Supporting Information

An in-cell spin-labeling methodology provides structural information on cytoplasmic proteins in bacteria.

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**CueR cloning & expression protocol**

CueR cloning & expression protocol: The CueR mutants were cloned into the pET28a expression vector by restriction-free cloning. The sequence was isolated by PCR using WT DNA as a template and the primers presented in Table S1. The clones were expressed in BL21 strain. A single colony was used to inoculate in terrific broth (TB) medium supplemented with kanamycin as selection factor and grown at 37 °C to an optical density of 0.6 (at 600 nm) and induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG, CALBIOCHEM) at 20°C overnight.

**Table S1. Primers used in this study.**

<table>
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<tr>
<th>Mutant</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tr>
<td>CueR_L60H_G64H</td>
<td>CAG GTG GGC TTT AAC CAC GAA GAG AGC CAC GAG CTG GTG AAT CTG TTT AAC GAC</td>
<td>GTC GTT AAA CAG ATT CAC CAG CTC G TG GCT C TC TTC GTG GTT AAA GCC CAC CTG</td>
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<tr>
<td>CueR_H94_L98H</td>
<td>GTG GCG GAG ATC GAA CGA CAC ATT GAG GAG CAC CAA TCC ATG CGG GAC CAG CTG CTG GCA C TG</td>
<td>CAG TGC CAG CAG CTG GTC GCG CAT GGA TTG GTG CTC CTC AAT GTG TCG TTC GAT CTC GCG CAC</td>
</tr>
<tr>
<td>CueR_E21H_L25H</td>
<td>ATT ACC GGC CTG ACC AGC AAA GCC ATT CGG TTC TAT CAT GAG AAG GGG CAC GTG ACG CCG CCG ATG CGC AGC GAA AAC GGT</td>
<td>ACC GTT TTG GCT GCG CAT CGG CGG GTG CAT CCC CTG ATG ATA GAA GCG AAT GCC TTG GCT GGT CAG GCC GTT AAT</td>
</tr>
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</table>

The data presented in this study for CueR_L60H_G64H mutant also contains $^{19}$F labelled F58 amino acid. Unfortunately, we were not able to detect the $^{19}$F signal using ESEEM and ENDOR measurements.

For this mutant, the forward primer is: ACC TTA CTG CGC CAG GCA CGG CAG GTG GGC TAG AAC CTG CGC CAG GCA CGG CAG GTG GGC TAG AAC CTG GG A GAG AGC GGC GAC GAG GTG GTG AAT, and the reverse primer is: ATT CAC CAG CTC GCC GCT CTC TTC CAG GTT CTA GCC CAC CTG CCG TGC CTG GCG CAG CAA GGT.

The clone was expressed in BL21 strain containing pDule-tfmF plasmid (ADDGENE). A single colony containing both pET28a and pDule-tfmF vectors was used to inoculate in terrific broth (TB) medium supplemented with kanamycin and tetracycline as selection factors and grown at 37 °C to an optical density of 0.4 (at 600 nm), then 1 mM of L-4-Trifluoromethylphenylalanine (tfmF, ALFA AESAR) was added. The cells were grown until optical density of 0.6 and induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG, CALBIOCHEM) at 20°C overnight.

For in-cell EPR measurements, nitrilotriacetic acid (NTA, Sigma-Aldrich) was incubated overnight with CuCl₂ at a 1:1 ratio to form a complex. The next day, 150 μM of Cu(II)-NTA was added together with IPTG to E. coli cells that were grown until optical density of 0.6 at 600 nm. 100 mL cultures were centrifuged at 1,200 g for 20 min, washed twice with 100 mL of TB and resuspended in 200 mL of TB. 100 μL of the sample was used for in-cell EPR measurements. Immediately after completing the EPR measurements, the supernatant was collected by centrifugation (2000 g at 4°C for 10 min) and its EPR spectrum was measured.

EPR measurements on lysate: The bacteria were harvested by centrifugation at 10,000 rpm for 30 min. The pellet was re-suspended in lysis buffer (25 mM Tris, pH 7.4, 250 mM NaCl, 20 mM PMSF protease inhibitor (Sigma-Aldrich), 1% Triton X-100 (Sigma-Aldrich)) and sonicated (10 min with a duty cycle of 5 sec on, 5 sec off at 35% amplitude). The clear lysate was collected after centrifugation at 14,000 rpm at 4°C for 30 min.
**CueR purification:** The bacteria were harvested by centrifugation at 10,000 rpm for 30 min. The pellet was re-suspended in lysis buffer (25 mM Tris, pH 7.4, 250 mM NaCl, 20mM PMSF, 1% Triton X-100,) and sonicated (10 min of 30 sec pulses at 35% amplitude). The cell lysate was then centrifuged at 4°C for 30 min at 14000 rpm. Next, the protein was purified from soluble fraction by Ni-NTA agarose beads (Thermo Fisher Scientific), according to the manufacturer’s protocol. The protein was eluted by addition of 250 mM imidazole to the lysis buffer. The obtained fractions were dialyzed overnight. The dialysis buffer is comprised of 25 mM Tris, pH 7.4, 250 mM NaCl. The protein purity was confirmed by 12% tricine SDS-PAGE and Coomassie blue staining (Figure S1).

![Figure S1](image)

**Figure S1.** A. SDS-gel picture of purified CueR mutated proteins. The purified protein (10 µl) was loaded onto 4% stacking gel and 14% separating gel. The arrow marks the 14 kDa mass of CueR. B. SDS-PAGE with uninduced and IPTG-induced E. coli BL21 and Cu(II)-NTA treatment. 10 µl of total protein extract was loaded onto 4% stacking gel and 14% separating gel. The arrow marks the 14 kDa mass of CueR. The 40 kDa band should be one of the house-keeping genes since it is present also in uninduced sample.

**CD characterization**
Circular Dichroism (CD) measurements were performed using a Chirascan spectrometer (Applied Photophysics, UK) at room temperature. Measurements were carried out in a 1 mm optical path length cell. The data were recorded from 200–260 nm with a step size and a bandwidth of 1 nm. Spectra were obtained after background subtraction. Figure S2 shows the CD spectra of WT_CueR and CueR mutants.
**Electrophoresis mobility shift assay (EMSA)**

EMSA experiments were carried out according to a published protocol. Briefly, 5% (wt/vol) non-denaturing gels were poured and stored at 4 °C until usage. The double stranded copA promoter DNA (5'- TTGACCTCCCTTGCTGGAAGGT -3') and protein were incubated at room temperature for 30 min. Glycerol was added to the sample reaching a final concentration of 15% (v/v) prior loading. Then, the gel was run with TAE buffer (40 mM Tris, 20 mM acetic acid, 1mM EDTA) at 4 °C, 80 V for 1 hour. Subsequently, the gel was added to an ethidium bromide staining, containing 1 μg/ml Ethidium bromide in TEA running buffer for 30 min. The gel was then washed with TEA running buffer for 30 min. The stained gel was analyzed with a Gel Doc EZ BioRad System using the EtBr protocol (Figure S3).
Cell Viability experiments
Unmodified BL21 strain of *E. Coli* bacteria were grown in LB at 37°C to an optical density of 0.4 (at 600 nm), then Cu(II) or Cu(II)-NTA at various concentrations were added. Absorbance at 600 nm was measured after 16 hours (Figure S4).

Moreover, the bacterial cells were washed, suspended in double distilled water, and injected into the flow cytometer instrument (cytek aurora) to test their viability (Figure S5).

Figure S4. *E. coli* cell viability experiments using absorption in the presence of Cu(II)-NTA and CuCl₂ salt.

Figure S5. *E. coli* cell viability experiments using cytometry in the presence of Cu(II)-NTA (red) and CuCl₂ salt (blue), three independent experiments were performed.
**Q-band DEER measurements**

The DEER experiment $\pi/2(n_{\text{obs}}) - t_1 - \pi(n_{\text{obs}}) - t' - \pi(n_{\text{pump}}) - (t_1 + t_2 - t') - \pi(n_{\text{obs}}) - t_2 - \text{echo}$ was carried out at $50 \pm 1.0$ K on a Q-band Elexsys E580 spectrometer (equipped with a 2-mm probe head) and 50 W AmpQ. A two-step phase cycle was employed on the first pulse. The echo was measured as a function of $t'$, whereas $t_2$ was kept constant to eliminate relaxation effects. The durations of the observer $\pi/2$ and $\pi$ pulses were 12 ns and 24 ns respectively. The duration of the $\pi$ pump pulse was 24 ns, and the time increment was 12-16 ns. $t_1$ was set to 200 ns and $t_2$ to 1000-2500 ns. To be able to compare between the various DEER signals, all data presented in the manuscript was acquired at the same frequencies and magnetic field: observer frequency: 33.83 GHz pump frequency: 33.73 GHz magnetic field: 11640 G. The samples were measured in 1.6-mm capillary quartz tubes (Wilmad-LabGlass). The DEER experiments in the cells were run for several days 3-5 days, with about 3000-5000 scans. The data was analyzed using the DeerAnalysis 2019 \textsuperscript{2,4}. 
Figure S6. Q-band time domain data for: A. CueR_L60H_G64H, B. CueR_E21H_L25H, and C. CueR_H94_L98H. The red lines represent the background functions.
**X-band CW-EPR experiments**

Continuous-wave electron paramagnetic resonance (CW-EPR) spectra were recorded using an E500 Elexsys Bruker spectrometer operating at 9.0−9.5 GHz equipped with a super-high-sensitivity CW resonator. The spectra were recorded at low temperature (130 ± 5 K) at microwave power of 20.0 mW, modulation amplitude of 2.0 G, a time constant of 120 ms, and receiver gain of 60.0 dB. The samples were measured in 1.0-mm outer diameter quartz tubes (Wilmad-LabGlass, Vineland, NJ) which was placed in 4.0 mm outer diameter quartz tube for cooling process.

**Figure S7.** X-band CW-EPR spectra carried out at 130 ± 5 K for Cu(II)-NTA bound to purified CueR_L60H_G64H (grey), and in *E. coli* cells (orange). The EPR spectrum of supernatant after washing the cells before EPR measurements (green).
**Q-band two-pulse experiments**

The two-pulse echo detected experiment were performed with $\pi/2$ pulse length of 12 ns, and a $t$ value of 200 ns at $g_\perp$ position. It was carried out at 20K, 33.83GHz, 11670 G.

**Figure S8. Q-band two-pulse experiments**  
**A.** Two-pulse field sweep of CueR_L60H_G64H purified (grey), in lysate (light blue) and *in cell* (orange). The grey rectangular marks the position where the DEER experiments were performed.  
**B.** Two-pulse decay of CueR_L60H_G64H purified (grey), and *in cell* (with (orange) and without (green) free Cu(II) ions).
Figure S9. EPR measurements on BL21 cells without overexpression of CueR, in the absence and presence of Cu(II)-NTA. A. Low temperature X-band CW-EPR spectra at 130K. B. Field sweep Q-band EPR spectra at 20K. C. Q-band two-pulse decay at 11670G at 20K. D. Q-band DEER time domain data of Cu(II)-NTA in BL21 cells. 150 mM of Cu(II)-NTA were added to BL21 cells at optical density of 0.6 at 600 nm.