Supporting Information

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SI Materials and Methods.

Strains and Plasmids. Primers containing EcoRI and XbaI restriction sites (Table S1, primers 1 and 2 for *mtrA*; primers 3 and 4 for *mtrCAB*), and Platinum Pfx Polymerase (Invitrogen) were used to amplify the respective sequences. After digestion with EcoRI and XbaI, these DNA fragments were ligated into the modified pET30a+ vector using T4 DNA Ligase (Roche). The modified pET30a+ vector had an NdeI site (CATATG) directly upstream of the EcoRI site used for insertion. The ATG in the NdeI site was used as the start codon, and the forward primers for PCR were designed to clone from the second codon of *mtrA and mtrC* in the Shewanella genome. This design adds the two codons from the EcoRI site coding Glu-Phe; however, the extra amino acids will be cleaved by the Sec system in the process signal sequence cleavage of MtrA and MtrC in plasmids *mtrA and mtrCAB*. respectively. The *ccm* plasmid, pEC86, encodes the genes *ccmA-H* under a tet promoter and carries a chloramphenicol resistance marker.

Construction of *napC* Deletion Strain. The plasmids used in the gene disruption process, pKD46, pKD3, pKD4, and pCP20, were obtained from the Coli Genetic Stock Center at Yale University. Primers 5 and 6 (Table S1) contain regions homologous to the region directly upstream and downstream, respectively. These primers were used to amplify a cassette containing kanamaycin resistance flanked by FLP recombinase recognition target sites off of pKD4 using Platinum Pfx Polymerase (Invitrogen). This PCR product was electroporated with BL21(DE3) expressing pKD46, induced with 10 mM L-arabinose, to replace *napC* in the *E. coli* BL21(DE3) genome. Cells were grown with antibiotic selection on kanamycin LB plates grown at 37 °C. Colony PCR using primers 7 and 8 (Table S1) was performed to verify the replacement in the genome. Kanamycin resistance was removed by electroporating the new strain with pCP20, which removes the sequence between the FRT sites. After pCP20 inactivation, colony PCR was performed using primers 7 and 8 to verify removal of the kanamycin resistance gene. The resulting strain is BL21 (DE3)Δ*napC*.

Cell Growth. All strains, unless otherwise specified, were grown in 2xYT media at 30 °C with 50 μg mL^{-1} kanamycin; strains containing the *ccm* plasmid were grown with an additional 30 μg mL^{-1} chloramphenicol. Glycerol stocks were used to inoculate 5 mL media, and cultures were grown overnight at 37 °C with 250-rpm shaking. Then 50 μL of overnight cultures were back-diluted into 5 mL media and grown with 250-rpm shaking for 12 h. For Fe(III) reduction assays, 50 mL of fresh 2xYT was inoculated with 250 μL of the previous culture and then grown 16 h with 200-rpm shaking. For periplasmic and membrane fractionations, 5 mL of overnight culture were used to inoculate 1 L media and were grown for 16 h.

Periplasmic Fractionation. The cells from a 1-L culture were pelleted by centrifugation for 15 min at 4,000 × *g* and 4 °C. The resulting cell pellet was slowly resuspended to homogeneity in 30 mL of ice-cold *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid, pH 8.0, by pressing the cells with a rubber policeman on the side of the flask. Chicken egg white lysozyme (Sigma) was added to the resuspended pellet to a final concentration 0.5 mg mL^{-1} and incubated at room temperature for 15 min. After addition of 30 mL of ice-cold water, the suspension was shaken horizontally on ice for 15 min at 100 rpm and then centrifuged at 12,000 × *g* for 20 min at 4 °C. The supernatant was collected as the periplasmic fraction while the outer membrane and intact cytoplasm was in the pellet. The periplasmic fraction was analyzed by SDS-PAGE heme-stained gels and by UV-Vis for redox spectral properties.

Membrane Fractionation. The cells from a 1-L culture were pelleted by centrifugation for 15 min at 4,000 × *g* and 4 °C and then washed in 1 L 10 mM Hepes, pH 7.4. The washed pellet was then resuspended in 120 mL ice-cold 10 mM Hepes, pH 7.4. Chicken egg white lysozyme was added to the cell suspension to a final concentration of 20 μg mL^{-1} and incubated at room temperature for 30 min. The protease inhibitor PMSF (Thermo Scientific) was added to a final concentration of 1 mM. The cells were disrupted via ultrasonication (power level 5, 9 min total duty in cycles of 30 s on, 30 s off, Misonix 3000, Misonix Inc.) in an ice bath. Unbroken cells were removed by centrifugation at 1,000 × *g* for 15 min, and the resulting supernatant was removed and centrifuged at 100,000 × *g* for 2 h at 4 °C to yield a pellet corresponding to the crude cell envelope, containing both the outer and inner membrane. This membrane fraction was solubilized in a solution of 5% (wt/vol) Triton X-100, 50 mM Hepes pH 7.4, 200 mM NaCl before analysis by SDS-PAGE, Western blotting, and UV-Vis spectroscopy. The supernatant of this spin was saved for analysis by SDS-PAGE.

TMBZ Peroxidase Stain of SDS-PAGE. The 3,3′,5,5′-tetramethylbenzidene (TMBZ) peroxidase stain method was adapted from Thomas (1) to identify cytochromes c. Protein samples were suspended in lithium dodecyl sulfate without β-mercaptoethanol. The samples were run in 12.5% Tris HCl polyacrylamide gel (BioRad) at 16 °C at 200 V for 60 min. TMBZ was dissolved in methanol to 6.3 mM and mixed 3:7 TMBZ solution:0.25 M Sodium acetate, pH 5. The gel was immersed in this mixture in the dark with occasional mixing for 2 h. Hydrogen peroxide was added to a final concentration of 30 mM, and bands were visualized 30 min after peroxide addition.

MtrB Western. Denatured membrane protein samples were electrophoresed in 12.5% polyacrylamide gel and transferred to nitrocellulose membranes. The primary antibody for MtrB (Rabbit Anti-MtrB) was kindly provided by Prof. Daad Saffarini (University of Wisconsin–Madison) and used at 1:10,000 dilution. The Immun-Star Goat Anti-Rabbit-HRP Conjugate kit was used as the secondary antibody at 1:60,000 dilution. The Western and visualization of bands was done as per the Immun-Star WesternC Chemiluminescent Kit (BioRad).

Visible Spectra of Cytochrome Samples. Samples from the periplasmic fraction and membrane fractionation were diluted such that the absorbance was within the linear range. Visible spectra of air-exposed samples were considered as fully oxidized protein samples. The protein was chemically reduced by adding sodium dithionite crystals (Sigma), and the spectrum was taken again. Membrane fractions were baseline subtracted to consider the scattering of light caused by Triton X-100 micelles.

Iron Reduction Assay Using Ferrozine. Cells from 50-mL cultures were pelleted, washed, and resuspended to an OD_{600} of 0.5 in anaerobic M9 minimal media (12.8 g L^{-1} Na_2HPO_4-7H_2O, 3.0 g L^{-1} KH_2PO_4, 0.50 g L^{-1} NaCl, 1.0 g L^{-1} NH_4Cl) (Difco) supplemented with 0.4% lactate (Alfa Aesar), 1 mM thiamine

HCl (Sigma), 0.2% casamino acids (Merck), 2.0 mM $MgSO_4$ (Aldrich), and 0.1 mM $CaCl_2$ (Aldrich). One aliquot of each culture was centrifuged at 4,000 × *g* for 5 min at room temperature in the anaerobic chamber to pellet the cells, and the supernatant was acid extracted in 0.5 M HCl for 1 h. The total iron concentration was determined by a separate acid extraction with 10% hydroxylamine hydrochloride (Aldrich) in 0.5 M HCl for 1 h. An aliquot of each acid extracted sample was then added to the dye, ferrozine (Acros Organics) buffered in 100 mM Hepes, pH 8.0, which absorbs at 563 nm ($\varepsilon_{563\,nm} = 27.9\ mM^{-1}\ cm^{-1}$) when bound to Fe (II). The absorbance of all samples was recorded at 563 nm with a UV-Vis spectrophotometer (Perkin–Elmer Lambda 35) and was used to determine the change of Fe(II) concentration over time as well as monitor total iron concentration available in the media. The concentration of Fe(II) in each culture was subtracted by any abiotic iron reduction observed in media controls at each time point and was normalized to the relative number of cells by multiplying by the factor $\frac{0.5}{OD600_i}$. Error bars represent standard deviation by triplicate cultures.

Cytochrome c Redox Assay in Intact Cells. After apparent recovery of the redox state of the cytochrome, an additional 25 μM of Fe(III) citrate was added. The spectrum was monitored again with similar results. All spectra were normalized to the average absorbance at 561 nm, an approximate isobestic point. $\Delta A_{552\,nm}$ was calculated by subtracting the initial $A_{552\,nm}$ from all time points.

Synthesis of Fe_2O_3 Nanoparticles. Iron(III) chloride hexahydrate (544 mg, 2.01 mmol), sodium oleate (1.826 g, 6.00 mmol), oleic acid (1.0 mL, 3.14 mmol), absolute ethanol (5.0 mL), and H_2O (8.0 mL) were combined in a 20-mL microwave reaction vial and stirred for 1 h at 25 °C, giving an opaque, dark reddish-brown organic top layer and a clear, pale yellow aqueous bottom layer in the microwave vial. The reaction vessel was transferred to a microwave synthesizer (Biotage Initiator 8) and heated at 80 °C for 8 h, then at 180 °C for 15 min. The contents of the vessel were transferred to a 50-mL conical tube and centrifuged at 4,400 × *g* for 30 min, affording a dark reddish-brown pellet of nanocrystals. The liquid portion was decanted off and the pellet washed sequentially with H_2O (5.0 mL) and absolute ethanol (5.0 mL). The pellet was resuspended in hexanes (50 mL), and the tube centrifuged at 4,400 × *g* for 5 min., giving a clear, dark reddish-brown hexane solution containing smaller, soluble Fe_2O_3 nanoparticles, and a reddish-brown pellet containing larger, insoluble Fe_2O_3 nanoparticles and Fe_2O_3 nanoparticle aggregates. The hexanes solution was filtered through an Acrodisc Syringe Filter (0.22 μm, Pall Corporation), giving a clear, dark reddish-brown solution containing oleic acid-coated Fe_2O_3 nanoparticles. Dynamic light scattering (DLS) measurements (Malvern Instruments) performed in hexanes at 20 °C showed that the particles were narrowly distributed, with a mean diameter of 13 nm.

Fe_2O_3 nanoparticles were heated with citric acid (480 mg) in anhydrous DMSO (2.5 mL) at 100 °C for 24 h with stirring. The resulting solution was added slowly to 100 mL of 100 mM sodium tetraborate buffer, pH 10.0, with periodic adjustment of pH to 10 with NaOH (1.0 M), giving a clear, reddish-brown aqueous solution. The nanoparticles were concentrated by spin dialysis (Amicon Ultra-15 10 K MWCO, Millipore Corporation), and dialyzed with 5 × 15 mL of 10 mM sodium tetraborate buffer, pH 8.3. DLS measurements (Malvern Instruments) performed in buffer at 20 °C showed that the citrate-coated Fe_2O_3 particles were narrowly distributed, with a mean diameter of 13 nm.

Reduction of Bulk and Nano Fe_2O_3. At each time point, the colony forming units and Fe(II) concentration for each culture was measured. To determine colony forming units, dilutions of each culture were plated on LB plates supplemented with Kanamycin (to test for the presence of the *mtrA* or *mtrCAB* plasmids). Plates were grown at 30 °C for 24 h. The concentration of Fe(II) was determined by the ferrozine assay and was normalized for each culture by cfu. Error bars represent standard deviation by triplicate cultures.

1. Bose S, et al. (2009) Bioreduction of hematite nanoparticles by the dissimilatory iron reducing bacterium *Shewanella oneidensis* MR-1. *Geochim Cosmochim Acta* 73:962–976.

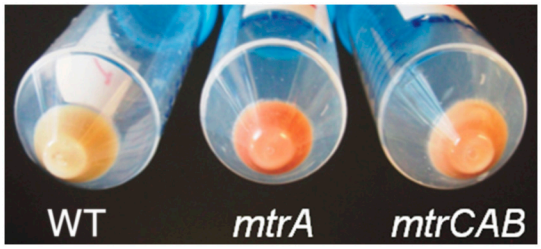

Fig. S1. Cell pellets of uninduced WT, *mtrA*, and *mtrCAB* strains. The red color is caused by the hemes. Interestingly, addition of as little as 10 μM IPTG to *mtrA* and *mtrCAB* cultures resulted in a less intense red color of the cell pellets and less intense MtrA and MtrC bands in the TMBZ stained whole cell extracts as compared to cultures containing no IPTG. Thus, basal expression of the *mtrA* and *mtrCAB* plasmids yields a higher concentration of correctly folded cytochrome c than induced expression.

Table S1. Primers

Primer no.	Sequence (5′–3′)
1	GGCCGAATTCAAGAACTGCCTAAAAATGAA
2	AACCTCTAGATTAGCGCTGTAATAGCTTGCCA
3	CCGCGAATTCATGAACGCACAAAAATCAAAAATCGC
4	CCGGTCTAGATTAGAGTTTGTAACTCATGCTC
5	CGCTCACAAAGTAACTCTCTGGCTTCAAGCATACCCACGCAATAACCCTG GTGTAGGCTGGAGCTGCTTC
6	TGGGGAATACCTTTACCCCATCAAAAGGTTACGGGAAATAAGAGGTTATT ATGGGAATTAGCCATGGTCC
7	CGCTCACAAAGTAACTCTCTGGCTT
8	TGGGGAATACCTTTACCCCATCAAA

Table S2. Plasmids

Plasmid	Promotor	Protein coding regions(s)	Antibiotic resistance	Source
pSB1ET2 (pET)	T7 lac	n/a	Kan	(This paper)
I5023	T7 lac	*mtrA*	Kan	(This paper)
I5024	T7 lac	*mtrCAB*	Kan	(This paper)
pEC86	Tet	*ccmA-H*	Chl	Steve Singer (Lawrence Berkeley National Lab, Berkeley, CA)
pKD46	P_{araB}	λ-red recombinase	Amp	Coli genetic stock
pKD3	NA	NA	Amp, Chl	Coli genetic stock
pKD4	NA	NA	Amp, Kan	Coli genetic stock
pCP20	NA	FLP	Amp	Coli genetic stock

NA, not applicable.

Table S3. Strains

Strain	Cell line	Plasmids	Gene(s)
wt	BL21(DE3)	pSB1ET2 empty vector	None
ccm	BL21(DE3)	pSB1ET2_empty + pEC86	*ccmA-H*
mtrA	BL21(DE3)	pSB1ET2_MtrA + pEC86	*mtrA, ccmA-H*
mtrCAB	BL21(DE3)	pSB1ET2_MtrCAB + pEC86	*mtrCAB, ccmA-H*
Δ*napC*	BL21(DE3)Δ*napC*	pSB1ET2 empty vector	None
Δ*napC ccm*	BL21(DE3)Δ*napC*	pSB1ET2_empty + pEC86	*ccmA-H*
Δ*napC mtrA*	BL21(DE3)Δ*napC*	pSB1ET2_MtrA + pEC86	*mtrA, ccmA-H*
Δ*napC mtrCAB*	BL21(DE3)Δ*napC*	pSB1ET2_MtrCAB + pEC86	*mtrCAB, ccmA-H*