Supporting Information for World Wide Web Edition

Heme O Synthase and Heme A Synthase From *Bacillus subtilis* and *Rhodobacter sphaeroides* Interact in *Escherichia coli*

by

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Cloning of heme O synthase (HOS) and heme A synthase (HAS) from *R. sphaeroides* and preparation of expression plasmids

Cloning of cox10 (RSP1827). The *cox10* gene (0.9 kb) was amplified from plasmid pCF102 by PCR using standard conditions. *Nde*I and *Xba*I restriction sites were introduced into the 5' and 3' ends of *cox10* using the forward primer 5'-TGGATTCCATATGACCGACATCCGTATCACC-3' and reverse primer 5'-GCTCTAGATCACCAGCCTCCCAGTCC-3'. The PCR product was ligated into the pGEM vector and subcloned into pET-15b, pET-9d, and pET-29a(+). The sequence of *cox10* was confirmed using the forward primer 5'-ATCGGGCTCGCGCTCTC-3', the reverse primer 5'-GCCGGCGTCGGAATAGTC-3', and the PCR primers. Standard methods were used for all the transformations, and transformants were selected on LB-Agar plates containing appropriate antibiotic(s).

Cloning of cox15 (RSP3831). ORF RSP3831 (1.2 kb) was amplified from the genomic DNA of *R. sphaeroides* 2.4.1 [Pappas, 2004 #932] by PCR using standard conditions. *Nde*I and *Kpn*I restriction sites were introduced into the 5' and 3' ends of *cox15* (RSP3831) using the forward primer 5'-AGCCATATGGCAGTGAAAAAGCGC-3' and the reverse primer 5'-GCGACGGGTACCTCATAGCGTTCCTTTCCGG-3'. A C-terminally tagged derivative of *cox15* was also prepared using the reverse primer 5'-GATCCGGAAAGGAACTCTAATGGCTAGCATGACTGGTGGACAGCAAATGGGTTGAATCGGTACCAGACCGAG-3' (C-terminal T7 epitope tag). The PCR products were inserted into the *Nde*I and *Kpn*I restriction sites of pUC18. The sequence of *cox15* was confirmed with the forward primers 5'-TTGTCTGTAAGCGGATGCC-3' and 5'-TTCCTGCCCGCCGAGATG-3', and the reverse primer 5'-GTGACGGGGCGCCATTC-3'. Expression plasmids were constructed by inserting *Nde*I/*Eco*RI fragments of *cox15* into pET-21a(+).

Table S1: Characteristics of plasmids used in this study.

Plasmid	Vector	Insert	Selection	Cloning Sites	Epitope
pUC18-Cox15	pUC18	*cox15* (1.2kb)	*Amp*	NdeI, KpnI	none
pUC18-Cox15-cT7	pUC18	*cox15* (1.2kb)	*Amp*	NdeI, KpnI	c-T7[a]
pET21a(+)-Cox15	pET-21a(+)	*cox15* (1.2kb)	*Amp*	NdeI, EcoRI	none
pET21a(+)-Cox15-cT7	pET-21a(+)	*cox15* (1.2kb)	*Amp*	NdeI, EcoRI	c-T7[b]
pET21a(+)-Cox15-cHis$_6$	pET-21a(+)	*cox15* (1.2kb)	*Amp*	NdeI, EcoRI	c-His$_6$[b]
pGEM-Cox10	pGEM	*cox10* (0.9kb)	*Amp*	A-T sites	none
pET15b-Cox10-nHis$_6$	pET-15b	*cox10* (0.9kb)	*Amp*	NdeI, NdeI	n-His$_6$
pET9d-Cox10-nHis$_6$	pET-9d	*cox10* (0.9kb)	*Kan*	BglII, BlpI	n-His$_6$[c]
pET29a(+)-Cox10	pET-29a(+)	*cox10* (0.9kb)	*Kan*	NotI, NcoI	none
pET3d-CtaA	pET-3d	*ctaA* (0.9kb)	*Amp*	NcoI, BamHI	none
pET9d-CtaA	pET-9d	*ctaA* (0.9kb)	*Kan*	NcoI, BamHI	none
pET9d-CtaA-cHis$_6$	pET-9d	*ctaA* (0.9kb)	*Kan*	NcoI, BamHI	c-His$_6$[a]
pET29a(+)-CtaB	pET-29a(+)	*ctaB* (0.9kb)	*Kan*	NdeI, BamHI	none
pET9a-CtaB	pET-9a	*ctaB* (0.9kb)	*Kan*	NdeI, BamHI	none
pET3a-CtaB	pET-3a	*ctaB* (0.9kb)	*Amp*	NdeI, BamHI	none
pET3a-CtaB-nT7	pET-3a	*ctaB* (0.9kb)	*Amp*	BamHI, BamHI	n-T7

[a]This epitope tag was introduced via appropriately designed PCR primers.
[b]This insert was subcloned from pUC18-cox15-cT7/cHis$_6$.
[c]This insert was subcloned from pET15b-cox10-nHis.

Table S2: A summary of all strains used for the figures of this paper**.** All expression studies were executed in BL21(DE3) strain containing pLysS.

Figure	HOS plasmid	HAS plasmid
1A chromatogram 1	none	none
1A chromatogram 2	pET29a(+)-Cox10	none
1A chromatogram 3	pET29a(+)-Cox10	pET21a(+)-Cox15
1B chromatogram 1	none	none
1B chromatogram 2	pET29a(+)-CtaB	none
1B chromatogram 3	pET29a(+)-CtaB	pET3d-CtaA
2A column 1	pET29a(+)-Cox10	none
2A column 2	pET29a(+)-Cox10	empty pET-21a(+)
2A column 3	pET29a(+)-Cox10	pET21a(+)-Cox15
2A column 4	pET29a(+)-Cox10	pET3d-CtaA
2B column 1	pET29a(+)-CtaB	none
2B column 2	pET29a(+)-CtaB	empty pET-3d
2B column 3	pET29a(+)-CtaB	pET3d-CtaA
2B column 4	pET29a(+)-CtaB	pET21a(+)-Cox15
3A,B	pET3a-CtaB-nT7	pET9d-CtaA-cHis_6
3C,D	pET3a-CtaB-nT7	pET9d-CtaA-cHis_6
4	none	pET9d-CtaA-cHis_6
4	pET3a-CtaB-nT7	none
5A	none	pET9d-CtaA-cHis_6
5B	pET3a-CtaB-nT7	none
5C	pET9d-Cox10-nHis_6	none
5D	none	pET21a(+)-Cox15-cT7
6A,B	none	pET9d-CtaA-cHis_6, pET21a(+)-Cox15-cT7
6C,D	none	pET9d-CtaA-cHis_6
6C,D	none	pET21a(+)-Cox15-cT7
7A,B	pET9d-Cox10-nHis_6	pET21a(+)-Cox15-cT7
7C,D	pET3a-CtaB-nT7	pET21a(+)-Cox15-cHis_6
S2	pET29a(+)-Cox10	pET21a(+)-Cox15
S3	pET29a(+)-Cox10	pET21a(+)-Cox15
S4 column 1 (left)	pET3a-CtaB-nT7	none
S4 column 2	pET3a-CtaB-nT7	pET9d-CtaA-cHis_6
S4 column 3	none	pET9d-CtaA-cHis_6
S4 column 4	none	pET21a(+)-Cox15-cT7
S4 column 5	pET9d-Cox10-nHis_6	pET21a(+)-Cox15-cT7
S4 column 6 (right)	pET9d-Cox10-nHis_6	none
S5	pET3a-CtaB-nT7	pET9d-CtaA-cHis_6

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CLUSTAL W (v. 1.82) multiple sequence alignment.

S. cerevisiae  ------MLFRNIEVGRQAAKLLTRTSSRLAWQSIGASRNISTIRQQIRKTQLYNFKKTVS 54
H. sapien      ---------MQRLLFPPLRALKGRQYLPLLAPRAAPRAQCDCIRRPLRPGQYS------- 44
S. pombe       MNISRSSGLMRQFLLQPLRKGCDISCLGRSSWRMSRSFSGSSVLNEINLSRTKNLFLNDC 60
RSP3831        -----------------MAVKKRSIFEEVGQGAKAPVPQGGSIDRGHGG----------- 32
                                                .   . . : .

S. cerevisiae  IRPFSLSSPVFKPHVASESNP------IESRLKTSKNVAYWLIGTSGLVFGIVVLGGLTR 108
H. sapien      ----TISEVALQSGRGTVSLP------SKA---AERVVGRWLLVCSGTVAGAVILGGVTR 91
S. pombe       KFNKNSFEKFFARRLSNSVAPTPGGILQETEKIPSKKVAFWLLGSSALVLAIVVVGGITR 120
RSP3831        --------------------------------ARRGIRLWLMALFLLVMAMIVVGGLTR 59
                                             . : :  **:     * . :::**:**

S. cerevisiae  LTESGLSITEWKPVTGTLPPMNQKEWEEEFIKYKESPEFKLLNSHIDLDEFKFIFFMEWI 168
H. sapien      LTESGLSMVDWHLIKEMKPPTSQEEWEAEFQRYQQFPEFKILNHDMTLTEFKFIWYMEYS 151
S. pombe       LTESGLSITEWKPITGVIPPLTDEQWNQEFELYKKSPEFEKLNSHMTVDEFKNIFFWEWF 180
RSP3831        LTDSGLSITEWRPVTGAVPPLNETQWAAEFDKYRDSPQYRLMNAGMTLAEFQRIYWWEWG 119
               **:****:.:*: :.   ** .: :*  **  *:. *::. :*  : : **: *:: *:

S. cerevisiae  HRLWGRAIGAVFILPAVYFAVSKKTSGHVNKRLFGLAGLLGLQGFVGWWMVKSGLDQEQL 228
H. sapien      HRMWGRLVGLVYILPAAYFWRKGWLSRGMKGRVLALCGLVCFQGLLGWYMVKSGLEEK-- 209
S. pombe       HRVLGRGIGLTILLPSIYMIVTKRASPWLSKRLIGLTGLVGLQGVIGWWMVKSGLSEELF 240
RSP3831        HRQLGRVIGLVWAVGFLGFLAARRIPRGWWPRLLALGALGGLQGGIGWWMVASGLEGD-- 177
               **  ** :* .  :    :      .     *::.* .*  :** :**:** ***. .

S. cerevisiae  DARKSKPTVSQYRLTTHLGTAFFLYMGMLWTGLEILRECKWIKNPVQAISLFKKLDNPAI 288
H. sapien      SDSHDIPRVSQYRLAAHLGSALVLYCASLWTSLSLL------------LPPHKLPETHQL 257
S. pombe       SDGS-HPRVSHYRLATHLAAAVALYIGLVWTGHGILQRHAFLKSMKSGSTSQLTSMVSSV 299
RSP3831        -----KVTVESTRLATHLGLAFIILGLIAWQALLLG-------------RSESDLLQARR 219
                      *.  **::**. *. :     * .  :

S. cerevisiae  GPMRKISLAL---LAVSFLTAMSGGMVAGLDAGWVYNTWPKMGER-WFPSSRELMDENFC 344
H. sapien      LQLRRFAHGT---AGLVFLTALSGAFVAGLDAGLVYNSFPKMGES-WIPEDLFTFSP--- 310
S. pombe       QKMKGFRTSVNSFVGLVLITLLSGAFVAGLDAGMIYCTFPEMGEGRLAPSKSELFDQRFC 359
RSP3831        QKDGRLVTLTTVLIGVAFLQIVLGALVAGIDAGRGFPTWPDMNGT-FLPAEMFYVPG--V 276
                    :        .: ::  : *.:***:***  : ::*.*.     * .   .

S. cerevisiae  RREDKKDLWWRNLLENPVTVQLVHRTCAYVAFTSVLAAHMYAIKKKAVIPRNAMTSLHVM 404
H. sapien      --------ILRNVFENPTMVQFDHRILGITSVT--AITVLYFLSRRIPLPRRTKMAAVTL 360
S. pombe       RKDDKSDLIWRNMIDNPSLVQLEHRILAITTFVAACGLFIFSRAKRNILPKKIKTSINVV 419
RSP3831        ETDWRNPAWWLGLLQNPGFVQFLHRMAGYTLAALGLIFWIFGRRSR---HRATRGAFDLL 333
                         .:::**  **: **  . .  .      ::    :    :    :   :

S. cerevisiae  MGVVTLQATLGILTILYLVPISLASIHQAGALALLTSSLVFASQLRKPRAPMRNVIITLP 464
H. sapien      LALAYTQG-----PVLFNFTFKISDLDEG----------------------IRNI----- 388
S. pombe       TGVVTAQATLGIMTLIYVVPVPLAALHQAGSLVTLTAALSLAQR-LHPEYALKNIRSWTK 478
RSP3831        AMALLAQILLGVGTVLSAAEWQVAIAHQVG-----------------AVVIWVLILHAR 375
                     *      .::      ::  .:                       :  :

S. cerevisiae  HSSKVTSGKILSEASKLASKPL------------------------------------- 486
H. sapien      ------------------------------------------------------------
S. pombe       LISSPPKSSISSSILTQQRQFHTFRPSFHSEIKKPLPGTGIKVFFVTPEGREIMIEGNEG 538
RSP3831        HLALYPRVGSIRKGTL---------------------------------------- 391
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Figure S1: An alignment of the expected gene product of ORF RSP3831 from *R. sphaeroides* and heme A synthases from various eukaryotes (Cox15p). Heme A synthase from *S. pombe* has been truncated to remove a fused C-terminal mitochondrial ferredoxin domain (Barros et al. *FEBS Lett.* **2001**, *492*, 133-138). We propose that ORF RSP3831 should be named *cox15* based on this sequence similarity and protein function. Three His residues and two Arg residues (highlighted) are conserved across multiple eukaryotic and prokaryotic organisms and may be important for function.

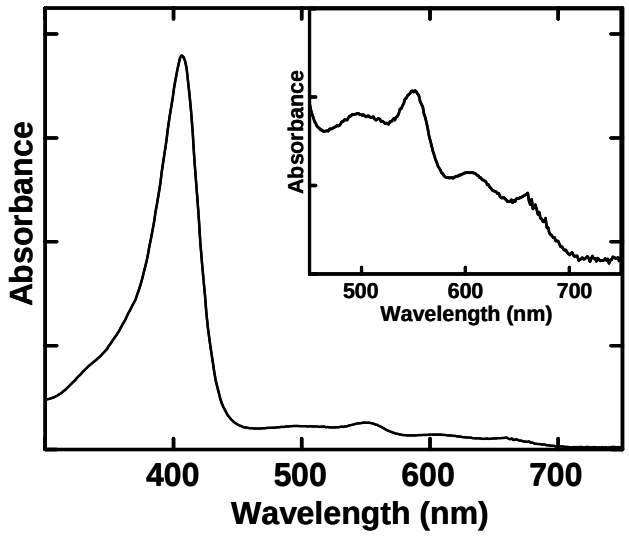

Figure S2: Optical spectra of heme A isolated from *E. coli* cells expressing both HOS (pET29a(+)-Cox10) and HAS (pET21a(+)-Cox15) from *R. sphaeroides*.

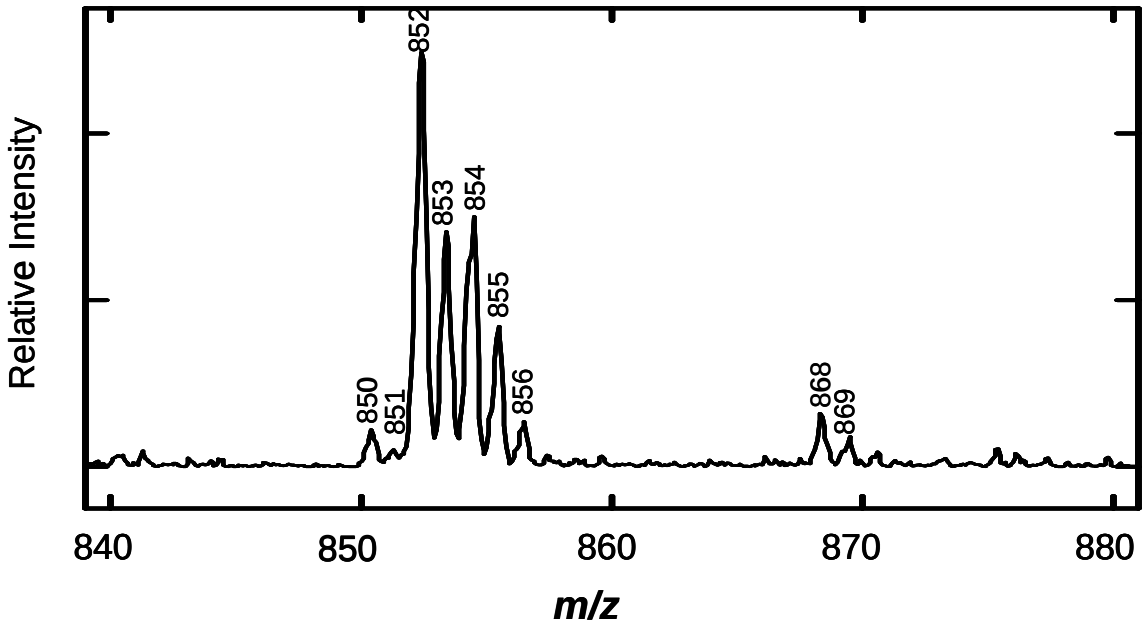

Figure S3: Mass spectral analysis of heme A isolated from *E. coli* cells expressing both HOS (pET29a(+)-Cox10) and HAS (pET21a(+)-Cox15) from *R. sphaeroides*. The molecular weight of heme A is 852 g/mol. Overlaying the isotopic pattern of heme A is the C8 alcohol derivative of heme O which has a molecular weight of 854 g/mol. Also seen in the mass spectrum is the C8 carboxylate derivative of heme O which has a molecular weight of 868 g/mol. All of these heme derivatives have been previously identified and characterized by our lab (see Brown et al. *Biochemistry* **2002**, *41*, 10906-10913).

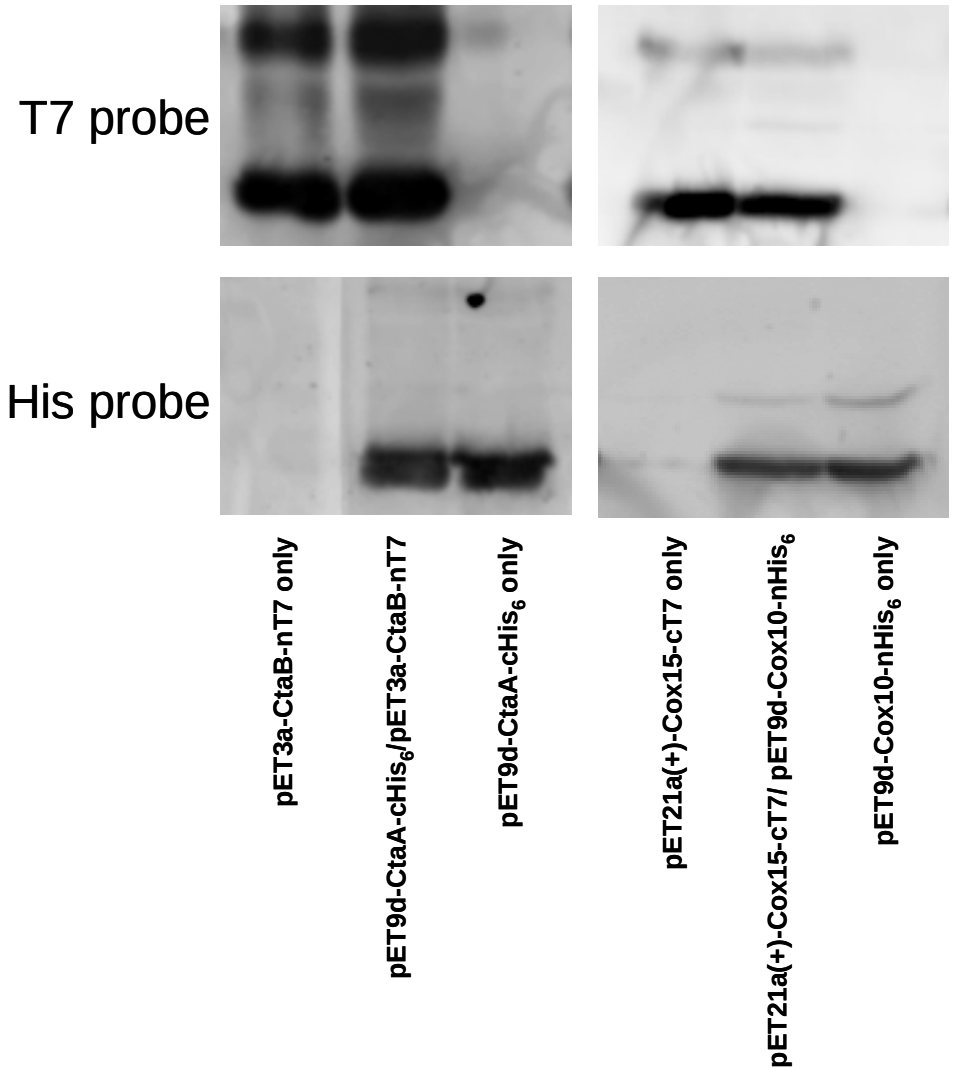

Figure S4: Western blot analysis of lysates from cells expressing epitope-tagged HOS and HAS either individually or in combination. The results of the T7 antibody demonstrate that CtaB-nT7 (bottom band) and Cox15-cT7 (bottom band) are expressed at the same level regardless of the presence or absence of the expression of its physiological partner. Likewise, the results of the anti 6x His antibody demonstrate that CtaA-cHis$_6$ and Cox10-nHis$_6$ are expressed at the same level regardless of the presence or absence of CtaB-nT7 and Cox15-cT7, respectively. Furthermore these experiments also demonstrate that the antibodies do not recognize these proteins in the absence of the appropriate epitope tag.

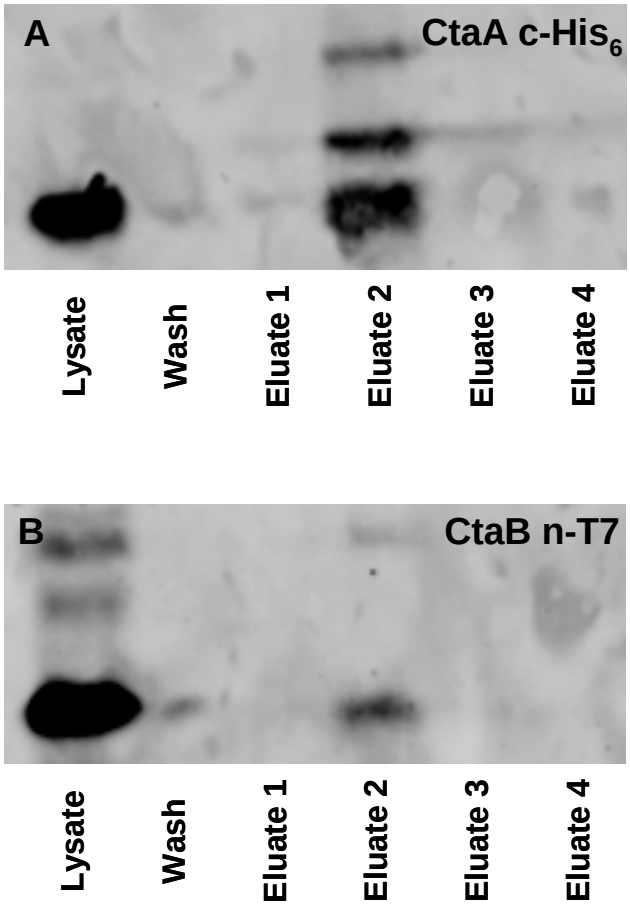

Figure S5: Western blot analysis of proteins purified from *E. coli* cells coexpressing CtaA-cHis$_6$ (pET9d-CtaA-cHis$_6$) and CtaB-nT7 (pET3a-CtaB-nT7). Cell Lysates were solubilized with 0.5% n-dodecyl β-D-maltoside. (A) CtaA-cHis$_6$ (bottom band) purified on a Ni-NTA column. (B) The same fractions probed with a T7 antibody demonstrate that CtaB-nT7 (bottom band) copurifies with CtaA-cHis$_6$ when n-dodecyl β-D-maltoside is used as the detergent.