a

MAQRIFTLILLLASTSVFAGLFDAPGRSQFVPADQAFAFDFQQNQHDLNLTWQIKDGYYLYRKQIRITPEHAKI
ADVQLPQGVWHEDEFYGKSEIYRDRLTLPVTINQASAGATLTVTYQGCADAGFCYPPETKTVPLSEVVANNAAP
QPVSVPQLVPR*GSTSQEQPTAQ*LMAFALGILSVFSPCVLPVVPLIFAGSRGRALDAFLIVAGLTISMLILGYTA
SLFFGFFRVVAMLFLLIFALILLSDELDEKVSIFASRMTSGLSWKIQTLPSFFFGMLLAFLWLPCILPFAGIAI
SQTLLSENPLVMLSYGLGMAVTIAAVFKMGEKFVKANFQLIRKVTGAIVLLYLAYFALTEVLL*LEHHHHHH*

b

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                        1          11   *     21         31         41
AfCcdA(CC)  GSTSQEQPTAQLMAFALGILSVFSPCVLPVVPLIFAGSRGRALDAFLIVAGLTISMLILG
AfCcdA(AA)  GSTSQEQPTAQLMAFALGILSVFSPAVLPVVPLIFAGSRGRALDAFLIVAGLTISMLILG
                        TM1                          TM2
                        H1                           H2

            51         61         71         81         91         101
AfCcdA(CC)  YTASLFFGFFRVVAMLFLLIFALILLSDELDEKVSIFASRMTSGLSWKIQTLPSFFFGML
AfCcdA(AA)  YTASLFFGFFRVVAMLFLLIFALILLSDELDEKVSIFASRMTSGLSWKIQTLPSFFFGML
                        TM3
                        H3                    H4

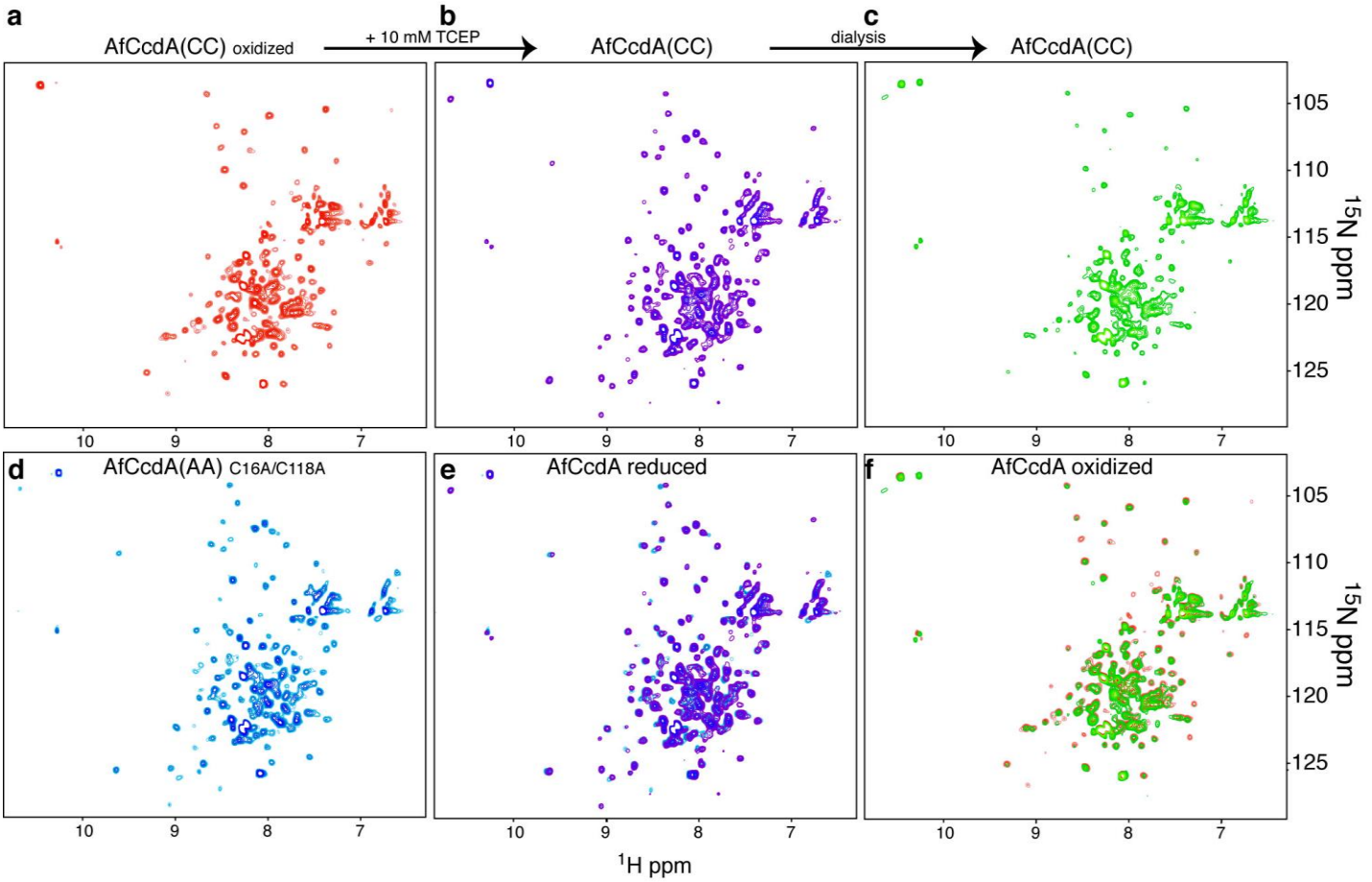
            111    *   121        131        141        151        161
AfCcdA(CC)  LAFLWLPCILPFAGIAISQTLLSENPLVMLSYGLGMAVTIAAVFKMGEKFVKANFQLIRK
AfCcdA(AA)  LAFLWLPAILPFAGIAISQTLLSENPLVMLSYGLGMAVTIAAVFKMGEKFVKANFQLIRK
                        TM4                          TM5
                        h          h'                H5

            171        181        191
AfCcdA(CC)  VTGAIVLLYLAYFALTEVLLLEHHHHHH
AfCcdA(AA)  VTGAIVLLYLAYFALTEVLLLEHHHHHH
                        TM6
                        H6
```

Supplementary Figure 1

***Af*CcdA sequences and topology.**

a. Amino acid sequence of the *Af*CcdA expression construct. *E. coli* DsbDα (gray) is at the N-terminus, followed by a thrombin cleavage site (underlined) and a 9-residue spacer, followed by the *Af*CcdA sequence with a C-terminal His-tag. Non-native residues in the final NMR protein sample are *italicized*.**b**. *Af*CcdA oxidized (CC) and reduced state (AA) sequences marked with actual and predicted TM helices. Amino acid sequences of the NMR constructs used in this study are aligned and colored as follows: unassigned positions are indicated in red; helical regions predicted by TALOS+ are underlined; and non-native sequences are *italicized* and the positions of the functional cysteines are marked with (*). For *Af*CcdA(AA), open boxes below the sequence indicate the TMpred predicted TM helices and colored boxes correspond to the structure presented in Figure 2.



Supplementary Figure 2

^{1}H-^{15}N TROSY-HSQC spectra of interchanging redox conformations of *Af*CcdA.

a. Wild-type AfCcdA(CC), prepared so that the disulfide bond is oxidized (red).

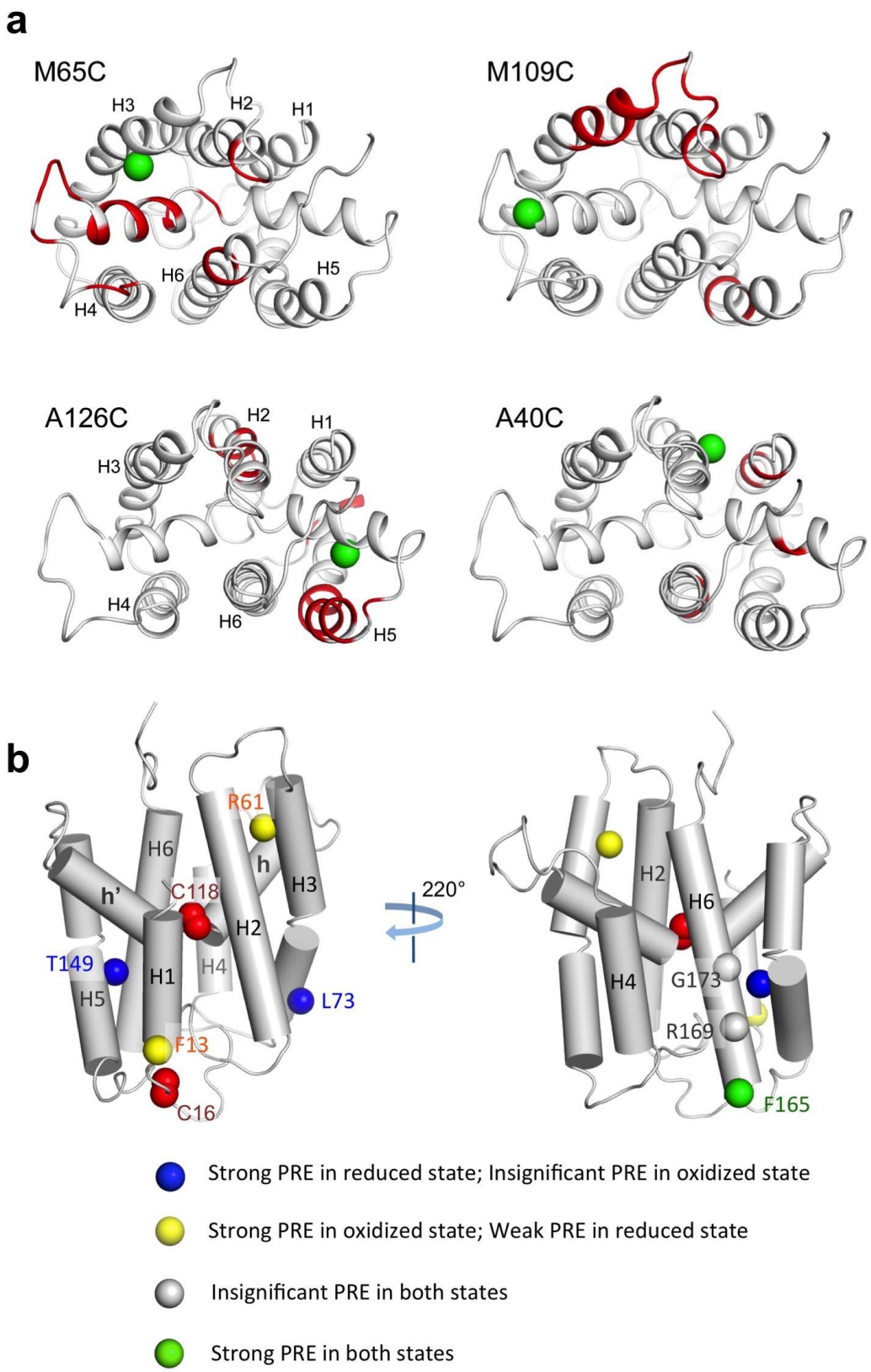
b. AfCcdA(CC) with 10 mM TCEP to reduce the disulfide bond (purple).

c. AfCcdA(CC) after dialysis to remove TCEP (green).

d. Double mutant AfCcdA C16A/C118A (AA) (blue).

e. Overlay of AfCcdA(AA) with AfCcdA(CC) + TCEP showing the equivalent reduced states of AfCcdA.

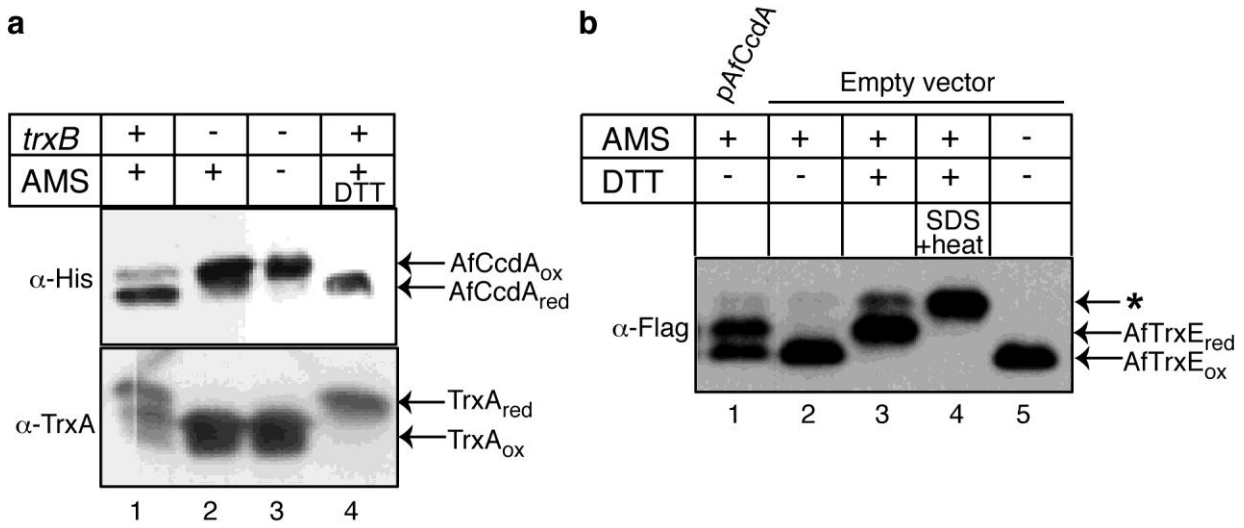
f. Overlay of oxidized AfCcdA(CC) and AfCcdA(CC) post-dialysis illustrating the return to the oxidized state in the NMR conditions.



Supplementary Figure 3

PRE analyses of CcdA.

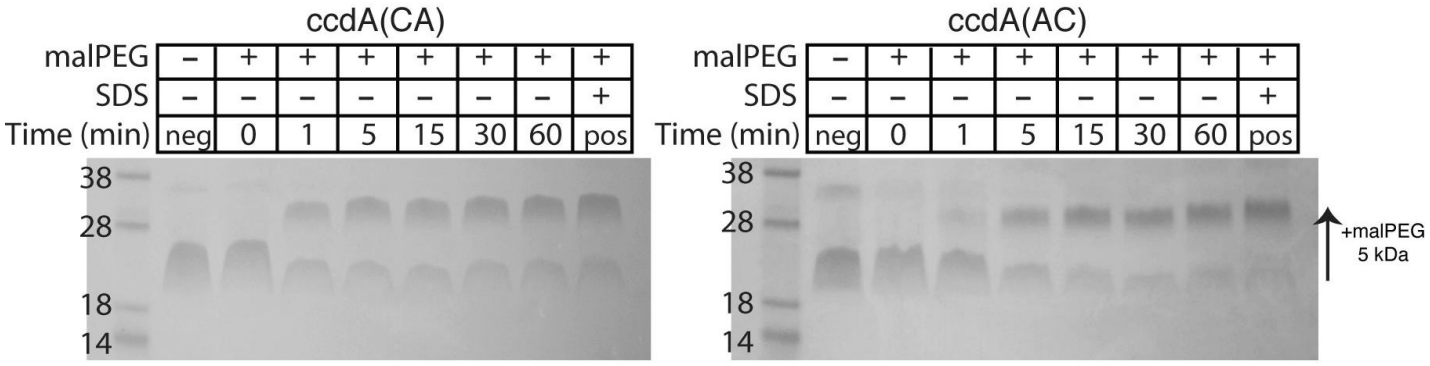
a. **Validation of NOE-derived structure with PRE.** Ribbon representations of AfCcdA(AA), with green spheres denoting the residue positions labeled with MTSL. PRE effects were measured as the ratio of peak intensities before and after reduction of MTSL with ascorbate. Strong long-range PREs are mapped onto the structure in red (PRE ratios < 0.2). The PRE effects are only shown for residues that are not in the same helix as the MTSL label.**b**. **Alternating accessibility of AfCcdA oxidized and reduced states probed by Gd-DOTA titration.** The AfCcdA(AA) reduced state structure is shown in grey with the cysteine positions indicated with red spheres. A strong PRE is indicative of solvent accessibility. Positions in the structure are indicated as follows: blue spheres indicate positions with greater accessibility in the reduced state; yellow spheres indicate positions with greater accessibility in the oxidized state; grey spheres indicate positions with insignificant accessibility in both states; and green spheres indicate strong accessibility in both states. PRE values were calculated as the ratio of the amide peak intensity with Gd-DOTA to the reference intensity without the paramagnetic.



Supplementary Figure 4

The *in vivo* functionality of *Af*CcdA.

a. **TrxA can reduce AfCcdA**. AfCcdA without the DsbDα fusion was expressed in wild-type (lanes 1 and 4) and Δ*trxB* (lanes 2 and 3) *E. coli* strains. AfCcdA is significantly in the reduced state in the wild-type strain (lane 1), while TrxA is in the oxidized state and keeps AfCcdA oxidized in the Δ*trxB* strain (lane 2). AMS alkylation was used to discriminate the redox states of the proteins. No AMS (lane 3) and DTT prior to AMS (lane 4) were added to the samples as controls. Note that the gel shift to show the redox states of AfCcdA is reverse of the shift for those of Dα-AfCcdA (Fig. 3a). Anti-His and anti-TrxA antibodies were used to detect AfCcdA-His and TrxA, respectively.**b**. **AfTrxE is the periplasmic substrate of AfCcdA**. AfTrxE was expressed with AfCcdA (lane 1) or alone (lanes 2-5) in the Δ*dsbD* strain. AfTrxE reduction depends on the presence of AfCcdA (compare lane 1 with lane 2). Lanes 3 to 5 are controls. In lanes 3 and 4, AfTrxE was reduced with DTT prior treatment with AMS. Two bands are observed because in addition to the active site CXXC, AfTrxE has an additional pair of cysteines that form a structural disulfide. This structural disulfide is partly reduced in lane 3 but fully reduced in lane 4 (* indicates reduction of the structural disulfide). The sample shown in lane 4 was prepared by reducing the protein in the presence of SDS, which denatures the protein and facilitates the reduction of the disulfide. Lane 5 shows the oxidized, non AMS-modified sample. Anti-Flag antibody was used to detect AfTrxE-3xFlag.



Supplementary Figure 5

AfCcdA single-cysteine mutants' time course of labeling by malPEG.

SDS-PAGE of AfCcdA-C118A (CA) and AfCcdA-C16A (AC) labeling by 5 kDa malPEG as a function of time (0-60 minutes) versus negative (no malPEG) and positive (SDS-denatured) controls.

a

Strains or Plasmids	Relevant Genotype or Features	Source or reference
Strains		
XL1-Blue	*endA*1 *gyrA*96(*nal*^{R}) *thi*-1 *recA*1 *relA*1 *lac gln*V44 F'[::Tn10 *proAB*$^+$ *lacI*^{q} Δ(*lacZ*)M15] *hsdR*17(r_K^- m_K^+)	Stratagene
C43(DE3)	*F- ompT hsdS*$_B$ (r_B- m_B-) *gal dcm* (DE3)	[31]
SEN212	C43(DE3) Δ*trxB* ::Cam^r	this study[32]
SEN256	C43(DE3) *dsbD*::mini-Tn*10* Cam^r	this study[5]
Plasmids		
pBAD43	Arabinose regulation, pSC101-based, Spectinomycin^r	[43]
pSC209	pBAD43 with 3×Flag	[15]
pET28a	IPTG-regulated T7 promoter, Kan^r	Novagen
pET22b	IPTG-regulated T7 promoter, Amp^r	Novagen
pSC124	pET28a with DsbDα^{thrombin}	this study
pSC148	pSC124 with *Af*CcdA	this study
pSC148AC	pSC148 with C16A in *Af*CcdA	this study
pSC148CA	pSC148 with C118A in *Af*CcdA	this study
pSC148AA	pSC148 with C16A and C118A in *Af*CcdA	this study
pSC148AA40C	pSC148AA with A40C in *Af*CcdA	this study
pSC148AA65C	pSC148AA with M65C in *Af*CcdA	this study
pSC148AA109C	pSC148AA with M109C in *Af*CcdA	this study
pSC148AA126C	pSC148AA with A126C in *Af*CcdA	this study
pSC175	pBAD43 with *Af*1675-3×FLAG	this study
pSC180	pET22b with *Af*CcdA	this study

b

Primers	Sequence (5' to 3')	Plasmids constructed
AfCcdA(NdeI)_F	AAAAAACATATGATGCTGATGGCATTCGCC	pSC180
AfCcdA(XhoI)_R	AAAAAACTCGAGCAGCAGGACCTCGGTCAG	pSC180
DsbDα(NcoI)_F	gagCCATGGCTCAACGCATCTTTAC	pSC124
DsbDα(BamHI)_R	gagGGATCCACGCGGAACCAGCTGCGGAACAGACA CAGGCTG	pSC124
AfCcdA(BamHI)_F	gagGGATCCACTAGTCAAGAGCAGCCCACCGCGCA ACTGATGGCATTCGCCCTGGGTATC	pSC148
Af1675(NheI)_F	gagGCTAGCAGGAGGAATTCACCATGAAGGCAATC TACGCTCTAC	pSC175
Af1675(XbaI)_R	gagTCTAGATTTCTCAAGCAGCATCATAAGCTC	pSC175
AF9ccda-C1F	TCCTGAGTGTGTTTAGTCCT**GCA**GTGCTGCCGGTA GTGCCGCT	pSC148AC pSC148AA
AF9ccda-C1R	AGCGGCACTACCGGCAGCAC**TGC**AGGACTAAACA CACTCAGGA	pSC148AC pSC148AA
AF9ccda-C2F	TGGCGTTTCTGTGGCTGCCG**GCA**ATCCTGCCGTTT GCGGGCAT	pSC148CA pSC148AA

AF9ccda-C2R	ATGCCCGCAAACGGCAGGAT**TGC**CGGCAGCCACA GAAACGCCA	pSC148CA pSC148AA
alphaA50CF	TGGACGCCTTTCTGATCGTCTGCGGGCTGACAATT AGTATGCT	pSC148AA40C
alphaA50CR	AGCATACTAATTGTCAGCCCGCAGACGATCAGAA AGGCGTCCA	pSC148AA40C
preM75CF	GTTTCTTCCGCGTGGTGGCTTGCCTGTTCCTGCTGA TCTTCG	pSC148AA65C
preM75CR	CGAAGATCAGCAGGAACAGGCAAGCCACCACGCG GAAGAAAC	pSC148AA65C
M119CF	TGCCATCTTTCTTCTTTGGTTGTCTGCTGGCGTTTC TGTGGCT	pSC148AA109C
M119CR	AGCCACAGAAACGCCAGCAGACAACCAAAGAAGA AAGATGGCA	pSC148AA109C
preA136CF	TCCTGCCGTTTGCGGGCATTTGTATCAGCCAGACT CTGCTGAG	pSC148AA126C
preA136CR	CTCAGCAGAGTCTGGCTGATACAAATGCCCGCAAA CGGCAGGA	pSC148AA126C

Supplementary Table 1

Strains and plasmids (a) and primers (b) used in this study

43. Guzman, L. M., Belin, D., Carson, M. J. & Beckwith, J. Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. J. Bacteriol. 177, 4121–4130 (1995).