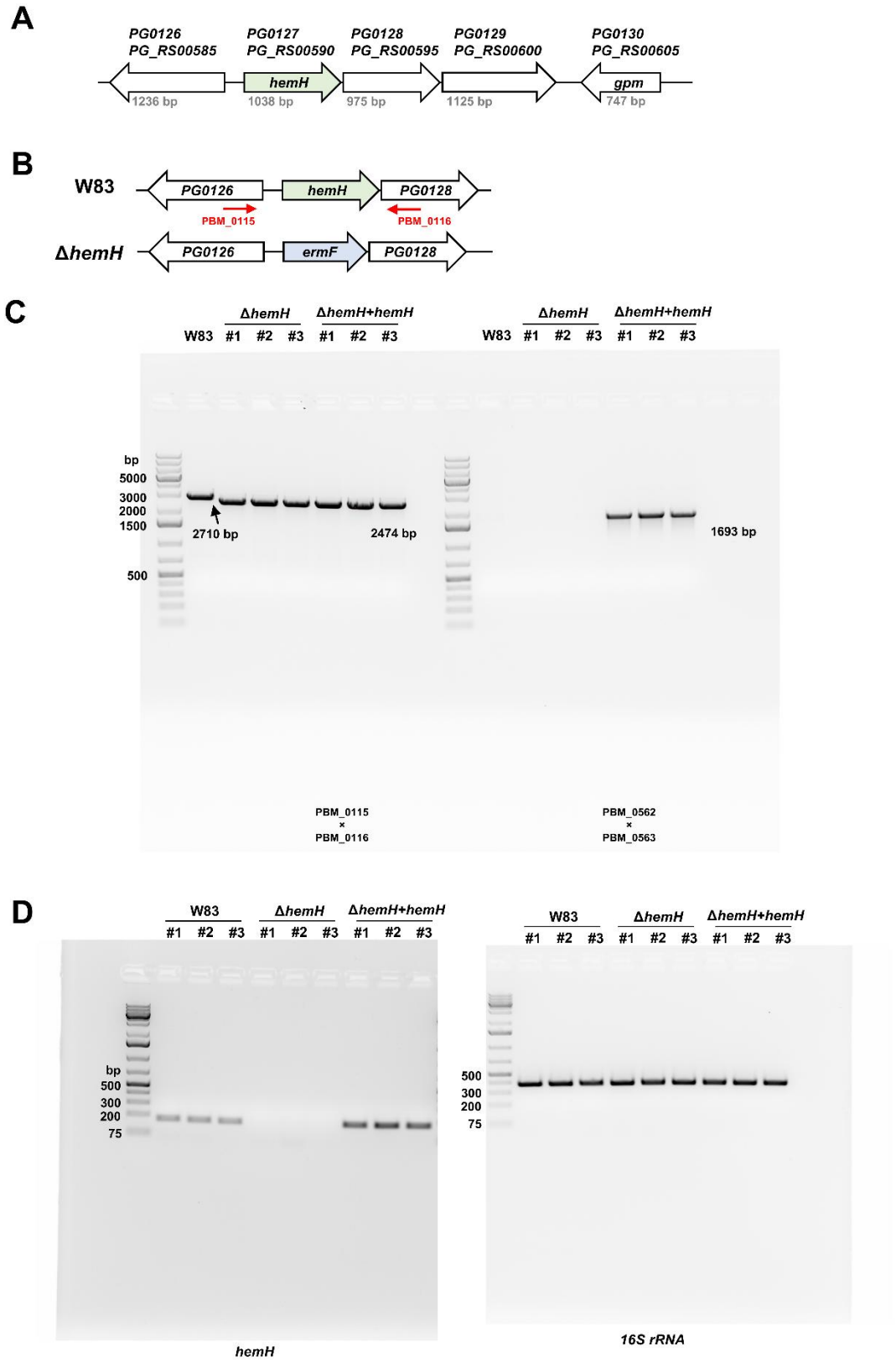
Supplementary data for:

Porphyromonas gingivalis produces a functional HemH ferrochelatase important for its survival in a heme-limited environment

Michał Śmiga[a]*, Ewa Roszkiewicz[a], Natalia Wojtal[a], Teresa Olczak[a]

[a] Laboratory of Medical Biology, Faculty of Biotechnology, University of Wrocław, Wrocław, Poland.

*Corresponding author: michal.smiga@uwr.edu.pl.


A
PG0126
PG_RS00585
PG0127
PG_RS00590
PG0128
PG_RS00595
PG0129
PG_RS00600
PG0130
PG_RS00605
hemH
gpm
1236 bp
1038 bp
975 bp
1125 bp
747 bp
B
W83
PG0126
hemH
PG0128
PBM_0115
PBM_0116
ΔhemH
PG0126
ermF
PG0128
C
ΔhemH
ΔhemH+hemH
W83 #1 #2 #3 #1 #2 #3
W83 #1 #2 #3 #1 #2 #3
bp
5000
3000
2000
1500
500
2710 bp
2474 bp
1693 bp
PBM_0115
×
PBM_0116
PBM_0562
×
PBM_0563
D
W83
ΔhemH
ΔhemH+hemH
#1 #2 #3 #1 #2 #3 #1 #2 #3
bp
500
300
200
75
hemH
16S rRNA

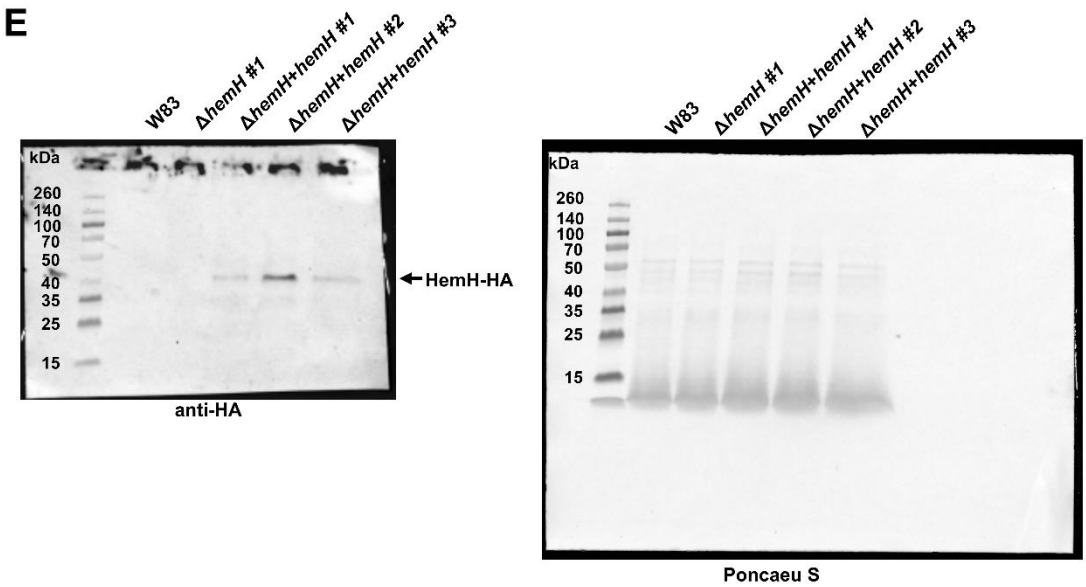


Fig. S1. Generation of Δ*hemH* mutant and Δ*hemH*+*hemH* complemented strains. (A) Location of the *hemH* gene in the genome of the *P. gingivalis* wild-type W83 strain. (B) The schematic presentation of the changes made in the genomic DNA of the Δ*hemH* mutant strain. The sequence encoding the entire open reading frame of the *P. gingivalis hemH* gene was replaced with the *ermF* antibiotic resistance cassette, lacking its native promoter sequence. (C) Genetic modification in the Δ*hemH* mutant and Δ*hemH*+*hemH* complemented strains was verified by PCR using genomic DNA and primers (PBM_0115 and PBM_0116), which amplify the flanking regions of the modified DNA fragment (shown in B). The reduced length of the amplified region in the Δ*hemH* mutant strain results from the deletion of the entire open reading frame from the *hemH* gene and the insertion of the *ermF* gene, lacking its native promoter sequence (top panel). The pTIO-*hemH* plasmid, introduced only into the Δ*hemH*+*hemH* complemented strain, was detected using PBM_0562 and PBM_0563 primers (bottom panel). (D) The *hemH* transcript was analyzed using RT-PCR with primers specific for the *hemH* gene. The *16S rRNA* gene was used as a control. (E) Production of the HemH-HA protein in the Δ*hemH*+*hemH* complemented strain was verified using Western blotting with anti-HA antibodies. The efficiency of protein transfer was visualized on nitrocellulose membranes by staining proteins with Ponceau S, and prior to antibody incubation, the membrane was cut to reduce membrane area and antibody consumption, removing only regions where no signal was expected.

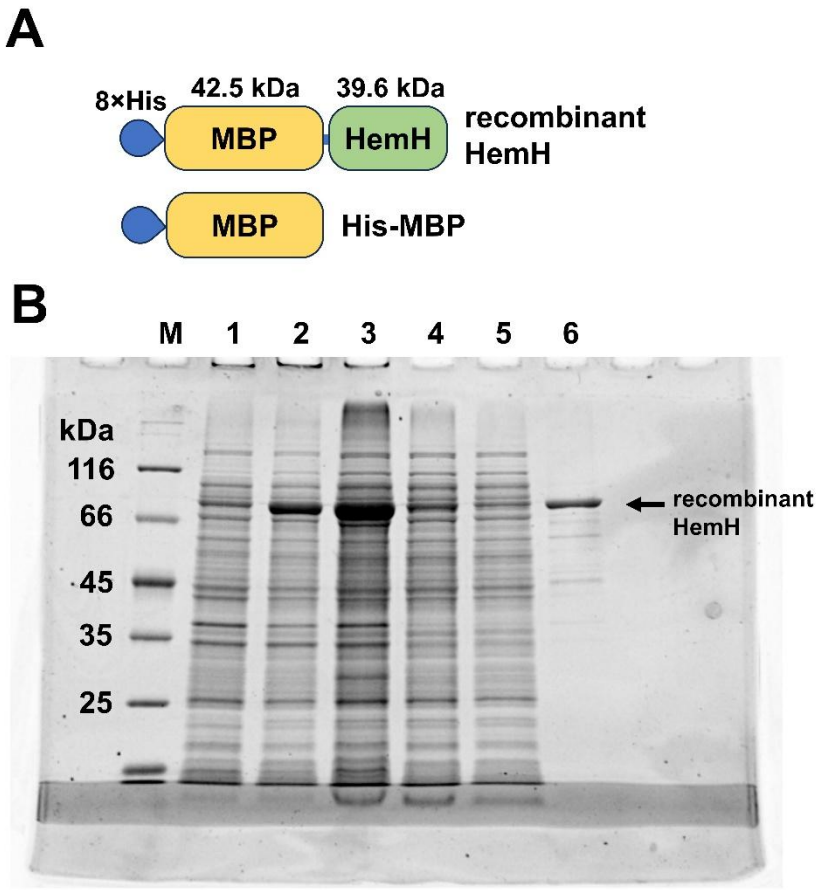


Fig. S2. Overexpression and purification of recombinant HemH protein. (A) HemH protein was produced in a fusion with the N-terminal 8×His-maltose-binding protein (His-MBP). The His-MBP protein alone was overexpressed and used as a control. (B) SDS-PAGE gel stained with CBB G-250 showing protein samples obtained during the HemH purification process. M – protein molecular weight marker; 1 – *E. coli* cell lysate before induction with IPTG; 2, 3 – *E. coli* cell lysate after protein overexpression; 4 – soluble protein fraction obtained from *E. coli* cell lysate (fraction loaded onto amylose resin); 5 – fraction unbound to amylose resin; 6 – recombinant HemH protein (~3 μg per lane) eluted from amylose resin with 25 mM maltose.

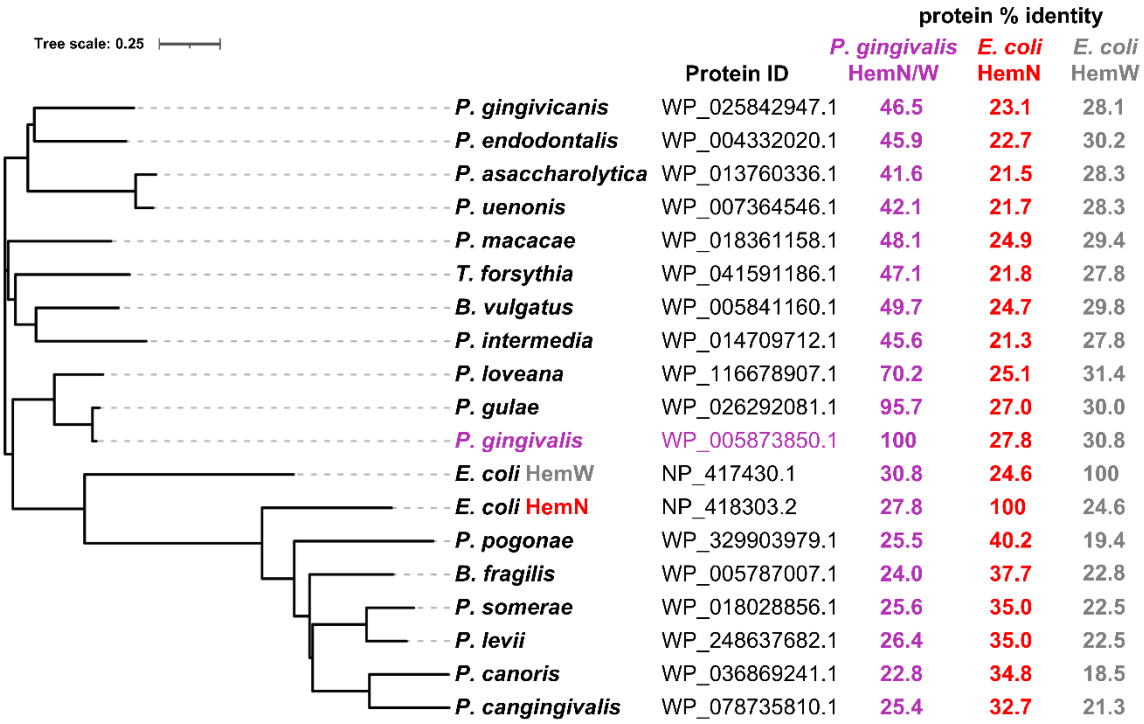


Fig. S3. Comparison of selected members of the radical SAM (S-adenosylmethionine) enzyme superfamily from the Bacteroidota phylum with HemN and HemW proteins from *E. coli*. A phylogenetic tree was constructed based on amino acid sequences. Protein access codes and % identity of amino acid sequences to the *P. gingivalis* HemN/W homolog, *E. coli* HemN, and *E. coli* HemW proteins are shown on the right.

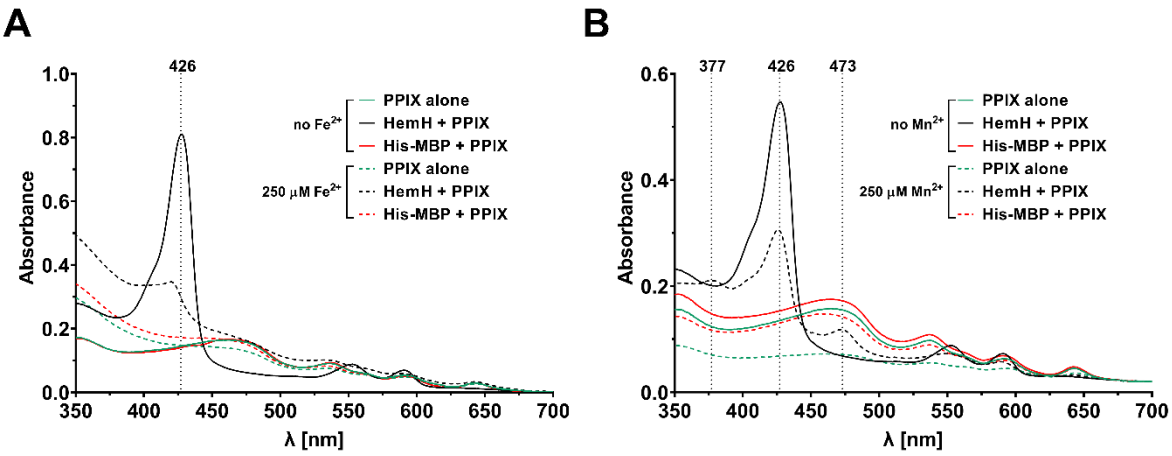


Fig. S4. Analysis of the specificity of *P. gingivalis* HemH activity. The recombinant HemH protein was incubated with PPIX and 250 µM iron (A) or 250 µM manganese (B) ions. The reactions were carried out in anaerobic conditions, in the presence of ascorbic acid in the dark. The enzymatic activity was determined by analyzing the reaction products through monitoring the UV-visible spectra of the reaction mixture with or without the addition of the ions. As a control, instead of recombinant HemH, His-MBP was added, or no protein was added.

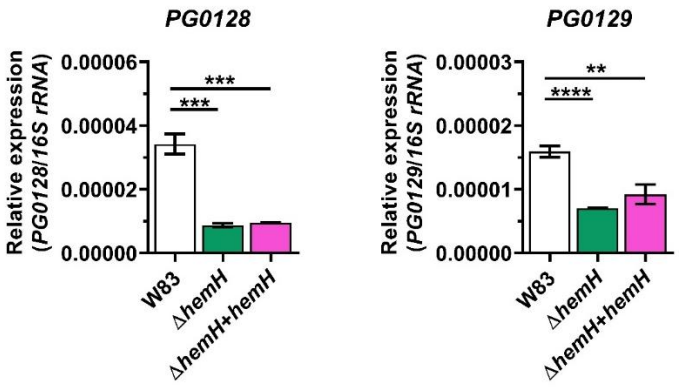


Fig. S5. Influence of the inactivation of the *hemH* gene and its complementation on *PG0128* and *PG0129* gene expression. Gene expression was examined in bacteria cultured in BM medium without added heme using RT-qPCR with the *16S rRNA* gene as a reference. $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

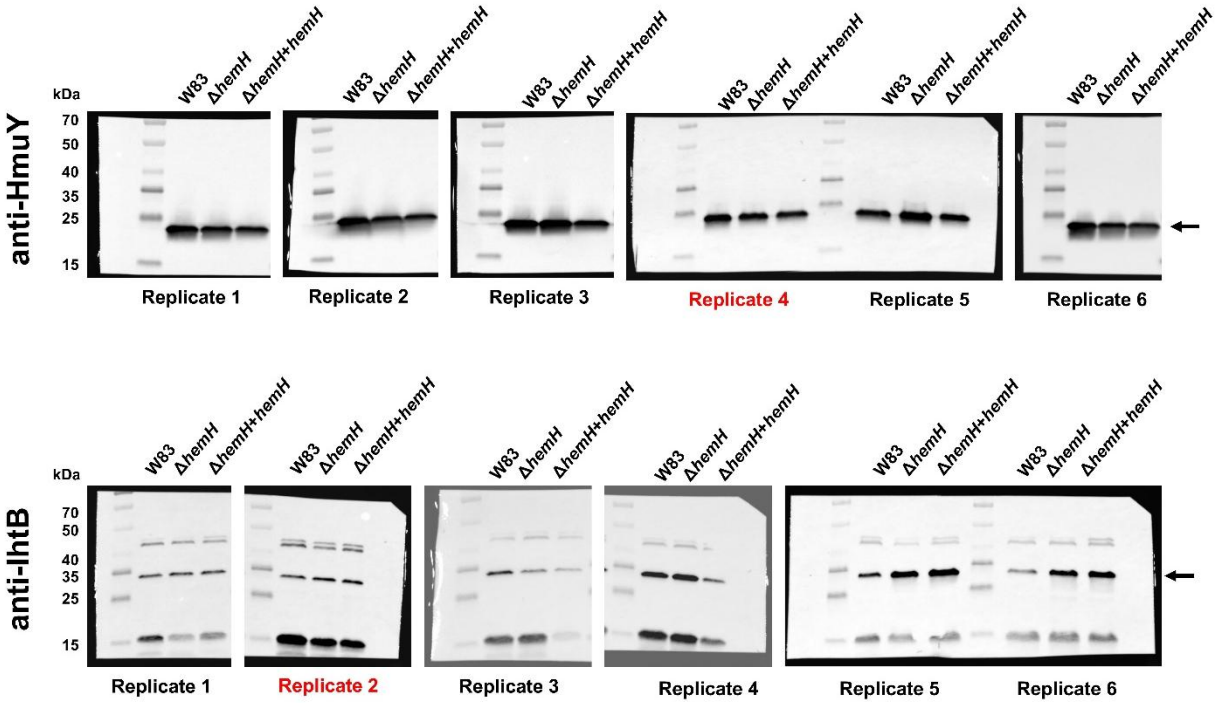


Fig. S6. Supplemental material for Fig. 5C. Analysis of the production of HmuY and IhtB proteins using Western blotting with appropriate antibodies. Prior to antibody incubation, the membrane was cut to reduce membrane area and antibody consumption, removing only regions where no signal was expected. The arrows indicate the band of the analysed protein. All replicates used for densitometric analyses are shown. The replicate presented in Fig. 5C is shown in red.

Table S1. Primers designed and used in this study.

Primer name	Sequence 5`→ 3`	Description	Reference
PBM_0109	TCCAAAGACAGGACTACG	Primer pairs (PBM_0109 × PBM_0110 and PBM_0113 × PBM_0114) amplify the flanking regions of the *hemH* (*PG_RS00590*/*PG0127*) gene; used for the generation of the Δ*hemH* mutant strain.	This study
PBM_0110	AATTTCTTTTTTGTCATAATTTCAGGACGAAGATAGG		
PBM_0113	AATTTCATCCTTCGTAGTACAGCATGCTCAGTTATC		
PBM_0114	GATCCGATTCGCTTTGTAC		
PBM_0111	ATCTTCGTCCTGAAATTATGACAAAAAAGAAATTGCC	Primers amplify the *ermF* gene without the native promoter sequence from the pTIO-1 plasmid; used for the generation of the Δ*hemH* mutant strain.	This study
PBM_0112	TAACTGAGCATGCTGTACTACGAAGGATGAAATTTTTC		
PBM_0115	GATTGGGAAATGTAAGCGAGG	Primers amplify the region affected by the *hemH* deletion and the *ermF* insertion; the reduced length of the amplified region in the Δ*hemH* mutant strain results from the deletion of the entire open reading frame from the *hemH* gene and insertion of the *ermF* gene, lacking its native promoter sequence; used to verify the construction of the Δ*hemH* mutant strain.	This study
PBM_0116	TAAGGCGTACCGAGCGATAG		
PBM_0160	GAGCCGATGATTTTCAGCG	Primers amplify the *hemH* gene (*PG_RS00590*/*PG0127*) together with its native promoter sequence and add the sequence encoding the HA tag at the *hemH* 5` end.	This study
PBM_0161	TTAAGCGTAATCTGGAACATCGTATGGGTATTCCTCTAAAATGTTTCGGAGAG		
PBM_0162	ACAAGGCAAGGGAAGAACGAGCCGATGATTTTCAGCG	Primers amplify the *hemH* gene (*PG_RS00590*/*PG0127*) together with its native promoter sequence, the latter obtained with the use of PBM_0160 and PBM_0161 primers, allowing cloning it into XhoI and BamHI restriction sites of the pTIO-tetQ plasmid, resulting in the pTIO-*hemH* plasmid.	This study
PBM_0163	CTCTAGAACTAGTGGATCC TTAAGCGTAATCTGGAACATCG		
PBM_0562	TCGGATGTATGGCATATAATAGC	Primers used to verify the presence of pTIO-*hemH* plasmid in *P. gingivalis* complemented strain.	This study
PBM_0563	GTCGTGAAGATTTGATGATAACC		
PBM_0100	CCTCGGGATCGAGGGAAGGATGGCAGATCGTAGAAGAACC	Primers used to amplify the *hemH* (*PG_RS00590*/*PG0127*) gene, enabling its cloning into XmnI and BamHI restriction sites of the pMAL-c5x plasmid, allowing overexpression of the recombinant HemH protein.	This study
PBM_0101	ATTACCTGCAGGGAATTCGGATCCTTATTCCTCTAAAATGTTTCGGAG		
PBM_0351	CGACAGGCTTTGAATCTCCG	Amplify the *hemH* (*PG_RS00590*/*PG0127*) gene fragment; used for the qPCR analysis.	This study
PBM_0352	TATGGCCTACACGTGACTGG		
PBM_0739	GCTTCGAAATACGAAACGTG	Amplify the *hmuY* (*PG_RS06840*/*PG1551*) gene fragment; used for the qPCR analysis.	**[S1]**
PBM_0740	TATATCCGTCTGTCGGAACG		
PBM_0743	AGGTGTACTTGGCATTCCGTC	Amplify the *hagA* (*PG_RS08090*/*PG1837*) gene fragment; used for the qPCR analysis.	**[S2]**
PBM_0744	CGTGTACGTGTAGTCGTTGGA		

PBM_0745	ATCGGCTATGCGAAGAAGC	Amplify the *husA* (*PG_RS08090*/*PG2227*) gene fragment; used for the qPCR analysis.	**[S3]**
PBM_0746	GAAGTAGGCTCGCGGATTAG		
PBM_0741	CTTGACTTCAGTGGCGGCAG	Amplify the *16S rRNA* (*PG_RS00460*/*PG_16SA*) gene fragment; used for the PCR and qPCR analysis.	**[S4]**
PBM_0742	AGGGAAGACGGTTTTCACCA		
PBM_0997	GTTCTTCCGCAGCAAAGTCC	Amplify the *PG_RS00595*/*PG0128* gene fragment; used for the qPCR analysis.	This study
PBM_0998	CAGGTCGCCCACTTCAAGAT		
PBM_0999	TCGTCTATCCTTCGCGCTTC	Amplify the *PG_RS00600*/*PG0129* gene fragment; used for the qPCR analysis.	This study
PBM_1000	GCCATCATTTCCGCATCGTC		

S1. Gmiterek A, Wójtowicz H, Mackiewicz P, Radwan-Oczko M, Kantorowicz M, Chomyszyn-Gajewska M, Frąszczak M, Bielecki M, Olczak M, Olczak T (2013) The unique *hmuY* gene sequence as a specific marker of *Porphyromonas gingivalis*. *PLoS ONE* **8(7)**:e67719. doi:10.1371/journal.pone.0067719.

S2. Ciuraszkiewicz J, Śmiga M, Mackiewicz P, Gmiterek A, Bielecki M, Olczak M, Olczak T (2014) Fur homolog regulates *Porphyromonas gingivalis* virulence under low-iron/heme conditions through a complex regulatory network. *Mol. Oral Microbiol*. **29(6)**:333-353. doi:10.1111/omi.12077.

S3. Śmiga M, Ślęzak P, Wagner M, Olczak T (2023) Interplay between *Porphyromonas gingivalis* hemophore-like protein HmuY and Kgp/RgpA gingipains plays a superior role in heme supply. *Microbiol. Spectr*. **11(2)**:e0459322. doi:10.1128/spectrum.04593-22.

S4. Maeda H, Fujimoto C, Haruki Y, Maeda T, Kokeguchi S, Petelin M, Arai H, Tanimoto I, Nishimura F, Takashiba S (2003) Quantitative real-time PCR using TaqMan and SYBR Green for *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *tetQ* gene and total bacteria. *FEMS Immunol. Med. Microbiol*. **39(1)**:81-86. doi:10.1016/S0928-8244(03)00224-4.

Table S2. Host and environmental niche of *Porphyromonas* species.

Species	Host	Occupied niche
P. gingivalis	human	oral cavity
P. gulae	mammals (canine, bovine)	oral cavity
P. loveana	marsupials	oral cavity
P. endodontalis	human	oral cavity
P. uenonis	human	gut
P. asaccharolytica	human	empyema
P. somerae	human	ulcer
P. pogonae	lizard/human	abscess, infected wound
P. cangingivalis	dog	oral cavity
P. canoris	dog	oral cavity
P. gingivicanis	dog	oral cavity
P. levii	bovine	rumen, abscess
P. macacae	feline/monkeys	oral cavity