Supporting information for:

The Copper Efflux Regulator CueR Is Subject to ATP-Dependent Proteolysis in \textit{Escherichia coli}

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Experimental procedures

*CueR* activity assays – *E. coli* Δ*cueR*, Φ(copA-lacZ) cells (Outten *et al.*, 2000) were transformed with plasmids encoding constitutively expressed CueR, CueR_C112S or the empty vector (pACYC184). Cultures were grown in plastic ware in copper-free M9 minimal medium treated with 50 g/liter Chelex 100 resin (Bio-Rad) to remove trace metals. Before usage trace metals (without copper component) were added to the medium, mixed and sterile-filtered. Cells were grown to an optical density (A$_{580}$) of 0.5 at 30 °C, defined copper concentrations (CuSO$_4$) were added to the cultures for 1 h and 1 ml culture was harvested for β-galactosidase activity assay. The assay was performed as described previously (Miller, 1972).

*In vivo* degradation experiments – To analyze the stability of Strep_CueR in the cueR mutant, corresponding cells containing an inducible expression plasmid encoding for Strep_CueR were grown overnight in M9 minimal medium containing corresponding antibiotics for selection at 30 °C. 15 ml M9 minimal medium supplemented with corresponding antibiotics were inoculated with the overnight culture to an optical density (A$_{580}$) of 0.05. Cells were grown to an A$_{580}$ of 0.5 and protein expression was induced by adding 15 ng/ml anhydrotetracycline (AHT) for 20 min. Translation was blocked by addition of 200 μg/ml Cm. Samples were taken at different time points, frozen in liquid nitrogen and subjected to SDS-PAGE, Western transfer and immunodetection as described below. To analyze the stability of endogenous CueR in the lon mutant, *E. coli* Δ*lon* cells were grown in M9 minimal medium to an optical density (A$_{580}$) of 0.05 and *in vivo* degradation experiments were performed as described above.

*Preparation of protein extracts and immunodetection* – Cell pellets were resuspended in TE buffer depending on their optical density (10 mM Tris/HCl, pH 8; 1 mM EDTA; 50 μl TE buffer per A$_{580}$ of 1.0) and mixed with protein sample buffer (final concentrations of 2 % SDS (w/v), 0.1 % (w/v) bromophenol blue, 10 % (v/v) glycerol, 1 % (v/v) β-mercaptoethanol, 50 mM Tris/HCl, pH 6.8). Samples were incubated for 5 min at 95 °C, centrifuged (1 min, 16,000 × g) and subjected to SDS-PAGE and Western transfer using standard protocols (Sambrook & Russell, 2001). Strep-tagged fusion proteins were detected using a Strep-tag-HRP conjugate (IBA GmbH). Protein signals were visualized using Luminata Forte Western HRP substrate (Millipore) and the Chemi Imager Ready (Alpha Innotec). Half-lives of proteins were calculated by pixel counting with AlphaEaseFC software (version 4.0.0, Alpha Innotec).
*In vitro degradation experiments* – 15 µM of Strep_CueR were incubated for 2 min at 37 ºC in the degradation buffer described in (Bissonnette *et al.*, 2010). *In vitro* degradation was initialized by addition of 20 mM ATP. Degradation experiments without addition of ATP were performed as controls. Results were visualized by SDS-PAGE and Western transfer using standard protocols (Sambrook & Russell, 2001).
Figure S1

**Figure S1.** Effect of increasing CuSO₄ concentrations on CueR activity. *E. coli ΔcueR, Φ(copA-lacZ) cells were transformed with plasmids encoding constitutively expressed CueR, CueR_C112S or the empty vector (pACYC184) and were grown to exponential growth phase in M9 minimal medium at 30 °C. Cells were stressed with increasing CuSO₄ concentrations for 1 h and β-galactosidase activity was measured in Miller Units (MU). Standard deviations were calculated from at least two independent experiments.

Figure S2

**Figure S2.** Plasmid-encoded Strep_CueR was expressed for 20 min in exponential growth phase (M9 minimal medium; 30 °C) in the cueR mutant. Translation was blocked by addition of Cm. Samples were taken at indicated time points, subjected to SDS-PAGE, Western transfer, and immunodetection. Half-lives (T₁/₂) and standard deviations were calculated from three independent experiments.
Figure S3. E. coli Δlon cells were grown to exponential growth phase (M9 minimal medium; 30 °C). Translation was blocked by addition of Cm. Samples were taken at indicated time points, subjected to SDS-PAGE, Western transfer, and immunodetection. Half-lives of endogenous CueR (T_{1/2}) and standard deviations were calculated from two independent experiments.

Figure S4. Stability of various CueR variants in E. coli.
Plasmid-encoded CueR variants were expressed for 20 min in exponential growth phase (M9 minimal medium; 30 °C). Translation was blocked by addition of Cm. Samples were taken at indicated time points, subjected to SDS-PAGE, Western transfer, and immunodetection. Half-lives (T_{1/2}) and standard deviations were calculated from at least three independent experiments. For comparison the Western blot for Strep_CueR was taken from Figure 1C.
Figure S5

| without Lon_His₆ |
|------------------|
| +ATP             |
| -ATP             |
| t [min]          |
| 0    | 60  | 120 | 180 |
| 0    | 60  | 120 | 180 |

Figure S5. Strep_CueR is stable in vitro.
Strep_CueR was purified and used for a control in vitro degradation experiment without the presence of the Lon protease. The effect of ATP addition (+ATP) or the approach without ATP (-ATP) was analyzed. Samples were taken at indicated time points, subjected to SDS-PAGE, Western transfer, and immunodetection. Data are representative of two independent experiments.
References

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