SUPPLEMENTARY MATERIAL

The CcmFH complex is the system I holocytochrome c synthetase: engineering cytochrome c maturation independent of CcmABCDE

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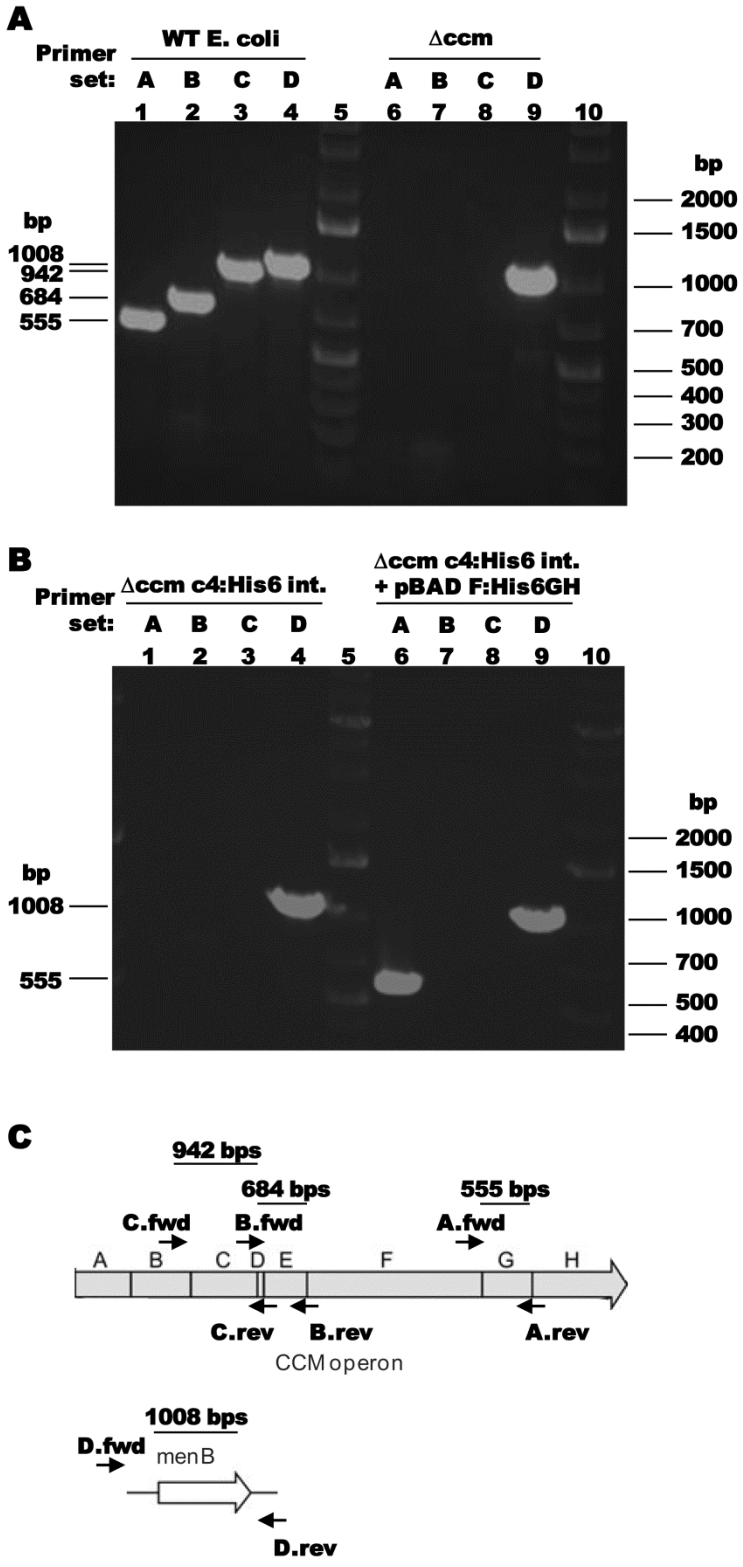
Fig S1. Confirmation of Δ*ccm* by genomic PCR. Ethidium bromide staining of the products from 4 separate PCRs using the indicated genomic DNA templates. Primer sets A, B, and C anneal to different regions of the ccm operon (panel C), and control primer set D anneals to regions flanking *E. coli* menB. For WT *E. coli*, all PCRs yielded products of the expected sizes (panel A, lanes 1-4). No products corresponding to the *ccm* operon were detected for any of the Δ*ccm* strains (panels A and B), with the exception of primer set A for strain Δ*ccm* c4:His6 int + pBAD CcmF:His6GH, as expected since this strain carries the ccmFGH genes on a plasmid. PCRs using control primer set D yielded products of the expected size for each strain. This confirms that our Δ*ccm* strains lack the endogeneous genes for cytochrome c synthesis; therefore, holocytochrome c formation in these strains is completely dependent on plasmid-based expression of the *ccm* genes. Lanes 5 and 10 in (A) and (B) contain MW markers; bp indicates base pairs.

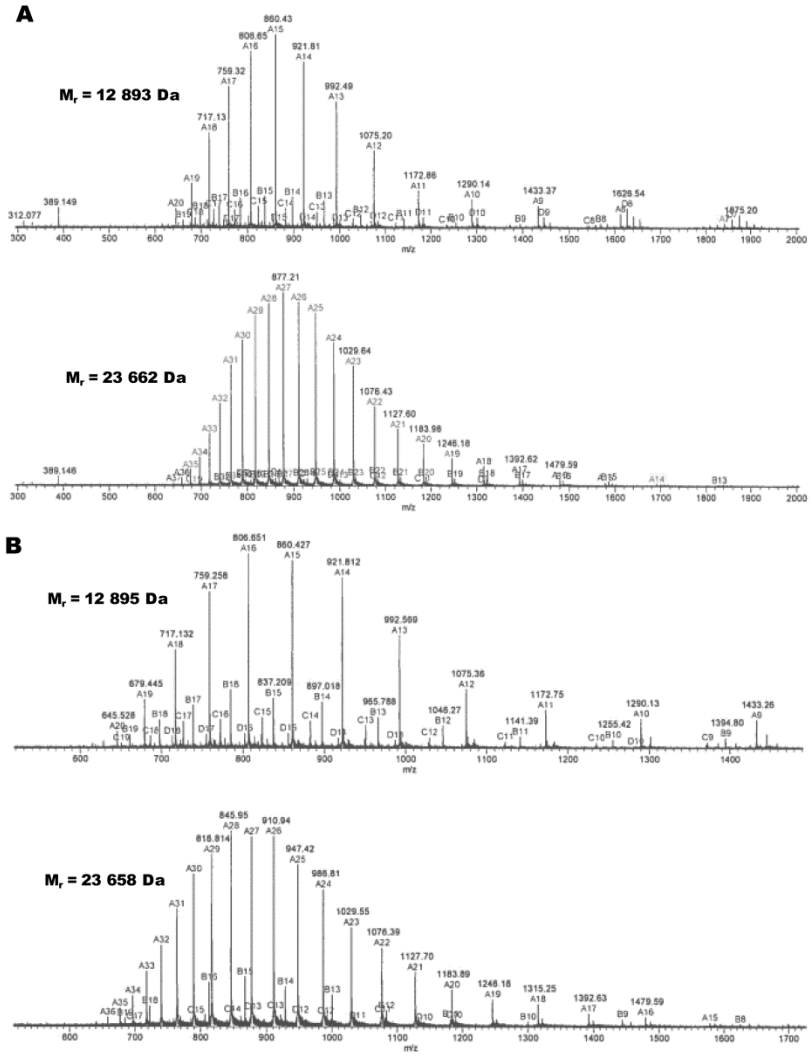
Fig S2. Mass spectral analysis of holocytochrome c4:His6 produced by CcmFGH in the presence (A) or absence (B) of CcmABCDE. After deconvolution of the positive ion mass spectra, two species were identified from each sample with the indicated molecular weights (each was within 2 Daltons of the reported average masses for full length holocytochrome c4:His6 and proteolyzed holocytochrome c4:His6', respectively). (C) Amino acid sequence of the *Bordetella pertussis* cytochrome c4:His6. The signal sequence is underlined and heme attachment sites (Cys-Xxx-Xxx-Cys-His) are in bold. Arrows indicate the periplasmic signal cleavage site and the site of endogeneous proteolysis giving rise to holocytochrome c4:His6'. The presence of identical molecular weight species in preparations of holocytochrome c4:His6 produced by full system I and by CcmFGH-ind confirms proper cleavage of the cytochrome c4 periplasmic secretion sequence, and covalent attachment of heme.

Fig S3. Full UV-Vis absorption spectra (360 to 800 nm.) of TALON-purified holocytochrome c4:His6 produced in the absence of Ccm proteins (blue line), in the presence of CcmABCDE (gray line), or by CcmFGH-only (purple line) under optimized conditions (i.e., in the presence of ALA (50 μg mL^{-1}); increased arabinose inducer (0.4 %); low rpm shaking (120 rpm); and plasmid-borne cytochrome c4 (p-c4)). Absorption maxima are indicated. No spectral evidence of covalent heme (i.e., holocytochrome c4) was detected in the absence of CcmFGH. Thus, cytochrome c4 does not self-assemble with heme, nor is CcmABCDE capable of synthetase activity; CcmFGH is the cytochrome c synthetase.

45 **Fig S1**


A
Primer set:
WT E. coli
Δccm
A B C D A B C D
1 2 3 4 5 6 7 8 9 10
bp
1008
942
684
555
bp
2000
1500
1000
700
500
400
300
200
B
Primer set:
Δccm c4:His6 int.
Δccm c4:His6 int. + pBAD F:His6GH
A B C D A B C D
1 2 3 4 5 6 7 8 9 10
bp
1008
555
bp
2000
1500
1000
700
500
400
C
942 bps
684 bps
555 bps
C.fwd
B.fwd
A.fwd
A B C D E F G H
C.rev
B.rev
A.rev
CCM operon
1008 bps
D.fwd
menB
D.rev

46 **Fig S2**



C

Signal cleavage site

MKRVLSRMLVASGLVLGASVHSMSFAADGAAGPAKPDAAK 40

GAQLYDQGDASRGVIA**CASCH**GAAGSSTIPANPNLAAQPH 80

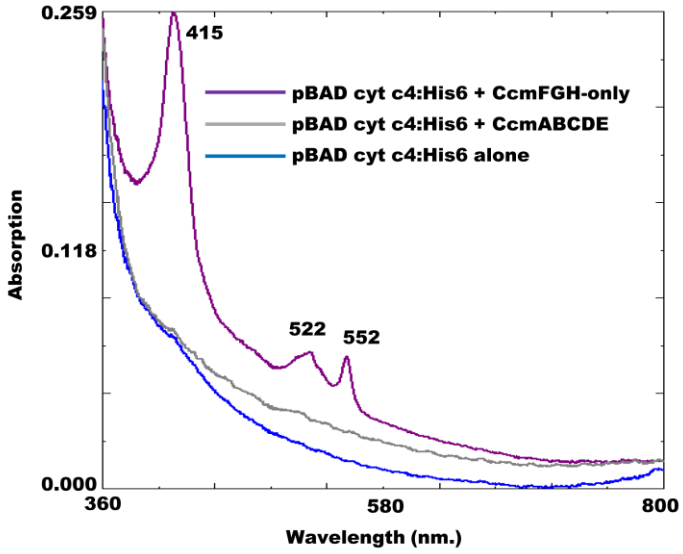
EYLVKQLTEFKVKEGEKLPLRMGPGGNPTPMTAMAQPLTA 120

QDMQNVALYLSQQPLKEPATAGHENLVELGQKIWRGGLAD 160

RNVPA**CAACH**GATGAGIPGQYPRLSGQFSSYIEEQLKLFR 200

SGERGNSVPMHDIADRMSDADIKAVADYAAGLRHHHHHH 239

47 **Fig S3**


0.259
0.118
0.000
Absorption
415
522
552
pBAD cyt c4:His6 + CcmFGH-only
pBAD cyt c4:His6 + CcmABCDE
pBAD cyt c4:His6 alone
360
580
800
Wavelength (nm.)

48

Table S1. Strains, plasmids, and oligonucleotide primers

Strain	Description	Reference
RK103	MG1655 ∆ccm	(Feissner *et al.*, 2006)
RK111	MG1655 ∆ccm *cyt c4*:His6 chromosomal integrate	(San Francisco *et al.*, 2011)

Plasmid	Description	Reference
--	pUCA6 *cyt c*2:Pho	(Beckman *et al.*, 1992)
pRGK332	pBAD *cyt c*4:His6	(Feissner *et al.*, 2006)
pRGK388	pBAD *ccmF*:His6*GH*	(Richard-Fogal *et al.*, 2009)
pRGK402	pGEX *ccmABCDE*	(San Francisco *et al.*, 2011)
pRGK434	pBAD *ccmF*(His173Ala):His6*GH*	(San Francisco *et al.*, submitted)
pRGK435	pBAD *ccmF*(His173Gly):His6*GH*	(San Francisco *et al.*, submitted)
pRGK436	pBAD *ccmF*(His173Cys):His6*GH*	(San Francisco *et al.*, submitted)
pRGK437	pBAD *ccmF*(His173Met):His6*GH*	(San Francisco *et al.*, submitted)
pRGK438	pBAD *ccmF*(His173Tyr):His6*GH*	(San Francisco *et al.*, submitted)
pRGK439	pBAD *ccmF*(His303Ala):His6*GH*	(San Francisco *et al.*, submitted)
pRGK440	pBAD *ccmF*(His303Gly):His6*GH*	(San Francisco *et al.*, submitted)
pRGK441	pBAD *ccmF*(His303Cys):His6*GH*	(San Francisco *et al.*, submitted)
pRGK442	pBAD *ccmF*(His303Met):His6*GH*	(San Francisco *et al.*, submitted)
pRGK443	pBAD *ccmF*(His303Tyr):His6*GH*	(San Francisco *et al.*, submitted)
pRGK444	pBAD ccmF(His261Gly):His6GH	This work
pRGK445	pBAD ccmF(His491Ala):His6GH	This work
pRGK446	pBAD *ccmF*:His6*G*(Cys80Ser/Cys83Ser)*H*	This work
pRGK447	pBAD *ccmF*:His6*GH*(Cys43Ser/Cys46Ser)	This work
pRGK448	pBAD *ccmF*:His6*GH—cyt c*4:His6	This work

Primer	Sequence (5'-3')	Purpose
C4:His_PstI_RBS_Fwd	GATCTGCAGAGGAGGAATATCATATGAAGCGTGTGCTGTCCCGG	pRGK448
C4:His_PstI_Rev	GATCTGCAGTCAGTGGTGGTGGTGGTGGTG	pRGK448
ccmG_C80A/C83A_Fwd	CTGGGCGACCTGGGCTCCGACCGCCCGTGCGGAACAT	pRGK446
ccmG_C80A/C83A_Rev	ATGTTCCGCACGGGCGGTCGGAGCCCAGGTCGCCCAG	pRGK446
ccmH_C43A/C46A_Fwd	CTCACTGAAGAACTGCGCGCCCCGAAAGCCCAGAACAACAGCATTGC	pRGK447
ccmH_C43A/C46A_Rev	GCAATGCTGTTGTTCTGGGCTTTCGGGGCGCGCAGTTCTTCAGTGAG	pRGK447
ccmF_H261G_Fwd	GGACTGCGCTGATGGGCTCACTGGCGGTCA	pRGK444
ccmF_H261G_Rev	TGACCGCCAGTGAGCCCATCAGCGCAGTCC	pRGK444
ccmF_H491A_Fwd	GGGATGGTGGCGGCTGCCCTTGGGCTGGC	pRGK445
ccmF_H491A_Rev	GCCAGCCCAAGGGCAGCCGCCACCATCCC	pRGK445
A.Fwd (delCcmF_right)	GCCGGAGGCCATATGAAGCGCAAAGTATTGTTA	gPCR
A.Rev (delCcmH_left)	GCGCCAATAAAAAGCTTATTGTGCGGCCTCCTT	gPCR
B.Fwd (delCcmC_right)	AAGAGGCCGCATATGACCCCTGCATTTGCTTCC	gPCR
B.Rev (delCcmF_left)	CCGAATTCTGGCATCATATGGCTGGGTCCTTAT	gPCR
C.Fwd (delCcmB_right)	GGTATCGAACATATGAGGAAAACACTGCATCAACT	gPCR
C.Rev (delCcmC_left)	AGTGTTTTCCACATATGTTCGATACCAGACTCG	gPCR
D.Fwd (menBflank_right)	CGCAGGCAAACATACAGCCC	gPCR
D.Rev (menBflank_left)	CGGACATAACGCGCATCGG	gPCR

REFERENCES

Beckman, D.L., Trawick, D.R., and Kranz, R.G. (1992) Bacterial cytochromes c biogenesis. *Genes Dev* 6: 268-283.

Feissner, R.E., Richard-Fogal, C.L., Frawley, E.R., Loughman, J.A., Earley, K.W., and Kranz, R.G. (2006) Recombinant cytochromes c biogenesis systems I and II and analysis of haem delivery pathways in Escherichia coli. *Mol Microbiol* 60: 563-577.

Richard-Fogal, C.L., Frawley, E.R., Bonner, E.R., Zhu, H., San Francisco, B., and Kranz, R.G. (2009) A conserved haem redox and trafficking pathway for cofactor attachment. *Embo J* 28: 2349-2359.

San Francisco, B., Bretsnyder, E.C., Rodgers, K.R., and Kranz, R.G. (2011) Heme ligand identification and redox properties of the cytochrome c synthetase, CcmF. *Biochemistry* 50: 10974-10985.