SUPPLEMENTAL DATA

Biochemical characterization and essentiality of *Plasmodium* fumarate hydratase

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Table S1. Oligonucleotide sequences used for various PCRs

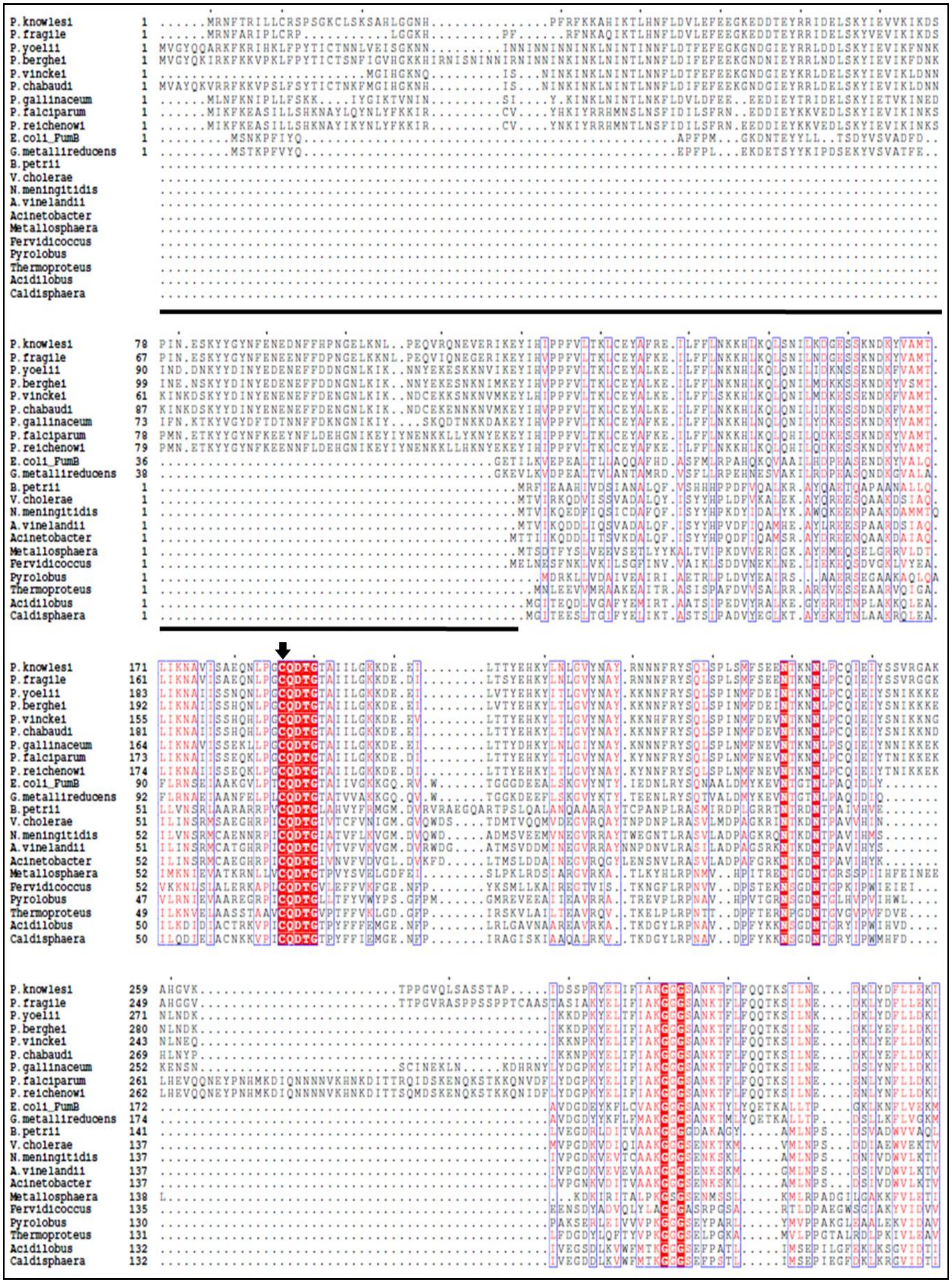
Primer Name	Primer sequence (5′ to 3′)	Application
PfFH FL _*BamH*1_FP	CGCGGATCCATAAAGTTTAAAGAAGCTTCC ATTTTG	Forward primer for cloning PfFHFL
PfFH_*Sal1*_RP	ACGCGTCGACTTATGATGGTAACCATTTATT ATAAAAATC	Common reverse primer for cloning PfFH
PfFHΔ40_*Bam H*1_FP	CGCGGATCCAGTTTAAATAGTTTTATAGACA TTTTAAGCTTTAG	Forward primer for cloning PfFHΔ40
PfFHΔ120_*Bam H*1_FP	CGCGGATCCAATTATGAAAAAGAATATATA CATATCCCACC	Forward primer for cloning PfFHΔ120
EcFumC_*BamH* I-FP	ACGCGGATCCATGAATACAGTACGCAGCGA AAAAGATTCG	Forward primer for cloning of EcFumC
EcFumC_*Sal*I-RP	ACGCGTCGACACGCCCGGCTTTCATACTGC	Reverse primer for cloning of EcFumC
EcFumA_BamH I-FP	GATGTAGGATCCATCAAACAAACCCTTTCAT TATCAGGCTC	Forward primer for cloning of EcFumA
EcFumA_SalI-RP	GCAATAGTCGACTTATTTCACACAGCGGGTG CATTGTG	Reverse primer for cloning of EcFumA
P1	CGGAACACCCGCCCAGAGCATAACCAAACC AGGCAGTAAGTGAGAGAACAGTGTAGGCTG GAGCTGCTTC	Generation and validation of *E.coli* strain ΔfumACB (in **Figure S3 a and b**)
P2	GCGCAGCCGCTTCGTTTGATCATTCCACGGC TGCACCTGTATGTTGCAGACTGTCAAACATG AGAATTAATTCCG	
P3	CTGAGTTAATGAGTTTTTGCATGATCAATCC CTG	
P4	CACAGCGGGTGCATTGTGTGAGTTG	
P5	GGCAGATAAGCTGTGGGGCGCAC	
P6	GCGTCTGGTACAAAGGAGATCAAAAACAAG TCC	
PfFHpGDB-*Xho*1-FP	GACTTACTCGAGATGATAAAGTTTAAAGAA GCTTCCATTTTGTTATC	Generation of the plasmid pGDB-PfFH
PfFHpGDB-*AvrI*I-RP	GACTTACCTAGGTGATGGTAACCATTTATTA TAAAAATCATTGCC	Generation of the plasmid pGDB-PfFH
P1 and P2	P1:GCCATTAAAAAATAGGATAACATATATA AAATGCACAATCC P2:CATATGATCTGGGTATCTCGCAAAGCATT G	5´ integration of RFA at PfFH genomic locus (**Figure 1a**)
P3 and P4	P3: GGAGACGGTCACAGCTTGTCTGTAAG P4:GTTGTGATATTGCACATGAATGGATCCAA TC	3´ integration of RFA at PfFH genomic locus (**Figure 1a**)

Qcup	CATTATTCTTTTTTCTTTTTTGCACATATTTA AAACG	Generation of *P. berghei fh* knockout construct (**Figure S4**)
QCdown	CCTTTTTCTTCAAACTCAAAAATATCTAAGA AATTG	
PheSR2	TCATTCTTCGAAAACGATCTGCG	
hdhfr	ACTTCTTAAACCTAATCTGTAGTAAGGAAGG GATTG	
RECupR2	GATTTTGTGTGAAATTTATTTAATAAATTTG TAAATTTTTGAACTCAACACCGCCTACTGCG ACTATAGA	
RECdownR1	TTAAAACTTATGAGTTTTTTTTTCCAACAAT GTACATATTTTTGGAAAAAAAGGCGCATAA CGATACCAC	
P1	CGAACTATGGGCATGGTAATAATAATGGAA ATAGC	Primers for diagnostic PCR *of P. berghei* clones. (**Figure 6 and 7**)
P2	GAAAAGCAGTAATGTTAGTACATCAAATGT GTATG	
P3	CAAGTCCAACTATTTATGAATCATTGAAGAG ACAAC	
P4	TTCTTAAACCTAATCTGTAGTAAGGAAGGG ATTG	
P5	GTGGATGAAAATATTACTGGTGCTTTGAGG GGTGAGC	
P6	ATGGTGAGCAAGGGCGAGGAG	
P7	CACATATTAACAATTTGACTCTCTGCATATT TTAGACTGATTTC	
P8	CGAGCTCTTTATGCTTAAGTTTACAATTTAA TATTC	
P9	ATGGTAGGATATCAAAAGATTAGAAAGTTT AAGAAGGTTCC	
P10	CTAAGAAGGTATCCATTTATTATAAAAGTCA TTTCCTTTATTATC	
Mqo_Fw	GGATAGCTATAGTTCCCTTCTTTTATTATATC TCAATAATTTTTGC	Primers for *mqo* gene (**Figure 7a**)
Mqo_Rv	GTAAAGCAGTACCTGTAACACCACCACC	

Table S2. Prediction of mitochondrial targeting sequence in Plasmodial FH

Organism	MitoProt II (Ref. 1)	TargetP (Ref. 2)	MitoFates (Ref. 3)	PlasMIT (Ref. 4)
P. falciparum	CS: NP (0.88)	CS:NP (0.34)	CS:38 (0.30)	Non-mito (99%)
P. reichenowi	CS:NP (0.97)	CS: NP (0.26)	CS:38 (0.29)	Non-mito (99%)
P. gaboni	CS:37 (0.92)	CS:NP (0.40)	CS:28 (0.24)	Non-mito (99%)
P. gallinaceum	CS:NP (0.85)	CS:NP (0.18)	CS:57 (0.01)	Mito (91%)
P. knowlesi	CS: 13 (0.99)	CS:11 (0.88)	CS:32 (0.69)	Mito (91%)
P. fragile	CS:21 (0.83)	CS:13 (0.83)	CS:12 (0.25)	Non-mito (99%)
P. vinckei	CS:NP (0.15)	CS:NP (0.09)	CS:45 (0.005)	Mito (91%)
P. chabaudi	CS:NP (0.89)	CS:9 (0.66)	CS:10 (0.17)	Mito (91%)
P. inui	CS:13 (0.97)	CS:50 (0.86)	CS:42 (0.32)	Mito (91%)
P. vivax	CS:13 (0.96)	CS:29 (0.816)	CS:21 (0.630)	Non-mito (99%)
P. berghei	CS:46 (0.85)	CS:NP (0.262)	CS:37 (0.251)	Mito (91%)
P. yoelii	CS:NP (0.435)	CS:NP (0.273)	CS:9 (0.078)	Mito (91%)
Pf DHODH#	CS:24 (0.50)	CS:NP (0.349)	CS:23 (0.996)	Mito (91%)

Fractional values in parentheses are probability values for either export to mitochondria (MitoProt II) or presence of mitochondrial targeting sequence (TargetP and MitoFates). Percentage values obtained from PlasMIT are an indication of the confidence for a specific localization. Boxes shaded grey in column 2 are for sequences for which MitoProt II has identified a targeting sequence and those in columns 3 and 4 are for sequences with probability values greater than 0.5 for presence of targeting sequence. In column 5, sequences predicted to have mitochondrial localization are shaded in grey. CS, cleavage site with the number adjacent corresponding to the predicted site of cleavage; mito, mitochondrially localized; non-mito, not localized to mitochondria; NP, not predicted. #As a positive control for the analysis, *P. falciparum* dihydroorotate dehydrogenase (PfDHODH), a well characterized protein with canonical mitochondrial targeting sequence was used.


P.knowlesi
P.fragile
P.yoelii
P.berghei
P.vinckei
P.chabaudi
P.gallinaceum
P.falciparum
P.reichenowi
E.coli_FumB
G.metallireducens
B.petrii
V.cholerae
N.meningitidis
A.vinelandii
Acinetobacter
Metallosphaera
Fervidicoccus
Pyrolobus
Thermoproteus
Acidilobus
Caldisphaera

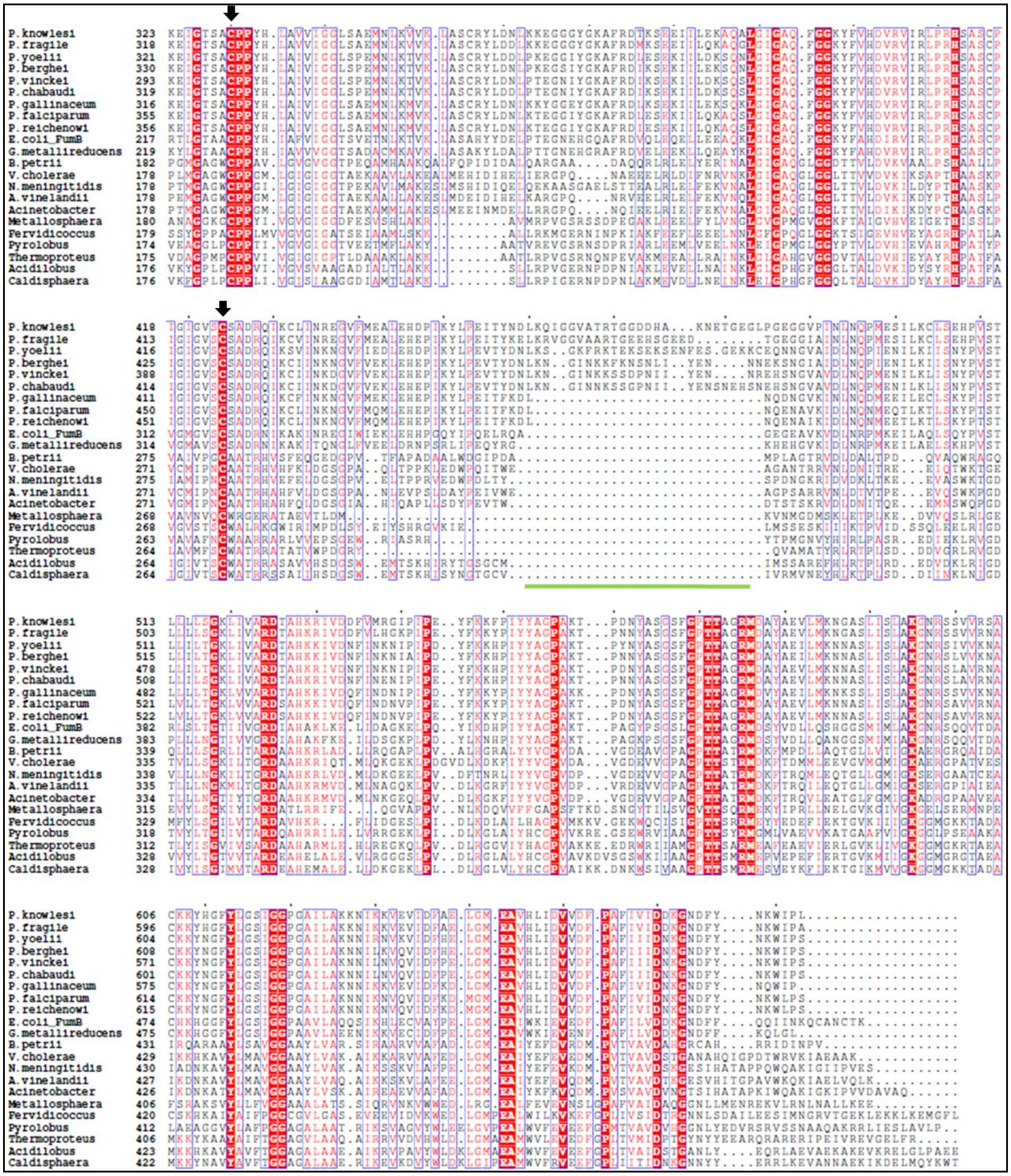


Figure S1. Multiple sequence alignment of *Plasmodial*, single-subunit and two-subunit bacterial and archaeal FH proteins sequences. The black solid line shows 120 amino acid insertion at the N-terminus in *Plasmodium* FH sequences. The black arrow shows invariant cysteine residues that are involved in Fe-S cluster ligation. The green bar indicates the boundary between the N- and C-terminal domains.

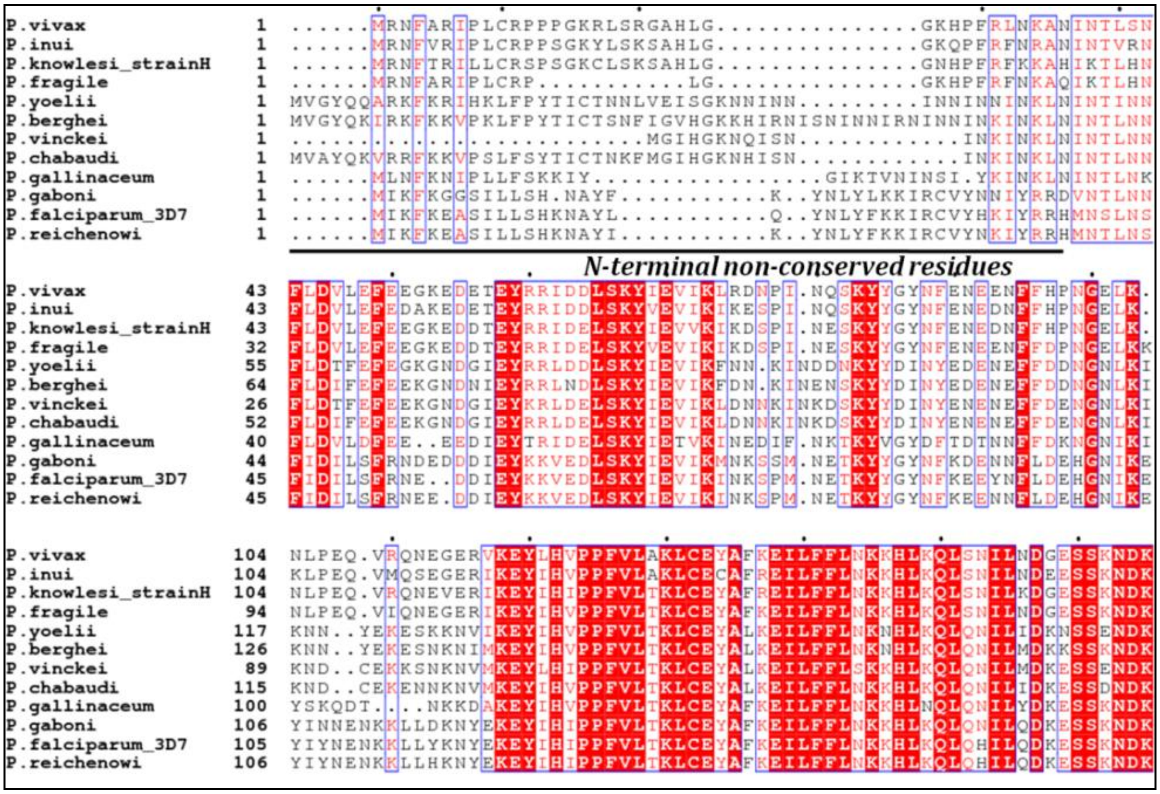


Figure S2. Multiple sequence alignment of FH sequences from different *Plasmodium* species. The initial 40 residues (Pf numbering) highlighted by a solid black line are not conserved. Many residues in the stretch of 41-120 residues, though not present in bacterial and two-subunit type FH sequences are highly conserved in all *Plasmodium* FH.

Methods S1. Generation of Δ*fumACB* strain of *E. coli*

To knockout *fumA* and *fumC* genes in the *E. coli* strain JW4083-1, established protocols were followed (5). Briefly, to remove the kanamycin cassette at the *fumB* locus, JW 4083-1 cells were transformed with the plasmid pCP20 that carries the gene for the enzyme flippase. Thereafter, the cells were cured of the plasmid by incubation at 42 °C for 12 h. Subsequently, the cells were transformed with the plasmid pSC101 to introduce the high-efficiency λ-phage recombination machinery. In order to knockout the genes *fumA* and *fumC*, oligonucleotide primers P1 and P2 (Table S1) were used to amplify a kanamycin cassette using plasmid pKD13 (5) as template, such that the PCR product carries the homologous regions corresponding to the 5´ flank of *fumA* and 3´ flank of *fumC* (Figure S3 a). The PCR product was DpnI treated and thereafter used for transformation. Transformation was performed by electroporation and cells were selected on LB medium containing kanamycin. Kanamycin resistant colonies were screened by PCR using oligonucleotides P3 and P6 to validate the insertion of the antibiotic cassette in the right locus as given in Figure S3 b and c. The cells were also checked for the absence of the wild-type genes, *fumA*, and *fumC* using oligonucleotide primers P3/P4 and P5/P6, respectively (Figure S3 d) The kanamycin cassette at the *fumA/C* gene locus was removed by expressing flippase in the strain resulting in the generation of marker free fumarate hydratase null strain.

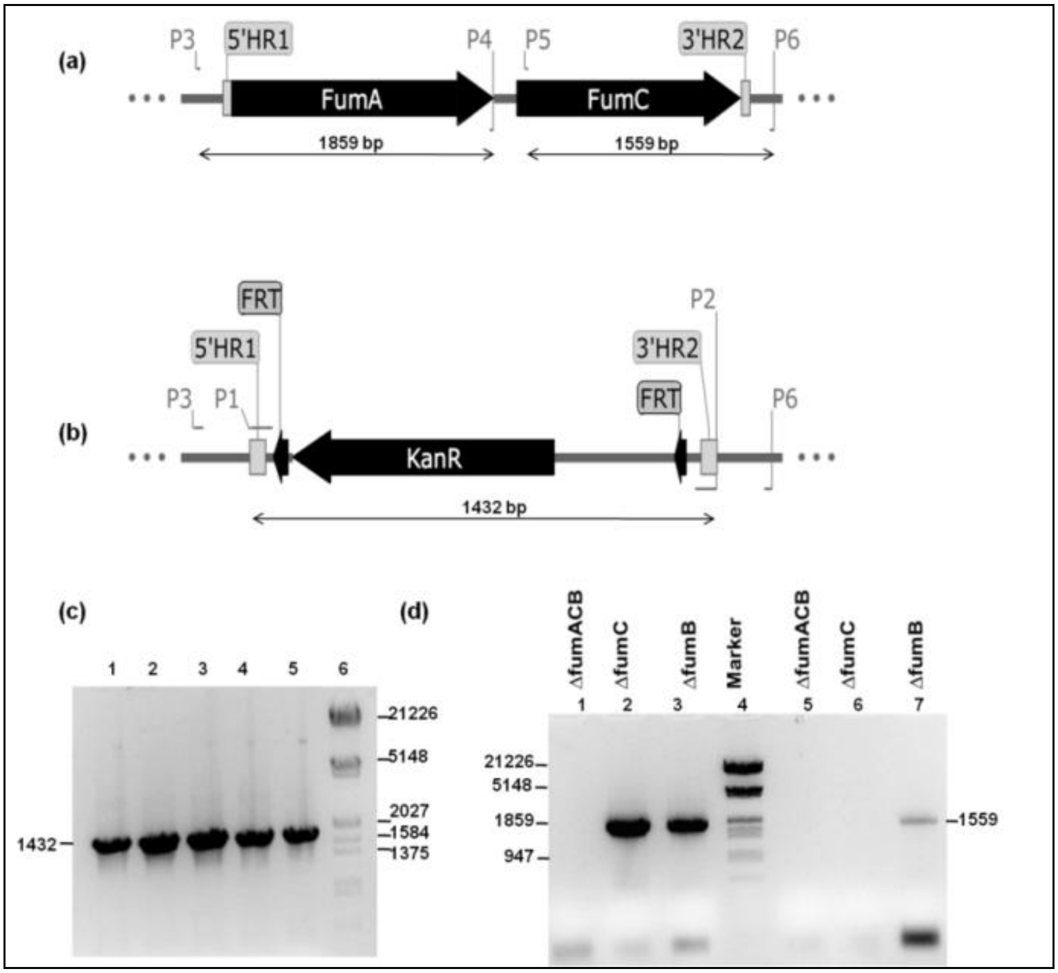


Figure S3. Genotyping of the *E. coli* strain Δ*fumACB.* (a) The relative orientation of *fumA* and *fumC* genes in the ΔfumB *E. coli* strain JW4083-1 and location of primers (P3-P6) used for validation of the knockout. 5′HR1 and 3′HR2 represent the 30 bp homologous regions used for gene replacement with kanamycin resistant marker. (b) fumA/C gene locus after homologous recombination and replacement with kanamycin resistance marker flanked by FRT sites. The orientation of the primers (P1 and P2) used for amplification by PCR of the kanamycin cassette is shown. (c) PCR amplified products using primers P1 and P2 and genomic DNA of 5 different colonies selected on kanamycin plate as template, showing the presence the kanamycin selection cassette integrated into the right locus. (d) Lanes 1-3 amplicons obtained on performing a PCR to check the presence/absence of *fumA* gene using primers P3 and P4 and genomic DNA from different *E. coli* strains (as mentioned in the figure) as template. Lanes 5-7, amplicons obtained on performing a PCR to check the presence/absence of *fumC* gene using primers P5 and P6 and genomic DNA from different FH knockout strains (as mentioned in the figure) as template. The size of DNA fragments is indicated in bp.

Text S1. *In vitro* enzyme inhibition studies

The small molecules examined for their effect on FH activity with fumarate as substrate were DL-mercaptosuccinic acid, meso-tartaric acid, succinic acid, aspartic acid, hadacidin (N-formyl-N-hydroxyglycine), alanosine, pyromellitic acid, itaconic acid, malonic acid, citric acid, trans-aconitic acid, D-malic acid, gamma-aminobutyric acid, alpha-ketoglutaric acid, oxaloacetic acid, DL-2 amino- 3 phenyl propionic acid, L-tartaric acid, sulfosuccinic acid, and ureidosuccinic acid. All molecules were tested at a concentration of 500 μM.

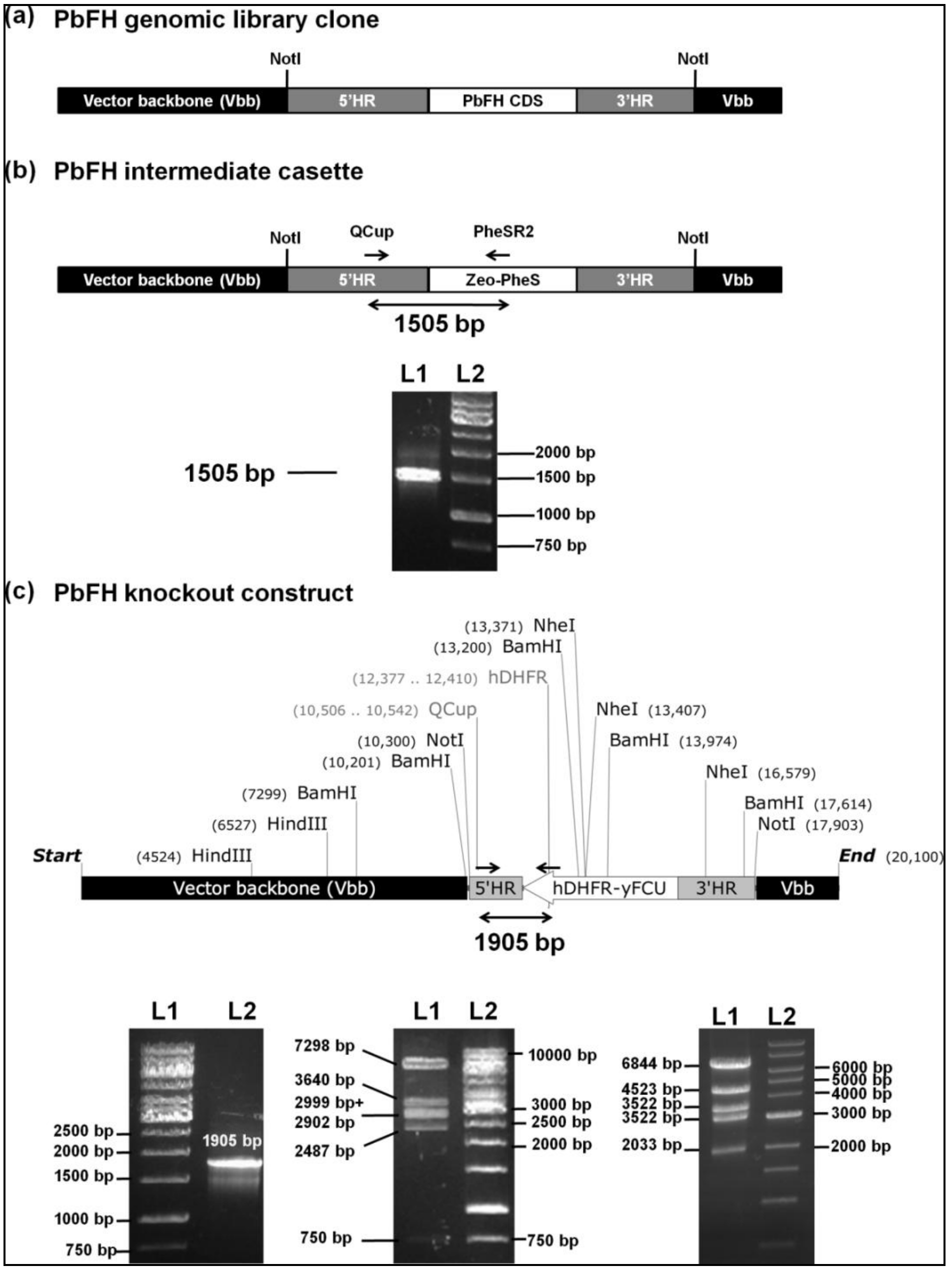


Figure S4. Generation of *P. berghei* FH knockout construct using recombineering. (a) Schematic representation of the PbFH genomic clone (PbG01-2466a09) obtained from PlasmoGEM. (b) Schematic representation of the intermediate vector in which the gene of interest is replaced by the bacterial selection marker, *ble* (resistant to zeocin). The position of validation primers QCup and PheSR2 are indicated. The agarose gel shows the band of expected size (1505 bp) obtained by PCR using QCup and PheSR2 oligonucleotide primers and the intermediate vector DNA isolated from a bacterial colony (L1) from the zeocin plate, L2, molecular weight marker. (c) Schematic representation of the final knockout construct in which the Zeo-Phe cassette is replaced by *Plasmodium* positive/negative selection cassette, hDHFR-yFCU. The position of validation oligonucleotide primers QCup and hdhfr are represented by black arrows. The agarose gel on the left shows the band of expected size (1905 bp) obtained by PCR using primers QCup and hdhfr and plasmid isolated from a bacterial colony (L2) (obtained upon negative selection in an LB-agar plate containing p-chlorophenylalanine) as template. L1, molecular weight marker. The agarose gels in the middle and right panels show the restriction mapping of the PbFHKO construct by BamHI and, NheI and HindIII digestion, respectively. All the bands of expected size are present. For transfection in *P. berghei,* this verified knockout construct was used.

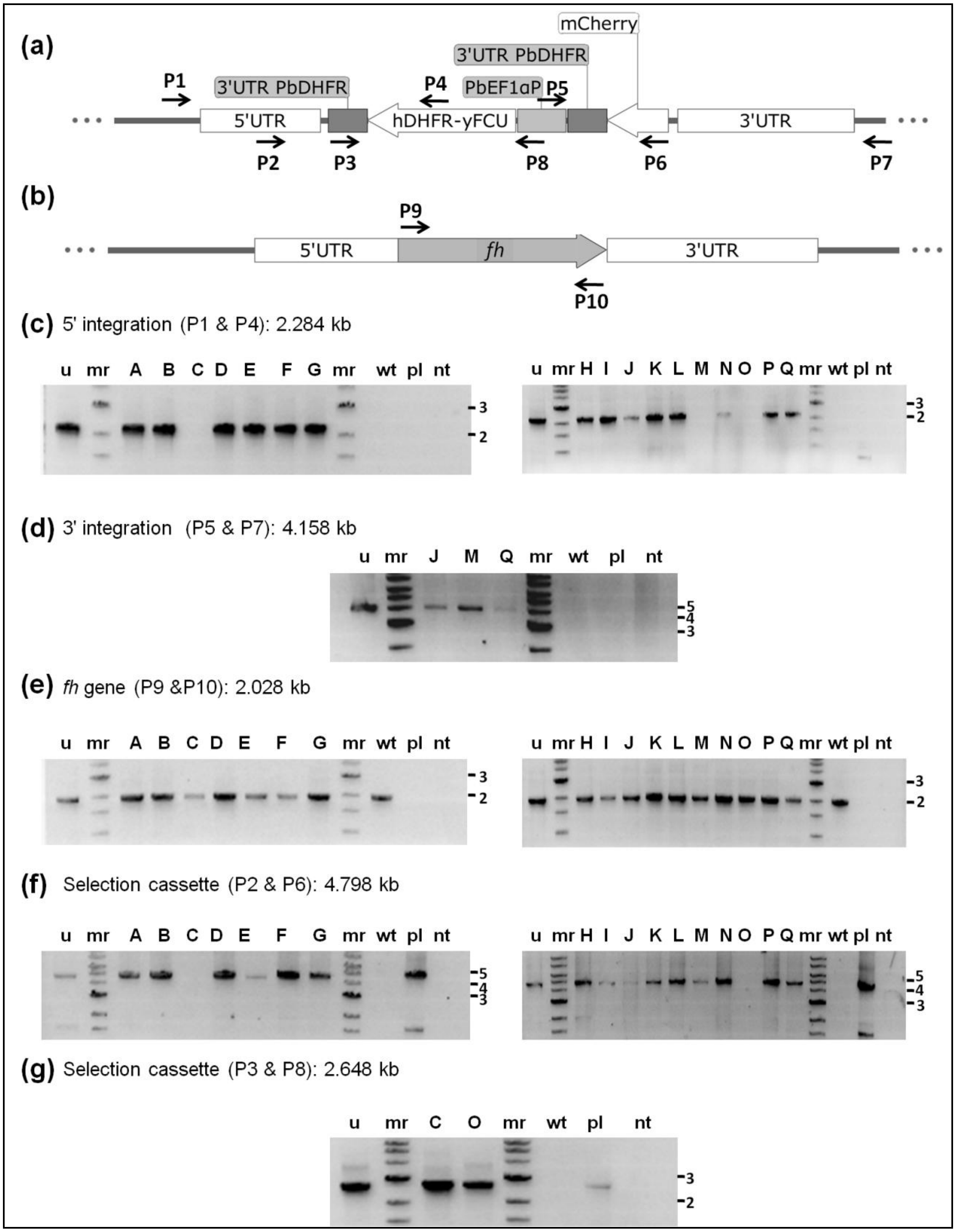


Figure S5. Genotyping of *P. berghei* clones of knockout of fumarate hydratase. (a) Schematic representation of the selectable marker cassette inserted into the *fh* gene locus of *P. berghei* genome. Primers (P1-P8) used for diagnostic PCRs are indicated. (b) Schematic representation of the *fh* gene (PBANKA_0828100) flanked by 5′ UTR and 3′ UTR showing the location of primers P9 and P10. Agarose gel electrophoresis of PCRs with genomic DNA from (c) clones A-G (left panel) and clones H-Q (right panel) for detection of 5´ integration; (d) clones J, M and Q for detection of 3´ integration (other clones did not answer for this PCR); (e) clones A-G (left panel) and clones H-Q (right panel) for the detection of *fh* gene; (f) clones A-G (left panel) and H-Q (right panel) for the presence of selectable marker cassette; (g) clones C and O using primers P3 and P8. Clones C, M and O did not answer for 5′ integration while only clones J, M and Q answered for 3′ integration. All clones answered for the presence of the *fh* gene (Panel d). All clones except C and O answered by PCR with primers P2 and P6 (left and right panels of f) indicating the integration of the entire selectable marker cassette into the genome. Clones C and O answered for a shorter fragment of the selectable marker cassette covered by primers P3 and P8 (panel g). hDHFR-yFCU, human DHFR-yeast cytosine and uridyl phosphoribosyltransferase, u, uncloned population, mr, molecular weight marker; wt, wild-type *P. berghei* genomic DNA; pl, pJAZZ-FH knockout construct (supplementary figure S4); nt, control PCR without template. Numbers to the right of panels d, e, f, g and h are the sizes of the marker DNA fragments in kbp.

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