Supplemental Information

Title: Biochemical mapping reveals a conserved heme transport mechanism via CcmCD in System I bacterial cytochrome *c* biogenesis

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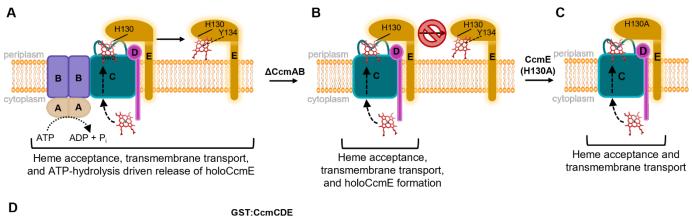
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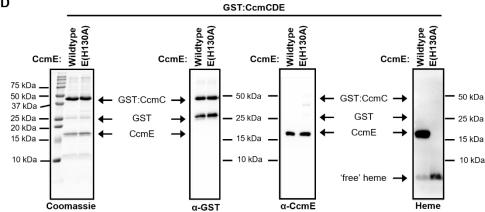
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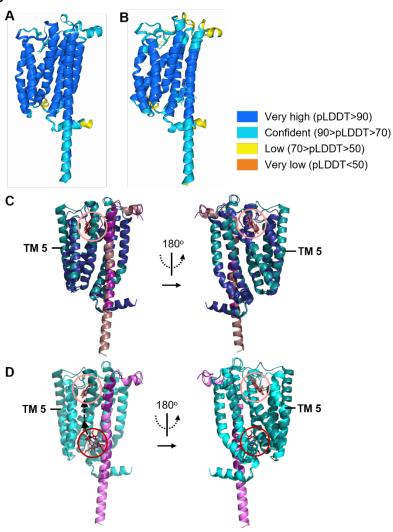
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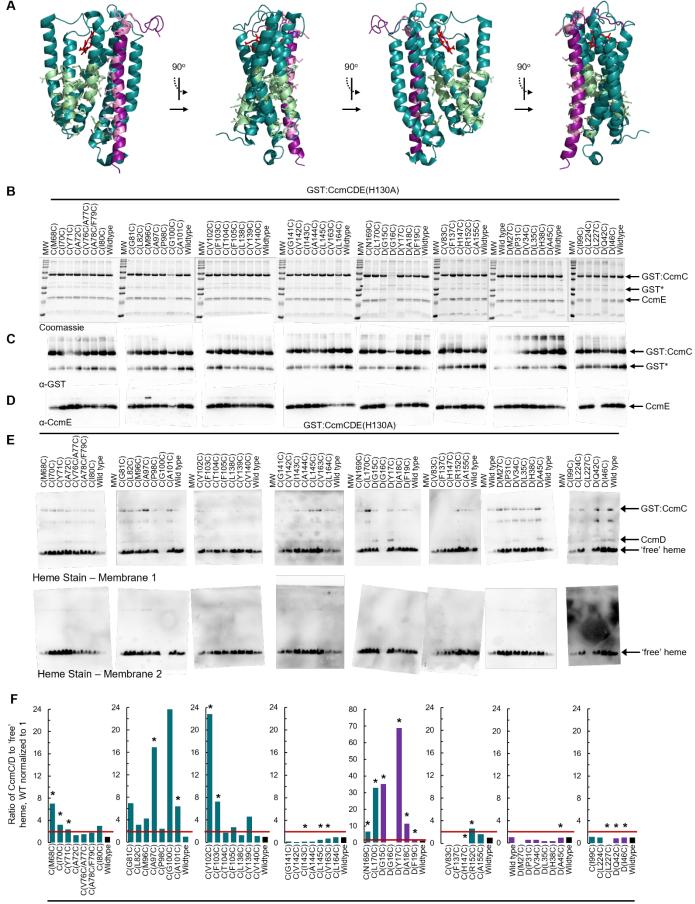


Supplemental Fig. 1. Known functions of CcmABCDE proteins in step 1 of System I. (A) CcmABCDE function as a protein subcomplex for System I. Heme is stereospecifically positioned in the CcmC WWD domain, covalently attached to CcmE at H130 and liganded by Y134. HoloCcmE is released from step 1 via ATP hydrolysis by CcmAB. (B) Deletion of CcmAB prevents release of holoCcmE. However, heme delivery to the CcmC WWD domain is still accomplished and holoCcmE formation occurs with only CcmCDE. (C) HoloCcmE covalent bond formation occurs at H130. Mutation of His130 to Ala inhibits holoCcmE formation, nonetheless heme is still delivered to the CcmC WWD domain. (D) GST:CcmCDE or GST:CcmCDE(H130A) were affinity purified, 5 µg affinity purified protein was separated by SDS-PAGE and assessed for protein stability via Coomassie stain and immunoblotted with antibodies for GST and CcmE. Heme co-purification was assayed via an ECL-based heme stain.

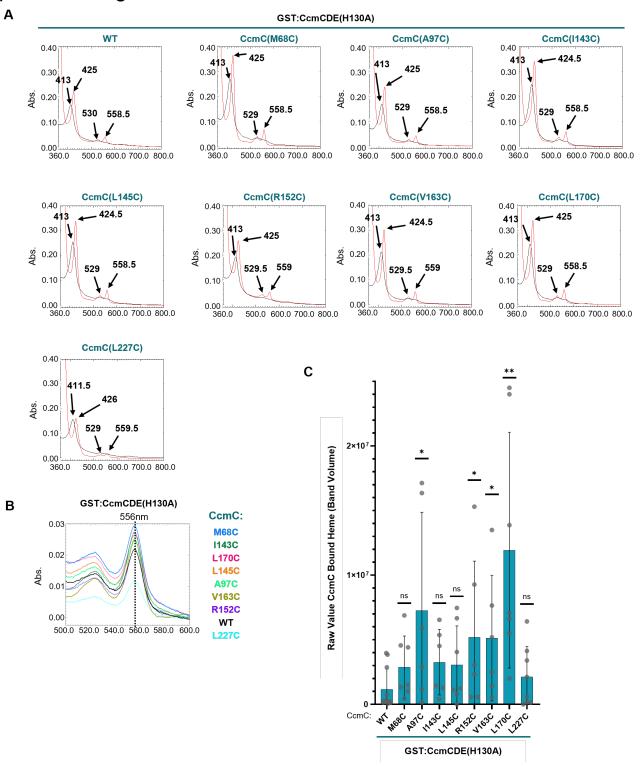


Supplemental Fig. 2. AlphaFold 3 heme placement correlates with Cryo-EM structures (A) AlphaFold 3 (AF3) *E. coli* CcmCD + 1 heme structural prediction confidence values pLDDT. (B) AF3 *E. coli* CcmCD + 2 heme structural prediction confidence values pLDDT. (C) AF3 structure of CcmCD (dark blue/dark pink) + 1 heme molecule (red; salmon circle) superimposed with CcmCD (PDB:7F04) cryo-EM structure (teal/violet); RMSD=0.754. (D) AF3 structure of CcmCD (cyan/light purple) + 2 heme molecules (red) superimposed with CcmCD (PDB:7F04) cryo-EM structure (teal/violet) displaying cytoplasmic heme acceptance domain (red circle), heme transport channel (black arrows), and WWD domain heme (red; salmon circle); RMSD=0.777.

Supplemental Fig. 3. Mechanism of cysteine/heme crosslinking between the cysteine thiol and heme vinyl. A single cysteine thiol is shown for simplicity, red arrows indicate a two electron transfer. The cysteine/heme crosslink is formed via a covalent thioether bond between the alpha carbon of heme and the reduced cysteine thiol. Figure is modified from ¹ Sutherland *et al.* 2018. Structurally Mapping Endogenous Heme in the CcmCDE Membrane Complex for Cytochrome c Biogenesis . Journal of Molecular Biology. 430(8):1065-1080.

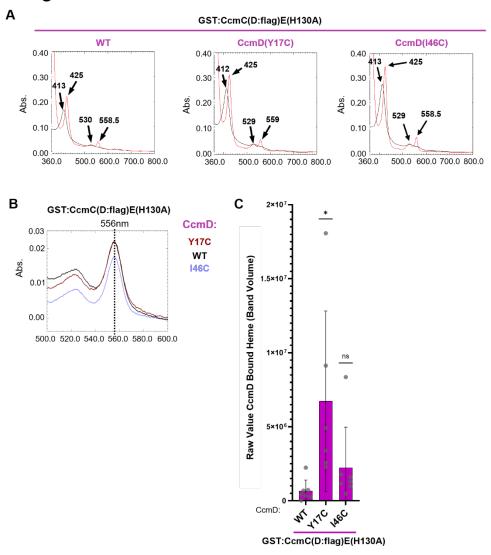


Supplemental Fig. 4. Analysis of fifty-one CcmC and CcmD cysteine variants. (A) CcmCD Cryo-EM structures (PDB:7F04) with cysteine variants indicated in light green (CcmC) or light pink (CcmD) with side chains displayed with sticks. Structure rotated around y-axis at 90° increments. (B-F) Cysteine variants were affinity purified in indicated groups. A wildtype was included in each group as an internal control. Data represents single initial screening of cysteine variants. (B-D) 5 μg of affinity purified protein was separated by SDS-PAGE and assessed for stability of protein complex via (B) coomassie total protein stain, (C) α-GST immunoblot, (D) α-CcmE immunoblot. (E) Heme stain. Free heme can transfer through the nitrocellulose membrane, therefore two membranes are layered to capture the total amount of *b*-type heme that co-purifies with GST:CcmCDE(H130A). (F) Quantitation of heme stain in Fig. S4E; representative of one independent biological replicate. Heme stains were quantified with AzureSpot Software (Azure, v.2.2.167) and the ratio of CcmC/D bound heme to 'free' heme was determined. The wildtype ratio was normalized to 1 and a ratio above 2 (red line) indicated formation of a cysteine/heme crosslink. Variants selected for additional study indicated with an *. Black bars – wild type; teal bars – CcmC; purple bars – CcmD.

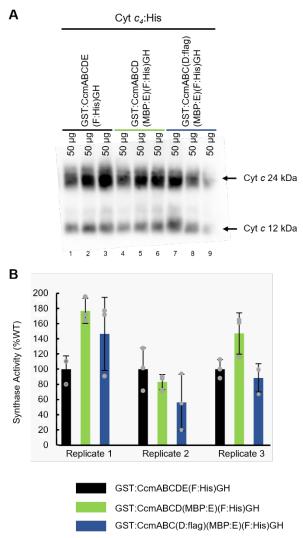


Supplemental Fig. 5. CcmC cysteine variants analysis. (A) UV-vis spectral analysis of as-purified (black) and sodium dithionite reduced (red) purified protein complex, $50 \mu g$ affinity purified protein. Wildtype and eight CcmC crosslinking cysteine variants individual UV-vis spectra. Data shown as representative of three independent affinity purifications (B) Pyridine hemochrome reduced spectra, $75 \mu g$ affinity purified protein. Wildtype and eight CcmC cysteine crosslinking spectra overlay. Black dotted line - $556 \mu g$ affinity purified protein. Single pyridine hemochrome assay was performed. (C) Formation of the cysteine/heme crosslink has historically used the ratio of cross-linked heme to free heme to account for the variability in cysteine/heme crosslink formation due to the transient nature of heme trafficking coupled with variability in heme co-purification with cytochrome c biogenesis proteins (see Fig. 2F). Here we complement this analysis with quantification of only the CcmC heme stained

polypeptide using a two-tailed unpaired t-test. Quantification of the heme stained GST:CcmC polypeptide from six independent purifications (grey dots). Teal bars represent the average GST:CcmC band intensity. Error bars indicate the standard deviation. Statistical analysis was performed with GraphPad Prism (v10.4.1) using a two-tailed unpaired t-test. * p < 0.05, ** p < 0.01, NS not statistically significant.

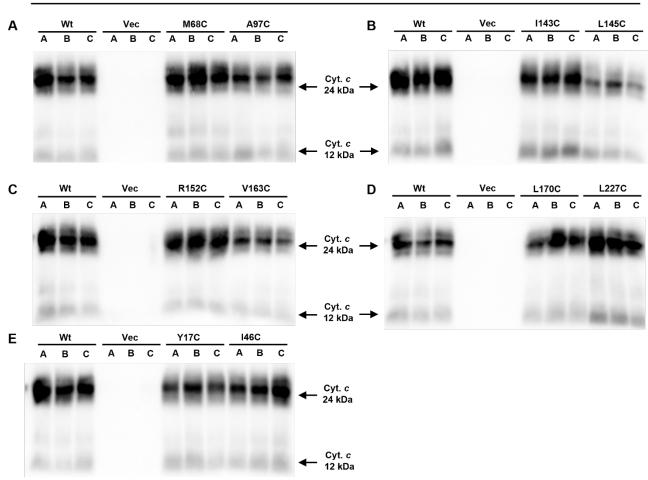


Supplemental Fig. 6. CcmD cysteine variants analysis. (A) UV-vis spectral analysis of as-purified (black) and sodium dithionite reduced (red) purified protein complex, 50 μ g affinity purified protein. Wildtype and two CcmD crosslinking cysteine variants individual UV-vis spectra. Data shown as a representative of three independent affinity purifications. (B) Pyridine hemochrome reduced spectra, 75 μ g affinity purified protein. Wildtype and two CcmD crosslinking spectra overlay. Black dotted line - 556 nm wildtype α -peak. Single pyridine hemochrome assay was performed. (C) Quantification of the heme stained CcmD:flag polypeptide as described for GST:CcmC in Fig. S5C. Violet bars represent the average CcmD:flag band intensity. Error bars indicate the standard deviation. Grey dots represent six independent purifications. Statistical analysis was performed with GraphPad Prism (v10.4.1) using a two-tailed unpaired t-test. * p < 0.05, ** p < 0.01, NS not statistically significant.

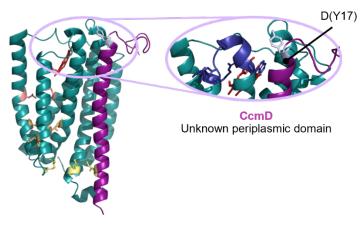


Supplemental Fig. 7. Differentially tagged System I expression constructs are functional for cytochrome c biogenesis. The indicated System I construct was co-expressed with cytochrome c_4 : His in E. $coli\ \Delta ccm$. 50 μg cell lysate was separated via SDS-PAGE and levels of cytochrome c biogenesis were determined by heme stain. (A) Heme stain, single biological replicate with three technical replicates. (B) Quantitation of heme stain. Three biological replicates, each with three technical replicates (gray dots) are shown. Error bars indicate standard deviation.



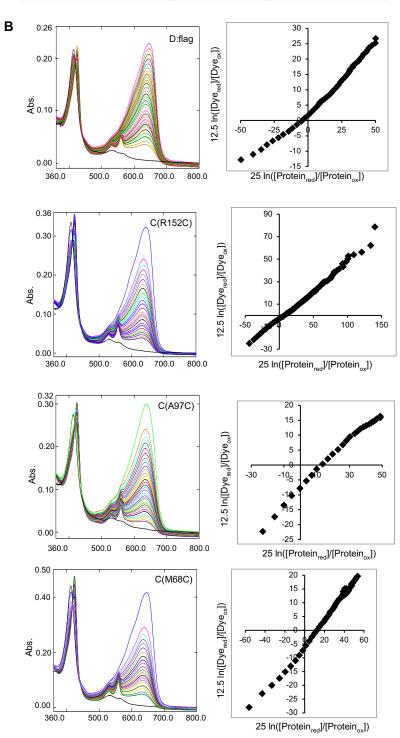


Supplemental Figure 8. Functional analyses of CcmCD cysteine variants. CcmC/D cysteine heme crosslinking variants were co-expressed with cytochrome c_4 :His in $E.\ coli\ \Delta ccm.$ 50 µg of total cell lysate was separated via SDS-PAGE, transferred to nitrocellulose membrane and heme stained. (A-E) Heme stains of cytochrome c biogenesis assay, 50 µg total protein cell lysate. Technical replicates labeled A-C. Data representative of three biological replicates, each containing three technical replicates. The same wildtype and vector samples are loaded on each blot and used as internal controls for quantitation.

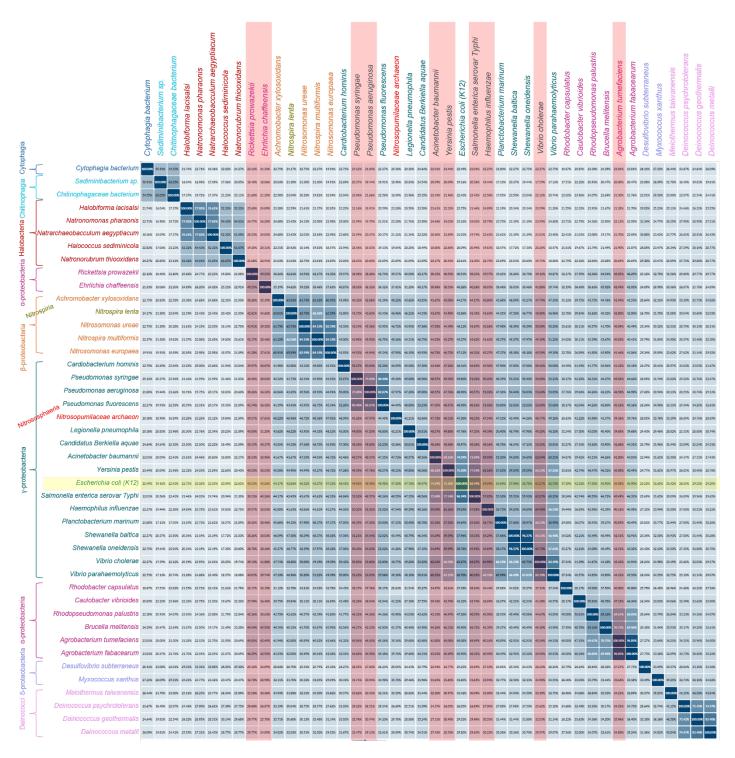


Supplemental Fig. 9. CcmD periplasmic heme interaction domain. Heme handling domain of unknown function CcmD(Y17C) crosslinking residue circled in purple. Image created from PDB:7F04. Cysteine/heme crosslinking variant is labeled.

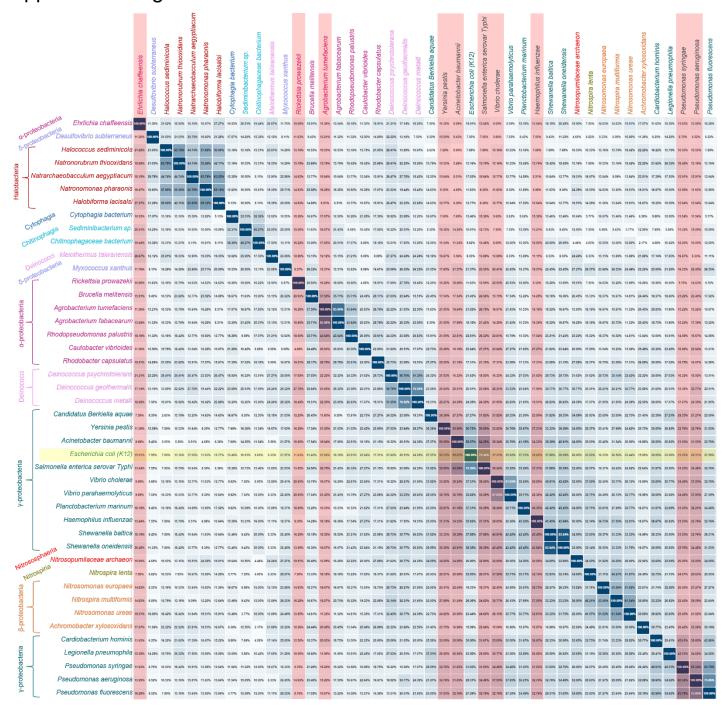
Variant	Average (std dev) mV	Ind. Redox mV
CcmD(Y17C)	-131.4 (±9.8)	-137.2, -117.6, -139.5
WT (GST:CDE(H130A))	-120.5 (±0.9)	-119.7, -120.1, -121.8
WT (GST:C(D:flag)E(H130A))	-120.0 (±1.1)	-119.2, -119.3, -121.6
CcmC(L170C)	-115.6 (±6.1)	-121.3, -107.1, -118.4
CcmC(M68C)	-114.1 (±2.7)	-114.2, -117.4, -110.8
CcmC(A97C)	-113.8 (±3.7)	-116.7, -108.6, -116
CcmC(R152C)	-109.7 (±5.5)	-114.7, -102.0, -112.3
CcmD(I46C)	-118.6 (±6.3)	-127.3, -115.7, -112.7



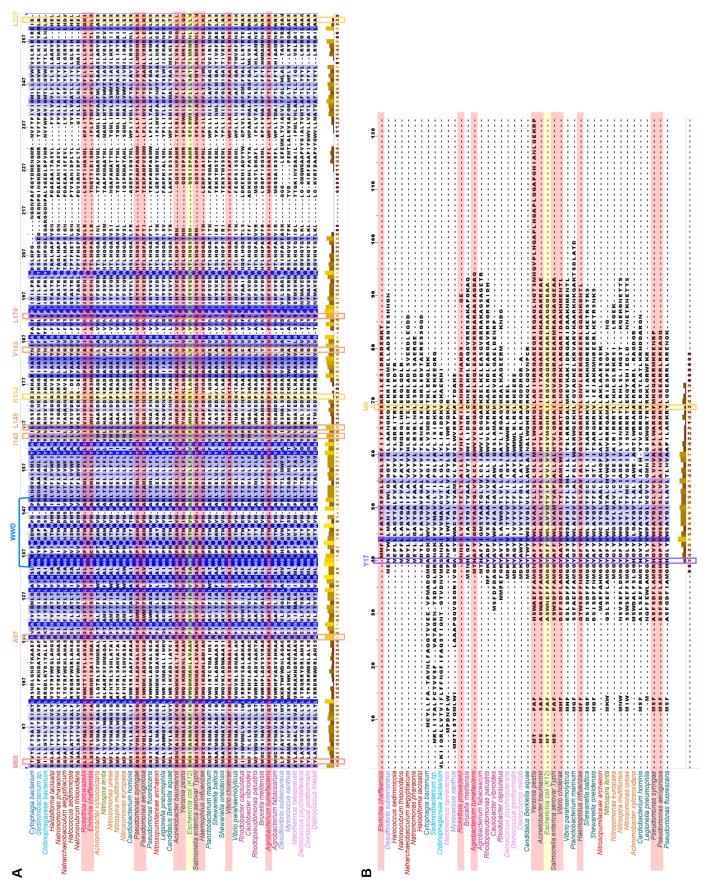
Supplemental Fig. 10. Redox potentials of key CcmCD variants. Heme redox potentials were determined use a modified Massey method. (A) Redox potentials of selected crosslinking variants in each heme-handling/transport domain across CcmCD. Shading indicates domain: purple – periplasmic domain of unknown function, blue – WT WWD domain, red – upper leaflet TMD, orange – lower leaflet TMD, yellow – cytoplasmic heme acceptance domain. (B) UV-vis spectra of redox titration time course and corresponding excel graphs for redox calculations. Representative spectra, excel graphs are shown. All redox titration were performed a minimum of three times, each with independently purified proteins.



Supplemental Fig. 11. Percent Identity Matrix of CcmC amino acid sequence alignment from *E. coli* + 43 organisms across System I. Sequences aligned utilizing UniProt Clustal O (1.2.4) interface and output determined from alignment. *E. coli* sequence was used as reference sequence for comparison of percent identity (highlighted in yellow). Organisms are labeled by taxonomic class: γ -proteobacteria (teal), β - (orange), α - (maroon), δ - (light purple), Cytophagia (royal blue), Chitinophagia (light blue), Halobacteria (dark red), Nitrospira (gold), Nitrososphaeria (bright red), Deinococci (light pink). Organisms highlighted in red are included in Table 1.



Supplemental Fig. 12. Percent Identity Matrix of CcmD amino acid sequence alignment from $E.\ coli$ + 43 organisms across System I. Sequences aligned utilizing UniProt Clustal O (1.2.4) interface and output determined from alignment. $E.\ coli$ sequence was used as reference sequence for comparison of percent identity (highlighted in yellow). Organisms are labeled by taxonomic class: γ -proteobacteria (teal), β - (orange), α - (maroon), δ - (light purple), Cytophagia (royal blue), Chitinophagia (light blue), Halobacteria (dark red), Nitrospira (gold), Nitrososphaeria (bright red), Deinococci (light pink). Organisms highlighted in red are included in Table 1.



Supplemental Fig. 13. Multiple Sequence Alignment (MSA) of CcmC and CcmD amino acid sequences from *E. coli* and forty-three organisms. Sequences aligned utilizing Clustal O (1.2.4) software through the UniProt interface and output

determined from alignment. *E. coli* CcmC (highlighted in yellow) represents model organism for this study. Conservation per residue calculated in JalView software (v2.11.4.1) and residues are colored by level of conservation >5 (light blue) to fully conserved (royal blue). Amino acids M68 to L227 are displayed with crosslinking residues outlined based on heme transport class defined in Fig. 5 (heme acceptance domain (yellow) C(R152, L227) and D(I46), lower leaflet transmembrane domain (orange) C(A97, I143, L145, V163), upper leaflet transmembrane domain (red) C(M68, L170), periplasmic domain of unknown function (purple) D(Y17). The highly conserved WWD domain is indicated with the blue bracket. Organisms highlighted in red are included in Table 1 (A) CcmC MSA. (B) CcmD MSA.

Supplemental Movie 1

Supplemental Movie 1. Model of CcmCD heme transport. Created from PDB:7F04 in PyMOL (version 2.4.1). Demonstrates predicted transmembrane heme movement through CcmCD based on identified cysteine/heme crosslinking residues (shown as sticks and colored via domain as follows: heme acceptance – yellow, TMD lower leaflet – orange, upper leaflet – red, WWD domain – blue). Briefly, heme is delivered from the cytoplasm to the heme acceptance domain of CcmCD. Next, heme transitions into the core CcmC heme channel composed of TMD 2/3/4/5, travels up the heme transport channel, and finally, is stereospecifically positioned in the WWD domain of CcmC.

Supplemental Table 1.

Supplemental Table 1. Forty-four total organisms containing System I cytochrome *c* biogenesis pathway whose CcmCD sequences were collected for bioinformatic analysis. Pathogens of interest are highlighted in red.

Class	Representative Species	GeneBank ID (reference genome)	CcmC reference #	CcmD reference #	Pathogen REF
gamma- proteobacteria	Escherichia coli (K12)	GCA_000005845.1	>NP_416703.1	>NP_416702.1	-
gamma- proteobacteria	Acinetobacter baumannii	UFMN01000003.1	>SST03238.1	>SST03237.1	WHO 2024 (Critical) ² + CDC 2019 (Urgent) ³
gamma- proteobacteria	Haemophilus influenzae	GCF_000931575.1	>WP_044330604.1	WP_005693416.1	WHO 2024 (Medium) ²
gamma- proteobacteria	Salmonella enterica serovar Typhi strain 721597	AAIFQV010000033.1	>ECD7321450.1	>ECD7321449.1	WHO 2024 (High) ² + CDC 2019 (Serious) ³
gamma- proteobacteria	Yersinia pestis Antiqua	AJJ78172.1	>AJJ80906.1	>AJJ78172.1	NIAID biodefense pathogen list ⁴
gamma- proteobacteria	Pseudomonas syringae strain MWU 16-30316	SEZV01000001.1	>TFZ38898.1	>TFZ38899.1	Plant pathogen of interest
gamma- proteobacteria	Candidatus Berkiella aquae	GCF_001431295.2	>WP_075065853.1	>WP_075065852.1	-
gamma- proteobacteria	Vibrio cholerae O1 serovar	CP047301.1	>QJS81051.1	>QJS81052.1	NIAID biodefense pathogen list ⁴
gamma- proteobacteria	Vibrio parahaemolyticus	GCF_000196095.1	>WP_005457654.1	>WP_005457743.1	-
gamma- proteobacteria	Shewanella baltica (strain OS223)	GCF_000178875.2	>WP_006079703.1	>WP_006079702.1	-
gamma- proteobacteria	Shewanella oneidensis MR-1	AE014299.2	>AAN53346.1	>AAN53345.1	-
gamma- proteobacteria	Planctobacterium marinum	AP027272.1	>BDX07708.1	>BDX07707.1	-
gamma- proteobacteria	Pseudomonas aeruginosa PA01	GCF_000006765.1	>NP_250168.1	>NP_250169.1	WHO 2024 (High) ² + CDC 2019 (Serious) ³
gamma- proteobacteria	Pseudomonas fluorescens	CP148752.1	>WXR69450.1	>WXR69451.1	-
gamma- proteobacteria	Legionella pneumophila	GCA_001753085.1	>WP_011213329.1	>WP_011945969.1	-
gamma- proteobacteria	Cardiobacterium hominis	GCF_900637305.1	>WP_004140696.1	>WP_004140695.1	-
beta- proteobacteria	Nitrospira multiformis ATCC 25196	GCF_000196355.1	>WP_011380556.1	>WP_011380555.1	-
beta- proteobacteria	Nitrosomonas europaea ATCC 19718	GCF_000009145.1	>WP_011111378.1	>WP_234988995.1	-
beta- proteobacteria	Nitrosomonas ureae	WP_062559647.1	>WP_062559647.1	>SNX58885.1	-
beta- proteobacteria	Achromobacter xylosoxidans	GCF_016728825.1	>WP_006384724.1	>WP_020927183.1	-
alpha- proteobacteria	Rickettsia prowazekii str. Chernikova	GCF_000277165.1	>WP_004596831.1	>AFE49388.1	NIAID biodefense pathogen list ⁴
alpha- proteobacteria	Ehrlichia chaffeensis	CP007480.1 GCA_000632965.1	>WP_006010829.1	>AHX10971.1	NIAID biodefense pathogen list ⁴
alpha- proteobacteria	Brucella melitensis bv. 1 str. 16M	GCA_000007125.1	>AAL53032.1	>AAL53031.1	-
alpha- proteobacteria	Agrobacterium tumefaciens strC58	NC_003062.2	>WP_006310864.1	>WP_006310865.1	Plant pathogen of interest
alpha- proteobacteria	Agrobacterium fabacearum P4	GCF_000442985.1	>WP_025594583.1	>WP_013636916.1	-
alpha- proteobacteria	Rhodopseudomonas palustris	GCF_016584445.1	>WP_011155773.1	>WP_012493979.1	-
alpha- proteobacteria	Caulobacter vibrioides (Caulobacter crescentus CB15)	GCF_000022005.1	>YP_002519159.1	>YP_002519160.1	-
alpha- proteobacteria	Rhodobacter capsulatus	GCF_000021865.1	>WP_013067511.1	>WP_013067512.1	-
delta- proteobacteria	Myxococcus xanthus	GCF_000012685.1	>WP_011553301.1	>WP_011553300.1	-
delta- proteobacteria	Desulfovibrio subterraneus	GCF_027558435.1	>WP_174406143.1	>WP_174405168.1	-
Nitrospiria	Nitrospira lenta	GCF_900403705.1	>WP_121987557.1	>WP_121987556.1	-
Chitinophagia	Sediminibacterium sp	PGC001000013.1	>PJE45959.1	>PJE45958.1	-
Chitinophagia	Chitinophagaceae bacterium	JACDBU010000083.1	>MBA2249319.1	>MBA2249320.1	-
Cytophagia	Cytophagia bacterium	REAB01000229.1	>TAG34495.1	>TAG34505.1	-

Deinococci	Deinococcus geothermalis	GCF_000196275.1	>WP_011530378.1	>WP_083807144.1	-
Deinococci	Deinococcus metalli	JACHFK010000001.1	>MBB5374879.1	>GHF33041.1	-
Deinococci	Deinococcus psychrotolerans	CP034183.1	>AZI43147.1	>AZI43146.1	-
Deinococci	Meiothermus taiwanensis	QWKX01000004.1	>RIH79654.1	>RIH79653.1	-
Nitrososphaeria	Nitrosopumilaceae archaeon	UYNY00000000.3	>CAI2287500.1	>CAI2287499.1	-
Halobacteria	Natronomonas pharaonis (strain ATCC 35678)	CR936257.1	>WP_011322744.1	>WP_011322743.1	-
Halobacteria	Natrarchaeobaculum aegyptiacum	CP019893.1	>ARS90949.1	>ARS90948.1	-
Halobacteria	Halobiforma lacisalsi	NZ_CP019285.1	>WP_007141830.1	>WP_007141831.1	-
Halobacteria	Halococcus sediminicola	GCF_000755245.1	>WP_237561118.1	>WP_079977575.1	-
Halobacteria	Natronorubrum thiooxidans	NZ_FTNR01000001.1	>WP_076607571.1	>WP_076607570.1	-

Supplemental Table 2.

Supplemental Table 2. Relevant strains and plasmids utilized in this study.

Plasmid, strain <i>E. coli</i>	Description	Reference
RK103	E. coli MG1655 Δccm::kanR , deleted for all ccm genes	Feissner et al, 2006 ⁵
NEB 5-α	fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17	
Plasmid	CEV MT CCT.ComCDE	Dishard Famal at at 20006
pRGK375 pRGK380	pGEX WT GST:CcmCDE pGEX WT GST:CcmCDE(H130A)	Richard-Fogal et al, 2009 ⁶
pMCS1243	pGEX WT GST.CcmC(D:flag)E(H130A)	Richard-Fogal <i>et al</i> , 2009 ⁶ This study
pRGK332	pBAD cytochrome c4:His	Feissner <i>et al</i> , 2006 ⁵
pronoz	CcmC cysteine variants	1 Clasher et al, 2000
pMCS612	pGEX GST:CcmC(M68C) DE(H130A)	This study
pMCS613	pGEX GST:CcmC(I70C) DE(H130A)	This study
pMCS614	pGEX GST:CcmC(Y71C) DE(H130A)	This study
pMCS615	pGEX GST:CcmC(A72C) DE(H130A)	This study
pMCS427	pGEX GST:CcmC(V76C/A77C)DE(H130A)	This study
pMCS425	pGEX GST:CcmC(A78C/F79C)DE(H130A)	This study
pMCS420	pGEX GST:CcmC(I80C)DE(H130A)	This study
pMCS422	pGEX GST:CcmC(G81C)DE(H130A)	This study
pMCS407	pGEX GST:CcmC(L82C)DE(H130A)	This study
pMCS424	pGEX GST:CcmC(V83C)DE(H130A)	This study
pMCS426	pGEX GST:CcmC(M96C)DE(H130A)	This study
pMCS408 pMCS440	pGEX GST:CcmC(A97C)DE(H130A) pGEX GST:CcmC(P98C) DE(H130A)	This study This study
pMCS409	pGEX GST:CcmC(I99C)DE(H130A)	This study
pMCS410	pGEX GST:CcmC(G100C)DE(H130A)	This study
pMCS616	pGEX GST:CcmC(A101C) DE(H130A)	This study
pMCS617	pGEX GST:CcmC(V102C) DE(H130A)	This study
pMCS618	pGEX GST:CcmC(F103C) DE(H130A)	This study
pMCS619	pGEX GST:CcmC(T104C) DE(H130A)	This study
pMCS620	pGEX GST:CcmC(F105C) DE(H130A)	This study
pMCS621	pGEX GST:CcmC(F137C) DE(H130A)	This study
pMCS411	pGEX GST:CcmC(L138C)DE(H130A)	This study
pMCS392	pGEX GST:CcmC(Y139C)DE(H130A)	This study
pMCS443	pGEX GST:CcmC(V140C) DE(H130A)	This study
pMCS431	pGEX GST:CcmC(G141C)DE(H130A)	This study
pMCS413 pMCS415	pGEX GST:CcmC(V142C)DE(H130A)	This study This study
pMCS419	pGEX GST:CcmC(I143C)DE(H130A) pGEX GST:CcmC(A144C)DE(H130A)	This study
pMCS417	pGEX GST:CcmC(L145C)DE(H130A)	This study
pMCS1119	pGEX GST:CcmC(H147C)DE(H130A)	This study
pMCS1108	pGEX GST:CcmC(R152C)DE(H130A)	This study
pMCS1109	pGEX GST:CcmC(A155C)DE(H130A)	This study
pMCS421	pGEX GST:CcmC(V163C)DE(H130A)	This study
pMCS423	pGEX GST:CcmC(L164C)DE(H130A)	This study
pMCS622	pGEX GST:CcmC(N169C)DE(H130A)	This study
pMCS623	pGEX GST:CcmC(L170C) DE(H130A)	This study
pMCS1110	pGEX GST:CcmC(L224C)DE(H130A)	This study
pMCS1111	pGEX GST:CcmC(L227C)DE(H130A)	This study
pMCS412	<u>CcmD cysteine variants</u> pGEX GST:CcmCD(G15C)E(H130A)	This study
pMCS414	pGEX GST:CcmCD(G16C)E(H130A)	This study
pMCS393	pGEX GST:CcmCD(Y17C)E(H130A)	This study
pMCS418	pGEX GST:CcmCD(A18C)E(H130A)	This study
pMCS416	pGEX GST:CcmCD(F19C)E(H130A)	This study
pMCS1112	pGEX GST:CcmCD(M27C)E(H130A)	This study
pMCS1113	pGEX GST:CcmCD(P31C)E(H130A)	This study
pMCS1114	pGEX GST:CcmCD(V34C)E(H130A)	This study
pMCS1115	pGEX GST:CcmCD(L35C)E(H130A)	This study
pMCS1120	pGEX GST:CcmCD(H38C)E(H130A)	This study
pMCS1116	pGEX GST:ComCD(Q42C)E(H130A)	This study
pMCS1117 pMCS1118	pGEX GST:CcmCD(A45C)E(H130A) pGEX GST:CcmCD(I46C)E(H130A)	This study This study
pMCS1256	pGEX GST.CcmC(I(46C)E(H130A)	This study This study
pMCS1250	pGEX GST:CcmC(D(I46C):flag)E(H130A)	This study
p00.201	CcmC/D cysteine variants – full System I	5 5.664
pRGK386	pGEX GST:CcmABCDE(F:His)GH	Richard-Fogal et al, 2009 ⁶
pMCS250	pGÉX GST:CcmABCD(MBP:E)(F:His)GH	This study

pMCS1311	pGEX GST:CcmABC(D:flag)(MBP:E)(F:His)GH	This Study
pMCS1247	pGEX GST:CcmABC(M68C)D(MBP:E)F:His(GH)	This study
pMCS1248	pGEX GST:CcmABC(A97C)D(MBP:E)F:His(GH)	This study
pMCS1249	pGEX GST:CcmABC(I143C)D(MBP:E)F:His(GH)	This study
pMCS1250	pGEX GST:CcmABC(L145C)D(MBP:E)F:His(GH)	This study
pMCS1251	pGEX GST:CcmABC(R152C)D(MBP:E)F:His(GH)	This study
pMCS1252	pGEX GST:CcmABC(V163C)D(MBP:E)F:His(GH)	This study
pMCS1253	pGEX GST:CcmABC(L170C)D(MBP:E)F:His(GH)	This study
pMCS1226	pGEX GST:CcmABC(L227C)D(MBP:E)F:His(GH)	This study
pMCS1235	pGEX GST:CcmABCD(Y17C)(MBP:E)F:His(GH)	This study
pMCS1233	pGEX GST:CcmABCD(I46C)(MBP:E)F:His(GH)	This study

Supplemental Table 3.

Supplemental Table 3. Relevant primers and templates utilized in this study.

MSP773	Oligonucleotide	Sequence (5' - 3')	Purpose	Template
MSP774 continatipatesianticopeaceaceaceageaceageaceageaceageaceageaceageaceageaceageageageageageageageageageageageageage	_	. , ,		
MSP775 gogatictgjorgetgtggoattiggoattiggoattig pMCS814 cloning pRCK380 MSP0215 gcatttigteateatggoattigtoggottlattiggottig pMCS814 cloning pRCK380 MSP0213 tatagcateataggoattigtoggottlattiggottigtoggangt pMCS427 cloning pRCK380 MSP0213 tatagcateataggoatgocateatgoatgoatgoatgoatgoatgoatgoatgoatgoatgo				
MSP776 cipocattajagocasaatopocatogactogo pMCS814 doining pRGR380 MSP0216 caatgocaataaaagogoaactagocattagocattagocattagocattagoattag				
MSP0215 b graditatipacetasalgoategoategotegoategoategoategoategoateg				•
MSP0216 caaagacaataaacqcqcaacatqacatqatgatgataataqc pMCS427 cloning pRCS330 MSP0214 cattqacaataaqaatqacatqacatqacatqatgatata pMCS425 cloning pRCS330 MSP0186 cacaaqaacaagacaataacaqaatqacatqacatqaatgatgata pMCS420 cloning pRCS330 MSP0187 cagacaagacaataaacqtqacactqaatgatgata pMCS420 cloning pRCS330 MSP0188 gacagacaagacaataaacqtqacactqa pMCS422 cloning pRCS330 MSP0189 gacagacaagacaataaacqtqacactq pMCS422 cloning pRCS330 MSP0197 catttitattgacagacaagacaataacqtqacact pMCS427 cloning pRCS330 MSP0197 gacagacagacaataacqcqcacatqaaga pMCS407 cloning pRCS330 MSP0197 gacacaatgagacaacqacacacaagaa pMCS407 cloning pRCS330 MSP0197 gacacaatgagacacacacacaagaa pMCS407 cloning pRCS330 MSP0197 gacacaatgagacacacacacaagaa pMCS407 cloning pRCS330 MSP0197 gacacaatgagacacacacacacaagaa pMCS407 cloning pRCS330 MSP0198 gacacaatgagacacacatacacacaaaaaaaaagaagaacaacaagaacaaaaaaaa				•
MSP0213 tatigacateageastgeastgeageageageageageageageageageageageageag				•
MSP01214 calcitopoagoaagocoastacegocotrotary pMCS425 cloning PRGK380 MSP0186 cagacaagocacaaaaacqtycacatgcattary pMCS420 cloning PRGK380 MSP0187 gadgacaagottattattyctgttgtage pMCS422 cloning PRGK380 MSP0189 gadgacaagottattattyctgttgtage pMCS422 cloning PRGK380 MSP0170 cattittattgcagacaagocaataaaacqtqccact pMCS407 cloning PRGK380 MSP0191 gagacacaagocaacaacaacqcagcaag pMCS426 cloning PRGK380 MSP0171 gatacaatgaggacaagocacqcaccaccacgag pMCS426 cloning PRGK380 MSP0172 gacacaatgaggagcacacqcaccaccacgag pMCS426 cloning PRGK380 MSP0173 gacacaatgaggaccacatgcacgoc pMCS400 cloning PRGK380 MSP0180 gacagacaatgacaactgcagcoc pMCS410 cloning PRGK380 MSP0180 gacagacaatgacaactgcagcaccagtcagcoc pMCS410 cloning PRGK380 MSP0180 gacagacaatgacaactgcagcaccagtcagcaccagtcagcaccagtcagcaccagtcagcaccagtcagcaccagtcagcaccagcactagcagcaccagtcagcaccagtcagcaccagtcagcaccagtcagcaccagtcagcaccagtcagcaccagtcagcaccagtcagcaccagtcagcaccagtcagcaccagcaccagtcagcaccagtcagcaccagtcagcaccagtcagcaccagtcagcaccagtcagcaccagtcagcaccagtcagcaccagtcagcaccagtcagcaccagtcagcaccagtcagcaccagtcagca				•
MSP0186 catacatagocatgugcagotttidtgocttigtdg pMCS420 doning pRCK380 MSP0187 cagacagocacaaaaacqtocacdigcadgad pMCS422 doning pRCK380 MSP0188 gcaggcaagogttattigtgdtgdegagtgasaatag pMCS422 doning pRCK380 MSP0189 agtggcacgtttattggctglcgtgdgagtgasaatag pMCS407 doning pRCK380 MSP0191 ggcgacgaacaacagcacaaacagcatagaacagcabaacagctgacaacagcagaacagcacaacagcacagaacagcaacagcag				•
MSP0186 cageaaggacaagacaasaacqtqccactqcatqtq pMCS422 doning pRGK380 MSP0188 gcaqtgacaaggatattattqctqttqtcgtg pMCS422 doning pRGK380 MSP0189 gcaqacaaggacaagaasaataagqtgcacttq pMCS407 doning pRGK380 MSP0170 cattitacttgcagacaagacaagtagaaatatq pMCS407 doning pRGK380 MSP01912 gogaqtgaggacqtgoccactgtggt pMCS426 doning pRGK380 MSP0171 gggacaatgaggacaatgacgagt pMCS426 doning pRGK380 MSP0171 gggacaatgaccatgacgcacgatt pMCS440 doning pRGK380 MSP0172 ggacacatgaggacaatgacgcact pMCS440 doning pRGK380 MSP0189 gacaggacatgacgactgacgcattgccgc pMCS410 doning pRGK380 MSP0189 ggacatgacgaccattgccgctgttt pMCS410 doning pRGK380 MSP0189 ggacatgatgaccactattgcagactacgcc pMCS411 doning pRGK380 MSP0189 ggacatgatacaccacatagaaacagacacagtcagt pMCS411 doning pRGK380 MSP0218 taaactgacacacacacaaaacagagaccacagtcagt pMCS412 doning pRGK380 MSP0219 taaacaggacacacacacaaaacagagaccacagtcagatacaaacagagaga				
MSP0187 geaglageageathatatgcetatglega pMCS422 cloning pRCK380 MSP0168 aglageageathatageagtgceactgc pMCS407 cloning pRCK380 MSP0191 aglageageagtutattgcetglgtdgeageageageageageageageageageageageageag				pRGK380
MSP0188 gocageaaggaaataaacgclgccactpc pMCS407 cloning pRGK380 MSP0170 cattitacitigccagacaagcaataaag pMCS407 cloning pRGK380 MSP0191 gacggilgcagepelgcqcoccategg pMCS426 cloning pRGK380 MSP0192 gacgalgacgaeglegacgcoccategge pMCS426 cloning pRGK380 MSP01972 galgacgacaaltgaccactgcccacc pMCS408, pMCS1248 cloning pRGK380, pMCS250 MSP01973 gacgacaaltgaggacategccactgccgc pMCS408, pMCS1248 cloning pRGK380, pMCS250 MSP01974 cacgacaattgaggacategccactgcc pMCS400 cloning pRGK380 MSP01984 cacgacaattgaaggacategcactgcc pMCS410 cloning pRGK380 MSP0288 gacgattgaccactategca pMCS410 cloning pRGK380 MSP0198 gacattgactgctgcttgtttgtatggttgtgttgt pMCS410 cloning pRGK380 MSP0198 gacattgacaccacaatacaacaacaacaacaacaacaacaacaaca	MSP0187			•
MSP0169 algtgogaegettattagedgtgtetgtegaagaagaaataa pMCS407 cloning PRCK380 MSP0191 geoggtgegegegegeegeeceadeagaag pMCS426 cloning PRCK380 MSP0192 geocaatagogogaegaegeegeegeegee pMCS426 cloning PRCK380 MSP0171 gelgogogaettgegeeceattgegee pMCS408 pMCS1248 cloning PRCK380 pMCS250 MSP0193 gegeacaataggggeactegeegee pMCS400 pMCS400 cloning PRCK380 pMCS250 MSP0193 gegegateactegeeceattgagegee pMCS401 cloning PRCK380 pMCS250 MSP0194 taaacaggecaaattgaggeactegee pMCS410 cloning PRCK380 MSP0197 gegateaceacageageacattgtgtgtagtgt pMCS411 cloning PRCK380 MSP0198 gegateaceacageageageacattgt pMCS411 cloning PRCK380 MSP0199 gegateaceacageageageageageageageageageageageageagea	MSP0188		pMCS422 cloning	pRGK380
MSP0170 cattlificatelgecagecageagecageageageageageageageageageageageageagea	MSP0169	agtggcagcgtttattggctgtgtctggcagatgaaaatg	pMCS407 cloning	pRGK380
MSP01912 geaceastggggocjaccocgocacgocacg pMCS488 pMCS1248 cloning pRGK380 pMCS250 MSP0172 gptgggocjactglatgoccatglugc pMCS488 pMCS1248 cloning pRGK388 pMCS250 MSP0194 cacaggaccaastgcaggcactcpccpc pMCS448 pMCS1248 cloning pRGK380 pMCS250 MSP0288 ggoggactggccaastgcacgc pMCS410 cloning pRGK380 pMCS250 MSP0288 goggactggccccatttggcqcgtgttatgc pMCS410 cloning pRGK380 MSP0197 gaactgggccccatttggtglatgtgattgc pMCS411 cloning pRGK380 MSP0198 ggcaattcaaccacaaaacagacaacagttc pMCS411 cloning pRGK380 MSP0199 daacaacaccacaacacaaaacagagcacacagttc pMCS322 cloning pRGK380 MSP0199 ctrictgaactgtgcdgctgttttitgtattgcgtgtgtatcctg pMCS322 cloning pRGK380 MSP0199 ctrictgaactgtgcdgctgttttttgattgcgtgtgtatcctg pMCS432 cloning pRGK380 MSP0195 tctgttttttttgattgtgtgtgtattgcctgt pMCS433 cloning pRGK380 MSP0196 tctgttttttttttttttttttttttttttttttttt	MSP0170		pMCS407 cloning	pRGK380
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MSP0194 cacegopaceastgogogocalogoco pMCS440 doning pRGX880 MSP0288 gagcatagocacaattgagocacatogoco pMCS410 doning pRGX880 MSP0197 gaactgglectgettttigcatglaggagtglattgc pMCS411 doning pRGX880 MSP0198 gaactgglectgetttittglagtgagtgatt pMCS411 doning pRGX880 MSP0218 gaactgatglectgettitttglagtgagtgat pMCS392 doning pRGX380 MSP0199 tacaacocacacacacacacagacacagttc pMCS392 doning pRGX380 MSP0199 tacaacocacatacacacacagacacagtecagt pMCS443 doning pRGX380 MSP0175 tuclgitttigtaltglugtgattgattgccttg pMCS431 doning pRGX380 MSP0175 tuclgitttigtaltglugtgattgatccttgg pMCS431 doning pRGX380 MSP0175 tuclgitttigtaltglugtgattgatcctgacacaca pMCS413 doning pRGX380 MSP0210 agoctgacacacacacacacacacacacacacacacacacaca	MSP0172	ggcaccaatggggcacatcgccgccacc		pRGK380, pMCS250
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MSP0197 gaactg/toctget/titt/catalg/agg/tipat/toc pMCS411 cloning pRG4380 MSP0198 gecatacaccaccatag-caaacagcaccagttc pMCS411 cloning pRG4380 MSP0217 atacaccaccaccacaaaacag-agcaccagttca pMCS392 cloning pRG4380 MSP0218 guaactgytet/cett/tittytatet/cgyteg-tittyttytitytytytytytytytytytytytytytyt		00 00000 0		•
MSP0187 gopaatcaaccacatagcaaacagaacaagtc pMCS411 cloning pRGK380 MSP0218 tacaacccacacaaaaacagaagcacagttca pMCS392 cloning pRGK380 MSP0193 tcttgaactglegtctgtttttgtattgoggtgtattcctg pMCS392 cloning pRGK380 MSP0190 ctttgaactglegtctgtttttgtattgoggtgtgattccctg pMCS43 cloning pRGK380 MSP0176 tcttgaattgtgtgtgtgttgtccttgtg pMCS431 cloning pRGK380 MSP0201 ccacaggacatacaaccacatacaaaaacaga pMCS431 cloning pRGK380 MSP0202 aggotgtccacaggacatacaaccacatacaaaaacaga pMCS413 cloning pRGK380 MSP0201 aggotgtccacaggacatacaaccacatacaaaacaga pMCS413 cloning pRGK380 MSP0202 aggotgtccacagggcatacacaccacatacaaaa pMCS415, pMCS1249 cloning pRGK380 MSP0203 ttttgatattgtoggtgtgttgtccttgtgcagcc pMCS415, pMCS1249 cloning pRGK380, pMCS250 MSP0204 gogtgtccacaaggcaatcacaccacacacacacacacacac				•
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MSP0218 tgaactgotgotgottgtttgtgtgdgtgat pMCS432 cloning pRGK380 MSP0199 citctgaactggtgctgtgtttttgtattgcgtgtgttgtattgcctg pMCS443 cloning pRGK380 MSP0175 ccacaggcaattacaacaccaatacaaaaacagaa pMCS431 cloning pRGK380 MSP0176 ccacaggcaattacacacatacaaaaacagaa pMCS431 cloning pRGK380 MSP0201 gottttttgtatgtgtgttgcctttggcacgct pMCS413 cloning pRGK380 MSP0202 agcctgtocacaagacacactacaaaaacaga pMCS413 cloning pRGK380 MSP0203 tttgtatgtggtgttgtgccttgtgcacqcc pMCS415 pMCS1249 cloning pRGK380 MSP0204 ggctgtccacaggcacacacacacatacaaa pMCS415 pMCS1249 cloning pRGK380, pMCS250 MSP0205 gctgtttttgtatgtgctgtgtgatgcacgcttcgacg pMCS419 cloning pRGK380, pMCS250 MSP0207 tgtggtgtgtattgctcgtgtgatgacgcactcacaca pMCS417, pMCS1250 cloning pRGK380 MSP0208 cgtcgaagagtatcgtcagacagcatacacaccaca pMCS417, pMCS1250 cloning pRGK380 MSP0210 ttcacacagcaataggcatacacacagatactgcacaca pMCS421 cloning pRGK380 MSP0210 ttcacacagcaatagaatactgcgacagagatactgcacacaca				•
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	MSP0725	gcgtaatttgattttgtgcatggaaaaacgccg	pMCS1111, pMCS1226 cloning	pRGK380, pMCS250

MCDOZOC		-MCC4440 -l	- DOK200
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Supplemental Methods

Protein purifications

Affinity purifications of GST:CcmCDE(H130A) were performed as previously described^{6–8} with minor modifications. RK103 *E. coli* Δ*ccm* was used for recombinant protein expression. Briefly, 10 mL starter culture was back diluted 1:100 in 1 L LB with appropriate antibiotics and grown at 37°C and 200 rpm for four hours. Cultures were induced with 1 mM IPTG, grown for an additional ~16-18 hours, harvested by centrifugation and cell pellets were stored at -80°C. Cells were resuspended in GST buffer (4.3 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 2.7 mM KCl, 140 mM NaCl, pH7.3), supplemented with 1 mM phenylmethansulfonyl fluoride (PMSF, Sigma-Aldrich, P-470-10) and 1 mg/mL egg white lysozyme (Sigma-Aldrich, L-040-10). Cells were lysed by sonication (BransonSFX250 sonicator), cleared of cell debris by centrifugation at 10,000 rpm for 1 hour at 4°C, followed by separation of soluble and membrane fractions via high-speed ultracentrifugation at 100,000*g* for 45 minutes at 4°C. Membrane pellets were stored at -80°C, then solubilized in GST buffer with 1% n-dodecyl-β-d-maltopyranoside (DDM, GoldBio, DDM25) and affinity purified with glutathione agarose (Pierce, G-250-5) via batch purification for 2 hours at 4°C. Columns were washed by gravity flow, eluted in 4 mL GST buffer supplemented with 0.02% DDM and 10 mM L-glutathione (Sigma-Aldrich) and then concentrated in a 30 kDa filter. Note: no exogenous heme was added to cultures or during purification. Protein concentrations determined by Bradford Assay (Sigma-Aldrich, PI23200).

UV-vis spectroscopy

UV-vis absorption spectra were collected on a UV-1900i with LabSolutions software (Shimadzu; LabSolutions UV-vis [v1.10]) and performed as described previously^{9,10}. Briefly, 50 µg affinity purified protein was assayed in the purification buffer. Reduction was performed with excess sodium hydrosulfite powder (Sigma, 157-953). Quantitation of relative heme co-purification levels were determined via as-purified Soret comparison. UV-vis spectra are representative of three independent protein purifications. Pyridine hemochrome spectra were performed as previously described ¹¹. Briefly, 75 ug purified protein, resulting in a Soret absorbance of at least 0.2 was used. The total volume was adjusted to 120 µl with GST buffer supplemented with 0.02% DDM. NaOH and pyridine were added to a final concentration of 100 mM NaOH and 20% pyridine (v/v). Samples were reduced as described above. Spectra were recorded from 500-600 nm. Pyridine hemochrome assays were performed once.

In vivo cytochrome c biogenesis assays and statistical analysis

The CcmC and CcmD cysteine variants were engineered in the full System I pathway (GST:CcmABCD(MBP:E)(His:F)GH), co-expressed with cytochrome *c*₄:His (pRGK332) in RK103 and cytochrome *c* biogenesis was monitored via heme stain as previously described^{12,13}. Briefly, 1 mL of saturated overnight cultures were back diluted 1:6 into 5 mL LB with appropriate antibiotics and grown for 3 hours at 37°C and 200 rpm. Protein expression was induced with 0.1 mM IPTG and 0.2% arabinose and grown for 3 hours at 37°C and 200 rpm. Cell pellets were collected by centrifugation at 3700 rpm for 10 minutes and frozen at -80°C. Bacterial Protein Extraction Reagent (B-PER, Thermo Scientific, PI78248) was used to lyse cells per manufacturer's instructions. 50 μg protein from total cell lysate was separated by SDS-PAGE and cytochrome *c* biogenesis (i.e., heme attachment to cytochrome *c*) was analyzed via enhanced chemiluminescent heme stain¹⁴. Heme stains were quantified with AzureSpot Software (Azure, v.2.2.167) and wildtype was normalized to 100%. Unpaired two-tailed t-test were performed with GraphPad Prism v10.4.1. A minimum of three biological replicates, each containing three technical replicates, were performed.

CcmCD structural predictions and modeling

Prediction of the CcmCD heme channel: CAVER 3.0.3 PyMOL plugin^{15,16} was utilized to model potential heme channels in CcmCD (PDB:7F04). The predicted cytoplasmic heme domain (Fig. 1B) was selected as a starting point; standard input values were utilized except the probe radius was decreased to 0.5 and the maximum distance (A) was increased to 5.

Analysis of CcmCD hydrophobicity: PyMOL (version 2.4.1) was used to classify and color residues in CcmCD as positively charged (blue: Arg, Lys, His), negatively charged (red: Asp, Glu), hydrophobic (grey: Ala, Gly, Val, Ile, Leu, Phe, Met, Pro) or polar (pink: Asn, Gln, Thr, Ser, Cys, Tyr, Trp). Analysis displayed as a cartoon with an overlay of transparent surface.

Predicted structures of CcmCD from other bacteria: Predicted structures of *E. coli* (K12) CcmCD with 1 or 2 heme ligands (CcmC:NP_416703.1 CcmD:NP_416702.1), *P. syringae* (CcmC: TFZ38898.1 CcmD: TFZ38899.1), *P. aeruginosa* (CcmC: NP_250168.1 CcmD: NP_250169.1), *R. prowazekii* (CcmC: WP_004596831.1 CcmD: AFE49388.1), and *A. tumefaciens* (CcmC: WP_006310864.1 CcmD: WP_006310865.1) were obtained in AlphaFold 3 DB version 2024-05-08. Most predicted structures had a high level of confidence (per residue confidence score, pLDDT, greater than 90) (Fig. S2). Predicted structural CIF files and PDB:7F04 of *E. coli* CcmABCD¹⁷ were uploaded and compared using PyMOL (version 2.4.1). Alignment of predicted structures to target CcmCD (PDB:7F04) were performed in PyMOL, one to one with outliers removed over 5 cycles and RMSD calculated.

Determination of heme redox potential

Redox potentials were determined as previously described⁸, utilizing the modified Massey method¹⁸ developed by Raven and colleagues 19,20. Please see Supplemental Methods for details. Briefly, the cysteine variants in the GST:CcmCDE(H130A) background were affinity purified, and heme redox potentials were determined via simultaneous reduction of the purified protein complex with a dye of known reduction potential in bulk solution. Nile blue was used as the reference dye and acted as a mediator ²⁰. Nile blue is suitable for use with heme complexes of the System I pathway and does not interfere with UV-vis spectral reading at ~420 nm, the Soret peak of heme 8. Likewise, heme does not interfere with the reference wavelength of 630 nm for the reduction of Nile Blue. Samples were placed in a Coy anaerobic chamber and allowed to equilibrate with N₂ (95%) and H₂ (5%) for approximately 3 hours. All assays were performed under anaerobic conditions in GST buffer supplemented with 0.02% DDM, 625 μ M xanthine (Millipore Sigma, X4002), and Nile blue chloride ($E_m = -$ 116mV) ²¹ as the reference dye. The reaction was initiated via the addition of 100 – 125 µM xanthine oxidase (Millipore Sigma X4376). Scanning UV-vis spectra was collected from 360 – 800 nm every minute utilizing a UV-1900i Shimadzu spectrophotometer with LabSolutions software (UV-vis [v1.10]). Reduction of heme was monitored via the absorbance change at 420 nm and 423 nm, and reduction of Nile blue was monitored via the absorbance change at 630 nm. Data analysis was completed in Excel utilizing the Nerst equation: one-electron reduction of heme with $E = 25 \text{ mV*In}(b \text{ heme}_{red}/b \text{ heme}_{ox})$ and two-electron reduction of Nile blue with E = 12.5mV*In(dye_{red}/dye_{ox}). Given the assumption that at the time point when the reduced/oxidized ratio is (A_{ox}- A_t)/(A_t-A_{red}) with A_{ox} being the absorbance from the most oxidized spectrum and A_{red} being the absorbance of the most reduced spectrum; the ratios of reduced/oxidized heme and dye were calculated for each absorbance spectral data point collected and represent the molar concentrations of ratio of reduced/oxidized state of heme and dye. If the ratio was greater than 10 or less than 0.1 as compared to the next recorded data point, then those points were excluded from the analysis. E_{heme} vs E_{dye} was plotted, and the y-intercept value represents the difference between the redox potential of Nile blue (-116 mV) and the redox potential of the purified protein. This difference was used to calculate the redox potential of the purified protein. Potentials are given versus the standard hydrogen electrode. Reduction potentials were calculated as the average of three independent assays with the standard deviation reported.

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