Surface sensing in Escherichia coli

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Chapter 5

CpxR is activated by and provides protection against toxic concentrations of copper

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Abstract

The CpxAR system can be activated by a range of signals, which all act via the cognate histidine kinase CpxA. For activation of CpxR by copper, it is not known where the signal enters the pathway. Here, we show that copper can activate the system in an NlpE- and CpxA-independent manner, involving the histidine kinase CusS of the copper-responsive two-component system CusSR. Further, we establish that CpxR is essential for the growth of *Escherichia coli* in the presence of even micromolar concentrations of copper. Further, we found that a mutation that is present in a number of laboratory strains of *E. coli*, which results in secretion of orotate, protects the bacteria against toxic concentrations of copper via conditioning of the medium. Our results illustrate how medium conditioning and interplay with another pathway affect the activity of CpxR, which plays a crucial role in copper tolerance.

Introduction

Copper is an essential element, but it is also toxic [1]. Copper causes damage to bacteria by displacement of metal ion cofactors from enzymes, mainly from iron-sulphur clusters [2], and by oxidative damage to lipids and proteins via generation of reactive oxygen species and oxidation of cysteine residues [3].

In the gram-negative model organism *Escherichia coli* several defence strategies for coping with high copper concentrations are known. The two-component system CusSR senses periplasmic copper and regulates the expression of the *cusCFBA* operon, with the CusCBA complex being a tripartite copper exporter that spans both membranes [4, 5]. Another copper sensor is the cytoplasmic CueR, with zeptomolar (10^{-21}) sensitivity towards Cu^{+} [6], which induces expression of the copper efflux P-type ATPase CopA and the periplasmic multi-copper oxidase CueO [5, 7]. A third copper-responsive regulator is ComR, which controls expression of ComC and thereby seems to decrease the copper-permeability of the outer membrane [8]. Finally, also the envelope stress-responsive two-component system CpxAR can sense the presence of copper and deletion of *cpxAR* was found to lower the concentration at which copper inhibits growth of *E. coli* [9–11].

A wide range of inducing signals for the CpxAR system are known, all of which ultimately lead to phosphorylation of the response regulator CpxR. All of these signals enter the system at the histidine kinase CpxA [12, 13]. Spontaneous (i.e. in absence of a signal) phosphorylation of CpxR by phosphate-carrying metabolites, such as acetyl-phosphate, has also been reported [14]. For one inducing signal, the presence of extracellular copper, it is not known how it leads to the activation of CpxR. One explanation could be that the signal transduction goes via NlpE, a well-studied activator of the Cpx system, as the *nlpE* (*cutF*) gene was originally identified as a copper tolerance gene [15] and this lipoprotein carries a CXXC motif, which may bind divalent cations [16]. However, it is also possible that copper-induced damage to membrane proteins and lipids is perceived as envelope stress by CpxA.

Here, we determined the requirements for copper-induced CpxR activation and we also coincidentally discovered a mechanism of copper protection via medium conditioning. We found that CpxR is essential for survival in the presence of low-micromolar concentrations of copper ions (provided as $CuCl_2$) and that such low concentrations are also sufficient for activation of CpxR. In absence of NlpE or CpxA, activation of CpxR was still possible. Our results suggest that the histidine kinase CusS was involved in signal transduction to CpxR. Furthermore, we found that *E. coli* can protect itself against copper ions by conditioning of the medium, by secretion of the copper-chelating compound orotate. Overall, our results demonstrate the crucial role of CpxR in copper tolerance, which is facilitated by the interplay with another copper-responsive two-component system and strongly affected by medium conditioning.

Results

Activation of Cpx by copper

While growing *E. coli* in M9 medium with 0.4% glucose for the experiments in Chapters 2 and 4, we noticed that the fluorescence intensity conveyed by the *yebE* transcriptional reporter, which is under control of CpxR [10, 11, 17], strongly correlated with the optical density of the culture up to an OD_{600} of 0.4-0.5 (Figure 1a), where growth was still exponential (Figure 1b). Notably, for reporters that are not regulated by CpxR, this correlation was not observed (Supplementary Figure S1). The decrease in fluorescence indicated that the activity of the Cpx system decreased during exponential growth in liquid culture. Thus, it seems like an inhibiting signal is produced or an activating signal consumed during growth of *E. coli*, such that the activity of the Cpx system decreases.

To identify the Cpx-affecting signal that is present in a growing culture, we first considered the changing glucose and acetate concentrations as the cause. We grew *E. coli* with a CpxR-regulated *gfp* gene in glucose minimal medium to an OD_{600} of 0.5 and diluted the cells into fresh medium with different glucose and acetate concentrations (Supplementary Figure S2). We observed only minor effects on fluorescence intensity.

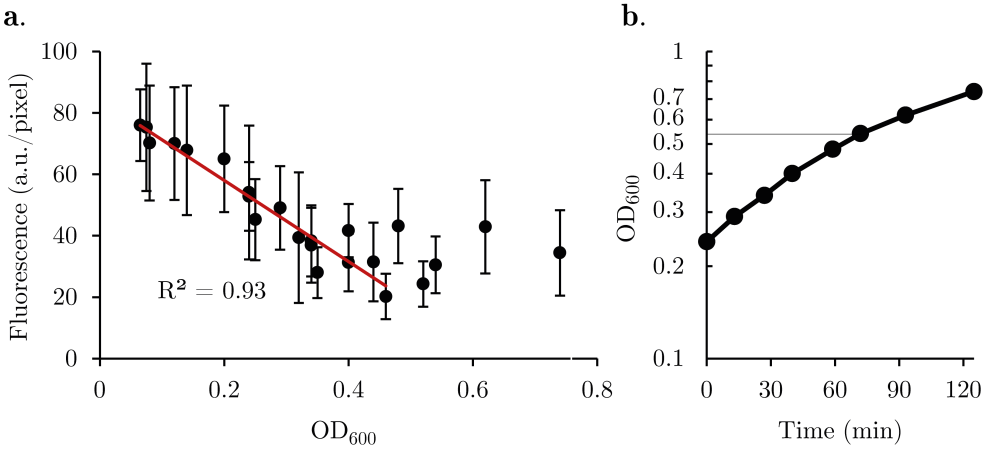

Figure 1: Dependence of Cpx activity on OD_{600}. (**a**) Bacteria carrying the *yebE* reporter plasmid were grown to various OD_{600} values and their fluorescence was immediately quantified under the microscope. The fluorescence intensities were background-corrected and shown as arbitrary units per pixel. The red line is a linear fit to the fluorescence values up to an OD_{600} of 0.46. The data are taken from multiple cultures. Data are mean ± standard deviation of an average of 39 cells. (**b**) Optical density plotted against time for a culture of the *yebE* reporter strain in M9 medium supplemented with 0.4% glucose. Growth starts to slow down around an OD_{600} of 0.5. Excluding the last two measurements, the growth rate is 0.67 h^{-1}.

We did not test medium with different pH, as we found the pH to be unchanged in spent medium from OD_{600} 0.5 cultures compared to fresh medium (data not shown). Our results indicate that glucose, acetate and pH are not responsible for the altered Cpx activity during growth.

As the medium composition included trace amounts of copper chloride, which is a known inducer of the Cpx system, we investigated whether the observed correlation between CpxR-regulated gene expression and optical density of the culture could involve copper. When *E. coli* were grown in medium with the trace concentrations of copper and diluted into medium with and without copper, distinct fluorescence responses were observed, indicating that the 7 μM $CuCl_2$ concentration in the medium is sufficient for activation of CpxR (Figure 2a). Therefore, if the availability of copper ions would change in growing cultures, this could cause the observed changes in CpxR activity.

The decreasing CpxR activity during growth is caused by orotate accumulation

To find out what might cause decreased copper availability during exponential growth, the composition of conditioned medium was determined. Separation and detection of

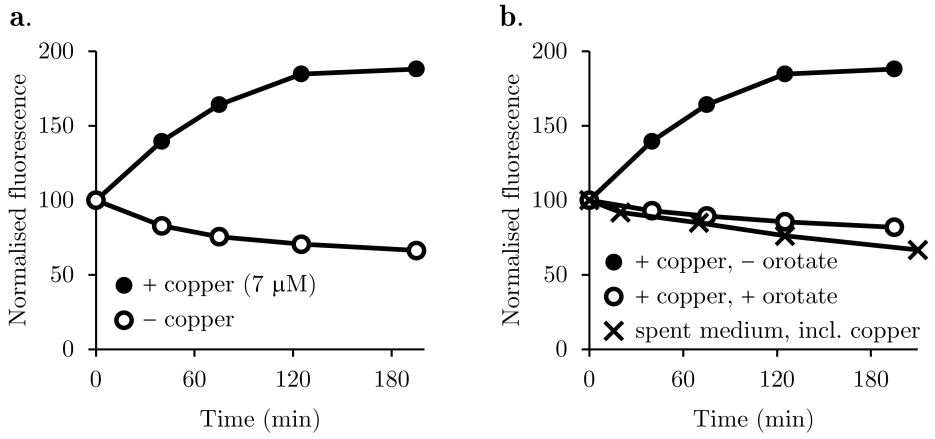

Figure 2: Induction of CpxR-regulated promoter by copper. (**a**) Bacteria carrying the *yebE* transcriptional reporter plasmid were grown in glucose minimal medium with copper to an OD_{600} of 0.5 and diluted into medium with or without 7 μM $CuCl_2$ at time point 0. The median fluorescence intensity normalised to the cell size (measured as the 'width') was determined by flow cytometry and set to a value of 100 at the initial time point. (**b**) Bacteria were diluted into copper-containing fresh medium, with or without supplementation with 90 μM orotate, or spent medium from an OD_{600} 0.5 culture, which also contained copper (7 μM $CuCl_2$).

extracellular metabolites using LC-MS yielded an indication that orotate, which might be capable of chelating copper, was present, based on the mass of this compound (Supplemental Figure S3a). With HPLC, the presence of this compound in conditioned medium was confirmed on the basis of equal retention time and UV absorption spectrum as a known orotate standard (Supplemental Figure S3b). The concentration of orotate in spent medium was found to be 90 μM at OD_{600} of 0.5, determined by integration of the peak area and comparison with samples of known concentration of this compound. Orotate is an intermediate in the pyrimidine biosynthesis pathway and in several *E. coli* strains, including the MG1655 strain used here, a mutation has been reported that leads to the accumulation and secretion of this compound [18, 19].

To determine whether the accumulation of orotate in the medium can explain the decreasing CpxR activity in growing cultures, the experiment of Figure 2a was repeated with addition of orotate. We found that when bacteria were diluted into fresh medium containing copper, the presence of orotate at the measured concentration prevented activation of the Cpx system (Figure 2b). In fact, in the presence of orotate, the expression of the CpxR-regulated *gfp* gene was the same as in conditioned medium and in fresh medium without copper (Figure 2), indicating that the decreasing CpxR activity can be explained solely by orotate accumulation. Thus, during growth, MG1655 secretes orotate into the medium, which chelates free copper ions, thereby preventing activation of the Cpx system by this metal ion.

NlpE and CpxA are not required for sensing copper

To define the pathway components that are necessary for the copper-mediated induction of CpxR-controlled genes, a few deletion strains were constructed. While the Δ*nlpE* and Δ*cpxA* mutants were able to grow in fresh M9 medium containing glucose and trace amounts of $CuCl_2$, the Δ*cpxR* mutant was not. Although there was no growth, the cells lacking this response regulator did remain viable, as transfer to LB medium restored growth. Besides in LB, the Δ*cpxR* strain could also grow in M9 medium supplemented with orotate, in conditioned M9 medium, or in absence of trace amounts of $CuCl_2$ (Table 1). Complementation with plasmid-borne *cpxR* restored the ability to grow in copper-containing medium. Thus, CpxR is essential for growth in the presence of free copper.

Since the Δ*cpxR* strain would not grow in presence of copper, the fluorescence response of this strain to copper could not be tested. For the other two deletion mutants, copper was still able to induce the CpxR-regulated *yebE* reporter (Figure 3a). While the relative increase in fluorescence intensity is smaller in the Δ*cpxA* strain, it

Medium	**Growth**
M9 + 7 μM $CuCl_2$	No
M9 + 7 μM $CuCl_2$ + 90 μM orotate	Yes
M9 without copper	Yes
Conditioned M9 + 7 μM $CuCl_2$	Yes
LB	Yes

Table 1: Growth of Δ*cpxR* in different media. M9 medium was always supplemented with 0.4% glucose.

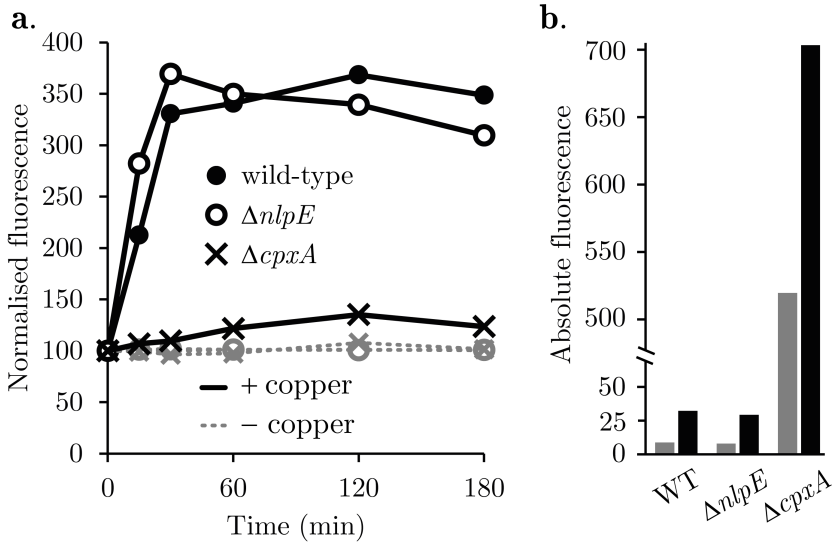

Figure 3: Effect of copper on *ΔnlpE* and *ΔcpxA* strains. (**a**) Wild-type, Δ*nlpE* and Δ*cpxA* carrying the *yebE* transcriptional reporter plasmid were grown in glucose minimal medium without copper and diluted into medium with or without 7 μM $CuCl_2$ at time point 0 (no orotate was added). The median fluorescence intensity divided by cell size (measured as the 'width') was determined by flow cytometry and normalised to a value of 100 at the initial time point. (**b**) Initial (grey) and maximum copper-induced (black) fluorescence without normalisation; same data as in **a**.

should be noted that the basal fluorescence level of this strain is much higher than of the wild-type (Figure 3b). The explanation for the difference in basal CpxR activities is that, in the wild-type, the phosphatase activity of CpxA predominates under non-inducing conditions to keep CpxR in an inactive unphosphorylated state [20]. Loss of the *cpxA* gene is then indeed expected to lead to higher activity of CpxR, as has been described before [21]. Thus, because the basal activity of CpxR is higher in the Δ*cpxA* background, the relative effect of copper addition is less pronounced, but still the effect of copper is clear also in this strain. Overall, our results indicate that copper induces expression of CpxR-regulated genes in a manner independent of NlpE and CpxA.

Involvement of CusS

Having established that NlpE and CpxA are not required for copper-sensing, we questioned how CpxR gets activated (i.e. phosphorylated) in the presence of copper. Since no mechanism for import of copper across the inner membrane is known in *E. coli* and the intracellular concentration is highly controlled to remain at a very low level [1, 3], we hypothesised that it was more likely that periplasmic copper was sensed than that the concentration in the cytoplasm would increase and activate CpxR

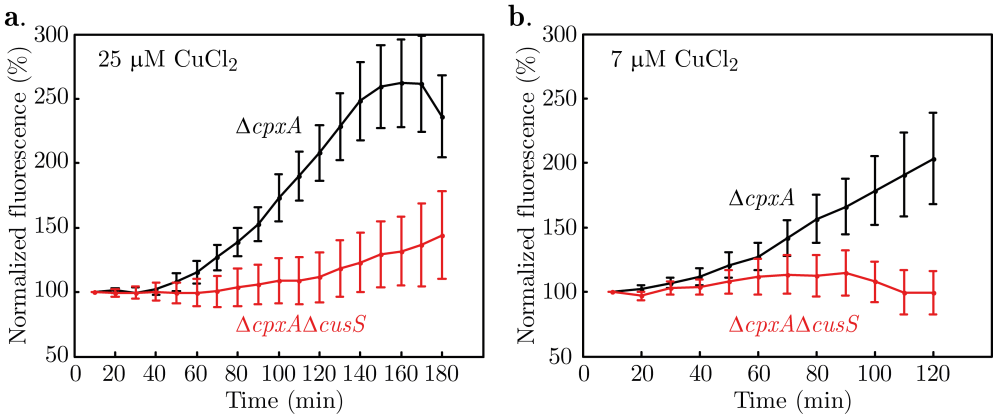

Figure 4: Role of CpxA and CusS in copper-mediated CpxR activation. Δ*cpxA* and Δ*cpxA*Δ*cusS* deletion strains carrying the *yebE* transcriptional reporter plasmid were grown in glucose minimal medium without copper and introduced into a microfluidic device. After growth for multiple hours, medium containing (**a**) 25 μM or (**b**) 7 μM $CuCl_2$ was perfused to the cells. These plots show the mean fluorescence intensities with 95% confidence intervals from the time that the copper-containing medium reached the cells. The 25 μM concentration affected survival: most cells stopped growing after 3 h.

directly. Therefore, we tested whether CpxR activation could occur upon the loss of the *cusS* gene, encoding a copper-sensing histidine kinase. We found that CpxR activation by $CuCl_2$ in the Δ*cusS* mutant was not impaired (Supplementary Figure S4). We also decided to test the *cusS* deletion in the Δ*cpxA* background, because of the possibility that both CpxA and CusS could redundantly mediate the copper-induced activation of CpxR. Indeed, while the single deletion strains could still induce the *yebE* gene upon addition of copper, we observed that this was not the case for the *cpxA cusS* double deletion, suggesting that either CpxA or CusS is sufficient for the copper-induced phosphorylation of CpxR (Figure 4). Thus, *E. coli* possess two redundant mechanisms for activation of CpxR in response to copper, which is consistent with the crucial role of CpxR in protection against copper, as described above.

Discussion

In this study, we report induction of the CpxR-regulated *yebE* promoter by copper and we provide evidence that neither the outer membrane lipoprotein NlpE, nor the cognate histidine kinase CpxA are required for recognition of the signal. We found that copper can activate CpxR via crosstalk with the CusS histidine kinase. Additionally, we found that medium conditioning by orotate secretion decreases the toxicity of extracellular copper ions and prevents the activation of CpxR by this metal ion. Our results can be summarised by the model in Figure 5.

Induction of CpxR-regulated genes by copper has previously been reported [9, 10], but no mechanism for the induction was provided, although Yamamoto and Ishihama did show that there was no induction when both *cpxA* and *cpxR* were deleted, proving that indeed CpxR is the transcriptional regulator. All known extracytoplasmic signals that activate CpxR are sensed via CpxA [13]. Here, we provide evidence that sensing of extracellular copper ions can proceed without NlpE or CpxA.

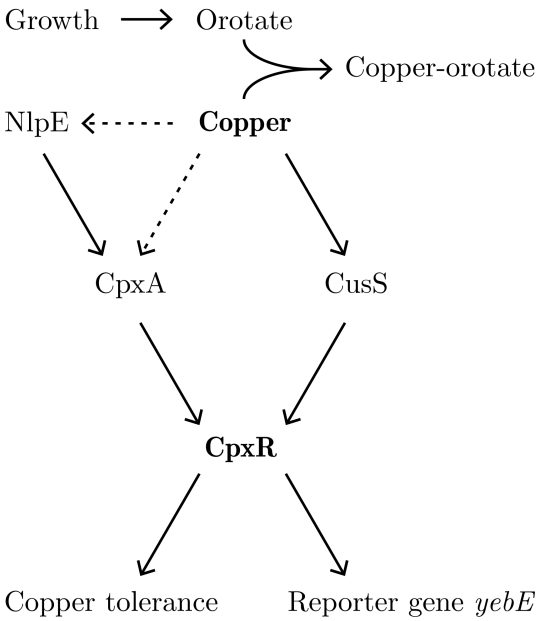

Figure 5: Model of copper-induced CpxR activation. In absence of orotate, which is secreted during growth of MG1655, or other chelators, exposure to copper leads to activation (i.e. phosphorylation) of CpxR, both via CusS and via the NlpE-CpxA pathway. One of the genes regulated by CpxR is *yebE*, which we used as a transcriptional reporter. Further, CpxR is essential for copper tolerance, allowing growth at otherwise toxic concentrations.

In Figure 3, we show that the wild-type and Δ*nlpE* strains have a very low basal activity of CpxR and that expression of the reporter gene is induced 3.5-fold with added copper. The basal reporter expression level in the Δ*cpxA* strain, on the other hand, is 59 times higher than in the wild-type, indicating that CpxR is highly active. Addition of copper could increase the expression even further. Why does induction with copper not increase the activity of CpxR in the wild-type to comparable levels as in the Δ*cpxA* strain? This might be understood by the loss of phosphatase activity in the Δ*cpxA* strain. It seems to be the case that, even in the presence of copper, the phosphatase activity of CpxA still plays an important role. Thus, while the phosphorylation of CpxR proceeds at a higher rate when copper is present (via CpxA and/or CusS), dephosphorylation by CpxA prevents a full activation of the Cpx system. In contrast, in the Δ*cpxA* strain, the majority of CpxR is phosphorylated (e.g. via acetyl-phosphate [14]) due to the lack of phosphatase activity, but a small increase in phosphorylated fraction can still be achieved by the action of CusS.

Our results implicate the copper-responsive histidine kinase CusS in the activation of CpxR. The involvement of CusS is supported by the lack of CpxR activation in the *cpxA cusS* double deletion strain. However, since disruption of the CusSR pathway might interfere with copper homeostasis, the effect of the *cusS* deletion could be indirect as well. Therefore, to ascertain that CpxR is activated by CusS, in future experiments an *in vitro* system of these two proteins will need to be tested for direct

phosphotransfer. Another possibility would be to employ FRET experiments to see whether there are direct interactions between CusS and CpxR upon addition of copper.

Previously, FRET experiments [22] have shown evidence of direct interactions between CpxR and a number of other response regulators, including CusR, the response regulator of the CusSR two-component system. The interaction required phosphorylation of both proteins. Here, we report interactions between the two pathways on a different level, namely activation of the response regulator CpxR by the histidine kinase CusS. While two-component systems are generally considered to be highly specific, a small extent of cross-talk between pathways is known to occur [23, 24]. It is unclear whether cross-talk between two-component systems provides an advantage to the cell, e.g. by allowing signal integration, or whether these interactions simply cannot be avoided due to the high similarity of the involved proteins. Overall, rather than being isolated pathways, two-component systems interact with each other on multiple levels, which complicates the study of individual systems, as also described in Chapter 1.

In our experiments, we used trace amounts of copper, namely 7 μM $CuCl_2$. This concentration was sufficient for induction of the CpxR-regulated *yebE* promoter, showing that the concentrations used in the literature to induce the CpxR regulon were rather high, at 500 μM [9, 10]. Additionally, we found that 7 μM prevents growth of the Δ*cpxR* strain. Again, in the literature higher concentrations were found to inhibit growth: 3 mM [10] and 8 mM [11]. Likely, the difference comes from the medium used in the different studies. Whereas we used minimal medium, in the other studies the bacteria were grown in LB or BHI. These complex media likely contain many compounds that, like orotate, could chelate copper, lowering the effective free copper concentration.

The chelating agent that we identified as being produced by growing *E. coli* cultures is orotate. Previously, secretion of this compound has been reported [18] and the reason for the secretion by some strains is that this intermediate in the pyrimidine biosynthesis pathway accumulates, as it cannot be efficiently utilised due to a mutation in the *rph* gene [19]. As laboratory strains are commonly grown in minimal medium containing copper ions, this *rph* mutation might exist because it provided a selective advantage to growth under laboratory conditions.

In summary, we have illustrated the importance of medium conditioning and how it can affect cellular responses and even growth. Additionally, we have demonstrated that CpxR is essential for growth in the presence of unchelated copper ions and that this protein can be activated by copper via CpxA and CusS.

Materials and methods

Bacterial strains and growth conditions

E. coli K12 strain MG1655 was used in this study. The fluorescent transcriptional reporter plasmids were obtained from the Promoter Collection [25]. The BW25113 deletion strains of *nlpE*, *cpxA*, *cpxR* and *cusS* were taken from the Keio Collection [26]. For transfer of these deletions to the MG1655 strain, P1-phage transduction was carried out. The pNTR-SD-*cpxR* plasmid with IPTG-inducible *cpxR* gene for complementation of the deletion strain, was taken from the Mobile Plasmid Collection [27].

Bacteria were grown at 37°C in an orbital shaker (300 rpm), in M9 minimal medium [28] supplemented with 0.4% glucose and optionally containing 7 μM $CuCl_2$. The medium was additionally supplemented with 25 μg/ml kanamycin. Conditioned medium was collected by spinning down bacterial cultures at 1000 g at 4°C and subsequent filtering of the supernatant through a 0.22 μm pore-size bottle-top filter made of PES (Thermo Scientific Nalgene).

Copper induction and flow cytometry analysis

Bacteria carrying the *yebE* reporter plasmid were grown until mid-exponential phase and diluted to an OD_{600} of 0.01 in medium of different compositions, i.e. with or without copper, with or without orotate, with various glucose or acetate concentrations and either freshly prepared or conditioned. Immediately after dilution and at several time points thereafter, samples were measured by flow cytometry (Accuri C6 flow cytometer, BD Biosciences; medium flow rate, FSC-H-threshold 8000, SSC-H threshold 500). The fluorescence intensities in the GFP channel (FL-1) were normalized to the size of each cell, measured as the 'width'.

Microfluidics

Bacteria were grown in M9 minimal medium without copper and introduced into the microfluidic device. We used a microfluidic device that was described previously [29]. The microfluidic device was constructed by bonding a cover glass to a PDMS slab moulded with two channels, followed by treatment of the channels with poly-D-lysine and flushing with medium for one hour before loading bacteria. When sufficient bacteria were surface-attached, a flow of 2.5 μL/min was initiated using a air-pressurised flow control system (OB1, Elveflow). During the microscopy time-lapse acquisition, the medium was switched to copper-containing medium (7 or 25 μM $CuCl_2$), which additionally contained the dye Alexa Fluor 594 to determine the time point at which the new medium arrived at the cells.

Microscopy

For image acquisition, a Nikon Eclipse Ti-E inverted microscope was used, with Nikon CFI Plan Apo Lambda DM 100X Oil objective, CoolLed pE-2 illumination system (470 nm LED for excitation of GFP, 565 nm LED for the Alexa Fluor 594 dye) and Andor iXon 897 EM-CCD camera. For detection of the GFP signal, the following filters were employed: excitation filter bandpass 470/40 nm, dichroic mirror 495 nm and emission filter 525/50 nm (AHF Analysentechnik F46-470). Focus was maintained by Nikon's PFS3 system. Every 10 min phase contrast, red and green channel images (200 ms exposure time) were acquired at multiple positions. The microscope was controlled by NIS Elements v4.51 software.

Image analysis

Microscopy images were segmented manually with in-house software written in Matlab (R2014a, MathWorks Inc.). The detected cells were analysed by applying the identified ROIs to background-corrected GFP images. The background correction was done by first subtracting the signal intensity of images without any bacteria, followed by division of each pixel by a correction factor to correct for uneven illumination. The correction factors were determined by smoothing the intensities on a position without cells with a 3x3 point moving average and then dividing the intensity of each pixel by the mean of all pixels.

Analysis of conditioned medium composition

Conditioned medium was prepared as described above. It was analysed by LC-MS, using a C18 column and ESI-TOF mass spectrometer. Further characterisation of conditioned medium was by HPLC, using a Hi-Plex H column and an Agilent 1260 Infinity HPLC system.

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References

[1] Osman D, Cavet JS (2008) Copper Homeostasis in Bacteria in *Advances in Applied Microbiology*, eds. Laskin AI, Sariaslani S, Gadd GM. (Elsevier Inc.) Vol. 65, pp. 217–247.

[2] Macomber L, Imlay JA (2009) The iron-sulfur clusters of dehydratases are primary intracellular targets of copper toxicity. *Proceedings of the National Academy of Sciences* 106(20):8344–8349.

[3] Dupont CL, Grass G, Rensing C (2011) Copper toxicity and the origin of bacterial resistance - New insights and applications. *Metallomics* 3:1109–1118.

[4] Munson GP, Lam DL, Outten FW, O'Halloran TV (2000) Identification of a Copper-Responsive Two-Component System on the Chromosome of *Escherichia coli* K-12. *Journal of bacteriology* 182(20):5864–5871.

[5] Outten FW, Huffman DL, Hale JA, O'Halloran TV (2001) The Independent *cue* and *cus* Systems Confer Copper Tolerance during Aerobic and Anaerobic Growth in *Escherichia coli*. *Journal of Biological Chemistry* 276(33):30670–30677.

[6] Changela A, et al. (2003) Molecular Basis of Selectivity and Sensitivity in Metal-Ion Recognition by CueR. *Science* 301:1383–1387.

[7] Outten FW, Outten CE, Hale J, O'Halloran TV (2000) Transcriptional activation of an *Escherichia coli* copper efflux regulon by the chromosomal MerR homologue, CueR. *Journal of Biological Chemistry* 275(40):31024–31029.

[8] Mermod M, Magnani D, Solioz M, Stoyanov JV (2012) The copper-inducible ComR (YcfQ) repressor regulates expression of ComC (YcfR), which affects copper permeability of the outer membrane of *Escherichia coli*. *BioMetals* 25:33–43.

[9] Yamamoto K, Ishihama A (2005) Transcriptional response of *Escherichia coli* to external copper. *Molecular Microbiology* 56(1):215–227.

[10] Yamamoto K, Ishihama A (2006) Characterization of copper-inducible promoters regulated by CpxA/CpxR in *Escherichia coli*. *Bioscience, Biotechnology and Biochemistry* 70(7):1688–1695.

[11] Price NL, Raivio TL (2009) Characterization of the Cpx regulon in *Escherichia coli* strain MC4100. *Journal of bacteriology* 191(6):1798–1815.

[12] Vogt SL, Raivio TL (2012) Just scratching the surface: an expanding view of the Cpx envelope stress response. *FEMS microbiology letters* 326(1):2–11.

[13] Raivio TL (2014) Everything old is new again: An update on current research on the Cpx envelope stress response. *Biochimica et Biophysica Acta* 1843:1529–1541.

[14] Wolfe AJ, Parikh N, Lima BP, Zemaitaitis B (2008) Signal integration by the two-component signal transduction response regulator CpxR. *Journal of Bacteriology* 190(7):2314–2322.

[15] Gupta SD, Lee BTO, Camakaris J, Wu HC (1995) Identification of *cutC* and *cutF* (*nlpE*) genes involved in copper tolerance in *Escherichia coli*. *Journal of Bacteriology* 177(15):4207–4215.

[16] Hirano Y, Hossain MM, Takeda K, Tokuda H, Miki K (2007) Structural Studies of the Cpx Pathway Activator NlpE on the Outer Membrane of *Escherichia coli*. *Structure* 15:963–976.

[17] Raivio TL, Leblanc SKD, Price NL (2013) The *Escherichia coli* Cpx envelope stress response regulates genes of diverse function that impact antibiotic resistance and membrane integrity. *Journal of bacteriology* 195(12):2755–2767.

[18] Womack JE, O'Donovan GA (1978) Orotic acid excretion in some wild-type strains of *Escherichia coli* K-12. *J.Bacteriol.* 136(2):825–827.

[19] Jensen KF (1993) The *Escherichia coli* K-12 "wild types" W3110 and MG1655 have an *rph* frameshift mutation that leads to pyrimidine starvation due to low *pyrE* expression levels. *Journal of Bacteriology* 175(11):3401–3407.

[20] Raivio TL, Silhavy TJ (1997) Transduction of envelope stress in *Escherichia coli* by the Cpx two-component system. *Journal of bacteriology* 179(24):7724–7733.

[21] Mahoney TF, Silhavy TJ (2013) The Cpx stress response confers resistance to some, but not all, bactericidal antibiotics. *Journal of Bacteriology* 195(9):1869–1874.

[22] Gao R, Tao Y, Stock AM (2008) System-level mapping of *Escherichia coli* response regulator dimerization with FRET hybrids. *Molecular Microbiology* 69(6):1358–1372.

[23] Laub MT, Goulian M (2007) Specificity in Two-Component Signal Transduction Pathways. *Annual Review of Genetics* 41:121–145.

[24] Agrawal R, Sahoo BK, Saini DK (2016) Cross-talk and specificity in two-component signal transduction pathways. *Future Microbiology* 11(5):685–697.

[25] Zaslaver A, et al. (2006) A comprehensive library of fluorescent transcriptional reporters for *Escherichia coli*. *Nature methods* 3(8):623–628.

[26] Baba T, et al. (2006) Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. *Molecular systems biology* 4474(2):2006.0008.

[27] Saka K, et al. (2005) A Complete Set of *Escherichia coli* Open Reading Frames in Mobile Plasmids Facilitating Genetic Studies. *DNA Research* 12(1):63–68.

[28] Kotte O, Volkmer B, Radzikowski JL, Heinemann M (2014) Phenotypic bistability in *Escherichia coli*'s central carbon metabolism. *Molecular Systems Biology* 10:736.

[29] Huberts DH, et al. (2013) Construction and use of a microfluidic dissection platform for long-term imaging of cellular processes in budding yeast. *Nature Protocols* 8(6):1019–1027.

Supplement

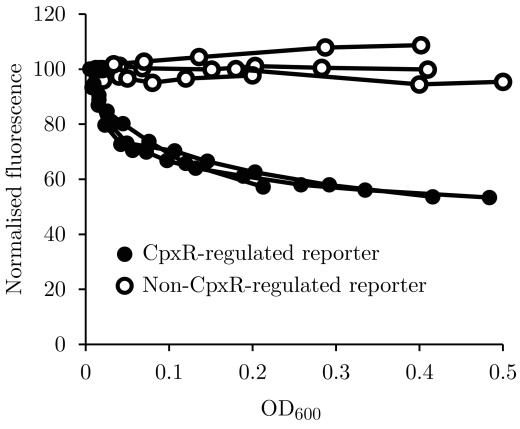

Figure S1: Expression of several reporters during growth. *E. coli* carrying a few different transcriptional reporter plasmids were grown in M9 medium with glucose and the fluorescence intensities were measured by flow cytometry at several time points. The data points are the median of the fluorescence intensity divided by cell size and normalised to a value of 100 at the initial time point. White circles are the *barA*, *mglB*, *osmB*, *rcsA* and *tppB* reporters, that are not regulated by CpxR. Black circles are the *ompC*, *yccA* and *yebE* reporters, that are regulated by CpxR.

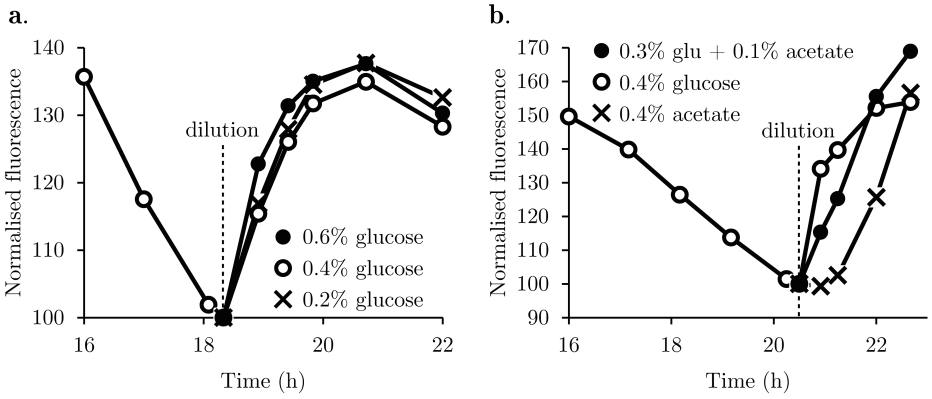

Figure S2: Effect of glucose and acetate on CpxR activity. Cells with CpxR reporter plasmid were grown to OD_{600} of 0.5 and then diluted in fresh medium with different compositions in terms of carbon source as indicated. The fluorescence intensity was determined by flow cytometry and set to a value of 100 at the time of dilution. The time on the x-axis is measured from the inoculation of the preculture.

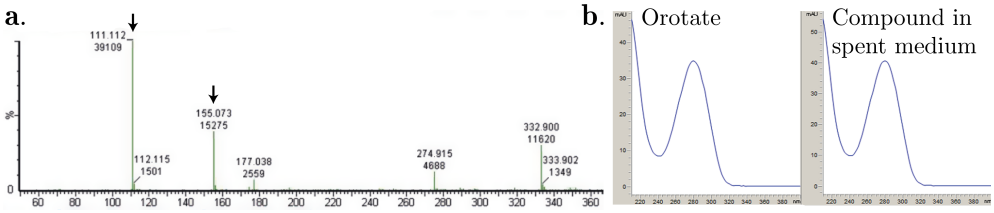

Figure S3: LC-MS and HPLC analyses identify orotate in spent medium. (**a**) LC-MS analysis of spent medium identified peaks at m/z of 155.1 and 111.1 that correspond to orotate, with and without its carboxylate group. (**b**) HPLC analysis found a compound in spent medium with the same retention time as an orotate standard (not shown) and with identical UV absorption spectrum (shown here). The concentration of orotate in spent medium was found to be 90 µM at OD_{600} of 0.5, determined by integration of the peak area and comparison with samples of known concentration of pure orotate.

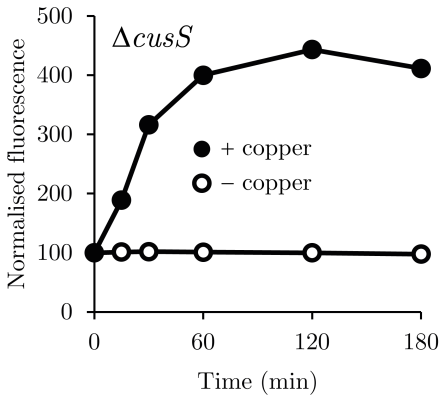

Figure S4: Effect of copper on the *ΔcusS* strain. Δ*cusS* carrying the *yebE* transcriptional reporter plasmid was grown in glucose minimal medium without copper and diluted into medium with or without 7 µM $CuCl_2$ at time point 0 (no orotate was added). The median fluorescence intensity divided by cell size was measured by flow cytometry and normalised to a value of 100 at the initial time point.