Electronic Supplementary Material (ESI) for Metallomics.

Supplementary information for

The CO dehydrogenase accessory protein CooT is a novel nickel-binding protein

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Supplementary Methods

Cloning, mutagenesis and expression

The *Rhodospirillum rubrum* CooT gene was obtained from DNA 2.0 in pET15b as a codon-optimized gene for expression in *E. coli*. To obtain the wild-type *Rr*CooT ORF, the additional glycine residue introduced by the initial cloning procedure was removed using the Q5 site-directed mutagenesis protocol from New England Biolabs (NEB) with primers CooT-gly_1 5' CTTTAAGAA GGAGATATACCATGTGTATGGCAAAAGTTGTTC 3' and CooT-gly_2 5' GAACAACTTTTGCCATACACA TGGTATATCTCCTTCTTAAAG 3'. For generation of the C2S mutant, cysteine 2 was mutated to serine using the same protocol and primers CooT-C2S_1 5' CTTTAAGAAGGAGAT ATACCATGTCTATGGCAAAAGTTGTTC 3' and CooT-C2S_2 5' GAACAACTTTTGCCATAGACATGGTAT ATCTCCTTCTTAAAG 3'. Mutagenesis success was confirmed by sequencing.

E. coli BL21 (DE3) cells harboring wild-type *Rr*CooT or the C2S mutant were cultured in M9 media, supplemented with appropriate antibiotics, at 37°C and 180 rpm. Protein expression was induced with 0.2 mM IPTG at OD_{600} ~ 0.6 and left overnight at 25 °C and 180 rpm before harvesting by centrifugation. The cell pellets were washed in PBS before storage at -20 °C.

Protein purification

Bacterial pellets from 3 L culture (overexpressing wild-type *Rr*CooT) were thawed on ice and resuspended in buffer QA (50 mM TrisHCl, pH 8.0, 1 mM EDTA, 5 mM DTT) containing one complete Protease Inhibitor cocktail tablet (Roche) per 100 mL buffer and lysed by sonication. The cell debris was removed by centrifugation at 7500 *g* for 45 min at 4 °C. The cleared lysate was loaded at 1.5 mL min^{-1} onto a 25 mL Q Sepharose column equilibrated in QA buffer. After washing with 100 mL QA buffer, protein was eluted with a 200 mL of a 0-50 % linear gradient of buffer QB (50 mM TrisHCl, pH 8.0, 1 M NaCl, 1 mM EDTA, 5 mM DTT). The fractions were analyzed by SDS-PAGE, and those containing *Rr*CooT were pooled and concentrated before loading onto a Superdex 75 16/600 size exclusion column (GE Healthcare) equilibrated in buffer S (25 mM TrisHCl, pH 8.0, 250 mM NaCl, 1 mM TCEP). The fractions were collected, analyzed by SDS-PAGE, and those containing pure *Rr*CooT were pooled and concentrated to 5.3 mg mL^{-1} before flash freezing in liquid nitrogen and stored at -80°C. *Rr*CooT C2S was purified using the same protocol as described for *Rr*CooT, except that DTT and TCEP were not added to buffers. Pure *Rr*CooT

C2S was concentrated to 10.6 mg mL^{-1} before flash freezing and storage at -80 °C.
The protein size and identity, as well as the presence of the N-terminal methionine were determined by electrospray ionization mass spectrometry (ESI-MS).

Data collection and X-ray structure solution

Diffraction data of both crystals, with and without heavy atom soak, were collected at the European Synchrotron Radiation Facility (ESRF) on beamlines BM30 and ID23. The images were processed with XDS[1] and AIMLESS from the CCP4 program suite[2]. Details of data collection, crystallographic data and statistics are summarized in Table S1. The structure was solved with the data set collected from the heavy atom soaked crystal (*Rr*CooT-NiI) by single anomalous dispersion using the SHELXC/D/E package.[3] Six iodide ions were initially located in the asymmetric unit (AU) and the automated model building resulted in a nearly complete model with six *Rr*CooT monomers in the AU. The model was completed and the protein sequence corrected manually in COOT.[4] Further heavy atoms could be identified during subsequent refinement. This corrected model was used for molecular replacement to obtain the apo-*Rr*CooT structure from the other data set (not soaked, apo *Rr*CooT) at a resolution of 1.9 Å. Refinement was performed using REFMAC5[5] with local NCS restraints automatically generated by REFMAC5. The last rounds of refinement were carried out with the jelly body option with sigma 0.02. The final model was validated in COOT and *MolProbity.*[6]

X-ray structure analysis

The closest structural homologues to *Rr*CooT were identified using the PDBeFold server (EMBL-EBI) and further homologues were identified by screening of the Pfam clans and families of the initial structure hits. Subsequent SSM superposition and calculation of the overall RMSDs of the respective proteins were carried out in CCP4*mg*.[7]

Rhodospirillum rubrum cultures

R. rubrum glycerol stock was used to inoculate 100 ml of SMN medium supplemented with 0.4% KH_2PO_4 and 100µg/ml of streptomycin. Cells were grown aerobically at 30°C for several days until cell suspension began turning pink. 20 ml were then collected (OD600nm = 2.7), centrifuged and the pellet was frozen in liquid nitrogen and stored at -20°C. 5 ml of culture were used to inoculate 50 ml of fresh SMN medium supplemented with 0.4% KH_2PO_4 and 100µg/ml of streptomycin under anaerobic conditions. Cells were grown anaerobically at 30°C under an incandescent light source until cultures became dark red. 25 ml were then

collected (OD600nm = 13), centrifuged and the pellet was frozen in liquid nitrogen and stored at -20°C. 5ml of anaerobic culture were used to inoculate 15 ml of fresh SMN medium supplemented with 1.6% KH_2PO_4, 100µg/ml of streptomycin and 1 µM of $NiSO_4$ under anaerobic conditions at 30°C. After 24 hours, 100%CO was sparged through cultures for 30-40 min after 0.01% Na_2S addition, every 12 hours during 2-3 days. (Ni and CO were added to induce the expression of *coo* operon). CODH activity measurements were performed to check the good production of the enzyme. Cells were collected (OD600nm = 12), centrifuged and the pellet was frozen in liquid nitrogen and stored at -20°C.

DNA/RNA extraction

Bacterial cells were re-suspend in 5ml of lysis buffer Tris 25mM-50mM NaCl) and vortex for 2 min before to be mixed with 5ml of premix phenol/Chlorophorme/AIA pH 7, vortex for 5 min and incubate for 30min at RT. After a centrifugation step 10000g at 4°c for 30 min, the supernatant was precipitated with 0.7 of cold isopropanol 100% and incubate at 4°c ON. Nucelotides are precipitates and centrifuges at 10000g at 4°c for 30 min, the pellet are wash with 2ml of ethanol 70%, and dry in SpeedVac before to be re-suspend in 100µl of pure DEPC water. 20µl of total extract are incubate with 20UI of DNAse (RNAse Free) or RNAse (DNAse free) in appropriate buffer at 37°c for 2h. Fianbl volume was ajust at 100µl with Pure DEPC water and treated by phenol/Chlorophorme/AIA pH 7 and precipitate at 2.5v of cold ethanol. Each sample was normalized at DO260nm: 10 with nanodrop.

EMSA (Electrophoretic mobility Shift Assays) and nucleotide binding

1µl of nucleotide extract at DO260nm: 10 were mixed with 1µl of Coot protein at 1.5mM in 10µl of binding buffer (50mM HEPES pH 7.5, 1mM TCEP +/- 1mM $NiSO_4$). After 30 min of incubation at room temperature, 10 µL of reaction mixtures were loaded on pre-run 10 % polyacrylamide (19/1) gel. The gel was pre-run 30 min at 75 V in TA buffer (40 mM Tris acetate pH 8.2) supplemented or not with 1mM of EDTA or 1mM of $NiSO_4$. Mobility shifts were revealed by soaking the gel in fresh gel red solution at 0,1%.

Supplementary Figures

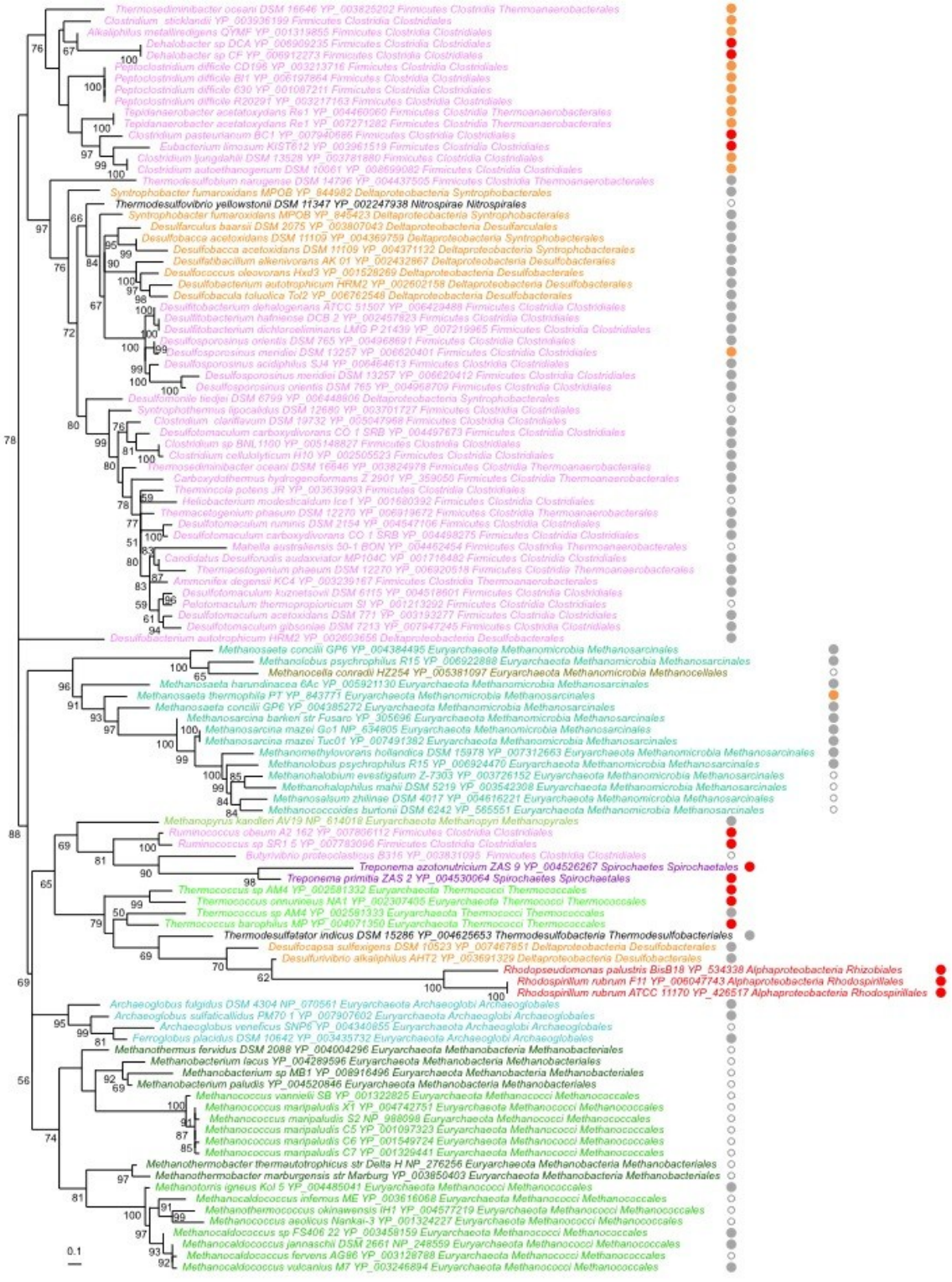


Figure S1A : Phylogentic Analysis of CooT. Shown is a maximum likelihood tree of 111 CooT sequences (111 sequences, 53 positions). The scale bar indicates the average number of substitution per site. Number at nodes corresponds to approximate bootstrap values (1,000 replicates of the original alignment). For clarity values lower than 50 are not shown. Colors correspond to taxonomic groups. An empty gray circle indicates that *cooS* is not present in the genome of the strain ; a filled gray circle indicates that a *cooS* gene is present in the genome but far away from *cooT* ; an orange circle indicates that a *cooS* gene is located in the neighborhood of

cooT (+/- 25 genes) an red circle indicates that a *cooS* gene is located in the direct neighborhood of *cooT* (+/- 10 genes).

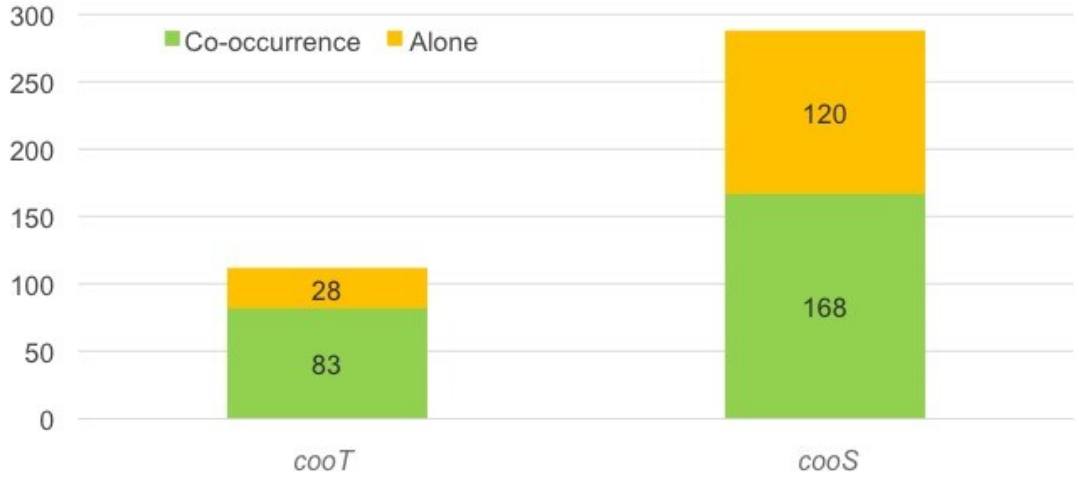
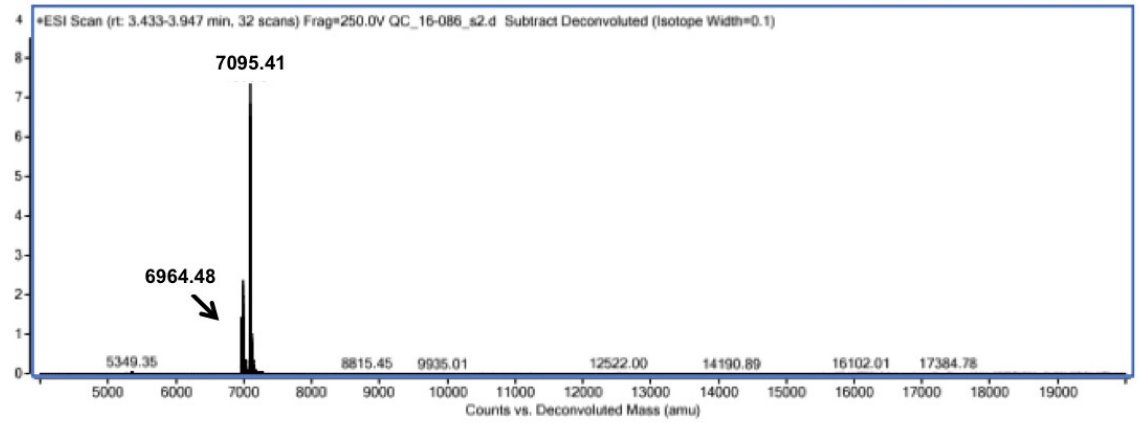


Figure S1B : Co-occurence of *cooT* and *cooS* in complete prokaryotic genomes. The histogram shows how many *cooT* sequences co-occur with *cooS* sequences and vice versa. The increased number of co-occurring *cooS* with *cooT* (168) arises from the presence of multiple isoforms of *cooS* in some species.

*Rr*CooT



*Rr*CooT-C2S

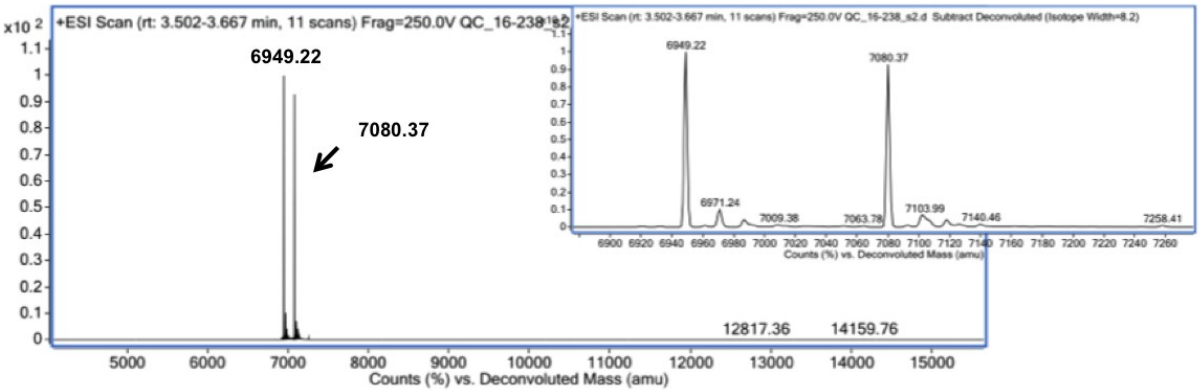


Figure S2 : ESI-MS spectra of *Rr*CooT and *Rr*CooT-C2S.

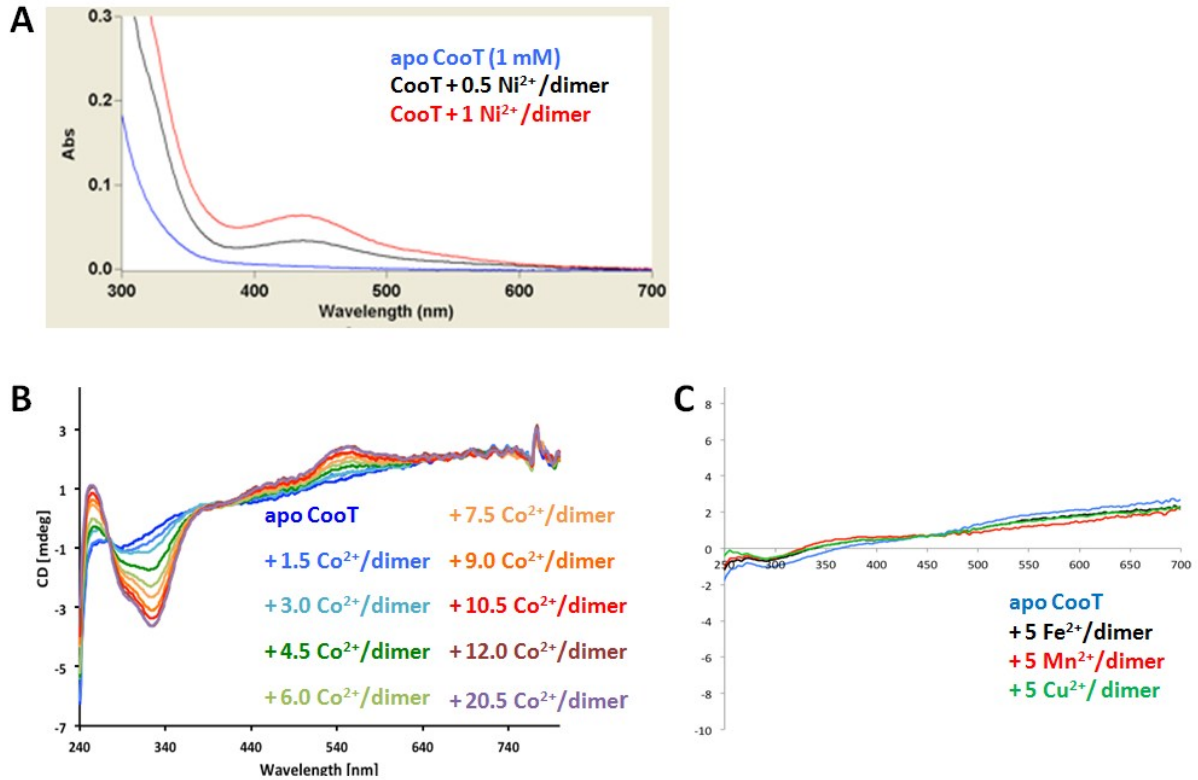


Figure S3 : UV/Vis spectra and circular dichroism (CD) in near-UV/Vis of *Rr*CooT with metal ions. (A) shows the absorption spectrum of purified *Rr*CooT in apo form and in complex with Ni^{2+} ions in the UV/visible range. The absorption peak at 420 nm indicates a square-planar coordination of the Ni^{2+} ions. The CD spectra in near-UV/Vis range of a *Rr*CooT titration with Co^{2+} salt, shown in (B), indicated specific binding of Co^{2+} ions to the protein as releaved by signals from the d→d charge transfers. In the CD spectra recorded in the near-UV/Vis range displayed in (C), addition of 5-fold molar excess of Fe^{2+}, Mn^{2+} and Cu^{2+} does not show the appearance of d→d transfer bands, indicating no specific binding events between these metals and *Rr*CooT. The CD spectra were recorded at a concentration of 7.5 µM *Rr*CooT dimers.

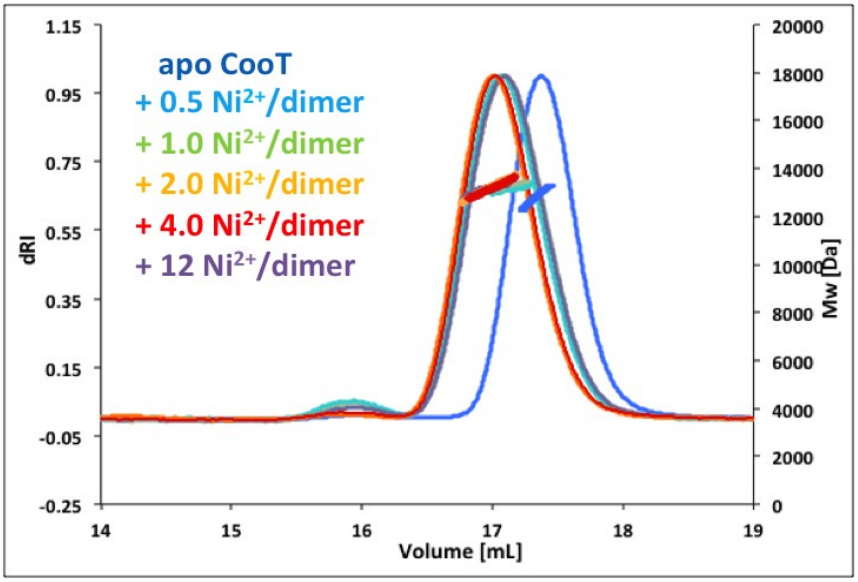


Figure S4 : SEC-MALLS analysis of *Rr*CooT Nickel titration. SEC-MALLS profile for apo-*Rr*CooT at 1.5 mg mL^{-1} (blue) and in addition of increasing molar ratios of Ni^{2+}. Shown are the traces of the differential refractive index (dRI, absolute) and the measured molecular weight (MW) in kDa over the elution volume.

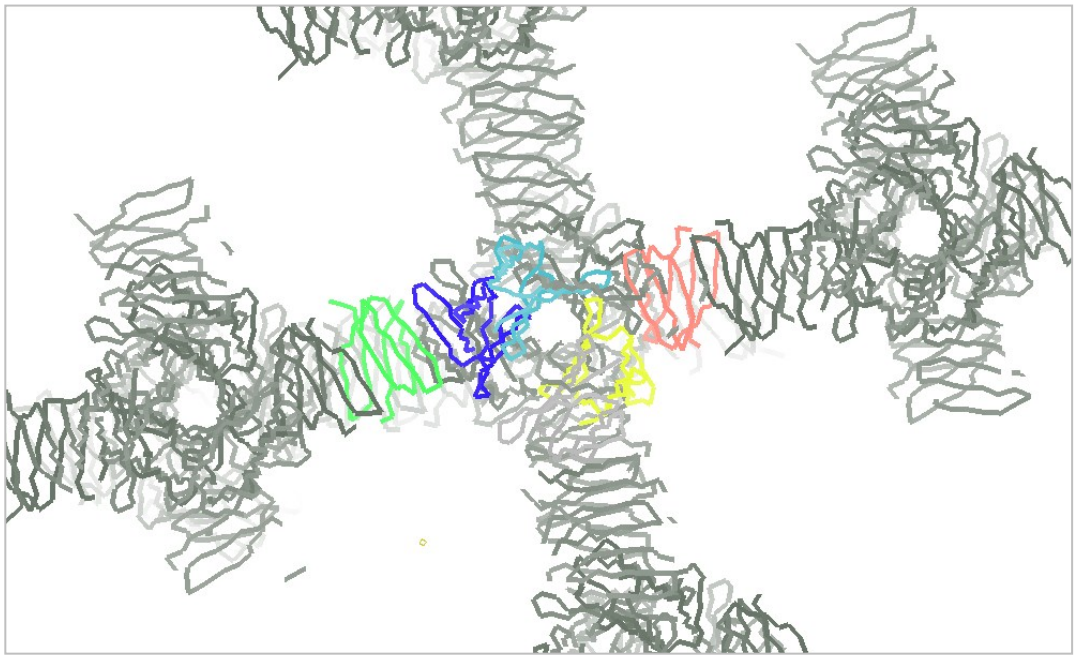


Figure S5 : Crystal packing of the *Rr*CooT crystals. The protein chains are displayed as backbone Cαs. Chains of the asymmetric unit are coloured by chain, the symmetry related molecules are displayed in grey. The display of all molecules in the crystal lattice reveals the large solvent channels and the formation of a continuous twisted β-sheet throughout the crystal. Chains and symmetry related molecules were displayed with COOT[4].

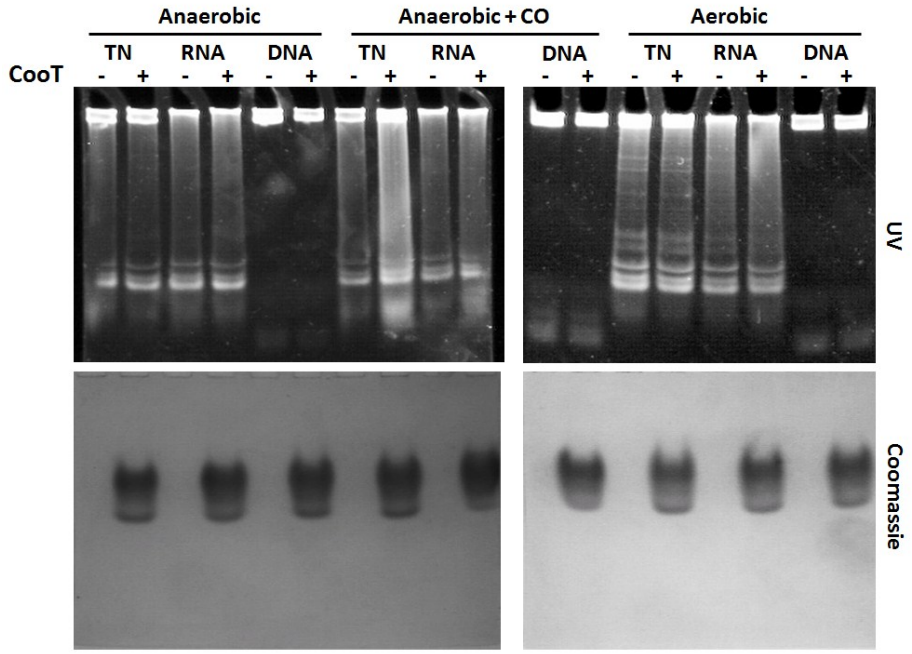


Figure S6 : Nucleotide binding of *Rr*CooT in absence of metal ions. Shown are electrophoretic mobility shift assays (EMSAs) of total nucleic acids (TN), RNA (DNAse treated) and DNA (RNAse treated) in presence and absence of *Rr*CooT on native PAGE gels. The running buffer of the EMSA was supplemented with 1 mM EDTA to ensure *Rr*CooT remaining metal-free. Tested were the nucleic acids isolated from *R. rubrum* cultures grown under anaerobic, anaerobic+CO and aerobic conditions. The top panels show the nucleic acids under UV light, the bottom panels show the exact same gels subsequently stained with coomassie, indicating the *Rr*CooT protein.

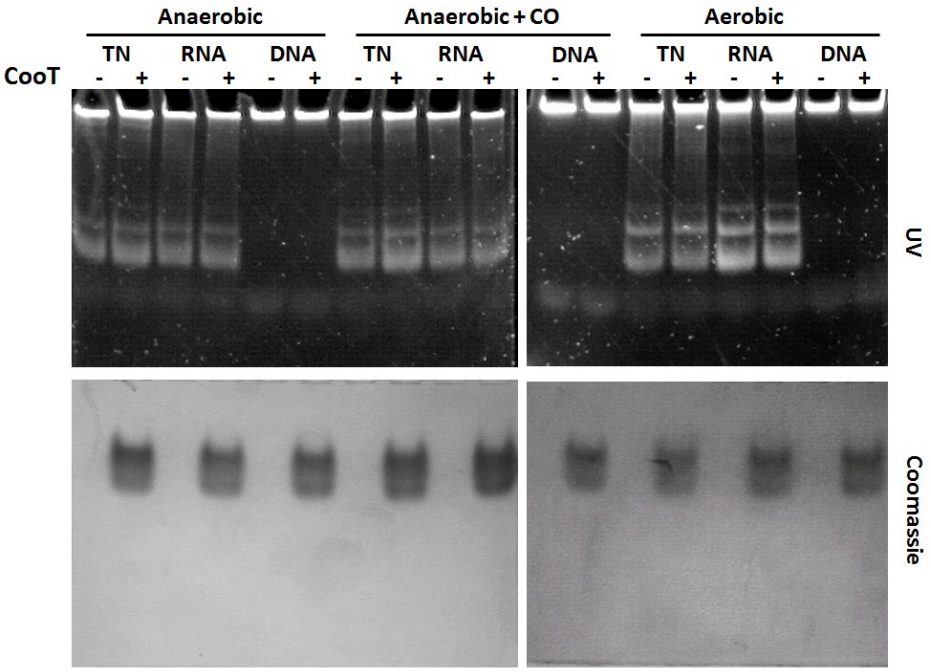


Figure S7 Nucleotide binding of *Rr*CooT in presence of $NiSO_4$. Shown are electrophoretic mobility shift assays (EMSAs) of total nucleic acids (TN), RNA (DNAse treated) and DNA (RNAse treated) in presence and absence of *Rr*CooT on native PAGE gels. The EMSA running buffer was supplemented with 1 mM $NiSO_4$ to analyze nucleotide binding of *Rr*CooT in its Ni^{2+} bound form. Tested were the nucleic acids isolated from *R. rubrum* cultures grown under anaerobic, anaerobic+CO and aerobic conditions. The top panels show the nucleic acids under UV light, the bottom panels show the exact same gels subsequently stained with coomassie, indicating the *Rr*CooT protein.

Supplementary Tables

Table S1 : Crystallographic Data and statistics for the *Rr*CooT crystals. The *Rr*CooT crystal soaked with nickel and iodide (NiI) was used for phasing and molecular replacement of the apo *Rr*CooT crystal, no further refinement was carried out.

Data collection	**apo *Rr*CooT**	***Rr*CooT-NiI**
Beamline	BM30A (ESRF)	ID23 (ESRF)
Wavelength (Å)	0.979790	1.5471
Space group	$P4_12_12$	$P4_12_12$
Unit cell parameters	a = 108.943 Å b = 108.943 Å c = 110.734 Å	a = 110.43 Å b = 110.43 Å c = 113.32 Å
Resolution range (Å)	44.59-1.90	79.09-2.0
R_{sym} (%)	10.6 [72.2]	25.0 [295.0]
Mean I/σ (I)	10.84 [2.88]	10.9 [2.60]
Completeness (%)	99.2 [100.0]	100 [100]
Redundancy	7.26 [7.35]	23.1[23.3]
$N_{measured}$	381912 [54607]	1105681 [81528]
N_{unique}	52618 [7429]	47954 [3503]
Refinement		
R factor/ R_{free} factor (%)	20.86 / 23.19	
No. atoms	3047	
No. water molecules	239	
average B-factor (Å^2)	32.5	
Rmsd bonds (Å)	0.022	
Rmsd angles (°)	1.926	
Ramachandran plot:		
Residues in most favorable region (%)	99.46	
Residues in disallowed region (%)	0.0	
PDB ID	**5N76**	

References

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