Supplementary information for

Coloring Dysbiosis: FetB-Dependent Mn-PPIX Production by *Porphyromonas gingivalis* Shapes the Oral Microbiome

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Supplemental Figures

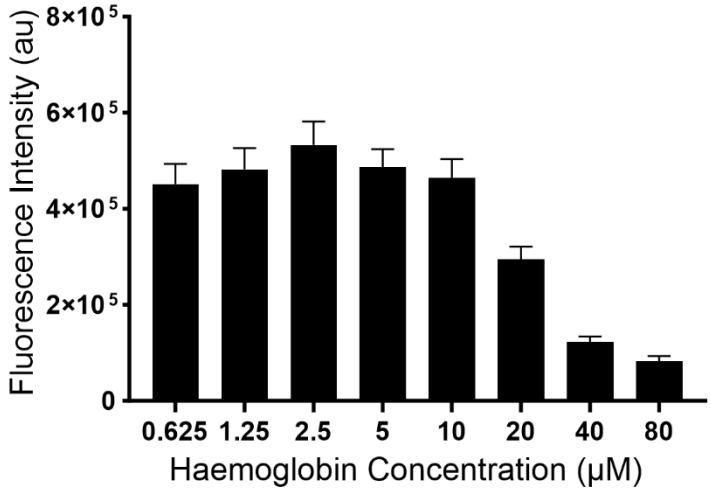

Fig. S1. *Porphyromonas gingivalis* strain W83 cultures grown on eTSB agar supplemented with various haemoglobin concentrations and 100 μM dipyridyl. Corresponding mean fluorescence intensity ± s.e.m. (excitation 365 nm and emission 638 nm) normalised to the same optical density at 600 nm is shown. Tukey's test results comparing different haemoglobin concentrations are presented in Table S2.

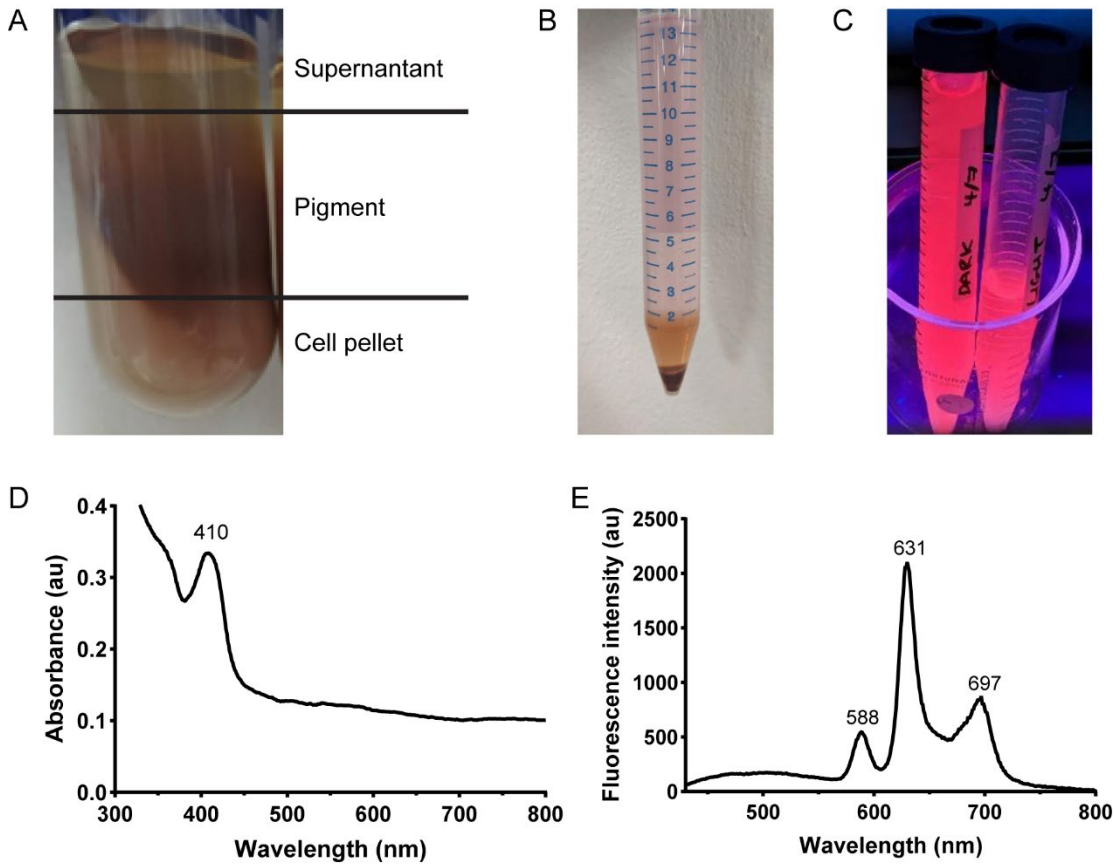

Fig. S2. A crude pigment extract from *Porphyromonas gingivalis.* (A) Harvested crude pigment in 20 mM Tris, 150 mM NaCl, pH 8 after ultracentrifugation. Only the pigment layer was collected and subjected to lyophilisation for further investigation. (B) Lyophilised crude pigment reconstituted in 75% acetonitrile observed under ambient light and (C) Wood's light. (D) UV-vis absorption spectrum of crude pigment extracts. (E) Fluorescence emission spectrum of crude pigment extracts in 75% acetonitrile (excitation = 410 nm).

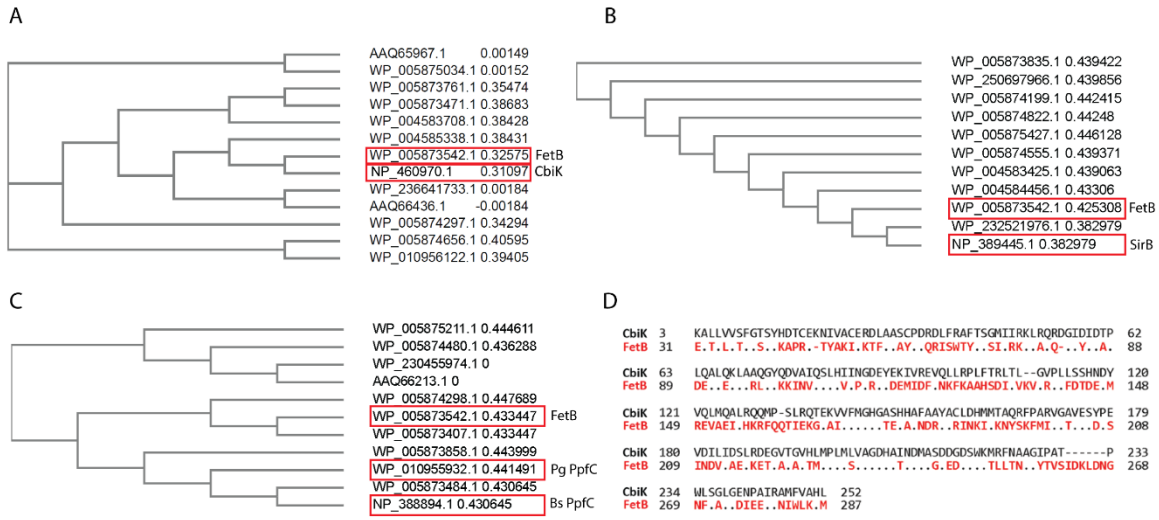

Fig. S3. Searching for chelatase homologues in *Porphyromonas gingivalis* using BLASTp. Guide trees of result from BLASTp search using (A) CbiK from *S. enterica* serovar Typhimurium (NP_460970.1), (B) SirB (NP_389445.1) and (C) PpfC (NP_388894.1) from *B. subtilis* against *P. gingivalis* strain W83. Tree was generated from an alignment of the resulted proteins using pairwise method on Clustal Omega website. (D) Alignment of CbiK and FetB amino acid sequence**.** Pg = *P. gingivalis*, Bs = *B. subtilis*, PpfC=ferrochelatase.

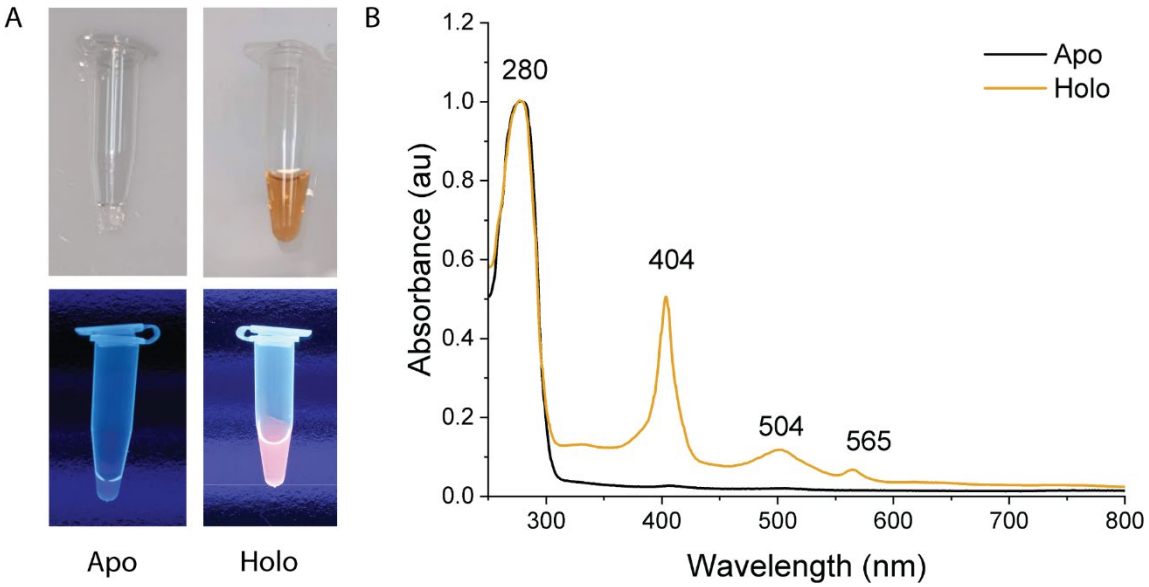

Fig. S4. Expression of recombinant apo FetB and holo-FetB using optimised conditions. (A) Recombinant FetB after the final step of purification observed under ambient light and Wood's light (B) UV-Vis absorbance spectra of purified recombinant apo-FetB and holo-FetB.

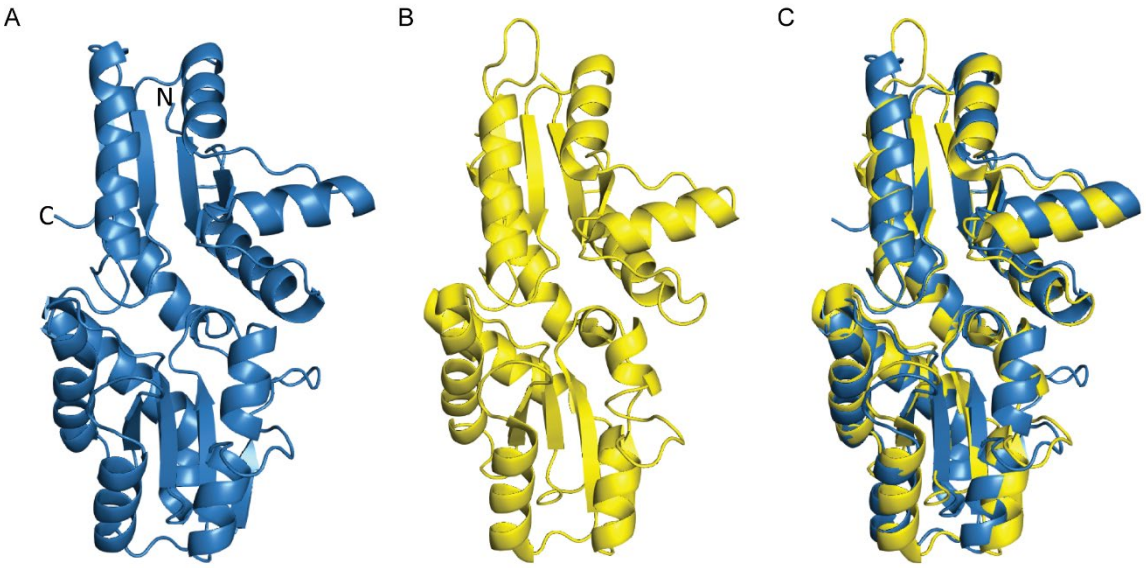

Fig. S5. Alignment of *Porphyomonas gingivalis* FetB and *Desulfovibrio vulgaris* periplasmic CbiK. (A) Recombinant FetB structure (B) Structure of *D. vulgaris* periplasmic CbiK (PDB ID: 2XVX) (C) Superimposition of FetB (Blue) with *D. vulgaris* periplasmic CbiKP (Yellow). The superimposition of the two proteins has an average root mean square deviation (RMSD) of 1.29 Å.

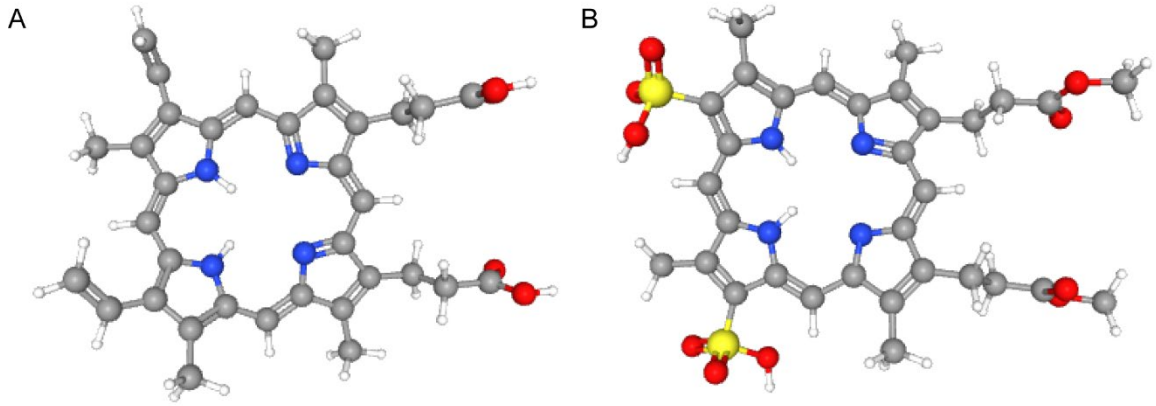

Fig. S6. Structures of (A) protoporphyrin IX (PubChem Identifier: CID 4971) and (B) deuteroporphyrin IX 2,4-disulfonic acid dimethyl ester (PubChem Identifier: CID 16414155).

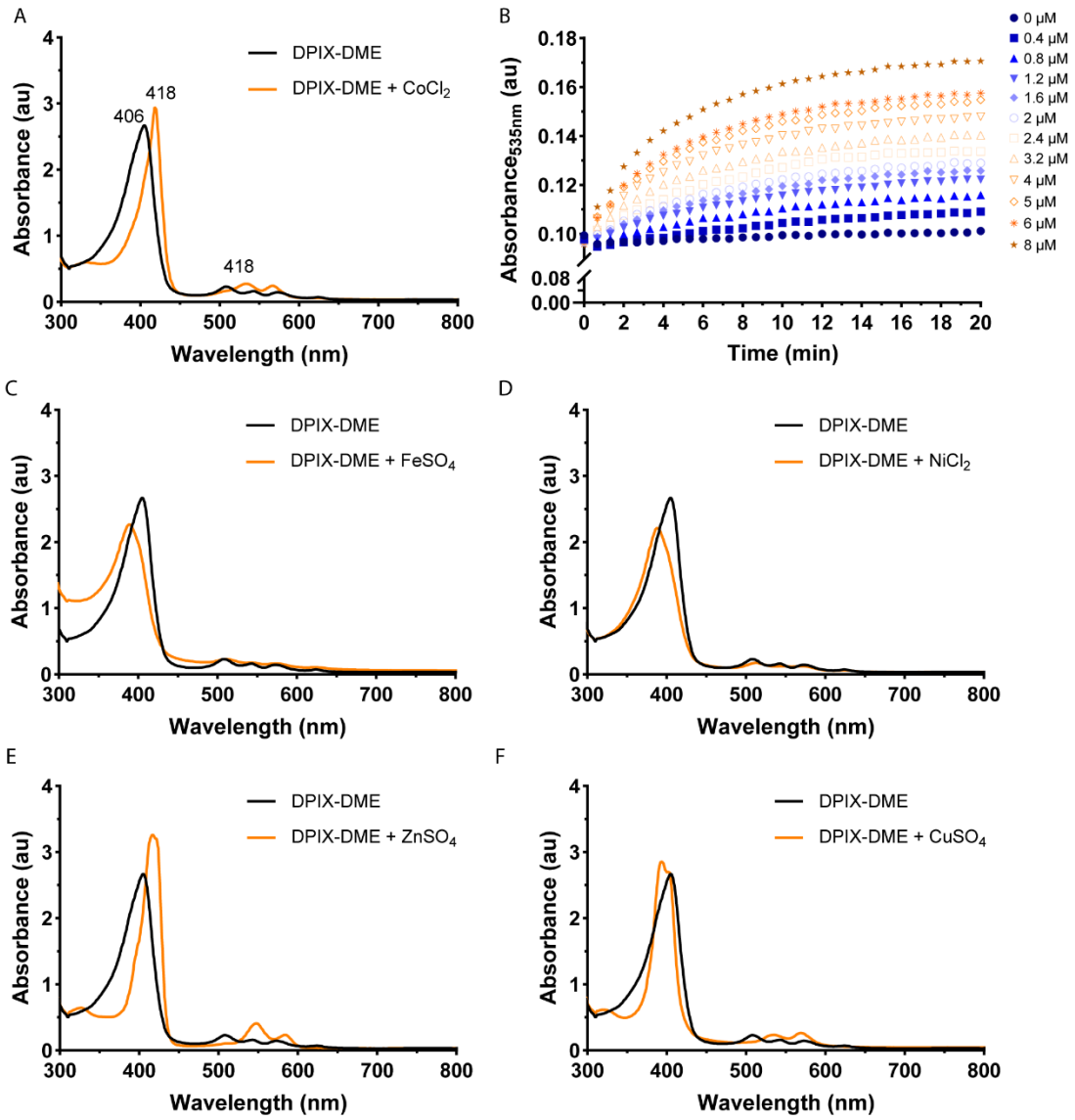

Fig. S7. FetB chelatase activity assays - (A) UV-Vis spectra of Deuteroporphyrin IX 2,4-disulfonic acid dimethyl ester (DPIX-DME) and cobalt-conjugated DPIX-DME. (B) Production of cobalt- DPIX-DME catalysed by increment titration of FetB measured at 535 nm. Spectrophotometric assays were performed using 20 µM DPIX-DME and 100 µM $CoCl_2$ in 1.5% Tween 20, 20 mM Tris pH 7.4. (C-F) Absorbance spectra of 200 µM DPIX-DME in the presence of 5 µM recombinant apo FetB and after the addition of 100 µM $FeSO_4$, $NiCl_2$, $ZnSO_4$, and $CuSO_4$. The spectra were recorded after 15 minutes for $CuSO_4$, and $ZnSO_4$ and after ten hours for $FeSO_4$, and $NiCl_2$. FetB chelatase activity for copper and zinc could not be determined as both metals formed complexes with DPIX spontaneously without the presence of chelatase.

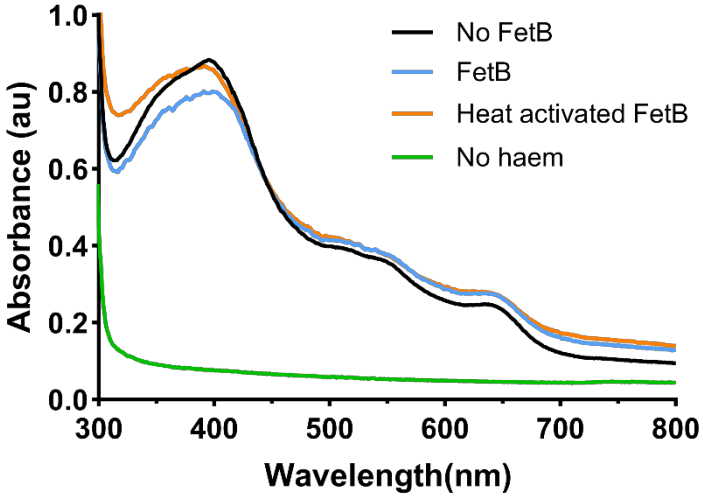

Fig. S8. FetB reverse chelatase activity assays A reverse chelatase assay was performed under anaerobic conditions at 37°C. The reaction mixture consisted of 1 mM DTT, 50 mM ATP (Sigma-Aldrich), 2.5 units/mL kinase (Sigma-Aldrich), 2 mM dithionite(Sigma-Aldrich), and 100 µM haem in 20 mM phosphocreatine (Sigma-Aldrich), 50 mM MOPS (pH 7.5), and 5% glycerol. The assay was initiated by adding 174.5 µM recombinant FetB and incubating the mixture in an aerobic chamber for 30 minutes. Control mixtures included samples without FetB, without haem, and with heat-inactivated FetB. No reverse chelatase activity was observed under these conditions, as there was no change in the absorbance pattern of the haem substrate.

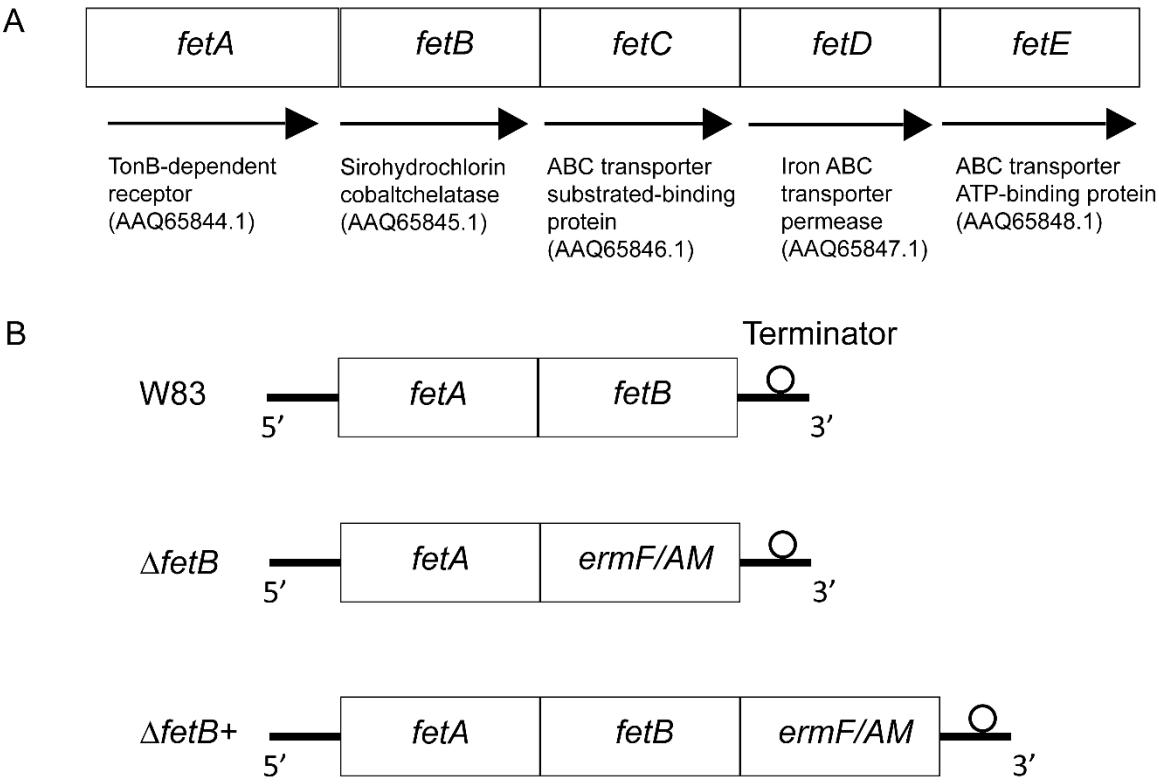

Fig. S9. Mutagenesis of *Porphyromonas gingivalis fetB*. (A) The *fetB* gene cluster in *P. gingivalis* strain W83 (B) The construction of *fetB* deletion mutant (Δ*fetB*) and *fetB* complementation mutant (Δ*fetB*+).

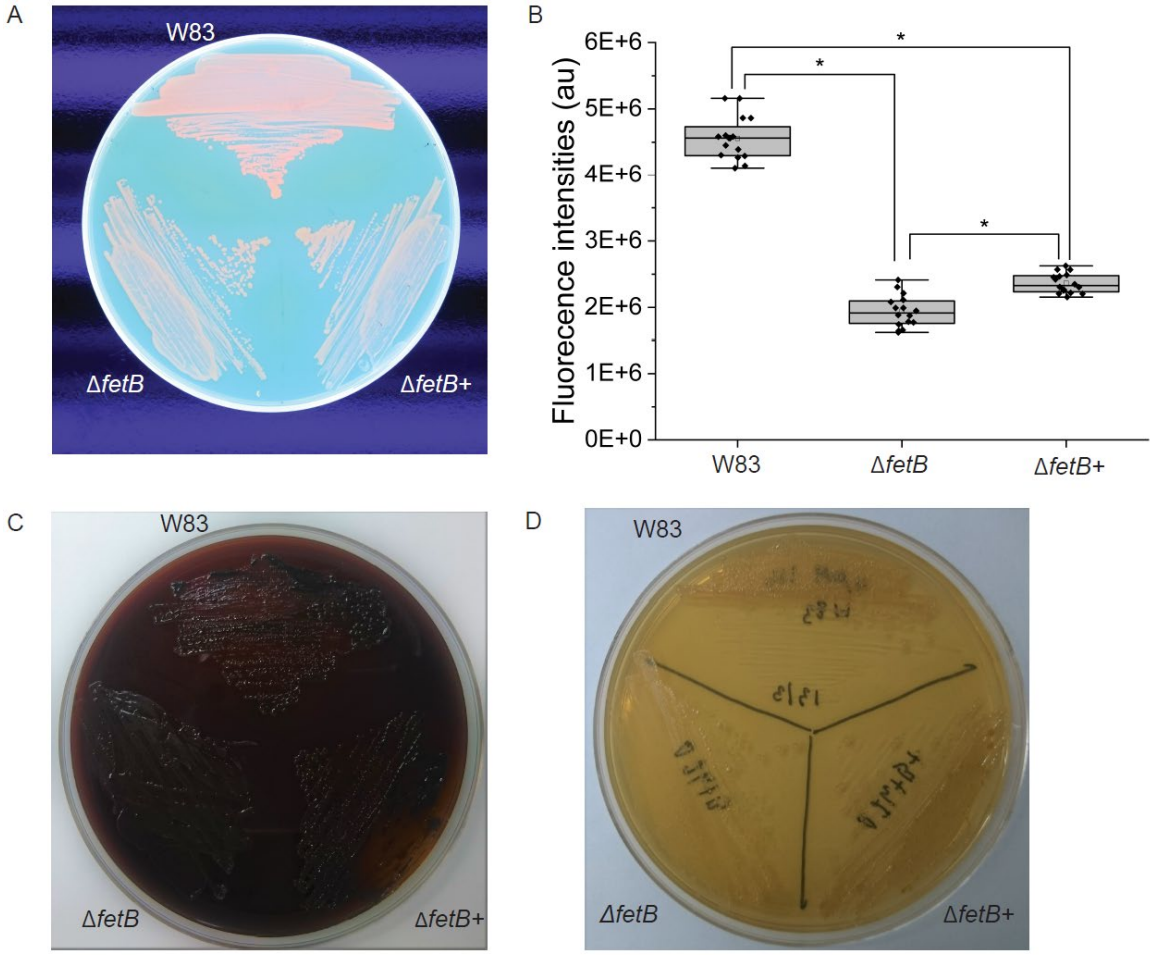

Fig. S10. Fluorescence production of the wild type W83, Δ*fetB*, and Δ*fetB*+ mutants. (A) Colonies grown on eTSB agar supplemented with 2 µM haemoglobin under Wood's light (Excitation at 365 nm). (B) Comparing the fluorescence emission between wild type W83, Δ*fetB*, and Δ*fetB*+ mutants, * = $p < 0.001$. (C) Colonies grown on eTSB agar supplemented with sheep blood. (D) Colonies grown on eTSB agar supplemented with 2 µM haemoglobin

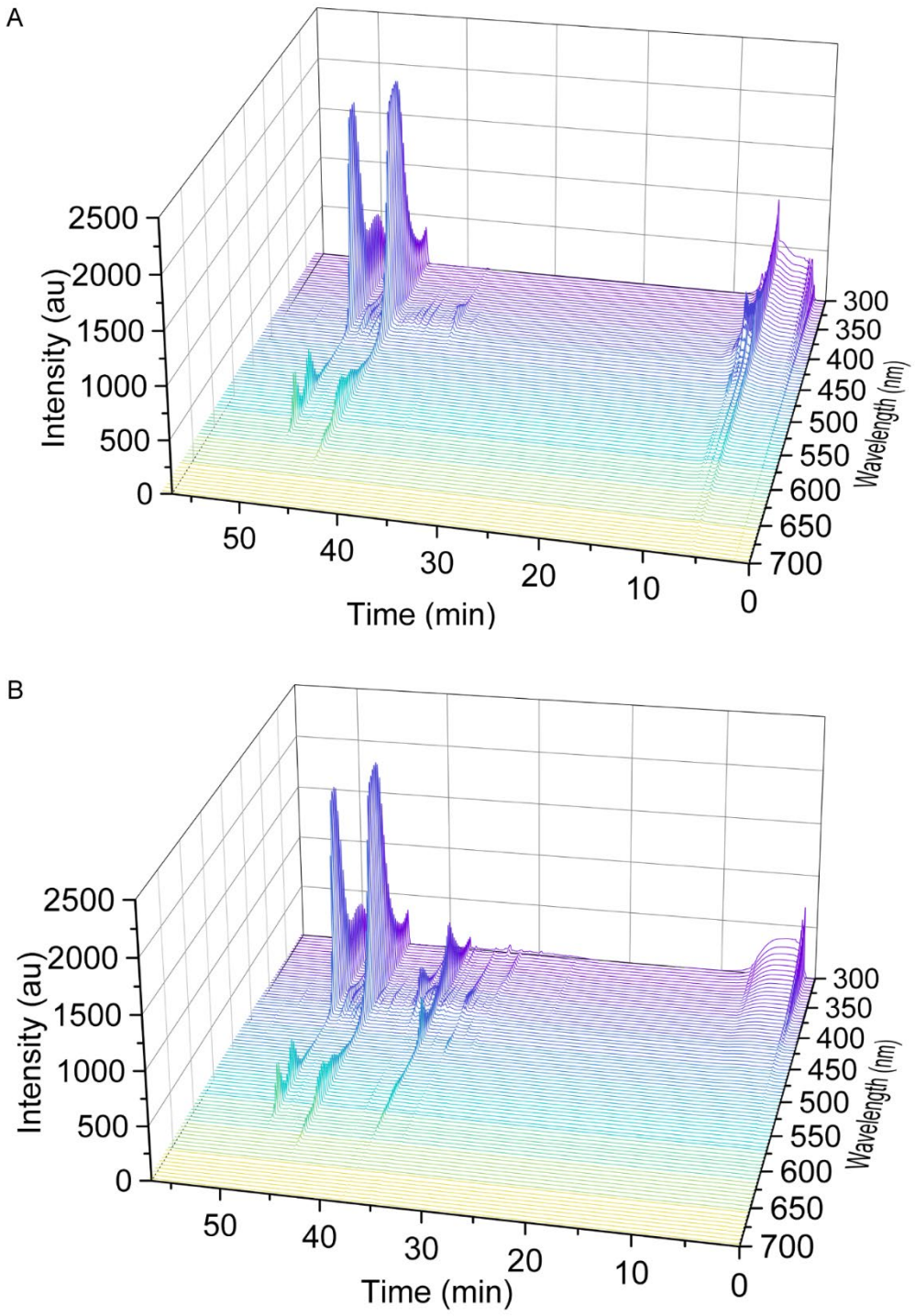

Fig. S11. Reversed-phase HPLC profiles of crude pigment extracted from *Porphyromonas gingivalis* mutants (A) Chromatographic profiles of pigment from Δ*fetB* shows absense of Mn-PPIX and haem accumulation. (B) Chromatographic profiles of pigment from Δ*fetB*+ mutant.

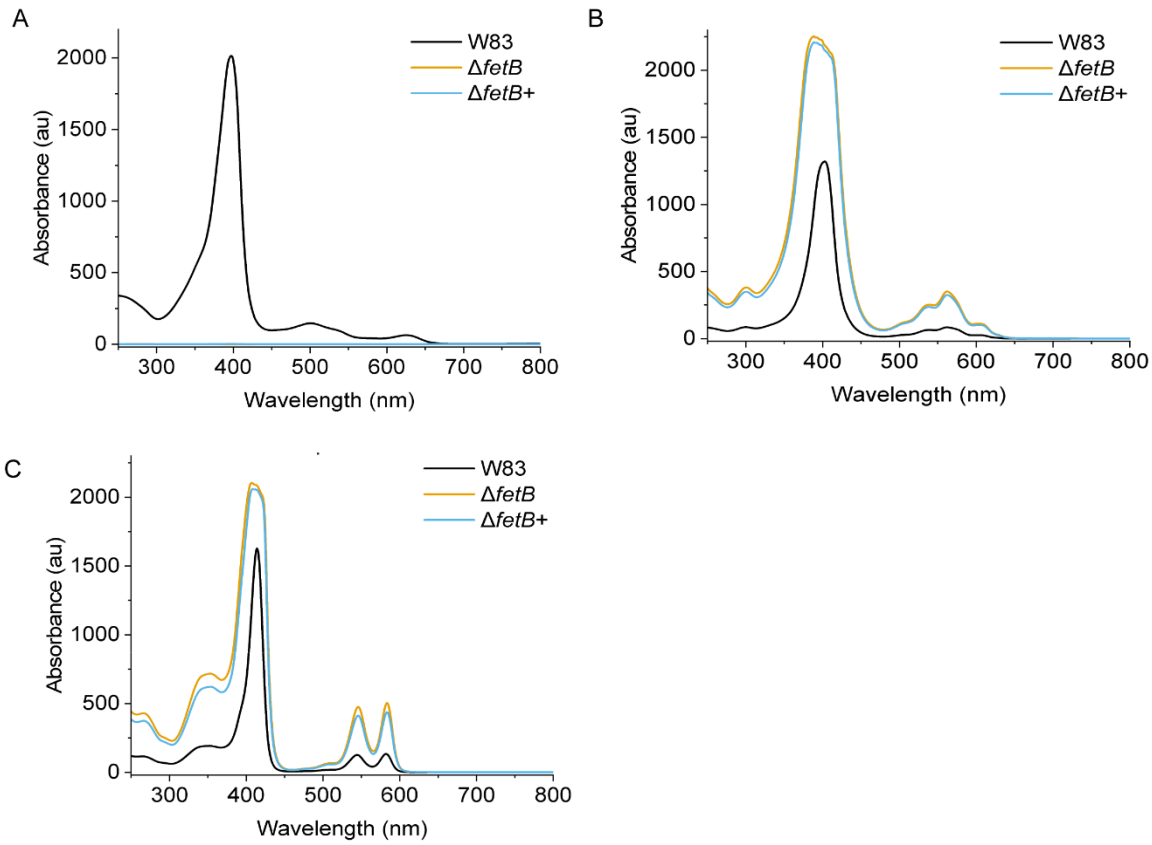

Fig. S12. Comparison of fractions from Reversed-phase HPLC of crude pigment between wild type W83, Δ*fetB*, and Δ*fetB* + mutant. (A) Fractions collected with a retention time of 42.0 min corresponding Haem. (B) Fractions collected with a retention time of 48.4 min corresponding PPIX. (C) Fractions collected with a retention time of 51.2 min.

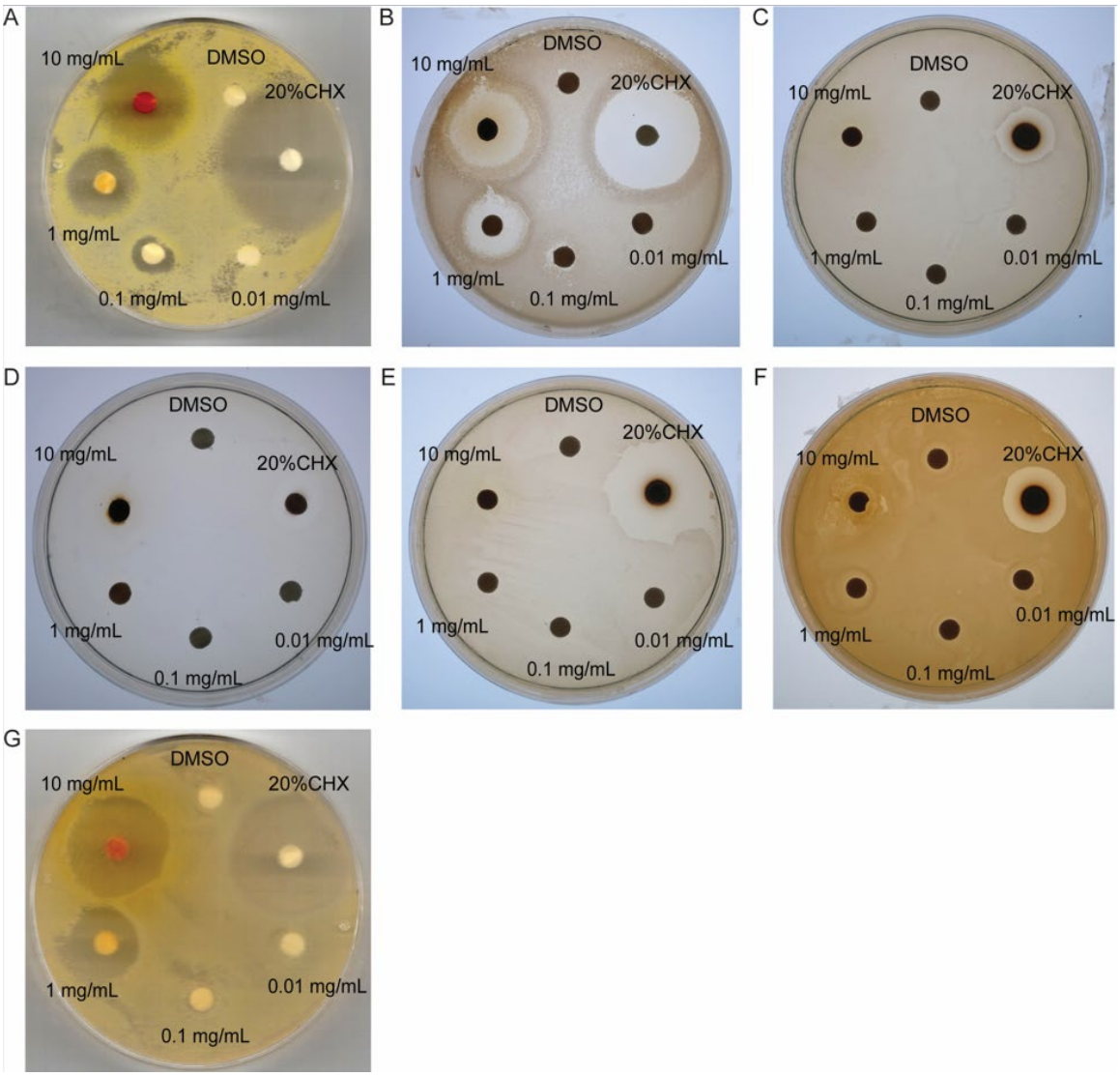

Fig. S13. Representative plate photos from zone of inhibition assay of Mn-PPIX against different bacterial strains. (A) *S. mitis* (B) *S. salivarius* (C) *S. oralis* (D) *S. sanguinis* (E) *S. mutans* (F) *S. marcescen* (G) *Lactobacillus*

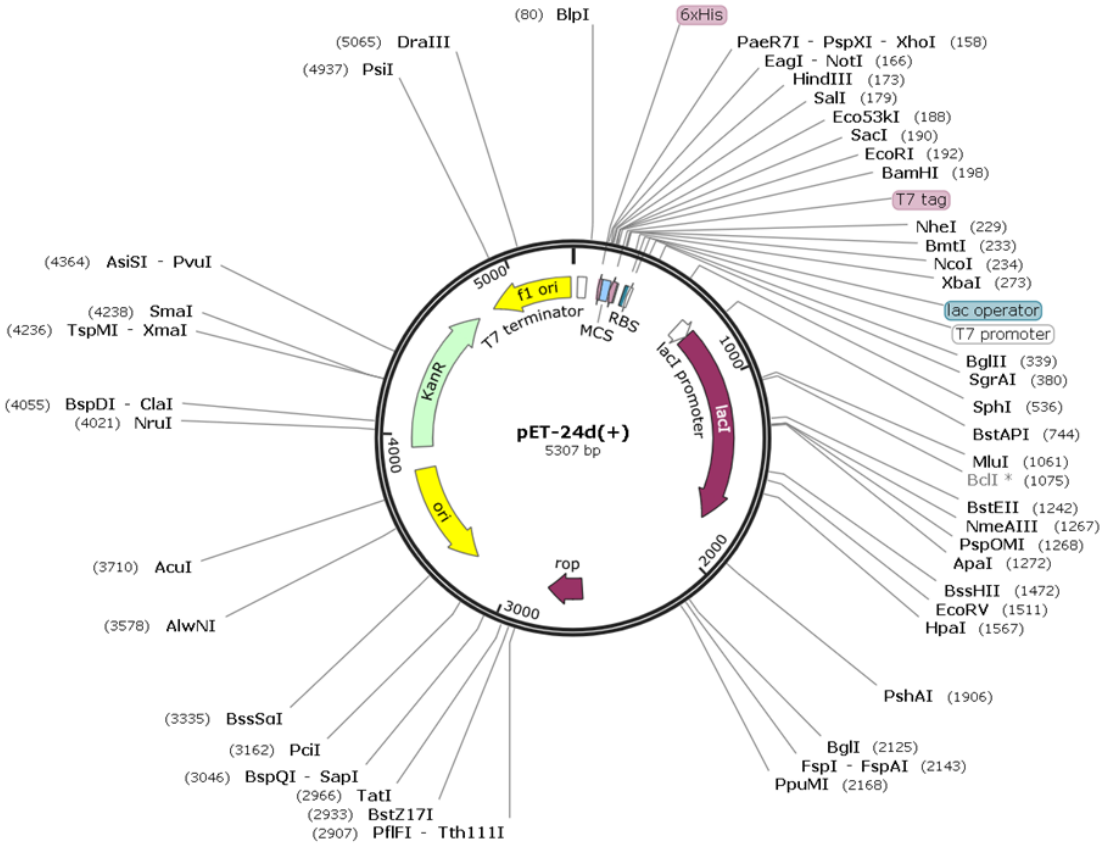

Fig. S14. The pET-24d (+) Vector (https://www.snapgene.com/resources/plasmid-files/?set=pet_and_duet_vectors_(novagen)&plasmid=pET-24d(%2B)).

Supplemental Tables

Table S1. Results of Tukey's test comparing fluorescence intensities of *Porphyromonas gingivalis* strain W83 grown on eTSB agar supplemented with various haemoglobin concentrations without dipyridyl

Groups (μM Hb)		p	95% confidence interval for mean	
			Lower bound	Upper bound
0.625	0.31	<0.05	3.46E+03	7.44E+04
1.25	0.31	<0.001	9.59E+04	1.67E+05
1.25	0.625	<0.001	5.69E+04	1.28E+05
2.5	0.31	<0.001	1.36E+05	2.07E+05
2.5	0.625	<0.001	9.73E+04	1.68E+05
2.5	1.25	<0.05	4.89E+03	7.59E+04
5	0.31	<0.001	6.52E+04	1.36E+05
5	0.625	<0.001	2.62E+04	9.72E+04
5	1.25	-	-6.62E+04	4.79E+03
5	2.5	<0.001	-1.07E+05	-3.56E+04
10	0.31	-	-1.22E+04	5.88E+04
10	0.625	-	-5.11E+04	1.98E+04
10	1.25	<0.001	-1.44E+05	-7.26E+04
10	2.5	<0.001	-1.84E+05	-1.13E+05
10	5	<0.001	-1.13E+05	-4.19E+04
20	0.31	-	-6.86E+04	2.38E+03
20	0.625	<0.001	-1.08E+05	-3.66E+04
20	1.25	<0.001	-2.00E+05	-1.29E+05
20	2.5	<0.001	-2.40E+05	-1.69E+05
20	5	<0.001	-1.69E+05	-9.83E+04
20	10	<0.001	-9.19E+04	-2.09E+04
40	0.31	<0.01	-7.83E+04	-7.32E+03
40	0.625	<0.001	-1.17E+05	-4.63E+04
40	1.25	<0.001	-2.10E+05	-1.39E+05
40	2.5	<0.001	-2.50E+05	-1.79E+05
40	5	<0.001	-1.79E+05	-1.08E+05
40	10	<0.001	-1.02E+05	-3.06E+04
40	20	-	-4.52E+04	2.58E+04
80	0.31	<0.001	-9.20E+04	-2.11E+04
80	0.625	<0.001	-1.31E+05	-6.00E+04
80	1.25	<0.001	-2.23E+05	-1.52E+05
80	2.5	<0.001	-2.64E+05	-1.93E+05
80	5	<0.001	-1.93E+05	-1.22E+05
80	10	<0.001	-1.15E+05	-4.44E+04
80	20	-	-5.89E+04	1.20E+04
80	40	-	-4.92E+04	2.17E+04

Table S2. Results of Tukey's test comparing fluorescence intensities of *Porphyromonas gingivalis* strain W83 grown on eTSB agar supplemented with various haemoglobin concentrations and 100 µM dipyridyl.

Groups (µM Hb)		p	95% confidence interval for mean	
			Lower bound	Upper bound
1.25	0.625	-	-7.06E+03	6.89E+04
2.5	0.625	<0.001	4.34E+04	1.19E+05
2.5	1.25	<0.01	1.25E+04	8.85E+04
5	0.625	-	-2.73E+03	7.33E+04
5	1.25	-	-3.37E+04	4.23E+04
5	2.5	<0.01	-8.41E+04	-8.16E+03
10	0.625	-	-2.43E+04	5.17E+04
10	1.25	-	-5.52E+04	2.08E+04
10	2.5	<0.001	-1.06E+05	-2.97E+04
10	5	-	-5.95E+04	1.64E+04
20	0.625	<0.001	-1.95E+05	-1.19E+05
20	1.25	<0.001	-2.26E+05	-1.50E+05
20	2.5	<0.001	-2.76E+05	-2.00E+05
20	5	<0.001	-2.30E+05	-1.54E+05
20	10	<0.001	-2.09E+05	-1.33E+05
40	0.625	<0.001	-3.66E+05	-2.90E+05
40	1.25	<0.001	-3.97E+05	-3.21E+05
40	2.5	<0.001	-4.48E+05	-3.72E+05
40	5	<0.001	-4.02E+05	-3.26E+05
40	10	<0.001	-3.80E+05	-3.04E+05
40	20	<0.001	-2.10E+05	-1.34E+05
80	0.625	0.001	-4.07E+05	-3.31E+05
80	1.25	<0.001	-4.38E+05	-3.62E+05
80	2.5	<0.001	-4.89E+05	-4.13E+05
80	5	<0.001	-4.43E+05	-3.67E+05
80	10	<0.001	-4.21E+05	-3.45E+05
80	20	<0.001	-2.50E+05	-1.74E+05
80	40	<0.05	-7.88E+04	-2.81E+03

Table S3. Expression conditions to maximise the purity of apo and holo form of recombinant FetB

Expression Condition	Recombinant Apo FetB	Recombinant Holo FetB
Expression media	1L LB	500 mL LB 10 mg/L 5-ALA before induction
Shake flasks	2L without baffles Cover with aluminium foil	2L with baffles
Expression time	3 hours	24 hours
Expression temperature	37°C	18°C

Table S4. Crystallographic statistics of the FetB structure

Space group	P 2_1 2_1 2_1
Cell dimensions	48.63 49.82 98.91
a, b, c (Å) **α, β, γ (°)**	90.00 90.00 90.00
Resolution (Å)	48.63 to 1.60
Observed reflections	431594
Unique reflections	32719
I/σ (I)	1.74
Completeness (%)	99.8
Rmerge (I)	0.067
Refinement	PHENIX
Reflections in **test set**	27169
$\mathbf{R_{free}}$	0.225
$\mathbf{R_{work}}$	0.202
Total number of non-H atoms	2242
r.m.s.d bonds (Å)	0.037
r.m.s.d angles (°)	0.59
Ramachandran plot allowed (%)	1.16
Ramachandran plot outliers (%)	0
Average B factor ($Å^2$)	29

Table S5. Antimicrobial activity test of Mn-PPIX. The results show zone of inhibition from three biological replicants

Test organism	Concentration (mg/ml)	Zone of inhibition (mm)		
		Replicate 1	Replicate 2	Replicate 3
S. mitis	DMSO	0	0	0
	20% CHX	38 ± 3.39	38 ± 1.39	26 ± 1.24
	10	26 ± 0.49	25 ± 1.97	23 ± 0.51
	1	21 ± 0.80	20 ± 0.44	21 ± 1.31
	0.1	12 ± 0.41	11 ± 0.78	0
	0.01	0	0	0
S. salivarius	DMSO	0	0	0
	20% CHX	29 ± 0.41	29 ± 0.74	26 ± 3.53
	10	20 ± 0.54	22 ± 0.91	20 ± 0.46
	1	16 ± 0.07	15 ± 0.47	16 ± 0.42
	0.1	7 ± 0.44	8 ± 0.23	4 ± 0.48
	0.01	0	0	0
E. faecalis	DMSO	0	0	0
	20% CHX	26 ± 0.94	26 ± 0.35	22 ± 0.46
	10	31 ± 0.57	28 ± 1.28	26 ± 0.89
	1	15 ± 0.74	11 ± 0.30	19 ± 0.80
	0.1	0	0	0
	0.01	0	0	0
Oral *Lactobacillus*	DMSO	0	0	0
	20% CHX	28 ± 0.37	29 ± 0.99	29 ± 4.89
	10	28 ± 1.80	27 ± 2.66	26 ± 0.63
	1	24 ± 0.72	22 ± 1.20	19 ± 0.38
	0.1	13 ± 0.96	10 ± 0.32	14 ± 0.95
	0.01	0	0	0

Table S6. Primers and probes used in this work

Name	Sequence (5' – 3', uppercase)*	Target genes
FetBFrASmaI R	AGCAATAGCGGAAGCTAT**CCCGGG**TGTGA GACATTAGTTGTTGAATCG	pFetBAeB construction
FetBFrCSmaI R	AGCAATAGCGGAAGCTAT**CCCGGG**CTTAG CGAGCAGAGGTGG	pFetBCeB construction
FetBFrASacIF	ACGACGGCCAGTGAATTC**GAGCTC**ACGAT CAGATCCGATACACG	pFetBAeB construction and pFetBCeB construction
FetBFrBXbaIF	ATACTACTGACAGCTTCG**TCTAGA**CGGAT ATGCAATGAGACAATCAAG	
FetBFrBHind3 R	TATGACCATGATTACGCC**AAGCTT**GATAG CTGTTGAGGATTCGC	
ermFAMXmaI F	atta**CCCGGG**ATAGCTTCCGCTATTGC	
ermFAMXbaIR	gc**TCTAGA**CGAAGCTGTCAGTAGTATACC	
rFetBNcoIF	aata**CCATGG**GTAAGGATTTGGAGAACAAA GGGGAG	pETFetBTEV6H construction
rFetBTEVXhoI R	taatt**CTCGAG**ACCCTGAAAGTACAGATTTTC GCGAGCAGAGGTGGCTTT	pETFetBTEV6H construction
RTfetF	ACCGAGCATGCTGCCAATGA	Real-time PCR
RTfetR	CCGGTTTCTTTCAGTTCGGCAAT	Real-time PCR
RT16sF	TCGGTAAGTCAGCGGTGAAAC	Real-time PCR
RT16sR	ATTGCGAAGGCAGCTTGC	Real-time PCR
TaqMan™ Probes		
FAMRTfetP	TCGTCGGAACCGTCGAGTCCGATCCCTCT	*fetB*
FAMRT16sP	AGCGCTCAACGTTCAGCCTGCCGTTGA	*16s RNA*

Table S7. rpHPLC Chromatographic conditions used for pigment separation and analysis

Stationary phase	**C-18**		
Mobile phase A	90 % MQ water (Millipore), 10 % methanol (Sigma-Aldrich), and 0.1 % Trifluoroacetic acid (Sigma-Aldrich) (v/v/v)		
Mobile phase B	90 % acetonitrile (Sigma-Aldrich), 10 % methanol, and 0.1 % Trifluoroacetic acid (v/v/v)		
Flow rate	1 mL/min		
Absorbance spectrum	210 – 800nm using HyStar software package (Agilent Technologies, Inc)		
Gradient	**Time (min)**	**A %**	**B %**
	0	90	10
	10	90	10
	45	0	100
	50	0	100
	55	90	10

Table S8. Parameters used in ESI-MS

Capillary Voltage	2500 - 4000 V
Nebulizer	27.3 psi
Dry gas	4 L/min
Dry temp	180c
Compound stability	80 - 100%
Scan range	200 - 1000
Accumulation time	200 ms
Scan mode	Ultra scan
Ion Mode	Positive
Syringe pump flow rate	100 µL/h

Table S9. Buffers used in recombinant protein expression and purification

Buffer	Components
Lysis buffer	50 mM sodium phosphate (Sigma-Aldrich), 300mM NaCl, 10 mM Imidazole (Sigma-Aldrich), pH 8
Wash buffer	50 mM sodium phosphate, 300mM NaCl, 20 mM Imidazole (Sigma-Aldrich), pH 8
Elution buffer	50 mM sodium phosphate, 300mM NaCl, 200 mM Imidazole (Sigma-Aldrich), pH 8
Storage buffer	50 mM sodium phosphate, 150mM NaCl, pH 8
SALTS medium	13 g/L KH_2PO_4, 10 g/L K_2HPO_4, 9 g/L Na_2HPO4, 2.4 g/L K_2SO_4
Minimal media	30 g/L Thiamine, 10mL/L of 1M $MgCl_2$, 10 mL/L trace metals, 10µL/L of 10% w/v yeast extract (Oxoid), 25 mg/L kanamycin (Sigma-Aldrich), 1g/L $^{15}NH_4C$ in 1 L of SALTS
Trace metals	6 g/L $FeSO_4.7H_2O$, 6g/L $CaCl_2.2H_2O$, 1.2 g/L $MnCl_2.4H_2O$, 0.8 g/L, $CoCl_2.6H_2O$, 0.7 g/L $ZnSO_4.7H_2O$, 0.3 g/L $CuCl_2.2H_2O$, 20 g/L H_3BO_3, 0.25 g/L $(NH_4)_6Mo_7O_{24}.4H2O$, 5 g/L EDTA (Sigma-Aldrich)

296 **Table S10.** Buffers used in SDS-PAGE

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Buffer	Components
10× Electrode Buffer	250 mM Tris (Sigma-Aldrich), 1.92 M Glycine, 1% (w/v) SDS, pH 8.3
Tris buffer for stacking gel	0.5 M Tris, pH 6.8
Tris buffer for resolving gel	1.5 M Tris, pH 8.8
5x SDS-PAGE sample buffer	62.5 mM Tris, 20% (v/v) Glycerol (Sigma-Aldrich), 10% (w/v) SDS (Sigma-Aldrich), 0.05% (w/v) Bromophenol blue, 5% (v/v) β-mercaptoethanol (Sigma-Aldrich), pH 6.8,

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