Supporting Information for

Biochemical and Spectroscopic Observation of Mn(II) Transfer Between Bacterial Mn(II) Transport Machinery and Calprotectin

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This Supporting Information includes:

Experimental Section

General Materials and Methods. Buffers used for titrations, EPR sample preparation, and pull-down assays were prepared using nitric acid-washed volumetric glassware using ULTROL grade HEPES (Calbiochem), TraceSelect NaCl (Sigma), and Milli-Q water (18.2 MΩ•cm, 0.22-μm filter). TraceSelect NaOH (Sigma) was used to adjust the buffer pH. Stock solutions of Ca(II) (1 M, 100 mL) and Mn(II) (1 M, 100 mL) were prepared from 99.999% $CaCl_2$ (Sigma) and 99.999% $MnCl_2$ (Alfa Aesar), respectively, and Milli-Q water using acid-washed volumetric glassware and the solutions were stored in polypropylene tubes. Working solutions of metal solutions were prepared by serial dilution of the stock solutions in Milli-Q water immediately prior to each experiment. Zinpyr-1 (ZP1) was synthesized as described elsewhere (kindly provided by Dr. Jacob Goldberg and Prof. Stephen J. Lippard).[1] Stock solutions of ZP1 (≈3 mM) were prepared in DMSO and stored in aliquots (≈15 μL) at -20 °C. The concentration of each stock solution was determined using the reported extinction coefficient of apo ZP1 (ε_{515} = 79,500 $M^{-1}cm^{-1}$).[1] Aliquots of ZP1 were thawed only once, immediately prior to use, and the solutions were handled in the dark. Oligonucleotide primers were synthesized by Integrated DNA Technologies (Coralville, IA) and used as received (standard desalting protocol*). Staphylococcus aureus* USA300 JE2 was obtained from the Network on Antimicrobial Resistance in *Staphylococcus aureus* (NARSA, BEI Resources). The CP protein used in this work is CP-Ser, which is the heterooligomer of S100A8(C42S) and S100A9(C3S).[2] CP-Ser is referred to as "CP" in the text. was overexpressed and purified as described previously.[2] Protein concentration was determined using the calculated extinction coefficient for the S100A8(C42S)/S100A9(C3S) heterodimer (ε_{280} = 18,450 $M^{-1}cm^{-1}$, calculated using the ProParam tool available at http://web.expasy.org/protparam/).). All B-CP and CP protein concentrations are for the heterodimer.

Preparation of Biotin-CP (B-CP). B-CP was prepared by conjugating biotin polyethyleneoxide iodoacetamide (BPEOIA, Sigma) to the Cys3 thiol moiety of the S100A9 subunit of CP(C42S) (the heterooligomer of S100A8(C42S) and native S100A9) using the procedure previously described for a biotinylated CP ΔHis_3Asp variant.[3] The Ca(II)-dependent oligomerization properties of the protein were evaluated by analytical size-exclusion chromatography using a reported procedure.[2,3] Protein concentration was determined using the calculated extinction coefficient of the CP-Ser heterodimer (ε_{280} = 18,450 $M^{-1}cm^{-1}$) and concentrations reported are for the heterodimer.

Cloning, Overexpression and Purification of MntC. The Wizard Genomic DNA Purification Kit (Promega, manufacturer protocol) was used to isolate genomic DNA from *S. aureus* USA300 JE2. A portion of the *mntC* gene that lacks the sequence for the N-terminal lipid anchor (*mntC*$^{19\text{-}309}$) was PCR amplified from *S. aureus* USA300 JE2 genomic DNA using the forward primer 5'-ggaatccatatgggtactggtggtaaacaaag-3' (*Nde*I restriction site underlined) and reverse primer 5'-gatcctcgag**tta**tttcatgcttccgtgtac-3' (*Xho*I restriction site underlined, stop codon bold). Polymerase chain reactions were performed using Pfu Turbo DNA polymerase (Stratagene). The amplified *mntC* gene sequence was digested with *Nde*I and *Xho*I (New England Biolabs), purified by 1% agarose gel electrophoresis, and subsequently isolated with the GE Illustra PCR and Gel Band Purification Kit. The resulting gene was ligated into the *Nde*I and *Xho*I sites of pET41a using T4 DNA ligase (New England Biolabs). Following the ligation reaction, the vector was transformed into chemically competent *Escherichia coli* TOP10 cells and isolated using a miniprep kit (Qiagen). The identity of the resulting pET41a-*mntC* construct was verified by DNA sequencing (MIT Biopolymers).

The plasmid pET41a-*mntC* was transformed into chemically competent *E. coli* BL21(DE3). Freezer stocks were prepared by combining a 1:1 ratio of an overnight culture (LB,

50 μg/mL kanamycin, 37 °C, 9 h, 150 rpm) and sterile 50% glycerol, and flash freezing the mixture in liquid nitrogen. Cell stocks were stored at -80 °C. For protein overexpression, 40 mL of LB containing 50 μg/mL kanamycin was inoculated from a freezer stock and grown overnight with shaking (37 °C, 150 rpm, 12-16 h). This overnight culture was diluted 1:100 into 1 L of LB medium containing 50 μg/mL kanamycin, and the resulting culture was incubated with shaking (37 °C, 150 rpm). At OD_{600} ≈0.6, protein expression was induced by addition of 500 μM IPTG (1 mL of a 0.5 M stock solution in water). The culture was grown for another ≈3.5 h at which time the cells were pelleted by centrifugation (3,000 rpm, 20 min, 4 °C), frozen in liquid nitrogen, and stored at -80 °C. Cell pellets weighed ≈2.5 g / L of culture.

A cell pellet obtained from 1 L of culture was thawed on ice. Lysis buffer A (50 mM MES, pH 6.5, 100 mM NaCl, 1 mM EDTA, 0.5% Triton X-100) was stored at 4 °C and supplemented with 1 mM PMSF (Amresco, prepared immediately before use by dissolving 21 mg in ≈1 mL EtOH) immediately prior to use. The pellet was suspended in 60 mL of cold lysis buffer A, and transferred to an ice-cold stainless steel beaker, and lysed on ice by sonication by using a Branson sonicator (30 sec pulse on, 10 sec pulse off, 2.5 min, 40% amplitude). The lysate was then centrifuged (10 min, 14,000 rpm, 4 °C) and the supernatant was transferred to a glass beaker on ice. The resulting cell pellet was resuspended in 60 mL of lysis buffer A and the sonication and centrifugation process was repeated with this material. The combined supernatants were placed in a 4 °C cold room and treated with 60% ammonium sulfate (Sigma) for ≈1 h with stirring. This procedure resulted in the precipitation of contaminating proteins. The mixture was centrifuged (20 min, 14,000 rpm, 4 °C) and vacuum filtered. The filtrate (≈130 mL) was transferred to dialysis tubing (Spectra/Por 3,500 kDa MWCO) and dialyzed against 4 L of 20 mM HEPES, pH 7.0 at 4 °C (2 x ≈12-24 h).

The dialyzed protein solution was centrifuged (10 min, 14,000 rpm, 4 °C) and filtered (0.2 μm filter). A GE ÄKTA Purifier housed in a 4 °C room was employed for ion exchange and size

exclusion chromatographic purification. MntC was first purified by cation exchange chromatography using a MonoS 10/100 cation–exchange column with elution buffers composed of 20 mM HEPES, pH 7.0, without (buffer A) or with (buffer B) 1 M NaCl. MntC was eluted with a gradient of 0–30% buffer B over 15 column volumes at a 2 mL/min flow rate. MntC-containing fractions (identified by SDS-PAGE) were combined, concentrated to ≈10 mL by centrifugation (3,700 rpm, 4 ^{o}C) using a 15-mL spin filter (Pall, 10 kDa MWCO) and loaded onto a HiLoad 26/600 Superdex-75 gel filtration column pre-equilibrated with 20 mM HEPES, pH 7.0, 200 mM NaCl. MntC was eluted from the column using a flow rate of 1 mL/min. Fractions containing MntC were combined, transferred to dialysis tubing (Spectra/Por, 3,500 kDa MWCO), and dialyzed against 4 L of de-metalating buffer (100 mM acetic acid, 20 mM EDTA, pH 3.7) at room temperature (2 x ≈12 h). MntC was then dialyzed once (≈12 h, 4 ^{o}C) against 4 L of storage buffer (20 mM HEPES, 200 mM NaCl, pH 7.0) and subsequently filtered (0.2-μm syringe filter; this step is optional), concentrated by centrifugation (3,700 rpm, 4 ^{o}C) using a 15 mL spin filter (Pall, 10 kDa MWCO), aliquoted, flash frozen in liquid nitrogen, and stored at -80 °C. Typical yields of MntC were ≈30 mg / L culture.

Cloning, Overexpression and Purification of PsaA. A fragment of *Streptococcus pneumoniae* D39 genomic DNA containing the *psaA* gene was provided by Professor David P. Giedroc (Indiana University). The NCBI reference sequence NC_011072.1 was used to design primers for amplification of a portion of the *psaA* gene that lacks the sequence for the N-terminal lipid anchor (PsaA$^{21\text{-}309}$). The forward primer 5'-ggaatccatatggctagcggaaaaaaagatac-3' (*Nde*I restriction site underlined) and the reverse primer (5'-gatcctcgag**tta**ttttgccaatccttcag-3' (*Xho*I restriction site underlined, stop codon in bold) were used to PCR amplify *psaA* as described above for *mntC*. The gene was ligated into the *Nde*I and *Xho*I sites of pET41a using T4 DNA ligase. Following the ligation reaction, the vector was transformed into chemically-competent

Escherichia coli TOP10 cells and isolated using a miniprep kit (Qiagen). The identity of the resulting pET41a-*psaA* construct was verified by DNA sequencing (MIT Biopolymers).

The plasmid pET41a-*psaA* was transformed into chemically competent *E. coli* BL21(DE3). Freezer stocks were prepared by combining a 1:1 ratio of an overnight culture (LB, 50 μg/mL kanamycin, 37 ^{o}C, 9 h, 150 rpm) and sterile 50% glycerol, and flash freezing the mixture in liquid nitrogen. Cell stocks were stored at -80 °C. For protein overexpression, 50 mL of LB containing 50 μg/mL kanamycin was inoculated from a freezer stock and grown overnight with shaking (37 °C, 150 rpm, 12-16 h). This overnight culture was diluted 1:100 into 2 L of LB medium containing 50 μg/mL kanamycin, and the resulting culture was incubated with shaking (37 ^{o}C, 150 rpm). Once the cultures reached OD_{600} ≈0.6, overexpression of PsaA was induced with 100 μM IPTG. The cultures were incubated at 37 °C and 150 rpm for an additional ≈3.5 h, and the cells were pelleted by centrifugation (3,000 rpm, 20 min, 4 ^{o}C). Cell pellets were frozen in liquid nitrogen and stored at -80 °C. Cell pellets weighed ≈4.6 g / 2 L culture.

A cell pellet from 2 L of culture was thawed on ice. PMSF (1 mM, prepared immediately before use by dissolving 17.4 mg in ≈1 mL EtOH) was added to ≈100 mL of Lysis buffer B (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Triton X-100) immediately before use. The pellet was suspended in 50 mL lysis buffer B and lysed by sonication with a Branson sonicator (30 sec pulse on, 10 sec pulse off, 2.5 min, 40% amplitude). The lysate was then centrifuged (10 min, 14,000 rpm, 4 ^{o}C) and the PsaA-containing supernatant was transferred to a glass beaker on ice. The lysis process was repeated on the remaining pellet with a second 50-mL aliquot of lysis buffer B. The supernatant fractions were combined and dialyzed (Spectra/Por 3,500 kDa MWCO) in 4 L of 20 mM HEPES, pH 8.0 at 4 °C (3 x ≈12-24 h).

The dialyzed protein solution was centrifuged (10 min, 14,000 rpm, 4 ^{o}C) and filtered (0.2 μm). A GE ÄKTA Purifier housed in a 4 ^{o}C room was employed for ion exchange and size exclusion chromatographic purification. PsaA was first purified by anion exchange

chromatography. A MonoQ 10/100 anion–exchange column was used with elution buffers composed of 20 mM HEPES, pH 8.0 without (buffer A) or with (buffer B) 1 M NaCl. PsaA was eluted with 0–30% buffer B over 15 column volumes with a flow rate of 2 mL/min. Two major peaks eluted, and SDS-PAGE revealed that both peaks contained PsaA. ICP-MS of the corresponding fractions indicated that one peak corresponded to apo PsaA whereas the other corresponded to Zn(II)-bound PsaA. All of the PsaA-containing fractions were combined, concentrated to ≈10 mL, and loaded onto a Hiload 26/600 Superdex-75 gel filtration column pre-equilibrated with 75 mM HEPES, 100 mM NaCl, pH 7.5. The fractions containing PsaA were transferred to a dialysis bag (Spectra/Pro 3,500 kDa MWCO) and dialyzed in 4 L of de-metalating buffer (100 mM acetic acid, 20 mM EDTA, pH 3.7) at room temperature (2 x ≈12-24 h). The PsaA solution was then dialyzed (Spectra/Por 3,500 kDa MWCO) once against the storage buffer (20 mM HEPES, 100 mM NaCl, pH 7.5) in a 4 °C cold room (≈12 hours) before it was filtered (0.2 μm), concentrated by centrifugation (Pall, 15 mL spin filter, 10 kDa MWCO) aliquoted, frozen in liquid nitrogen, and stored at -80 °C. The yield of protein was typically ≈100 mg / 2 L of culture.

Circular Dichroism Spectroscopy. A Jasco J-1500 circular dichroism spectrometer housed in the Biophysical Instrumentation Facility at MIT was used for all measurements. Proteins were thawed on ice and buffer-exchanged into 1 mM Tris-HCl, pH 7.5 using 10 kDa MWCO Amicon spin concentrators. CD samples (300 μL) were prepared using 10-41 μM protein with and without 1.0 equiv of Mn(II). Samples were transferred to a nitric acid-washed Hellma quartz cuvette (1-mm path length) for data collection. CD spectra were recorded from 195 to 260 nm using continuous scan mode (50 nm/min) and a 1-nm bandwidth. All data represent averages of three replicate baseline-subtracted scans, where the baseline scan was obtained from the buffer.

Liquid-Chromatography Mass-Spectrometry (LC-MS). An Agilent 1290 series LC system with an Agilent Jetstream ESI source and Agilent 6230 TOF system was utilized for protein mass spectrometry. An Agilent Poroshell 300SB-C18 column (5-μm pore size) was used for gradient elution (60–85% B over 13 min at 0.2 mL/min) with 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). Protein samples were thawed and diluted in Milli-Q water to a final concentration of ≈30-40 μM, and 10 μL was injected. The Agilent MassHunter Workstation Data Acquisition Software was used with the Agilent MassHunter Qualitative Analysis program for analysis.

Metal Analysis by Inductively-Coupled Plasma Mass Spectrometry (ICP-MS). An Agilent 7900 instrument outfitted with an auto-sampler housed in the Center for Environmental Health Sciences (CEHS) Bioanalytical Core Facility at MIT was employed for all ICP-MS. Metal ion concentrations were analyzed in He mode. The spectrometer was calibrated immediately prior to each sample analysis using an Environmental Calibration Standard mix (Agilent) serially diluted in ≈3% nitric acid. All standards and samples were spiked with internal standard (2 ppb Tb, Agilent) to monitor for sample effects. For analysis of the metal content in PsaA and MntC, samples were prepared by diluting a protein stock solution ≥35-fold in ≈3% nitric acid to afford a final concentration of 7-20 μM. For analysis of the metal content in samples obtained from the B-CP pull-down assay (*vide infra*), 200 μL of B-CP assay flow-through was diluted with 1,200 μL of ≈3% nitric acid, and 28 μL of Tb internal standard (2 ppb) was added. In all cases, ICP-MS samples were centrifuged (10 min, 13,000 rpm, 4 ºC) as a precaution to remove any particulates or precipitated material prior to analysis.

Mn(II) Competition Titrations with ZP1. Mn(II) competition titrations between the fluorescent metal sensor ZP1 and either MntC or PsaA were performed as described previously

for CP variants.[4] In brief, aliquots of MntC and PsaA were thawed and subsequently buffer-exchanged into titration buffer (75 mM HEPES, 100 mM NaCl, pH 7.0) using 0.5-mL Amicon spin filters (10 kDa MWCO). For each titration, a 2-mL solution of titration buffer containing ZP1 (1 μM) and either MntC (4 μM) or PsaA (4 μM) was prepared in a 1-cm path length nitric acid-washed quartz cuvette. The mixture was titrated with Mn(II) (2 or 4 μL of a 500-μM Mn(II) solution in Milli-Q water). After each Mn(II) addition, the solution was gently mixed and incubated for ≥4 min at room temperature prior to recording the fluorescence emission.

Emission spectra were collected on a Photon Technologies International QuantaMaster 40 fluorimeter outfitted with a continuous xenon source for excitation, autocalibrated QuadraScopic monochromators, a multimode PMT detector, and a circulating water bath maintained at 25 °C. This instrument was controlled by the FelixGX software package. The excitation wavelength was 490 nm. The emission spectra were collected and integrated over 500-650 nm.

Mn(II) Competition Assay Monitored by B-CP Pull-Down. To examine Mn(II) competition between CP and either MntC or PsaA, 300-μL solutions containing 20 μM MntC or PsaA, 20 μM B-CP, and 18 μM Mn(II) were prepared with or without 400 μM Ca(II) in plastic microcentrifuge tubes (75 mM HEPES, pH 7.5, 100 mM NaCl). In each case, Mn(II) was added last and the solutions were gently mixed by using a pipetman, capped, and incubated at room temperature for 10 h, at which time pull-down with streptavidin resin was performed. Immediately prior to use, streptavidin resin (Thermo Scientific, 200 μL) was transferred to a spin-X centrifuge tube with a 0.22-μm cellulose acetate filter (Corning) and washed. To wash the resin, 500 μL of buffer (75 mM HEPES, pH 7.5, 100 mM NaCl) was added, the sample was centrifuged (2 min, 13,000 rpm, 4 °C), and the flow-through was discarded. The resin was washed three times in this manner before application of the protein mixture. The samples (300

μL) were transferred to the filters containing the washed resin, and the filters were capped and mixed by inversion on a tube rotator for 45 min at room temperature. The samples were then centrifuged (3 min, 13,000 rpm, 4 °C) to separate the B-CP-containing resin and SBP-containing flow-through. Aliquots (200 μL) of the flow-through were prepared for ICP-MS as described above. The flow-through was also analyzed by SDS-PAGE. Samples for which the pull-down was not performed were prepared for ICP-MS analysis in the same manner to measure the total Mn(II) concentration in the starting mixtures.

EPR Sample Preparation of Mn(II)-MntC, Mn(II)-PsaA. MntC and PsaA were buffer-exchanged into 75 mM HEPES, 100 mM NaCl, pH 7.5. Protein concentrations were determined by absorbance at 280 nm (ε_{280} = 35,870 M^{-1} cm^{-1} for MntC and PsaA, calculated using the ProParam tool available at http://web.expasy.org/protparam/). To prepare 0.75:1 Mn(II)-MntC and Mn(II)-PsaA samples, 750 μM Mn(II) (1.6 μL from a 100 mM working solution in Milli-Q water) was added to MntC or PsaA (1 mM protein, 210 μL). The samples were incubated for 15 min before a 200-μL portion was transferred to an SQ EPR tube (≈4x3 mm OD x ID) and frozen in liquid nitrogen. We also examined the effect of PEG-200 on the Mn(II) EPR spectra of Mn(II)-MntC and Mn(II)-PsaA. Samples without PEG-200 contained 750 μM Mn(II) and 1 mM SBP (75 mM HEPES, 100 mM NaCl, pH 7.5). Samples with 20% PEG-200 contain 750 μM Mn(II) and 1 mM SBP for the Mn(II)-PsaA sample or 500 μM Mn(II) and 1.1 mM protein for the Mn(II)-MntC sample (75 mM HEPES, 100 mM NaCl, pH 7.5, 20% PEG-200 (v/v)). A comparison of the Mn(II) EPR spectra of samples prepared with and without PEG-200 showed that the presence of PEG-200 affected the signal for each protein (Figure S8). As a result, all EPR spectroscopy studies were performed without PEG in the sample conditions. The samples were shipped to the University of California Davis in a liquid-nitrogen cooled shipping dewar for EPR spectroscopic analysis.

Mn(II) Transfer Assay Monitored by EPR Spectroscopy. To investigate Mn(II) transfer from Mn(II)-MntC or Mn(II)-PsaA to CP-Ser, end-point and time-course experiments were performed.

For the end-point experiments, solutions of CP-Ser (1 mM) were prepared with or without 10 equivalents of Ca(II) (10 mM from a 1-M stock solution) in 75 mM HEPES, 100 mM NaCl, pH 7.5. This Ca(II):CP-Ser ratio was selected on the basis of prior EPR spectroscopic investigations of Mn(II)-CP.[4] Solutions containing 1 mM SBP and 1 mM Mn(II) were prepared in 75 mM HEPES, 100 mM NaCl, pH 7.5 and incubated for 15 min at room temperature. Subsequently, a 90 μL portion of the CP-Ser solution was added to 90 μL of the Mn(II)-SBP solution, which afforded 180-μL mixture containing 500 μM CP-Ser, 500 Mn(II)-SBP with or without 5 mM Ca(II). The solutions were gently mixed with a pipetman and incubated for 10 h at room temperature. Then, 170 μL of each solution was transferred to a PQ EPR tube (Wilmad-706-PQ-9.50; 3.8 x 2.8 mm OD x ID) and frozen in liquid nitrogen. This experiment was performed twice and representative spectra from one trial are presented. No discernible spectral differences were detected between the two trials.

For the time course experiments, solutions (≈1.5 mL) containing 1 mM CP-Ser and 10 mM Ca(II) were was prepared in 75 mM HEPES, 100 mM NaCl, pH 7.5 as described above. Solutions (360 μL) containing 1 mM SBP and 1 mM Mn(II) were prepared in 75 mM HEPES, 100 mM NaCl, pH 7.5 and incubated for 15 min at room temperature. A 360-μL portion of the CP-Ser solution was then added to the Mn(II)-SBP solution to afford a 720-μL mixture containing 500 μM CP-Ser, 500 μM Mn(II)-SBP, and 5 mM Ca(II). The solution was mixed for ≈10 sec with a pipetman and a 170-μL aliquot was transferred to a PQ EPR tube. At t = 30 sec after addition of the CP-Ser solution, this sample was frozen in liquid nitrogen. The remaining assay solution was incubated at room temperature, and additional 170-μL aliquots were taken, transferred to PQ EPR tubes, and frozen in liquid nitrogen at t = 5, 10, and 30 min. After a

sample was frozen, the next aliquot was added to a PQ EPR tube and frozen at the indicated time point. In each case, freezing of an EPR sample began at the indicated time point and took ≈40 seconds to freeze completely. The samples were shipped to the University of California Davis in a liquid-nitrogen cooled shipping dewar for EPR spectroscopic analysis. Following optimization, this time course experiment was performed in duplicate and spectra from one representative trial are presented. No discernible spectral differences were detected between the two trials.

EPR Spectroscopy. All low-temperature continuous-wave (CW) electron paramagnetic resonance (EPR) spectra were collected on a Bruker E500 spectrometer (Billerica, Ma) equipped with a super-high QE (SHQE) resonator and an Oxford Instruments ESR900 cryostat. A data point was collected every 0.34 mT (1 mT = 10 G) with a conversion time of 80 ms and 0.5 mT of field modulation at 100 kHz. The microwave power was 200 μW and the frequency was 9.4 GHz. All spectra presented were collected under slow passage conditions at 10 K.

Antimicrobial Activity Assay. The growth inhibitory activity of B-CP against *Escherichia coli* ATCC 25922 was evaluated using a reported protocol. The antimicrobial activity assay medium was a 32:68 (v/v) mixture of tryptic soy broth (TSB) and antimicrobial assay buffer (20 mM Tris-HCl, 100 mM NaCl, pH 7.5 supplemented with 3 mM Ca(II) and 5 mM β-mercaptoethanol).

Table S1. Metal content of SBP samples at stages during representative protein purifications.

Metal	*MntC Purification Steps*			*PsaA Purification Steps*		
	Dialysis[a] (4.9 μM)	**Post-IEX[b] (5.4 μM)**	**Post-SEC[c] (4.9 μM)**	**Dialysis[a] (12.4 μM)**	**Post-IEX[b] (6.7 μM)**	**Post-SEC[c]** *(n.d.[d])*
[Mn] (μM)	0.029	0.067	0.064	0.059	0.101	*n.d.[d]*
equivalents	0.006	0.012	0.013	0.005	0.015	*n.d.[d].*
[Fe] (μM)	0.324	0.223	0.150	0.574	0.939	*n.d.[d].*
equivalents	0.066	0.041	0.031	0.046	0.141	*n.d.[d].*
[Co] (μM)	0.001	0.001	0.001	0.001	0.001	*n.d.[d].*
equivalents	0.000	0.000	0.000	0.000	0.000	*n.d.[d].*
[Ni] (μM)	0.112	0.108	0.101	0.021	0.002	*n.d.[d].*
equivalents	0.023	0.02	0.021	0.002	0.003	*n.d.[d].*
[Cu] (μM)	0.008	0.006	0.007	0.008	0.008	*n.d.[d].*
equivalents	0.002	0.001	0.001	0.001	0.001	*n.d.[d].*
[Zn] (μM)	0.169	0.878	0.872	0.718	2.19	*n.d.[d].*
equivalents	0.034	0.163	0.178	0.058	0.329	*n.d.[d].*

[a]Metal analysis (ICP-MS) was performed after lysis and dialysis into the IEX buffer before column purification. [b] Metal analysis (ICP-MS) was performed after ion-exchange chromatography (IEX). [c] Metal analysis (ICP-MS) was performed size-exclusion chromatography (SEC). [d] Not determined.

Table S2. Metal content of representative purified SBPs.[a]

Metal	Protein	
	MntC (8 μM)	**PsaA (19.5 μM)**
[Mn] (μM)	0.004	0.011
equivalents	0.001	0.001
[Fe] (μM)	0.096	0.167
equivalents	0.012	0.009
[Co] (μM)	0.0004	0.0003
equivalents	0.000	0.000
[Ni] (μM)	0.036	0.102
equivalents	0.004	0.005
[Cu] (μM)	0.005	0.006
equivalents	0.001	0.000
[Zn] (μM)	0.184	0.137
equivalents	0.023	0.007

[a] Metal content was determined by ICP-MS.

Table S3. Summary of Results from Protein Mass Spectrometry.[a]

Protein	Calculated Mass ± ^{N}Met (Da)[b]	Observed Mass (Da)[c]
MntC$^{19-309}$	32967.33 32836.13 (-Met1)[d]	n.f.[e] 32837.99
PsaA$^{21-309}$	32596.75 32465.55 (-Met1)[d]	n.f.[e] 32466.17
B-CP (S100A8(C42S) subunit)	10,818.4	10,818.93
B-CP (biotinylated S100A9 subunit)	13,525.3 (-Met1)[d]	13,525.64

[a] A denaturing protocol on an Agilent Poroshell 300SB-C18 column over a 60-85% gradient of acetonitrile in 0.1 % formic acid was utilized for LC-MS. [b] Molecular weights were calculated by using the ProtParam tool found on the ExPASy site (http://web.expasy.org/protparam). [c] Masses were calculated with the Agilent MassHunter BioConfirm software package. [d] The N-terminal methionine can be cleaved during overexpression in *E. coli*. [e] Not found; the mass was not found following deconvolution of the raw data.

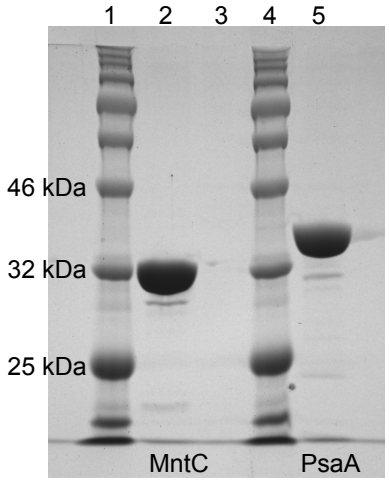

Figure S1. SDS-PAGE gel (12% Tris-glycine) of purified MntC and PsaA. Lanes: (1) ladder P7712S (New England Biolabs), (2) MntC, (3) empty lane, (4) ladder P7712S (New England Biolabs), (5) PsaA. The SBPs were overexpressed as MntC$^{19\text{-}309}$ (32.8 kDa) and PsaA$^{21\text{-}309}$ (32.5 kDa) to achieve soluble protein. The N-terminal region of each full-length protein contains a hydrophobic lipid anchor.

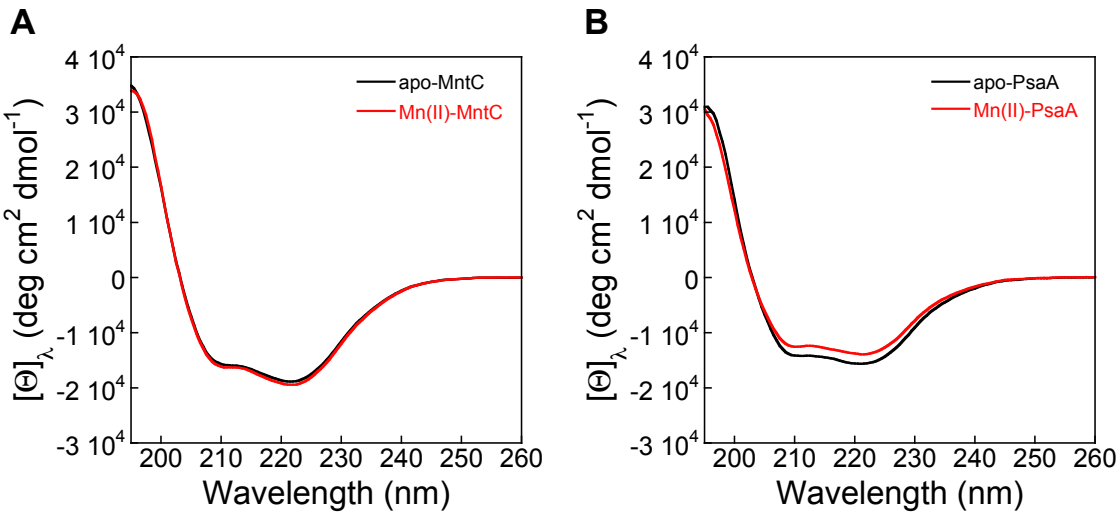

Figure S2. Circular dichroism spectra of 10 μM MntC and 40 μM PsaA with no metal added (black) or 1 equivalent of Mn(II) added (red) in 1 mM Tris-HCl, pH 7.5.

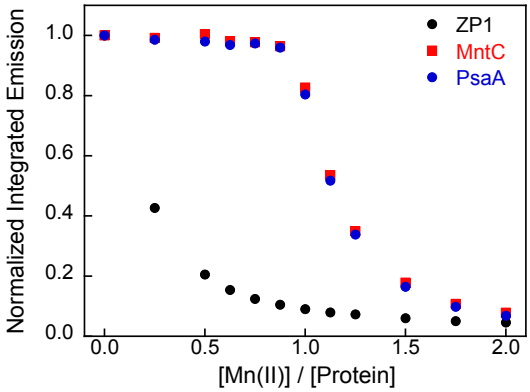

Figure S3. Representative Mn(II) competition titrations of ZP1 (1 μM) and MntC (4 μM) or PsaA (4 μM) in 75 mM HEPES, 100 mM NaCl, pH 7.0. Black circles: titration of ZP1 with Mn(II) in the absence of a SBP; red squares: titration of ZP1 and MntC with Mn(II); blue circles: titration of ZP1 and PsaA with Mn(II). For ZP1, the reported apparent $K_{d1,Mn(II)}$ is 550 nM at pH 7.0.[5]

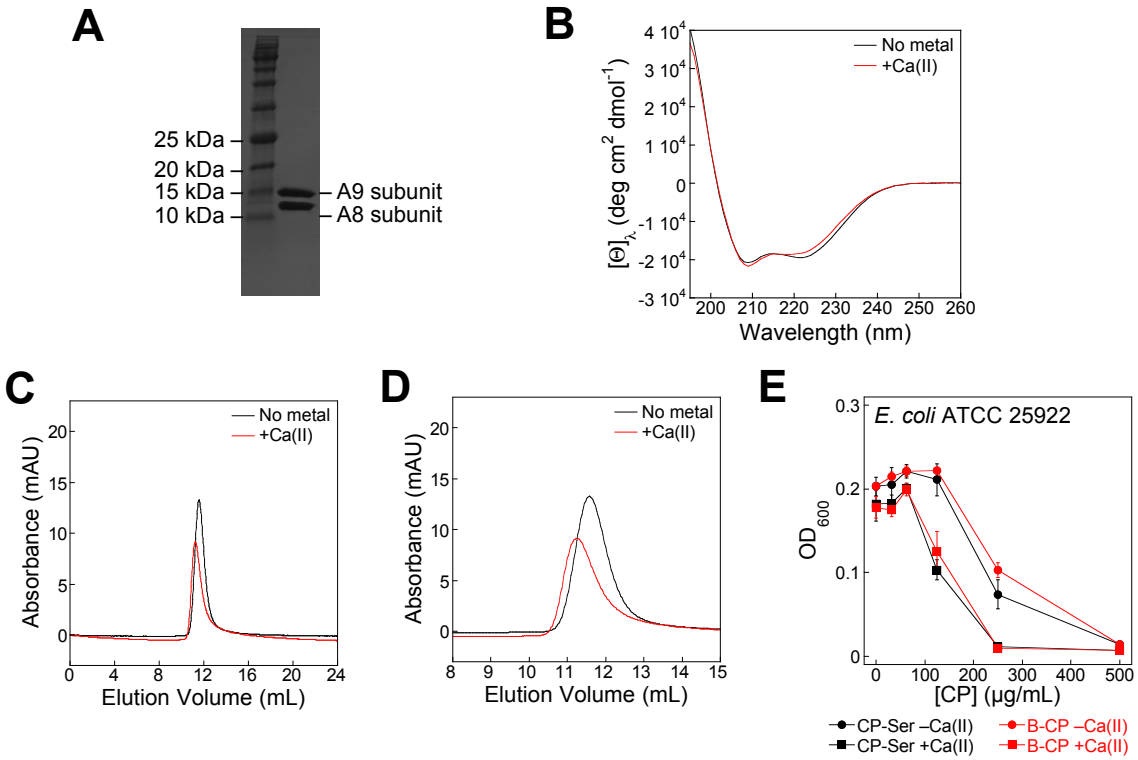

Figure S4. Characterization of B-CP. (A) SDS-PAGE gel (15% Tris-glycine) of purified B-CP with the P7704 protein ladder (New England BioLabs). (B) Circular dichroism spectra of 10 μM B-CP in the absence and presence of 2 mM Ca(II) in 1.0 mM Tris-HCl, pH 7.5. (C) Analytical size-exclusion chromatography of 20 μM B-CP in the absence and presence of 2 mM Ca(II) in 75 mM HEPES, 100 mM NaCl, pH 7.0. B-CP elutes at ≈11.7 mL (–Ca(II)) and ≈11.1 mL (+Ca(II)), and these peak elution volumes correspond to the αβ heterodimer and $\alpha_2\beta_2$ heterotetramer, respectively.[2,3] (D) Zoom-in view of analytical SEC chromatograms presented in panel D. (E) Antimicrobial activity of CP-Ser (positive control) and B-CP against *E. coli* ATCC 25922. The mean OD_{600} values and SEM are shown (n = 3; t = 20 h, T = 30 ºC, 150 rpm).

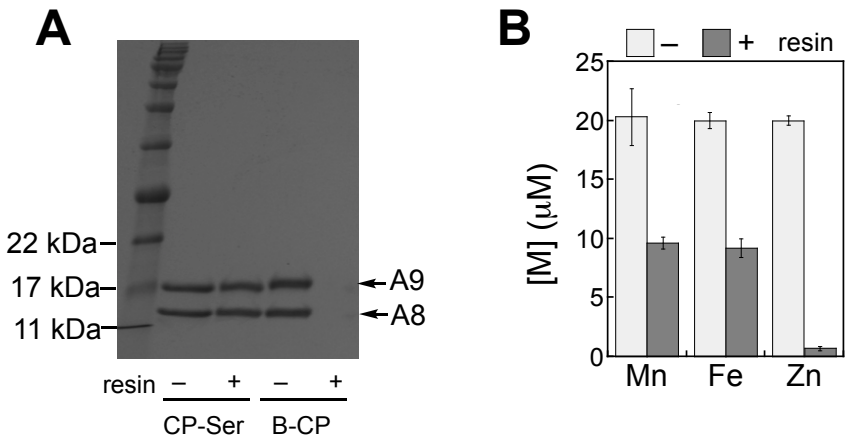

Figure S5. B-CP binds to streptavidin agarose resin and complexes transition metals. (A) SDS-PAGE gel (15% Tris-glycine) of 10 μM CP-Ser and B-CP in 75 mM HEPES, 100 mM NaCl, pH 7.0 before and after pull-down with streptavidin agrose resin. (B) B-CP (10 μM) was pre-incubated with 20 μM Mn(II), 20 μM Fe(II) or 20 μM Zn(II) and each mixture was treated with streptavidin agarose resin (75 mM HEPES, 100 mM NaCl, pH 7.0, 2 mM Ca(II)). The metal content of each solution was measured before (light gray bars) and after (dark gray bars) pull-down with streptavidin agarose resin by ICP-MS.

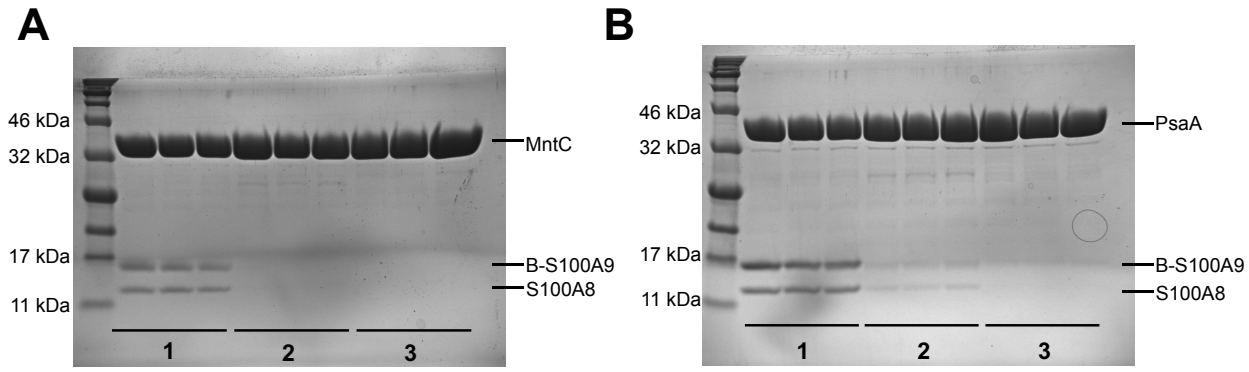

Figure S6. SDS-PAGE (15% Tris-glycine) analysis of B-CP pull-down assays. (A) The gel corresponds to the competition between MntC and B-CP for Mn(II). (B) The gel corresponds to the competition between PsaA and B-CP for Mn(II). The samples labeled as 1-3 are from mixtures of B-CP, SBP and Mn(II) (1) prior to pull-down, (2) the soluble portion after pull-down with streptavidin agarose resin for experiments performed without added Ca(II), and (3) the soluble portion after pull-down with streptavidin agarose resin for experiments performed in the presence of added Ca(II). The three lanes per type of sample correspond to three independent replicates. The results from metal analysis are presented in Figure 1 of the main text.

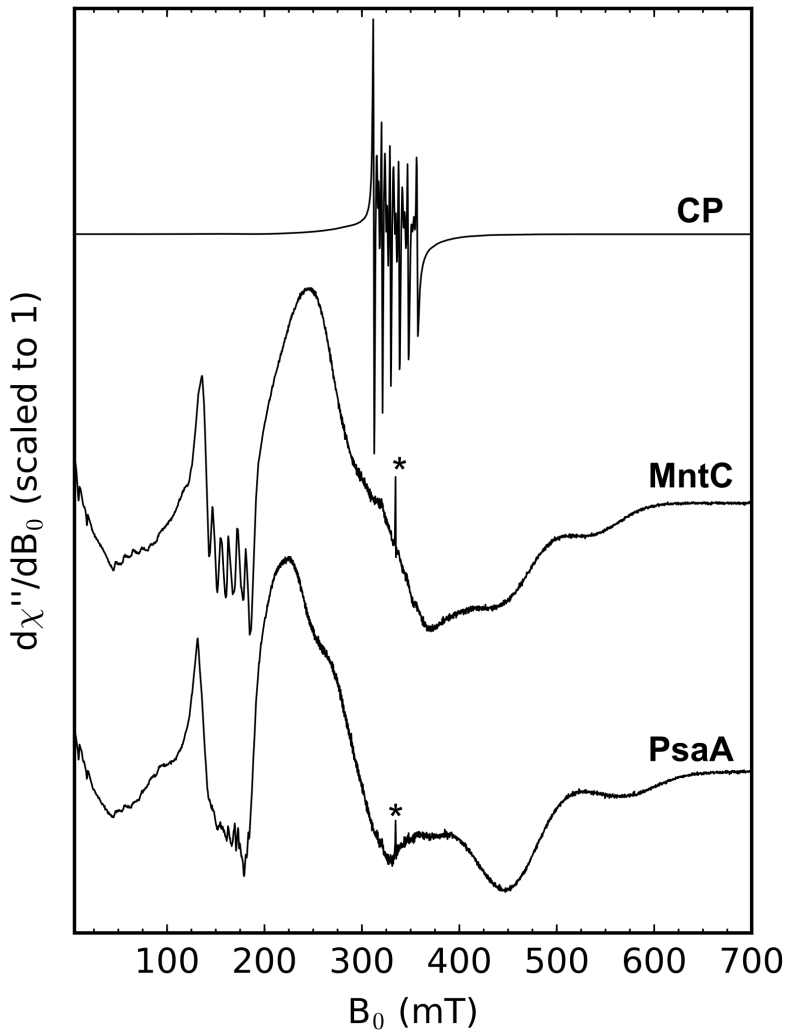

Figure S7. Comparison of X-band Mn(II) EPR spectra of Mn(II)-bound CP-Ser in the presence of 10 equivalents of Ca(II), Mn(II)-bound MntC, and Mn(II)-bound PsaA (75 mM HEPES, 100 mM NaCl, pH 7.5). The MntC and PsaA spectra correspond to the spectra shown in Figure 2 of the main text. The asterisk denotes a quartz background radical. For the Mn(II)-CP-Ser spectrum, the sample contained 600 μM CP-Ser, 500 μM Mn(II), and 6 mM Ca(II). Spectrometer settings: ν_{mw} = 9.4 GHz, 0.5 mT modulation amplitude at 100 kHz, power = 200 μW, temperature = 10 K.

This presentation of the spectra shows that the Mn(II) EPR signals for CP-Ser in the *g* = 2 region (≈335 mT) are readily distinguishable from the Mn(II) EPR signals of MntC and PsaA in the *g* = 4 region (≈150 mT).

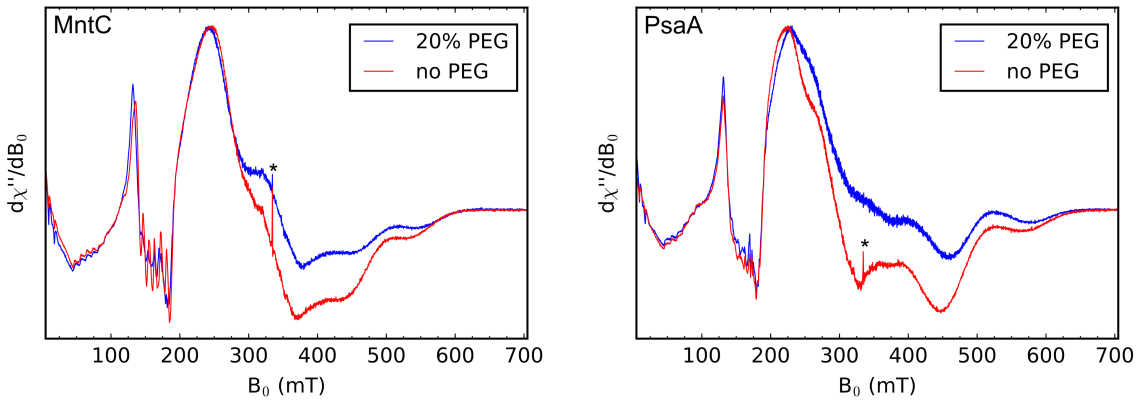

Figure S8. EPR spectra of Mn(II)-MntC (left) and Mn(II)-PsaA (right) with and without 20% (v/v) PEG-200 in the sample. Samples without PEG-200 contain 750 μM Mn(II) and 1 mM protein (75 mM HEPES, 100 mM NaCl, pH 7.5). Samples with 20% PEG-200 contain 500 μM Mn(II) and 1.1 mM protein for Mn(II)-MntC or 750 μM Mn(II) and 1.0 mM protein for Mn(I)-PsaA (75 mM HEPES, 100 mM NaCl, pH 7.5, 20% PEG-200 (v/v). The asterisk denotes a quartz background signal. Spectrometer settings: ν_{mw} = 9.4 GHz, 0.5 mT modulation amplitude at 100 kHz, power = 200 μW, temperature = 10 K.

These spectra demonstrate that caution should be exercised when using glassing agent with the SBPs. We elected to not use any glassing agent in the low-temperature EPR experiments presented in this work.

Supporting References

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