Supporting Information

Leveraging Oxidative Stress to Regulate Balance-Based, *In Vivo* Growth Selections for Oxygenase Engineering

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A. Methods

B. Supporting Tables and Figures

C. References

A. Methods

Strain Construction. Construction of *E. coli* strain MX203 was described in a previous work[1] and served as the template strain for the deletion of genes associated with oxidative stress defense, *ahpC*, *katG*, and *sodA*. Plasmid pCP20 was used to eliminate kanamycin resistance in between knockouts performed[2]. Knockouts Δ*ahpC*, ΔkatG, and Δ*sodA* on MX203 were generated using the P1 phage transduction method[3]. Keio collection strains[2] JW0598-2 (*ΔahpC::kan*), JW3914-1 (*ΔkatG::kan*), and JW3879-1(*ΔsodA::kan*) served as donors for the generation of P1 lysate with gene knockout cassettes containing a kanamycin resistance marker. All knockouts were confirmed by MyTaq Colony PCR (Bioline).

Plasmid Construction. All PCR fragments were generated using PrimeSTAR Max DNA Polymerase (TaKaRa) unless otherwise noted. After PCR and gel extraction, all gene fragments were inserted into the pQElac vector backbone which contains a 6×His tag at the N-terminus (ColE1 ori, Amp^r), using Gibson isothermal DNA assembly method[4]. The *Bacillus megaterium BM3* gene was amplified from previously constructed plasmid pSM101[5]. Plasmid pLS202 and pLS203 carrying variants of the *BM3* gene were generated using plasmid pSM101 as a template and using site directed mutagenesis to introduce the following mutations F87V and R47A-Y51A-F87V, respectively. Plasmid pLS206 was generated using plasmid pLS202 as a template and using site directed mutagenesis to introduce the mutation L188Q.

The *Acinetobacter sp.* NCIMB 9871 *chnB* gene was amplified from an *E. coli* codon optimized gBlock (IDT). After PCR and gel extraction, the *Ac chnB* gene fragment was inserted into the pQE vector backbone using Gibson isothermal DNA assembly method, resulting in plasmid pLS201.

In order to facilitate downstream ligation in BM3 library construction, a silent AvrII site was introduced within 60 bp after site A328 codon and an AvrII site present on the standard vector (outside the gene) was eliminated. Both changes were introduced via single base-pair mutations using site-directed mutagenesis and previously described cloning methods to generate plasmid pLS206.

BM3 Library Construction. All PCR reactions with degenerate primers were generated with KOD Xtreme Hot Start DNA Polymerase (Novagen). To construct the *Bm BM3* NNK substrate binding site library (pLS206), a forward primer containing an NNK codon at position V78 was used in a PCR reaction with a reverse primer containing an MNN codon at position A328 to generate a DNA insert. The gel purified insert fragment and plasmid pLS206 (backbone) were separately digested with AvrII and AflII. Both digestion products were again gel purified and the appropriate length fragments were assembled by ligation, purified, and transformed into ElectroMAX DH10β cells (Invitrogen) using electroporation.

To construct the *Bm BM3* NNK substrate electron transport library (pLS217), a forward primer containing an NNK codon at position F393 was used in a PCR reaction with a reverse primer containing an MNN codon at position M490 to generate a DNA insert. The gel purified insert fragment and PCR generated backbone were assembled using the Gibson Assembly method with a 5:1 insert to backbone ratio. Plasmid generated was subsequently transformed into ElectroMAX DH10β cells (Invitrogen) using electroporation.

The following procedure was applied to both library constructions: After electroporation, cells were rescued with SOC medium at 37 °C for 1 hour and then added to 20 mL 2xYT medium (16 g/L Tryptone, 10 g/L Yeast Extract and 5 g/L NaCl) with appropriate antibiotics. 2 µL, 20 µL,

and 200 µL of culture was sampled from the culture and plated on 2xYT agar plates with appropriate antibiotics. The remaining liquid culture was incubated at 37 °C with 250 rpm agitation for 10 hours, before extraction of the library DNA. The plates were incubated at 37 °C overnight, and colonies formed were counted to estimate the library size. Six single colonies were also cultured individually to extract plasmids, which were sequenced to sample the sequence space of the library. All six plasmids sequenced contained unique mutation patterns in the targeted sites.

Growth Rescue Conditions. The growth rescue condition used for Figure 2B, 3B, S1, and S2 was as follows[1]: Briefly, the strains were first cultured in 2xYT under aerobic conditions at 30 °C overnight with appropriate antibiotics and inducers. Next, overnight cultures were washed 3 times and re-suspended in M9 Wash Buffer. For liquid growth, a 0.1% (v/v) volume of washed culture was used to inoculate to an OD_{600nm} of ~0.02 in 3 mL of M9 Selection Media, with appropriate antibiotics, inducers, and substrates. Culture tubes were incubated at 30 °C in a rotary shaker, and OD_{600nm} was measured using a 96-well plate reader.

Transformation of BM3 library by Electroporation. To generate electro-competent cells of *E. coli* MX203, cells were cultured in 200 mL SOB medium with appropriate antibiotics at 30 °C with shaking at 250 rpm until OD_{600nm} reached 0.4-0.6. The culture was chilled on ice for 15 min and the cells were pelleted at 4 °C, 4000×g. The cells were washed at 4 °C three times with 40 mL 10% glycerol in water (sterile, ice cold). After, cells were finally resuspended with 500 µL 10% glycerol in water (sterile, ice cold), and aliquoted for transformation.

The transformation was performed as follows: After MX203 electro-competent cells were prepared 20 µL of library DNA was added to 200 µL competent cells. Cell-DNA mixture was added to four ice chilled 1 mm gap electroporation cuvettes (55 µL of per electroporation cuvette). Cells were electroporated at 2 kV, 129 Ω, 50 µF, resistance 2.5 kV; 200 µL of SOC medium was immediately added and transferred to a microcentrifuge tube at room temperature. This step was repeated twice more. Cells were rescued at 37 °C with shaking for 1 hour. Serial dilution of the cells was performed and then plated on 2xYT agar plates with appropriate antibiotics. After incubation at 37 °C overnight, colonies formed were counted to estimate transformation efficiency.

Determination of Library Size Selected. After electroporation and rescue, all transformants culture containing the library were added to a flask containing 20 mL 2xYT. From this culture, 2 µL was plated and incubated overnight. The number of isolated MX203 colonies formed on this plate was counted, ~844 colony forming units (CFU) and ~463 for libraries pLS206 and pLS217, respectively. The library size in the entire culture was calculated as ~8.4×10^6 and 2.3×10^6 using the following equation.

$$Library\ Capability = \frac{844\ CFU}{2\ \mu L} \times \frac{1000\ \mu L}{mL} \times 20\ mL\ \approx 8.4 \times 10^6\ individual\ transformants$$

Selection of *Bm* BM3 Library. M9 Wash Buffer consisted of 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, trace metal mix A5 with Co (H_3BO_3 2860 µg/L, $MnCl_2 \cdot 4H_2O$ 1810 µg/L, $ZnSO_4 \cdot 7H_2O$ 222 µg/L, $Na_2MoO_4 \cdot 2H_2O$ 390 µg/L, $CuSO_4 \cdot 5H_2O$ 79 µg/L, $Co(NO_3)_2 \cdot 6H_2O$ 49 µg/L), and BD Difco M9 salts (Na_2HPO_4 6.78 g/L, KH_2PO_4 3g/L, NaCl 0.5 g/L, NH_4Cl 1 g/L). M9 Selection Media shared the same composition of M9 Wash Buffer with the inclusion of 2 g/L D-glucose, 0.01 g/L thiamine, 0.04 g/L $FeSO_4 \cdot 7H_2O$. For solid media M9 Selection Plates, 15 g/L agar was added in addition to M9 Selection Media composition.

E. coli MX203 was transformed with the libraries by electroporation as described above. After rescue in SOC medium for 1 hour, transformed cells were combined in 20 mL 2xYT with appropriate antibiotics in a 250 mL baffled shake flask. Controls were added to 5 mL 2xYT in a 50 mL conical tube (cap loose to allow increased aeration) and grown at 30 °C for ~7-8 hours or until OD_{600nm} = 0.6 was reached. Subsequently, controls and the library were induced by addition of IPTG and 0.5 mM 5-Aminolevulinic Acid was added. Cultures were grown for an additional 4 hours or until OD_{600nm} = ~1.68.

To prepare cells for the selection condition, 1 mL of each culture was pelleted in 2 mL microcentrifuge tubes and washed three times with M9 Wash Buffer. After wash, cells were finally re-suspended in 1 mL M9 Wash Buffer.

For liquid selection of substrate binding site library pLS206, ten culture tubes of 3 mL selection media with 0.5 mM 5-Aminolevulinic Acid, and 0.2 g/L of acenaphthene were inoculated to OD 0.02 and grown at 30°C for ~63 hr. Subsequently a small volume of culture from each library culture was plated on individual 2xYT media plates with appropriate antibiotics to isolate single variants. At time of harvest negative controls, three library cultures without substrate and a GVQ culture with substrate, did not show apparent growth.

For plate selection of electron transport library pLS217, washed library culture was diluted to a final cell concentration of ~10^7 cells/mL in M9 Wash Buffer. 100 µL of this cell suspension was plated on M9 Selection Plates (Supplemented with 0.2 g/L acenaphthene or lauric acid (For positive control) and 0.5 mM 5-Aminolevulinic Acid. Plates were incubated, and monitored periodically for roughly 72 h. Despite significantly reduced growth, a few colonies appeared to grow on negative control (pLS204) plate and indicated low level of background. Approximately 5 to 20 colonies appeared on each library plate. A diverse set of colonies were picked and re-streaked onto fresh selection plates were incubated at 30 °C to obtain single colonies. Three colonies were cultured in liquid media to extract plasmids using QIAprep Spin Miniprep kit (Qiagen) to yield pLS218, pLS219, and a wild type plasmid.

Subsequently, isolated variant plasmids were individually transformed into fresh MX203 cells. Selection procedure steps were repeated to confirm restoration of growth. After re-suspension in M9 Wash Buffer, washed cultures were used to inoculate 3 mL of M9 Selection media with 0.5 mM 5-Aminolevulinic Acid, and appropriate antibiotics and inducers. Cultures were grown either with or without supplementation of 0.2 g/L acenaphthene at 30 °C (Figure S2).

Protein Expression and Purification of *Bm BM3* Variants. Plasmids containing *Bm* BM3 variants were transformed into BL21 (DE3) and cultured in 2xYT medium with appropriate antibiotics at 37 °C overnight. Overnight cultures were then used to inoculate 100 mL 2xYT medium with antibiotics at 37 °C in a 500 mL baffled flask, agitated at 250 rpm to an OD_{600nm} =~1. The cultures were induced with 0.25 mM IPTG and 0.5 mM 5-Aminolevulinic Acid Hydrochloride Salt (Santa Cruz Biotechnology) and incubated at 30 °C (250 rpm) for 24 h. Recombinant protein was purified using His-Pure Ni-NTA Miniprep kit (Thermo Fisher) according to the manufacturer's instructions. The concentrations of purified proteins were quantified by Bradford assay against a BSA standard curve.

B. Supporting Table and Figures

Table S1. Strains and plasmids used in this study

Strains	Description	Reference
XL-1 blue	Cloning strain	Stratagene
BL21 (DE3)	Protein expression strain	Invitrogen
BW25113	*E. coli* F-, DE(araD-araB)567, lacZ4787(del)::rrnB-3, LAM-, rph-1, DE(rhaD-rhaB)568, hsdR514	Datsenko *et al.*[2]
DH10β	Electrotransformation strain	Invitrogen
JW0598-2	BW25113 Δ*ahpC::kan*	Coli Genetic Stock Center
JW3914-1	BW25113 Δ*katG::kan*	Coli Genetic Stock Center
JW3879-1	BW25113 Δ*sodA::kan*	Coli Genetic Stock Center
MX203	BW25113 Δ*pgi* Δ*edd* Δ*qor* Δ*udhA::kan*	Maxel *et al.*[1]
MX401	MX203 Δ*ahpC::kan*	This study
MX402	MX203 Δ*katG::kan*	This study
MX403	MX203 Δ*sodA::kan*	This study
MX404	MX203 Δ*ahpC* Δ*katG::kan*	This study
Plasmids	**Description**	**Reference**
pCP20	Temperature-inducible yeast Flp recombinase gene controlled by λ*cIts857* in a temperature-sensitive replicon	Datsenko *et al.*[2]
pQElac	Amp^r ; ColE1 ori; P_{LlacO1}. Expression vector	Li *et al.*[6]
pSM101	pQElac 6xHis *Bm BM3* A74G, Amp^r	Black *et al.*[5]
pSM105	pQElac 6xHis *Empty*, Amp^r	Black *et al.*[5]
pLS201	pQElac 6xHis *Ac chnB*, Amp^r	This study
pLS202	pQElac 6xHis *Bm BM3* A74G-F87V, Amp^r	This study
pLS203	pQElac 6xHis *Bm BM3* R47A-Y51A-A74G-F87V, Amp^r	This study
pLS204	pQElac 6xHis *Bm BM3* A74G-F87V-L188Q, Amp^r	This study
pLS205	pLS204 + modified AvrII sites	This study
pLS206	pLS205 V78-A328 NNK library, Amp^r	This study
pLS207	pQElac 6xHis *Bm BM3* V78S-A74G-F87V-L188Q-A328F, Amp^r	This study
pLS208	pQElac 6xHis *Bm BM3* V78F-A74G-F87V-L188Q-A328F, Amp^r	This study
pLS209	pQElac 6xHis *Bm BM3* V78S-A74G-F87V-L188Q-A328F, Amp^r	This study
pLS210	pQElac 6xHis *Bm BM3* V78S-A74G-F87V-L188Q-A328F, Amp^r	This study
pLS211	pQElac 6xHis *Bm BM3* V78A-A74G-F87V-L188Q-A328F, Amp^r	This study
pLS212	pQElac 6xHis *Bm BM3* V78A-A74G-F87V-L188Q-A328L, Amp^r	This study
pLS213	pQElac 6xHis *Bm BM3* V78S-A74G-F87V-L188Q-A328L, Amp^r	This study
pLS214	pQElac 6xHis *Bm BM3* V78A-A74G-F87V-L188Q-A328F, Amp^r	This study
pLS215	pQElac 6xHis *Bm BM3* V78S-A74G-F87V-L188Q-A328F, Amp^r	This study
pLS216	pQElac 6xHis *Bm BM3* V78S-A74G-F87V-L188Q-A328F-R148C, Amp^r	This study
pLS217	pLS204 F393-M490 NNK library, Amp^r	This study
pLS218	pQElac 6xHis *Bm BM3* A74G-F87V-L188Q-D222N, Amp^r	This study
pLS219	pQElac 6xHis *Bm BM3* A74G-F87V-L188Q-K224N, Amp^r	This study

Abbreviations indicate source of genes: *Bm Bacillus megaterium*, *Ac Acinetobacter sp.* NCIMB 9871

Table S2. Sequences of BM3 variants obtained from the active site liquid selection

Plasmid	Variant	Val 78		Ala 328	
		Codon	Residue	Codon	Residue
pLS205	GVQ (WT)	GTA	V	GCT	A
pLS207	GVQ-SF	TCG	S	TTT	F
pLS208	GVQ-FF	TTT	F	TTT	F
pLS209	GVQ-SF	AGT	S	TTT	F
pLS210	GVQ-SF	AGT	S	TTT	F
pLS211	GVQ-AF	GCT	A	TTT	F
pLS212	GVQ-AL	GCT	A	CTG	L
pLS213	GVQ-SL	AGT	S	TTG	L
pLS214	GVQ-AF	GCG	A	TTT	F
pLS215	GVQ-SF	TCT	S	TTT	F
pLS216*	GVQ-SFC	TCG	S	TTT	F

*Complete gene sequencing revealed a random mutation R148C on pLS216

Sequencing of the ten candidates revealed six unique residue combinations at the mutation sites. Based on the sequencing results we observed consensus of smaller amino acids alanine or serine at site 78 and bulky hydrophobic amino acids phenylalanine or leucine at site A328. All variants contained a combination of those amino acids with the exception of pLS208, which included a phenylalanine mutation at site 78 and pLS216 which included random mutation R148C.

Interestingly, although all the of variants selected restored growth, selection strains expression carrying pLS212 (GVQ-AL), pLS213 (GVQ-SL), and pLS216 (GVQ-SFC) demonstrated strong acenaphthene dependent growth as seen in Figure S2.

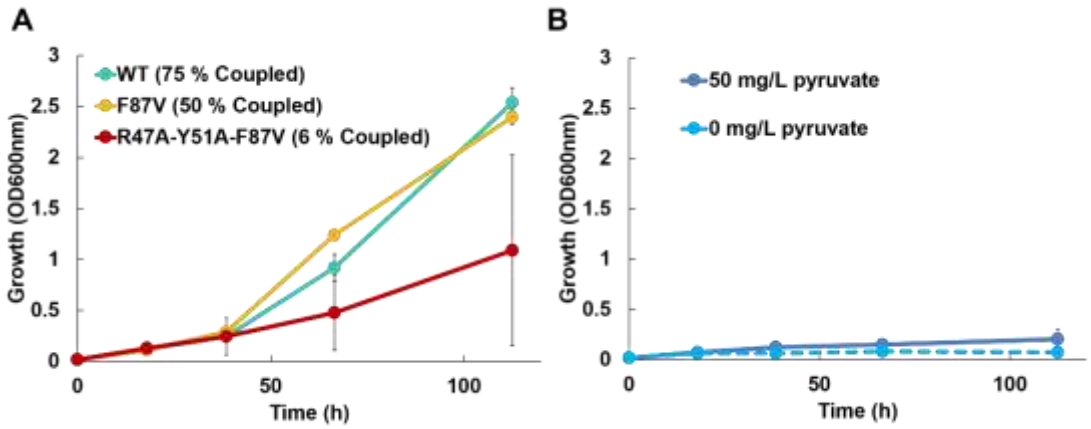

Figure S1. Growth of BM3 panel with pyruvate supplementation. (A) With the addition of 50 mg/L pyruvate as an H_2O_2 antioxidant to the selection media with 0.2 g/L of the substrate lauric acid, the BM3 panel displayed an apparent growth trend that correlates to their respective NADPH oxidation activity. BM3 WT and F87V, which share similar activity with lauric acid, demonstrated similar growth to each other with pyruvate. The BM3 variant R47A-Y51A-F87V, which has reduced activity with lauric acid, demonstrated elevated growth with pyruvate, but still had slower growth compared to WT and F87V. (B) In M9 minimal media with 2 g/L glucose with 0.2 g/L lauric acid, strain MX203 carrying an empty plasmid vector (pSM105) grew similarly with or without 50 mg/L pyruvate supplementation. Metabolism of pyruvate as a source carbon source can bypass the pentose phosphate pathway and minimize NADPH accumulation; however, addition of pyruvate did not restore strain growth without expression of NADPH consuming enzymes.

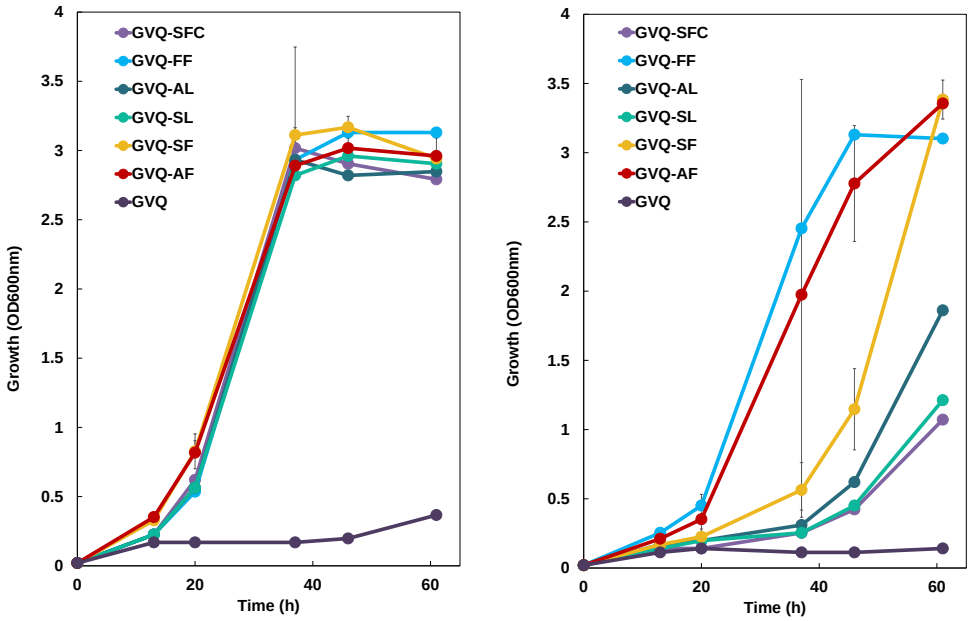

Figure S2. Selected variants displayed diverse growth phenotypes in M9 Selection Media with or without addition of substrate. (Left) In M9 minimal media with glucose as the sole substrate all GVQ variants isolated from liquid selection restored growth in the presence of 0.2 g/L acenaphthene. (Right) In M9 minimal media with glucose as the sole substrate all GVQ variants improved growth without acenaphthene in comparison to template GVQ. Growth restoration of with variants GVQ-FF (blue) and GVQ-AF (red) displayed little to no difference with and without substrate. Therefore, these variants were not characterized further. Expression of the remaining variants demonstrated improved growth with the substrate present and were all evaluated further to determine coupling efficiency. Observed substrate sensitivity was validated by improved coupling of variants GVQ-AL and GVQ-SL (dark and light teal, respectively) which both displayed significantly slower growth without the substrate.

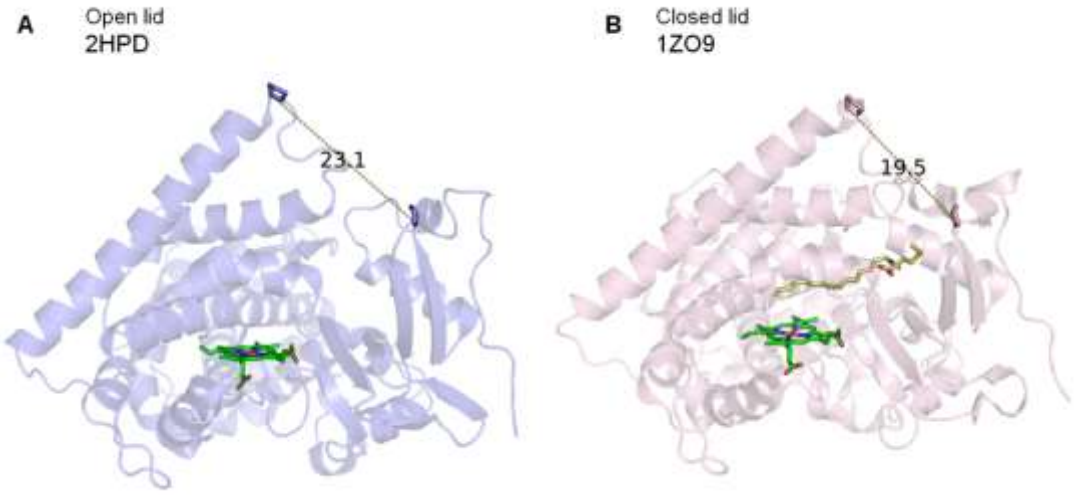

Figure S3. P450-BM3 structural flexibility. The lid opening motion of P450-BM3 is described by the alpha carbon distance from P196 located on the F/G loop to P45 across the substrate channel. The heme group is highlighted in green. A) PDB 2HPD[7] captures P450-BM3 in the inactive, open state with raised F and G helices. The lid opening distance is measured to be 23.1 Å. B) PDB 1ZO9[8] illustrates P450-BM3 in the substrate bound, closed form that is catalytically active. The bound substrate N-palmitoylmethionine is colored yellow, and the lid opening distance is measured to be 19.5 Å, indicating downward movement of the F and G helices by roughly 3.6 Å.

C. References

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