, 2010), i.e. CusS acts as a bifunctional kinase (Figure S2 and equations 11–16 in Materials and Methods). Consequently, we derived the mathematical model including 16 variables (see Materials and Methods).

Model fitting to data

Model fitting to data including our Lux data and other published data has been accomplished using the Metropolis-Hastings algorithm for parameter inference. Convergence plots from the simulations and posterior distributions for all parameters are shown in Figure S3. Point estimates and ranges for all parameter values are shown in Table S1. Independently of copper concentrations, P_{copA} and P_{cueO} activities exhibited the linear increase in the $\Delta copA$ strain (Figures 2D, 2E,

253 S1A, and S1B), indicating that CueR could be continuously activated by
 254 accumulated copper in the cytoplasm. We thought that deletion of CopA caused
 255 Cu(I) accumulation in the cytoplasm due to lack of cytoplasmic copper export, and
 256 P_{copA} and P_{cueO} inductions occurred before addition of $CuSO_4$ (Figure S1E). These
 257 data were excluded from the parameter inference to focus on the concentration-
 258 dependent regulations.

259

260 **The model fits a zeptomolar sensitivity of the transcriptional factor *in vitro* data**
 261 The model fits very closely (Figure 3A) to the published zeptomolar sensitivity of
 262 CueR for Cu(I) (Changela et al., 2003), providing confidence in the model results,
 263 especially given the high sensitivity of the molecular switch. The binding of two
 264 Cu(I) ions is required to convert inactive CueR to active CueR at rate l_1 ; the
 265 median value was $0.136 \text{ nM}^{-2}\text{s}^{-1}$. The active form reverts to the inactive form at
 266 rate l_2 ; the median value was $4.06 \times 10^{-25} \text{ s}^{-1}$ (Table S1 and equation 4 in Materials
 267 and Methods). Consequently, the half-maximal induction of CueR was estimated at
 268 $1.72 \times 10^{-21} \text{ M}$, consistent with the reported data, $2 \times 10^{-21} \text{ M}$ (Changela et al. 2003).

269

270 **The model fits the P_{copA} , P_{cueO} , and P_{cusC}**

271 The model replicated experimental data for *in vivo* inductions of P_{copA} , P_{cueO} and
 272 P_{cusC} in all strains (Figures 3B and S4), with a very high goodness of fit ($R^2 =$
 273 88.2%) associated with the promoter activities in all strains under all conditions.
 274 There were two exceptions where the fit was less good: the model predicts higher
 275 induction of P_{copA} in the $\Delta cueO$ strains, while the model fits to all inductions of
 276 P_{copA} in the WT and $\Delta cusC$ strains. In addition, although the model fits to the
 277 inductions of P_{cusC} in the WT and $\Delta cueO$ strains, the model fit of the induction of
 278 P_{cusC} in the $\Delta cusC$ strain is less good (Figure S4C): the model predicts greater
 279 induction (15.99 [log2 ratio] over the time course) than the experimental data (6.78
 280 [log2 ratio] over the time course). The model also gives a reasonable fit to the
 281 maximum inductions of P_{copA} , P_{cueO} and P_{cusC} (Figure 3C). Overall, these results give
 282 considerable confidence in the model processes and parameter estimations.

283

284 **Prediction of copper abundance in the periplasm from a stochastic model**

285 P_{cusC} exhibited the highest level of induction among the genes we tested in the WT
 286 strain (Figures 3B and S1C). We observed a similar behavior of P_{cusC} in the $\Delta cueO$
 287 strain, indicating that Cu(I) accumulates in the periplasm following addition of
 288 copper regardless of whether CueO exists. In order to explore copper atom levels,

i.e. Cu(I) and Cu(II), in the periplasm in single cells, we developed a stochastic model. The model contains exactly the same processes as the ODE model described above, but uses a discrete chemical reaction scheme to describe them (Gibson and Bruck, 2000), and thus captures intrinsic variability due to molecular events (Swain et al., 2002).

To reveal the dynamics of copper in the WT and $\Delta cueO$ strains, we conducted stochastic simulations following addition of a wide range of copper concentrations from 0 μ M to 6,000 μ M. We adopted a copper concentration of 6,000 μ M as the upper limit to examine the dynamics under extremely high copper concentration, where the growth was experimentally confirmed (Kershaw et al. 2005). In the WT and $\Delta cueO$ strains, Cu(I) and Cu(II) abundance in the periplasm increased with the increase in the external concentrations of copper (Figures 4A and B). In contrast, the cytoplasmic free Cu(I) was at most one molecule per cell under all tested conditions, indicating that copper homeostasis in the cytoplasm could be achieved.

***cueO* contributes both to protection against Cu(I) toxicity and to incorporating**

307 **copper in the periplasm**

308 The abundance of Cu(II) was higher in the WT strain than in the $\Delta cueO$ strain
 309 (Figure 4A). This could be caused by the oxidation of Cu(I) to Cu(II) by CueO in the
 310 WT strain. Under external copper concentrations ranging from 0 to 500 μ M
 311 (designated “Lower”), the abundance of Cu(I) was lower in the WT strain than that
 312 in the $\Delta cueO$ strain (Figure 4B). This could be because Cu(I) is exported by CusC in
 313 the $\Delta cueO$ strain. Looking at the profiles of CopA and periplasmic Cu(I) abundance
 314 in the $\Delta cueO$ strain, the induction of expression of CopA and the increase in the
 315 Cu(I) level occurred simultaneously (Figures 4E and F), followed by the expression
 316 of CusC (Figure 4G), resulting in lower Cu(I) levels in the $\Delta cueO$ strain. In
 317 contrast, the expression of CopA was followed by the oxidation of Cu(I) by CueO in
 318 the WT strain. Consequentially, the timing of expression of CusC was delayed
 319 (Figure 4G). Interestingly, we observed higher abundance of the Cu(I)-bound
 320 reservoir in the WT strain than that in the $\Delta cueO$ strain. In other words, the WT
 321 strain maintains higher levels of copper in the periplasm. This is likely due to the
 322 delay of expression of CusC caused by Cu(I) oxidation through CueO.

323

324 We assumed the existence of the reservoir molecules bound to Cu(I) in the

periplasm, such as CusF, which transfers Cu(I) to other proteins, leading to incorporation of copper required for various functions. This led us to the hypothesis that the abundance of the Cu(I)-bound reservoir is linked to the growth of *E. coli*. To address the relationship between the growth and copper levels in the periplasm, we measured the growth (OD₅₉₅) of WT and $\Delta cueO$ strains in DMA with different concentrations of copper (Figure 4D). We confirmed no differences of cell sizes between two strains (data not shown). Optimum growth of WT and $\Delta cueO$ strains occurred in DMA⁷⁵⁰ consistent with the previous report (Kershaw et al., 2005). The $\Delta cueO$ strain had a similar growth profile to the WT strain, although the $\Delta cueO$ strain exhibited poor growth compared with that of the WT strain. We found that the growth was associated with the abundance of the Cu(I)-bound reservoir (Figure S6A). The growth data for the WT strain in DMA¹⁰⁰, DMA²⁵⁰, and DMA⁵⁰⁰ was almost identical to those of the $\Delta cueO$ strain in DMA²⁵⁰, DMA⁵⁰⁰, and DMA⁷⁵⁰, respectively, indicating that the growth of the WT strain was similar to that of the $\Delta cueO$ strain if the WT and $\Delta cueO$ strains accumulate a similar abundance of Cu(I) in the Cu(I)-bound reservoir.

In contrast with the “Lower” conditions, there were no differences in the abundance

of Cu(I)-bound reservoir between the WT and $\Delta cueO$ strains under external copper concentrations over 1,000 μM (designated “Higher”) (Figure 4C). Instead, we observed a relationship between the growth and free Cu(I) abundance (Figure S6B). The model predicted the median of 149 free Cu(I) atoms per cell in the WT strain in DMA³⁰⁰⁰, where the growth of WT strain exhibited the OD₅₉₅ value of 0.127. These values were close to those of the $\Delta cueO$ strain in DMA²⁰⁰⁰, that is, the OD₅₉₅ value of 0.120 and the median of 146 free Cu(I) atoms per cell. This indicates that the strain with a particular OD₅₉₅ value could be characterized by the Cu(I) abundance in the periplasm. Taken together, CueO may contribute both to protection against Cu(I) toxicity and to incorporating copper into the periplasmic components/proteins, resulting in maximizing the growth.

Comparative genome analysis shows *cueO* and *cusC* were acquired in

Proteobacteria

Finally, we investigated how the transporters involved in copper and zinc homeostasis are evolutionally conserved among four bacteria in three different phyla including *Proteobacteria*, *Cyanobacteria* and *Firmicutes*, i.e. *E. coli*, *Synechococcus elongatus*, *Bacillus subtilis*, and *Staphylococcus aureus*. We

identified 1,989 orthologous groups. Interestingly, no orthologous genes to *cusC* was found in *S. elongatus*, *B. subtilis*, and *S. aureus*, while *copA* is a single-copy gene among all tested species (Figure 5). *zntA* was found in *E. coli* and *B. subtilis*, while two orthologues of *znuC* in *S. elongatus*, *B. subtilis*, and *S. aureus* were classified into the same cluster. Although the orthologous gene of *cueO* was *cotA* in *B. subtilis*, CueO and CotA had 23% amino acid sequence identity. Since it is conceivable that *cotA* functions as laccase (Hullo et al. 2001), we concluded that *cueO* exists only in *E. coli*. Taken together, bacteria with periplasm could acquire *cusC* which exports Cu(I) in the periplasm and the cytoplasm to protect against Cu(I) toxicity, resulting in expansion of living niche such as copper-replete environments. Furthermore, acquirement of *cueO* makes it possible to safeguard against Cu(I) toxicity and to incorporate copper, resulting in rapid growth.

Discussion

We aimed to develop a detailed understanding of the responses of copper homeostasis in *E. coli* through a Systems Biology approach. Copper homeostasis is achieved through two export systems, namely, the *cue* and *cus* systems. First, to derive a mathematical model, we confirmed four operons, that is, *copA*, *cueO*,

379 *cusCFBA*, and *cusRS* are regulated by the *cue* and *cus* systems by ChIP-chip
380 experiments. Second, we generated novel experimental data on four strains,
381 including time course transcriptional responses of copper efflux genes following
382 addition of copper to the growth media in the wild type *E. coli* strain and a series of
383 deletion mutant strains. These data, along with literature data, have been
384 integrated with a detailed, mechanistic, mathematical model, using a Monte Carlo
385 approach to fit this model to the data. Our model comprising of 16 equations fits
386 the experimental data well; the model reproduced the zeptomolar sensitivity of
387 CueR for Cu(I), and the model fits had an R^2 value of 88.2% for time course data.
388 Furthermore, we validated our model in regard to the protein abundance. It was
389 reported that the abundance of CusB transcribed together with CusC was
390 approximately 144 molecules per cell by using LC-MS/MS (Ishihama et al., 2008).
391 Concordantly, our simulation predicted the abundance of CusC ranging from 0 to
392 450 molecules per cell (Figure S5). Thus, we concluded that our model describes
393 copper homeostasis in *E. coli*, in particular, the copper stress response and the
394 number of molecules in the periplasm.

395

396 We assumed the CusS is bifunctional. For the dephosphorylation activity of CusS

397 m_8 , the median value was 46.8 s^{-1} , while for the kinase (phosphorylation) activity
398 of that m_6 , the median value was 49.9 s^{-1} (Table S1), indicating the phosphatase
399 and kinase activities of CusS was nearly identical. Although we observed that P_{cusC}
400 was induced after a few minutes following the inductions of P_{copA} and P_{cueO} (Figures
401 2A–I and S1A–C), the model did not reproduce the delay of P_{cusC} when the
402 parameters of kinase activity m_6 and dephosphorylation activity m_8 were set to
403 be 49.9 s^{-1} and 0.1 s^{-1} , respectively. Furthermore, the model without the
404 assumption of CusS bifunctionality was not able to reproduce the delay of P_{cusC}
405 (data not shown). This result suggests that the bifunctional activity of CusS is
406 important in determining the timing of *cusC* induction. Modulation of phosphatase
407 and/or kinase activities of histidine kinase may affect the equilibrium between
408 active (phosphorylated) and inactive (unphosphorylated) forms of the response
409 regulator, and therefore alter the dynamics of target genes. It is reported that other
410 histidine kinases phosphorylate and dephosphorylate their partner response
411 regulators, for example, the histidine kinase PhoQ and its partner response
412 regulator PhoP (Miyashiro and Goulian, 2008), EnvZ and OmpR (Batchelor and
413 Goulian, 2003), and the TorS and TorR systems (Carey et al., 2018) in *E. coli*,
414 supporting the bifunctional activity of CusS.

415

416 We introduced the reservoir molecules for the periplasmic Cu(I). This is reasonable
 417 because a small (110-aa) periplasmic protein CusF binds with high affinity to Cu(I)
 418 but not Cu(II) (Kittleson et al., 2006), and may function as a metallochaperone,
 419 bringing Cu(I) from the periplasm to other proteins (Loftin et al., 2005). That is, a
 420 possible role of CusF may be connected with the specific selection of Cu(I) to
 421 prevent its undesirable removal (Bagai et al., 2008; Loftin et al., 2009). We
 422 considered that the predicted abundance of reservoir molecules was reasonable,
 423 because 110 reservoir molecules was close to the abundance of CusC (Figure S3 and
 424 Table S1), consistent with the report that the abundance of CusF is almost the
 425 same as that of CusB (Loftin et al., 2005). Given that any molecules have not been
 426 reported for sequestering the periplasmic Cu(II), we did not assume any reservoir
 427 molecules for the periplasmic Cu(II). Assuming existence of the reservoir molecules
 428 for the periplasmic Cu(II) in our pilot model, the model fit was worse (data not
 429 shown). We considered that Cu(II) is not targetted to be sequestered because
 430 Cu(II) is less toxic to the cell.

431

432 Little is known about the role of CueO in copper homeostasis, although it is

essential and the biochemical activity of CueO is deeply understood. Our simulation shows that the oxidation of Cu(I) by CueO moderates the accumulation of periplasmic Cu(I), leading to the delay of CusC induction. Thus, the abundance of Cu(I)-bound reservoir was higher in the WT strain than that in the $\Delta cueO$ strain (Figure 4C). Moreover, as the *cueO* disruption is expected to generate a higher level of Cu(I) in the periplasm, the induction of P_{cusC} could occur at lower concentration of CuSO₄ in the $\Delta cueO$ strain compared to the WT strain. That is, CueO may contribute to the delay of induction of CusC. This could be a mechanism for incorporating Cu(I) in the periplasm under the lower copper condition. It is suggested that CueO modulates sequestering or exporting of Cu(I) by regulating the timing and level of *cusC* induction in response to the environmental copper condition, and the acquirement of *cueO* makes it possible to safeguard against Cu(I) toxicity and to incorporate copper, resulting in rapid growth.

It has been reported that there are diversified systems for copper homeostasis in gamma proteobacteria (Hernández-Montes et al., 2012). Particularly, the periplasmic proteins working with CopA and CusC are diversified, probably to adapt to the different environments, during the evolution of gamma proteobacteria.

451 Multicopper oxidases are encoded in the genomes of other Gram-negative bacteria.

452 For example, *Salmonella enterica* sv. Typhimurium has a homologous CueO (alias

453 CuiD), which demonstrates 80% identity over 516 amino acids with CueO (Osman

454 and Cavet, 2011). Similar to the biochemical activity of CueO, CuiD has high

455 oxidase activity for Cu(I), and reduces the concentration of periplasmic Cu(I) to

456 avoid its toxic effects (Osman et al., 2010). The *cus* system is not present in *S.*

457 *enterica* sv. Typhimurium (Pontel and Soncini, 2009). It is conceivable that instead

458 of the *cus* system, another periplasmic protein CueP is involved in copper

459 sequestration under anaerobic conditions, and constitutes a major cellular copper

460 pool (Pontel and Soncini, 2009; Dupont et al. 2011). Although the mechanism by

461 which CueP contributes to copper tolerance has not been fully understood, the

462 CueP-like proteins are found in other Gram-negative bacteria such as *Yersinia* sp.,

463 *Citrobacter* sp., *Erwinia* sp., and *Corynebacterium* sp. (Osman and Cavet, 2011),

464 indicating that most Gram-negative bacteria may have a multicopper oxidase and

465 other periplasmic proteins participated in the copper homeostasis.

466

467 Thus, Gram-negative bacteria could acquire periplasmic proteins worked with

468 CopA and CusC to protect the cells against Cu(I) toxicity. In particular, the

acquisition of multicopper oxidase may help to utilize the periplasm as a sensor and store for copper, resulting in expansion of living niche with a wide range of environmental copper concentrations.

Materials and Methods

Strains and Culture Condition

The *E. coli* K-12, MG1655 and MC4100 strains were used as the parental strains, and all strains and plasmids used in this study are presented in Tables S3 and S4, respectively. Primers used for genetic manipulations are presented in Table S2. The gene disruptions were performed with one-step inactivation method (Datsenko and Wanner, 2000). BW25113 pKD46 was used to construct $\Delta cueR::cm$, $\Delta cueR::km$, $\Delta copA::cm$, $\Delta copA::km$, $\Delta cueO::cm$, $\Delta cueO::km$, $\Delta cusCFBA::cm$ and $\Delta cusCFBA::km$ by transformation using PCR products amplified with the template plasmids, pKD3 and pKD4 and the primers, copA-del-f, copA-del-r, cueO-del-f, cueO-del-r, cusCFBA-del-f and cusCFBA-del-r. The $\Delta cusRS::km$ strain has already been constructed by the authors (Oshima et al., 2002). The strains expressing 3x Flag-tagged CusR and CueR were constructed by the epitope tagging procedure (Uzzau et al., 2001). BW25113 pKD46 was transformed with the DNA fragments encoding

487 3x Flag followed by the kanamycin resistant gene amplified with the template
 488 plasmid, pSUB11, and the primers, CueR-3xFLAG, CueR-RV, CusR-3xFLAG and
 489 CusR-RV. The derivatives of MG1655 and MC4100 were constructed by P1
 490 transduction using P1 lysates prepared from the BW25113 derivatives disrupted
 491 genes or expressed Flag-tagged genes. The strains for the β -galactosidase analysis
 492 of the *cusR* promoter activity were constructed by the transduction of the lambda
 493 phage carrying the *cusR-lacZ* gene fusion into MC4100. The lambda phage was
 494 prepared by *in vivo* transfer of the DNA segment from the pMC1403 plasmid
 495 carrying the DNA fragment from 182 bp upstream to 27 bp downstream
 496 (corresponding to N-terminal 9 amino acids of CusR protein including the first Met)
 497 from the first adenine in the *cusR* gene, which expressed CusR (9 amino acids) –
 498 LacZ fusion protein, to λ RZ5 phage (Carter-Muenchau and Wolf, 1989) by the
 499 RecA-mediated recombination as described previously (Ito et al., 1993). The DNA
 500 fragment to make the *cusR-lacZ* gene fusion was amplified by PCR with the *E. coli*
 501 chromosome DNA and the primers, ylcA3 and ylcA5, and then cloned into
 502 pMC1403 as *Bam*HI digested fragment. The strains used for the Lux assay were
 503 prepared by the transformation of MG1655 and its derivatives with the luciferase
 504 reporter plasmids, constructed from pLUX. The DNA fragments including the

promoters of *copA*, *cueO* and *cusC* were amplified by PCR using the primers, *copA*-promoter-01, *copA*-promoter-02, *cueO*-promoter-01, *cueO*-promoter-02, *cusC*-promoter-01 and *cusC*-promoter-02, and the *E. coli* chromosome DNA, and then cloned into pLUX as *XhoI/BamHI* fragments.

Luria-Bertani broth was used for the strain constructions. ChIP-chip and Lux assay were performed using Defined Medium A (DMA: 11.3 g K₂PO₄, 5.4 g NaH₂PO₄, 200 mg MgSO₄, 10 mg CaCl₂, 5 mg FeSO₄, 0.5 mg ZnSO₄, 0.5 mg MnSO₄, 0.1 mg CuSO₄, 0.1 mg CoCl₂, 0.1 mg sodium borate, 0.1 mg sodium molybdate, 0.26 g EDTA, 2 g NH₄Cl per litre) (Pirt, 1967). DMA was supplemented with 0.4% (w/v) glucose, and CuSO₄. CuSO₄ was buffered at pH 7 using 4 M glycine to avoid the precipitation of Cu(II) salts (Kershaw et al., 2005). MacConkey agar plate (BD Difco, Franklin Lakes, NJ) was used to monitor the *cusR* expression.

Lux Assay

The luciferase (Lux) assay was performed according to the method used in our previous analysis of the response to zinc (Takahashi et al., 2015). MG1655 (a parental strain) and its derivatives, $\Delta copA::cm$ (SK14), $\Delta cusCFBA::cm$ (SK16), and

ΔcueO::cm (SK18) (Table S3) were transformed with the plasmids, pLUX, pLUX*copA*, pLUX*cueO* and pLUX*cusC*. The strains were pre-cultivated in 1 mL of DMA with 0.4% glucose, 25 µg/mL kanamycin, and 8 mM glycine at 37°C overnight. The strains were then diluted to the OD₆₀₀ value of 0.04 in fresh 2 mL of DMA and re-cultivated for 2.5 h. After the cultures reached at early log phase (OD₆₀₀ = 0.1~0.4), 180 µL of each culture solution was transferred to Greiner 96-well microwell white plate (Greiner, Frickenhausen, Germany) and pre-incubated with shaking at 37°C for 15 min. Adequate amount of copper and glycine solution prewarmed at 37°C was added to each well at final concentrations from 0 µM (only glycine solution was added) to 400 µM of copper so that the total volume became 200 µL. Following addition of copper, the luciferase activities by absorbance (OD₅₉₅) were measured every 30 seconds for one hour using a Mithras LB940 (Berthold Technologies, Bad Wildbad, Germany) (Table S5). To normalize the bioluminescent activity per unit cell mass, the luminescence activity was divided by the absorbance of cell culture.

ChIP-chip Analysis

All strains used for ChIP-chip analysis were grown in 10 mL of DMA with aeration

at 37°C until the culture reached at the OD₆₀₀ value of 0.8. Each culture was fixed with formaldehyde at a final concentration of 1% for 20 min at 37°C. The excess formaldehyde was quenched with 1.5 mL of 3 M glycine for 5 min. Cultures were harvested and subjected to successive washing steps with TBS and lysis buffer (10 mM Tris-HCl pH 8.0, 20% sucrose, 50 mM NaCl, 10 mM EDTA). Cells were suspended in 500 µL of lysis buffer containing 20 mg/mL lysozyme and incubated for 30 min at 37°C and keep them at -30°C until the next step. 4 mL of IP buffer (50 mM HEPES-KOH pH 7.5, 300 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 5% glycerol) and 50 µL of 100 mM PMSF were added to the samples. The samples were then sonicated by a XL2020 (Astrason, Plainview, NY) on ice for 2 min (4 x 30 s with 30 s intervals) to prepare CueR samples or 10 min (20 x 30 s with 30 s intervals) to prepare CusR samples. To prepare the supernatant (whole cell extract) including chromatin, cell samples was clarified by centrifugation at 20,000 g for 30 min at 4°C. The supernatant was mixed with 15 µL of protein A Dynal Dynabeads 100.02 (Invitrogen, Waltham, MA) coated with anti-Flag antibody; to coat the protein A beads with antibody, the beads were washed twice with 500 µL of cold TBS containing 5 mg/mL BSA, and coated with anti-FLAG M2 antibody (Sigma-Aldrich, St. Louis, MO) by gentle mixing with

20 μ L of cold TBS containing 5 mg/mL BSA added 1.1 μ L of antibody solution (4.6 μ g/ μ L) for 6 h at 4°C before use. The mixture was further incubated at 4°C overnight with rotation. The beads were rinsed once with 1.5 mL of IP buffer, once with 1 mL of IP buffer for 5 min with rotation at 4°C, once with 1 mL of IP salt buffer (IP buffer containing 500 mM NaCl), three times with 1 mL of wash buffer (10 mM Tris-HCl pH8.0, 250 mM LiCl, 1 mM EDTA, 0.5% Nonidet P-40, 0.5% sodium deoxycholate) and then once with 1 mL of TE (10 mM Tris-HCl, 1mM EDTA pH 8.0). After the rinses, the protein-DNA complexes bound on the beads were eluted by 100 μ L of elution buffer (250 mM Tris-HCl pH 7.5, 50 mM EDTA pH 8.0, 5% SDS). DNA fragments crosslinked with proteins were decrosslinked by heating at 65°C for 20 min to prepare purified DNA fragments from whole cell extract and immunoprecipitated fractions. After decrosslinking, proteins in whole cell extract and immunoprecipitated fractions were digested with 2 mg/mL proteinase K (Takara Bio Inc., Kusatsu, Japan) at 42°C for 2 h, followed by incubation at 65°C for 6 h to inactivate proteinase K. Free DNA fragments in whole cell extract and immunoprecipitated fractions were purified by the QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany) and eluted with 50 μ L of elution buffer provided by the kit.

577

578 Random amplification of purified DNA fragments was performed according to
 579 Katou et al. (2006). Terminal labeling of amplified DNA purified from whole cell
 580 extract and immunoprecipitated fractions, and hybridization to the microarray
 581 were performed according to the Affymetrix instruction (Affymetrix, Santa Clara,
 582 CA). The custom Affymetrix oligonucleotide array (ECTILE2b520514F) was
 583 employed to obtain raw data (CEL.file). Raw data was visualized by In Silico
 584 Molecular Cloning program array edition (In Silico Biology, Yokohama, Japan) to
 585 identify the protein binding regions. Signal intensities of mismatch probes were
 586 subtracted from those of perfect match probes. Probes with a negative value for
 587 signal intensity were excluded from further analysis. The mean values of signal
 588 intensities of DNA isolated from immunoprecipitated fraction and whole cell
 589 extract fraction without purification (control DNA) were normalized to 500. Then,
 590 the signal intensities of DNA in the immunoprecipitated fraction were divided by
 591 those of control DNA. In order not to use the abnormal values caused by the low
 592 signal intensities of control DNA signal intensities in the analysis, we filtered 10%
 593 of probes with lower intensities of control DNA samples by the global rate
 594 trimming function in In Silico Molecular Cloning program. As we found the high

background noise likely due to the PCR amplification (especially CusR data), the binding sites were identified by eye inspection.

Growth Measurements

Optical density measurements were taken from each strain type in each media type in triplicate using a SmartSpec™ Plus spectrophotometer (Bio-Rad Laboratories, Inc., Hercules, CA). 2 μ L of stationary-phase culture grown in DMA was added to 2 mL of DMA with final concentrations of either 0 μ M, 50 μ M, 100 μ M, 250 μ M, 500 μ M, 750 μ M, 1,000 μ M, 2,000 μ M, 3,000 μ M, 4,000 μ M, 5,000 μ M, or 6,000 μ M in a 15 mL sterile plastic tube. The cultures were grown for 8 h at 37°C with shaking at 185 rpm.

Mathematical Model

We developed a mathematical model that describes the molecular process of copper homeostasis representing the dynamics of Cu(I) concentration in the cytoplasm and the periplasm (Figure 1A). There are 16 variables; the concentration of CopA, the concentration of CueO, the concentration of CusC, the concentration of active (copper-bound) CueR (active CueR), the concentration of CusS, the concentration of

613 active (phosphorylated) CusS (CusS-P), the concentration of free (ionic) Cu(I) in the
 614 cytoplasm (Cu_c^+), the concentration of free Cu(I) in the periplasm (Cu_p^+), the
 615 concentration of free Cu(II) in the periplasm (Cu_p^{2+}), the concentration of copper
 616 bound to the reservoir molecules, i.e. any other copper-binding molecules in the
 617 periplasm (R), the concentration of CusR, the concentration of phosphorylated
 618 CusR (CusR-P), the concentration of phosphorylated CusR dimer (CusR-P dimer),
 619 the concentration of protein complex of phosphorylated CusS and CusR (CusS-P·
 620 CusR), the concentration of protein complex of CusR and phosphorylated CusR
 621 (CusS·CusR-P), and the concentration of active (copper-bound) phosphorylated
 622 CusS (CusS-2Cu_p⁺-P). The equations are

623

$$624 \quad \frac{d[CopA]}{dt} = r_1 + b_1 \cdot [\text{active CueR}] - g \cdot [CopA], \quad (1)$$

$$625 \quad \frac{d[CueO]}{dt} = r_2 + b_2 \cdot [\text{active CueR}] - g \cdot [CueO], \quad (2)$$

$$626 \quad \frac{d[CusC]}{dt} = r_3 + b_3 \cdot \frac{K \cdot [CusR-P \text{ dimer}]}{1 + K \cdot [CusR-P \text{ dimer}]} - g \cdot [CusC], \quad (3)$$

$$627 \quad \frac{d[\text{active CueR}]}{dt} = I_1 \cdot [Cu_c^+]^2 \cdot (Xt - [\text{active CueR}]) - I_2 \cdot [\text{active CueR}]$$

$$628 \quad -g \cdot [\text{active CueR}], \quad (4)$$

$$629 \quad \frac{d[CusS]}{dt} = r_4 + b_4 \cdot \frac{K \cdot [CusR-P \text{ dimer}]}{1 + K \cdot [CusR-P \text{ dimer}]} - m_1 \cdot [Cu_p^+]^2 \cdot [CusS] + m_6 \cdot [CusS \cdot CusR-P]$$

$$630 \quad -m_7 \cdot [CusS] \cdot [CusR-P] + m_8 \cdot [CusS \cdot CusR-P] - g \cdot [CusS], \quad (5)$$

$$\begin{aligned} 631 \quad \frac{d[\text{CusS-P}]}{dt} = & m_9 \cdot [\text{CusS} \cdot 2\text{Cu}_p^+ \cdot \text{P}] - m_2 \cdot [\text{CusS-P}] \cdot [\text{CusR}] + m_3 \cdot [\text{CusS-P} \cdot \text{CusR}] \\ 632 \quad & - g \cdot [\text{CusS-P}], \quad (6) \end{aligned}$$

$$\begin{aligned} 633 \quad \frac{d[\text{Cu}_c^+]}{dt} = & A \cdot [\text{Cu}_p^{2+}] - C \cdot [\text{Cu}_c^+] - k_1 \cdot [\text{Cu}_c^+] \cdot [\text{CopA}] - k_3 \cdot [\text{Cu}_c^+] \cdot [\text{CusC}] \\ 634 \quad & - 2 \cdot I_1 \cdot [\text{Cu}_c^+]^2 \cdot (Xt - [\text{active CueR}]) + 2 \cdot I_2 \cdot [\text{active CueR}] - g \cdot [\text{Cu}_c^+], \quad (7) \end{aligned}$$

$$\begin{aligned} 635 \quad \frac{d[\text{Cu}_p^+]}{dt} = & C \cdot [\text{Cu}_c^+] + k_1 \cdot [\text{Cu}_c^+] \cdot [\text{CopA}] - k_2 \cdot [\text{Cu}_p^+] \cdot [\text{CueO}] - k_3 \cdot [\text{Cu}_p^+] \cdot [\text{CusC}] \\ 636 \quad & - 2 \cdot m_1 \cdot [\text{Cu}_p^+]^2 \cdot [\text{CusS}] + 2 \cdot m_9 \cdot [\text{CusS} \cdot 2\text{Cu}_p^+ \cdot \text{P}] - (Rt - R) \cdot [\text{Cu}_p^+] \cdot r_{\text{on}} \\ 637 \quad & + R \cdot r_{\text{off}} - g \cdot [\text{Cu}_p^+], \quad (8) \end{aligned}$$

$$638 \quad \frac{d[\text{Cu}_p^{2+}]}{dt} = B \cdot \text{Cu}_{\text{ext}} - A \cdot [\text{Cu}_p^{2+}] + k_2 \cdot [\text{Cu}_p^+] \cdot [\text{CueO}] - g \cdot [\text{Cu}_p^{2+}], \quad (9)$$

$$639 \quad \frac{d[R]}{dt} = (Rt - R) \cdot [\text{Cu}_p^+] \cdot r_{\text{on}} - R \cdot r_{\text{off}} - g \cdot [R], \quad (10)$$

$$\begin{aligned} 640 \quad \frac{d[\text{CusR}]}{dt} = & r_4 + b_4 \cdot \frac{K \cdot [\text{CusR-P dimer}]}{1 + K \cdot [\text{CusR-P dimer}]} - m_2 \cdot [\text{CusS-P}] \cdot [\text{CusR}] \\ 641 \quad & + m_3 \cdot [\text{CusS-P} \cdot \text{CusR}] + m_8 \cdot [\text{CusS} \cdot \text{CusR-P}] - g \cdot [\text{CusR}], \quad (11) \end{aligned}$$

$$\begin{aligned} 642 \quad \frac{d[\text{CusR-P}]}{dt} = & m_6 \cdot [\text{CusS} \cdot \text{CusR-P}] - m_7 \cdot [\text{CusS}] \cdot [\text{CusR-P}] - I_3 \cdot [\text{CusR-P}]^2 \\ 643 \quad & + 2 \cdot I_4 \cdot [\text{CusR-P dimer}] - g \cdot [\text{CusR-P}], \quad (12) \end{aligned}$$

$$\begin{aligned} 644 \quad \frac{d[\text{CusS-P} \cdot \text{CusR}]}{dt} = & m_2 \cdot [\text{CusS-P}] \cdot [\text{CusR}] - (m_3 + m_4) \cdot [\text{CusS-P} \cdot \text{CusR}] \\ 645 \quad & + m_5 \cdot [\text{CusS} \cdot \text{CusR-P}] - g \cdot [\text{CusS-P} \cdot \text{CusR}], \quad (13) \end{aligned}$$

$$\begin{aligned} 646 \quad \frac{d[\text{CusS} \cdot \text{CusR-P}]}{dt} = & m_4 \cdot [\text{CusS-P} \cdot \text{CusR}] - (m_5 + m_6) \cdot [\text{CusS} \cdot \text{CusR-P}] \\ 647 \quad & + m_7 \cdot [\text{CusS}] \cdot [\text{CusR-P}] - m_8 \cdot [\text{CusS} \cdot \text{CusR-P}] - g \cdot [\text{CusS} \cdot \text{CusR-P}], \quad (14) \end{aligned}$$

$$648 \quad \frac{d[\text{CusR-P dimer}]}{dt} = I_3 \cdot [\text{CusR-P}]^2 - I_4 \cdot [\text{CusR-P dimer}] - g \cdot [\text{CusR-P dimer}], \quad (15)$$

$$\begin{aligned} \frac{d[\text{CusS} \cdot 2\text{Cu}_p^+ \cdot \text{P}]}{dt} &= m_1 \cdot [\text{Cu}_p^+]^2 \cdot [\text{CusS}] - m_9 \cdot [\text{CusS} \cdot 2\text{Cu}_p^+ \cdot \text{P}] \\ &- g \cdot [\text{CusS} \cdot 2\text{Cu}_p^+ \cdot \text{P}]. \quad (16) \end{aligned}$$

CopA is produced at a basal rate r_1 and activated by active CueR with a constant of proportionality b_1 (equation 1). CopA and all other modeled components are diluted due to cell growth at rate g ; the four strains examined could potentially grow at different rates so the value of g is strain-dependent. CueO is produced at a basal rate r_2 and activated by active CueR with a constant of proportionality b_2 (equation 2). CusC is produced at a basal rate r_3 and activated by CueR-P dimer with a constant of proportionality b_3 and activation coefficient K (equation 3). X_T represents the total amount of CueR (assumed to be constant), and so inactive CueR is given by X_T - active CueR. The binding of two copper ions is required to convert inactive CueO to active CueO at rate I_1 . The active form can also revert to the inactive form at rate I_2 (equation 4). The kinetic parameters associated with the phosphorylation cycle model (Figure S2) are introduced (equations 5, 6, 11–16). Briefly, CusS is phosphorylated after binding to copper (equations 5, 6 and 16). CusR is phosphorylated by forming the complex with CusS-P (equations 11–14). CusR-P dimer activates the expression of CusC, CusS and CusR (equation 15).

667 CusS is produced at a basal rate r_4 and activated by CusR-P dimer with a constant
 668 of proportionality b_4 (equation 5). The binding of two copper ions is required to form
 669 the copper-CusS complex (CusS-2Cu_p^+) at rate m_1 (equations 5 and 16), and two
 670 copper ions dissociates from the copper-CusS complex to phosphorylate CusS at
 671 rate m_9 (equations 6 and 16). The phosphorylated CusS binds to CusR at rate m_2
 672 (equations 6, 11, and 13), and the protein complex can be dissociated at rate m_3
 673 (equations 6, 11, and 13). The phosphorylated CusS phosphorylates CusR at rate
 674 m_4 (equations 13 and 14), and the irreversible reaction can be proceeded at rate m_5
 675 (equations 13 and 14). The protein complex is dissociated at rate m_6 to produce the
 676 phosphorylated CusR (equations 5, 12, and 14). In this model, CusS also
 677 dephosphorylates CusR; CusS binds to the phosphorylated CusR at rate m_7
 678 (equations 5, 12, and 14), and dephosphorylates at rate m_8 (equations 5, 11, and
 679 14). In equation 15, phosphorylated CusR forms dimer at rate I_3 , and the dimer is
 680 dissociated at rate I_4 . In equations 7–9, the copper spatiotemporal dynamics in the
 681 cytoplasm and the periplasm is described. Cu(II) enters bacterial cells without any
 682 specific copper uptake system, and therefore, import of Cu_p^{2+} is proportional to the
 683 concentration of external copper with rate B (equation 9). The diffusion from the
 684 periplasm into the cytoplasm is proportional to the concentration of Cu_p^{2+} with rate

A , and Cu_p^{2+} is generated by the oxidation of Cu_p^+ by CueO with a constant probability k_2 (equations 8 and 9). The export of Cu_c^+ is proportional to the concentration of Cu_c^+ with a basal rate C , and linearly dependence on CopA with a constant of proportionality k_1 (equations 7 and 8). CusC exports Cu_p^+ and Cu_c^+ into the outside of cells with a constant of proportionality k_3 (equations 7 and 8). In equation 10, the Cu_p^+ -unbound and Cu_p^+ -bound reservoir are described. R_T represents the total amount of reservoir molecule in the periplasm (assumed to be constant), so Cu_p^+ -unbound reservoir molecule is given by $R_T - R$. The reservoir molecule binds to Cu_p^+ at rate r_{on} , and the Cu_p^+ -bound reservoir can be dissociated at rate r_{off} .

Monte Carlo Simulations

Parameter estimations were carried out using the Metropolis-Hastings algorithm (Hastings, 1970; Herman et al., 2011). For the majority of parameters, uninformative priors have been used. As proposal distributions, a lognormal distribution was used and the variances of the distributions were empirically chosen in order to ensure acceptance probabilities close to 0.25–0.30. The parameters were updated separately in each step, and 10,000,000 iterations were

carried out. ODE calculations were performed by the ccode solver with Newton iterations provided by the Sundials library (Cohen and Hindmarsh, 1996).

Calculations of ODE and Stochastic Simulations

ODE calculations were performed by the deSolve package (Soetaert et al., 2010) using the statistical environment *R*. Stochastic simulations based on the Gibson-Bruck algorithm (Gibson and Bruck, 2000) were performed until 1.1×10^6 s, i.e. 300 h, using Dizzy (ver. 1.11.3) (Ramsey et al., 2005).

Comparative Genome and Phylogenetic Analyses

Orthologous groups of all genes in *E. coli* (GCF_000005845.2), *Synechococcus elongatus* (GCF_000010065.1), *Bacillus subtilis* (GCF_000009045.1) and *Staphylococcus aureus* (GCF_000013425.1) were identified by using OrthoFinder (ver. 2.3.3) with default parameters (Emms and Kelly, 2019). Phylogenetic analysis was conducted based on 16S rRNA sequences. All 16s rRNA sequences were aligned by using MAFFT (ver. 7) with default parameters (Kato et al., 2019). The phylogenetic tree was constructed by the neighbor-joining method implemented the

MEGA software (ver. 10.1.5) with the Kimura two-parameter model (Kimura et al., 1980; Kumar et al., 2018). Bootstrap values were calculated with 100 resamples.

Calculation of R^2 value

We calculated the Pearson correlation coefficient between the experimental and theoretical values ($\log_2$ ratio) after addition of copper until 60 min.

Calculation of Coefficient of Variation, and Lower/Upper Bounds of Each Parameter

All model parameters were estimated from the posterior distributions of the MCMC runs. Table S1 shows the median value for each parameter, the coefficient of variation, and lower and upper bounds, that have been calculated by subtracting or adding 1.96 times the standard deviation from the median log value. This process has been carried out to avoid sampling errors at the extreme values of the Markov chain, assuming a Gaussian posterior centered on the median value.

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Authors' Contributions

J.I., T.O. and H.T. conceived the project. All members performed the experiments and analyzed the experimental data. J.I., T.O. and H.T. developed the model and performed the numerical simulation. J.I., T.O. and H.T. wrote the manuscript. TM, CK and SK equally contributed to wet works, including construction of most of strains used in this study, ChIP-chip, *lacZ* and Lux reporter assays. S.I. performed ChIP-chip experiments with T.M. H.A. constructed Flag tagged strains. T.O., S.I. and N.O. initiated the project.

Declaration of Interests

The authors declare no competing interests.

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Figure legends

Figure 1. (A) Schematic figure of copper efflux system, i.e. *cue* and *cus* systems in *E. coli*. Cu(II); white circle, Cu(I); black circle, CueR; aqua ellipse, CopA; pink cylinder, CueO; yellow ellipse, CusC; purple cylinder, CusS; light green ellipse, and CusR; yellow-green ellipse. (B) CueR and CusR binding sites on the genome in DMA⁰, DMA⁷⁵⁰, and DMA²⁰⁰⁰ identified by ChIP-chip. The values on the vertical axes indicate the binding magnitudes of ChIP DNA relative to those of supernatant DNA, and the horizontal axes indicate the genomic coordinate. (C) Expression of *cusR-lacZ* by β -galactosidase activity. MC4000, TM20 (MC4100 *cusR-lacZ* fusion) and TM21 (MC4100 *cusRS::km^r cusR-lacZ* fusion) were streaked on MacConkey agar plates supplemented without (left) and with 750 μ M CuSO₄ (right).

Figure 2. (A–I) Time series of P_{copA} , P_{cueO} , and P_{cusC} following addition of 100 μ M (black lines) and 400 μ M (red lines) copper (log to base 2 ratios to the value at $t=0$). The remaining experimental data are shown in Figure S1. Error bars indicate s.d. ($n=3$). (J) Maximum induction levels of P_{copA} , P_{cueO} , and P_{cusC} relative to the data at 0 min in the WT strain (log to base 2 ratios to the value at $t=0$).

Figure 3. (A) Model fit to CueR induction data from Changela et al, 2003. Black dot is the experimental value, and red line is the model value. (B) Time series of induction of P_{copA} , P_{cueO} , and P_{cusC} following addition of 100 μ M copper (black lines) and 400 μ M copper (red lines) (log to base 2 ratios). The solid line indicates the experimental data (error bar is s.d.), which are shown in Figure 2. The dotted line indicates the theoretical data with the best fit parameters. The remaining model fits are shown in Figure S4. (C) Experimental and theoretical maximum induction levels of P_{copA} , P_{cueO} , and P_{cusC} in the WT strain (log to base 2 ratios to the value at $t=0$).

Figure 4. (A, B, C) Boxplots of the abundance of periplasmic free Cu(II), reservoir-bound Cu(I), and free (reservoir-unbound) Cu(I) in the WT (black) and $\Delta cueO$ (gray) strains. (D) Effect of the copper concentration in DMA upon the growth of WT (●) and $\Delta cueO$ strains (○). The experiment was performed in triplicate, and error bars indicate s.d. (E–G) Realization of the stochastic simulation showing the abundance of CopA, periplasmic free Cu(I), and CusC in the WT (pink) and $\Delta cueO$ (light green) strains in DMA¹⁰⁰. The profiles from 0 min to 24 min were surrounded by the red line.

Figure 5. The presence of transporters involved in copper and zinc homeostasis. The phylogenetic tree was constructed based on 16S rRNA. The heatmap shows the number of genes in the orthologous groups, ranging from 0 to 2.

Figure 6. A schematic figure representing the relationship between the growth and periplasmic Cu(I) level. Under the lower copper condition, the growth is associated with the abundance of copper-bound reservoir (left). At the copper concentration of 750 μ M, all reservoir molecules are bound to Cu(I), resulting in the optimum growth (middle). Under the higher copper condition, Cu(I) becomes toxic, resulting in the inhibition of growth (right).

Figure S1. (A–C) Time series of induction of *copA*, *cueO*, and *cusC* promoters following addition of copper, log to base 2 ratios normalized to the value at $t=0$. Error bars indicate s.d. ($n=3$). (D) Maximum induction levels of P_{copA} , P_{cueO} , and P_{cusC} relative to the data at 0 min in the $\Delta copA$, $\Delta cueO$, and $\Delta cusC$ strains (log to base 2 ratios to the value at $t=0$). (E) Luminescence intensities of *copA*, *cueO* and *cusC* promoters in the WT, $\Delta copA$, $\Delta cueO$, and $\Delta cusC$ strains at $t=0$. The

luminescence intensities correspond to the data in DMA⁴⁰⁰. Error bars indicate s.d.
($n=3$).

Figure S2. A schematic figure of *cusRS* regulation. The phosphorylation and dephosphorylation ability of CusS, and the positive feedback by the phosphorylated CusR are indicated by arrows. P denotes being phosphorylated. The reaction scheme includes the association, dissociation, phosphorylation and dephosphorylation.

Figure S3. Trace plots for MCMC runs. 35 parameter (log to base 10) and natural logarithm likelihood were plotted. Blue line is the median value of posterior distribution.

Figure S4. Time series of induction of (A) *copA*, (B) *cueO*, and (C) *cusC* promoters following addition of copper, log to base 2 ratios normalized to the value at $t=0$. The solid line indicates the experimental data (error bar is s.d.), which are already shown in Figure S1. The dotted line indicates the theoretical data with the best fit parameters.

Figure S5. Boxplots of abundance of CopA, CueO, and CusC in the WT (black) and $\Delta cueO$ (gray) strains.

Figure S6. (A) Relationship between the growth and the abundance of reservoir-bound copper in the WT (black) and $\Delta cueO$ strains (red) in DMA⁰–DMA⁷⁵⁰. The vertical and horizontal axes correspond to the OD₅₉₅ values, and the median value of abundance of reservoir-bound copper, respectively. (B) Relationship between the growth and the abundance of periplasmic free copper in the WT (black) and $\Delta cueO$ strains (red) in DMA⁷⁵⁰–DMA⁶⁰⁰⁰. The vertical and horizontal axes correspond to the OD₅₉₅ values, and the median value of periplasmic free copper.

Table S1. Estimates of all model parameters

Table S2. A list of primers used in this study

Table S3. A list of bacterial strains used in this study

1098 **Table S4.** A list of plasmids used in this study

1099

1100 **Table S5.** Promoter activities of *copA*, *cueO*, and *cusC* in the WT, $\Delta copA$, $\Delta cueO$,

1101 and $\Delta cusC$ strains measured by the Lux reporter system

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Supplemental Figure 1

Supplemental Figure 2

Supplemental Figure 3

Supplemental Figure 4

Supplemental Figure 5

Supplemental Figure 6