

Supplementary information for:

Defining the role of Hmu and Hus systems in *Porphyromonas gingivalis* heme and iron homeostasis and virulence

Michał Śmiga^{a*}, Paulina Ślęzak^a, Michał Tracz^b, Patryk Cierpisz^a, Mateusz Wagner^{a#}, Teresa Olczak^a

^aLaboratory of Medical Biology, Faculty of Biotechnology, University of Wrocław, 50-383 Wrocław, 14A F. Joliot-Curie St., Poland

^bLaboratory of Protein Mass Spectrometry, Faculty of Biotechnology, University of Wrocław, 50-383 Wrocław, 14A F. Joliot-Curie St., Poland

*Corresponding author: Michał Śmiga; E-mail: michal.smiga@uwr.edu.pl

[#]Present address: Mateusz Wagner, Department of Molecular Biology and Genetics, Weill Institute for Cell and Molecular Biology, Cornell University, Ithaca, New York, USA

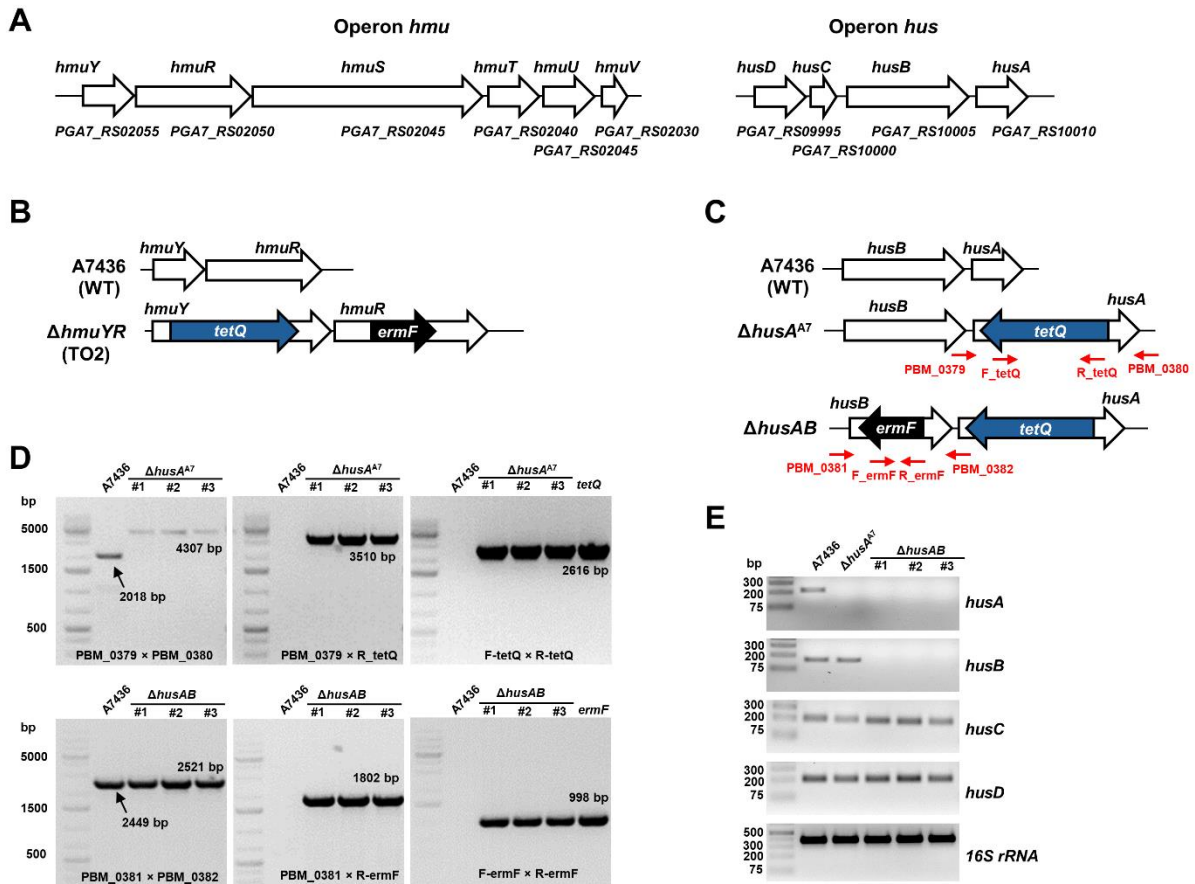
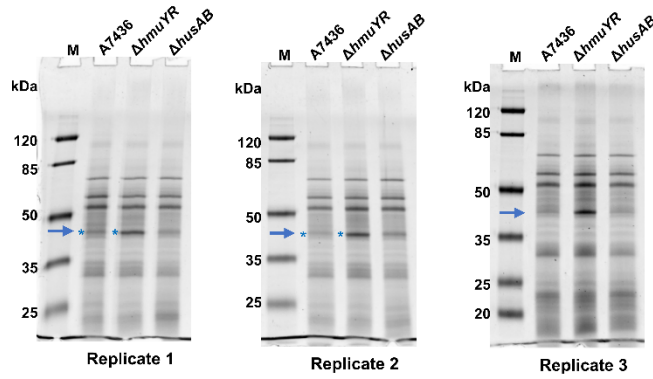
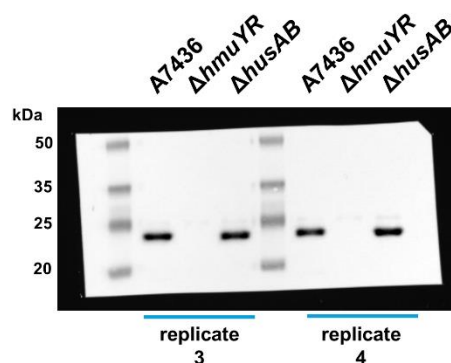
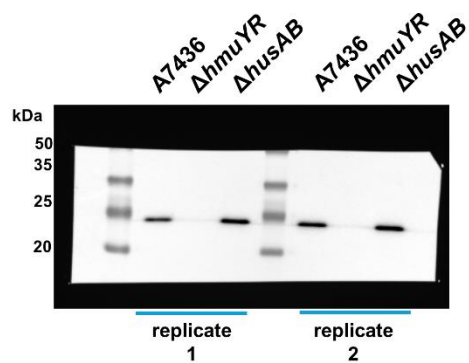


Figure S1. The construction and verification of mutant strains. (A) Schematic presentation of *hmu* and *hus* operons in the A7436 wild-type (WT) strain with respective gene IDs. (B) $\Delta hmuYR$ (TO2) mutant strain was generated in the A7436 wild-type strain by inserting *tetQ* and *ermF* antibiotic-resistant cassettes in the *hmuY* and *hmuR* genes, respectively [17]. (C) $\Delta husAB$ mutant strain was generated by partial deletion of the *husA* gene and replacement with a *tetQ* antibiotic-resistant cassette, creating the $\Delta husA^{A7}$ mutant strain, in which part of the *husB* gene was replaced with an *ermF* antibiotic-resistance cassette. (D) Construction of both $\Delta husA^{A7}$ and $\Delta husAB$ mutant strains was verified by PCR using primers listed in Table S2 and schematically shown in red in panel C. (E) Analysis of transcript encoding *hus* operon genes in $\Delta husA^{A7}$ and $\Delta husAB$ mutant strains using RT-PCR and primers listed in Table S2. 16S rRNA gene was used as a control.

A**B**

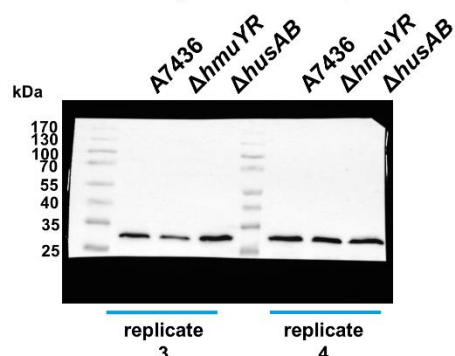
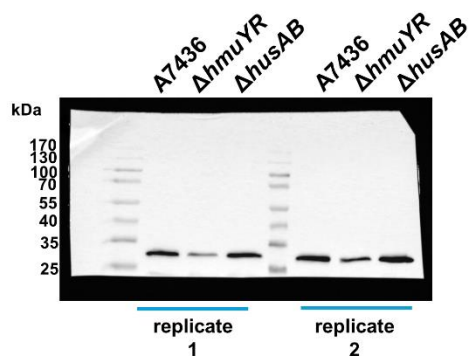
	Protein ID	Protein description	Protein MW (Da)	PLGS Score	No. of matched peptides	Protein sequence coverage (%)	No. of matched fragments	Protein relative amount (Top 3 peptides)
A7436 Replicate 1	Q7MT61	Glyceraldehyde-3-phosphate dehydrogenase	35992	1520	17	31.3	56	285760
	P59914	Major fimbrial subunit FimA type-4	41220	1272	14	17.8	62	968852
	P0C934	NAD-specific glutamate dehydrogenase	49167	334	17	18.2	54	59645
	Q7MVI3	Electron transfer flavoprotein alpha subunit	36780	152	7	15.5	19	43279
ΔhmuYR Replicate 1	P59914	Major fimbrial subunit FimA type-4	41220	6545	16	33.8	211	5440551
	P0C934	NAD-specific glutamate dehydrogenase	49167	1604	27	40.2	124	134711
	Q7MV30	Phosphoserine aminotransferase	40148	972	13	30.3	59	192165
	Q7MT61	Glyceraldehyde-3-phosphate dehydrogenase	35992	736	5	23.5	42	98085
	Q7MV21	branched-chain-amino-acid transaminase	37797	733	8	28.9	58	90838
	Q7MWD1	4-hydroxybutyryl-CoA dehydratase	53999	640	21	33.3	86	289951
	Q7MXK2	Fimbrial protein	36838	310	8	20.1	37	74837
	Q7MUR4	Uncharacterized protein	4404	279	1	27.8	11	13452
	Q7MXX1	Type IX secretion system protein PorV domain	43239	241	3	9.5	19	28681
A7436 Replicate 2	P59914	Major fimbrial subunit FimA type-4	41220	821	9	8.8	50	601436
	P0C934	NAD-specific glutamate dehydrogenase	49167	373	20	23.4	55	63759
	Q7MT61	Glyceraldehyde-3-phosphate dehydrogenase	35992	300	6	15.2	19	91328
	Q7MVI3	Electron transfer flavoprotein alpha subunit	36780	122	5	10.7	12	25955
	Q7MV30	Phosphoserine aminotransferase	40148	102	7	10.0	19	32374
ΔhmuYR Replicate 2	P59914	Major fimbrial subunit FimA type-4	41220	5117	12	32.0	186	4249984
	P0C934	NAD-specific glutamate dehydrogenase	49167	1496	30	42.2	130	109614
	Q7MV30	Phosphoserine aminotransferase	40148	745	12	29.4	65	166019
	Q7MWD1	4-hydroxybutyryl-CoA dehydratase	53999	655	23	25.3	89	331319
	Q7MT61	Glyceraldehyde-3-phosphate dehydrogenase	35992	595	9	24.4	44	111212
	Q7MV21	branched-chain-amino-acid transaminase	37797	425	13	29.8	49	69297
	Q7MVI3	Electron transfer flavoprotein alpha subunit	36780	271	8	19.1	28	44584

Figure S2. Protein analysis in *P. gingivalis* A7436 wild-type and $\Delta hmuYR$ or $\Delta husAB$ mutant strains. (A) Proteins present in whole cell lysates were analyzed by SDS-PAGE. After electrophoresis, proteins were visualized using staining with CBB G-250. The expression of the FimA protein in the protein band indicated by arrows has been verified by LC-MS analysis. (B) PLGS output protein identification list for the analyzed band samples indicated by asterisks in panel A for replicate 1 and 2. The trypsin match (identified in each case) was excluded. In both analyzed $\Delta hmuYR$ mutant replicates FimA was the highest scoring hit (mean score increase relative to the second-best scored hit: 3.7 $\times$) while being second (0.84 $\times$ score of the best-scored hit) and first (score increase relative to the second-best scored hit: 2.2 $\times$) in the two A7436 wild-type replicates used as a control.

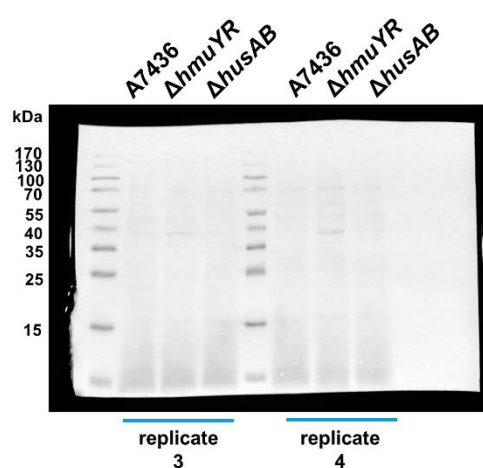
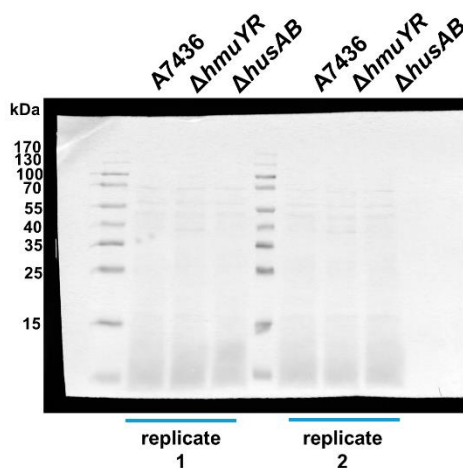


Hm

anti-HmuY



anti-IhtB



Ponceau S

DIP

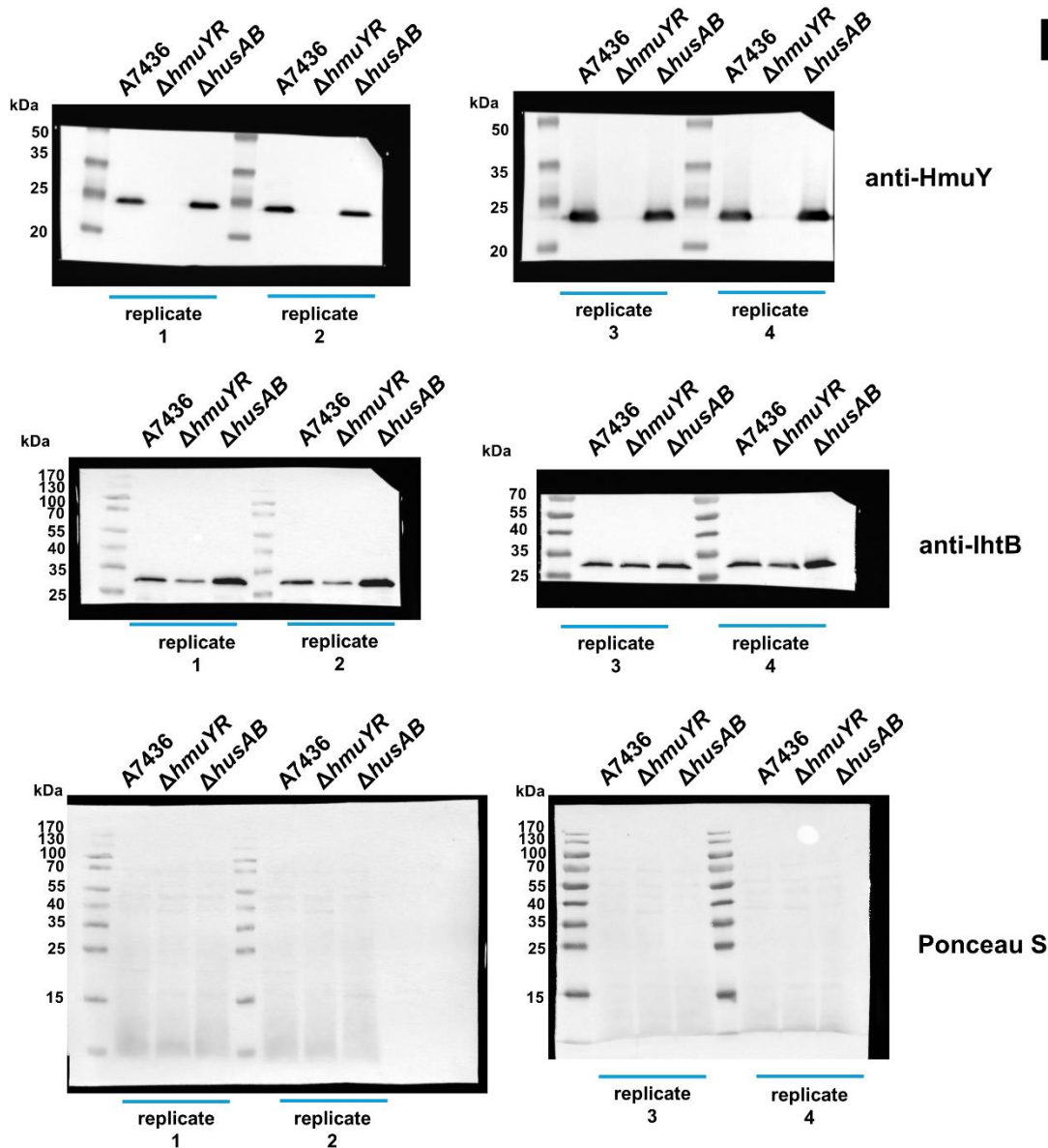


Figure S3. Supplemental data for Figure 4C. Expression of HmuY and IhtB proteins produced by *P. gingivalis* A7436 wild-type and $\Delta hmuYR$ or $\Delta husAB$ mutant strains was analyzed using Western blotting with anti-HmuY or anti-IhtB antibodies. The efficiency of protein transfer was visualized on nitrocellulose membranes by staining proteins with Ponceau S. Hm – iron and heme-replete conditions (Hm medium). DIP – iron and heme-depleted conditions (DIP medium).

Table S1. *Porphyromonas gingivalis* strains constructed and used in this study.

Strain name	Description/mutation	Antibiotic resistance (antibiotic concentration)	References
A7436	wild-type	none	Laboratory collection
$\Delta hmuYR$ (TO2)	$hmuY^{\Delta}Tc^r$ $hmuR^{\Delta}Em^r$	erythromycin (3 μ g/ml), tetracycline (1 μ g/ml)	[17]
$\Delta husA^{A7}$	$husA^{\Delta}Tc^r$	tetracycline (1 μ g/ml)	This study
$\Delta husAB$	$husA^{\Delta}Tc^r$ $husB^{\Delta}Em^r$	erythromycin (3 μ g/ml), tetracycline (1 μ g/ml)	This study

Table S2. Primers designed and used in this study.

Primer name	5'→3' DNA sequence	Gene abbreviation/locus ID	Description (reference)
PBM_0294	TACTACCTCTCCCATGGCATTG	husA/PGA7_RS10010	Amplification of DNA fragments used for the generation of ΔhusA ^{A7} mutant strain in A7436 wild-type strain [25]
PBM_0295	GAGATAGAAGCATTAGAACTTGGCACCTTGTGCGGATGTTGCAC		
PBM_0298	CGTACGTTAAGGAGATAATTCGTTGTAGCAGAGAGCAGCCGACAG		
PBM_0299	CGAAAGCCGACCAAGGCG		
PBM_0296	GTGCAACATCCGCACAAGGTGCCAAGTTCTAATGCTTCTATCTC		
PBM_0297	CTGTGCGGCTGCTCTCTGCTACAACGAATTATCTCCTTAACGTACG		
PBM_0379	CCGAGTCGGCTATTATCCTAC	husA/PGA7_RS10010	Verification of the construction of ΔhusA ^{A7} mutant strain [25 and this study]
PBM_0380	CAGATGGTTATAACCTTGAGATATAAAC		
F_tetQ	TGCCAAGTTCTAATGCTTCTATCTC	tetQ	
R_tetQ	ACAACGAATTATCTCCTTAACGTACG		
PBM_0369	GCATGTCCACATCAGGGATTG	husB/PGA7_RS10005	Amplification of DNA fragments used for generation of ΔhusB mutant strain in ΔhusA mutant strain, resulting in ΔhusAB mutant strain (this study)
PBM_0370	TGCCGCATAACGGCTGGCCCATAGCCTCCGGTGAG		
PBM_0373	AAAGAGAGACAACCGCGGTGGACCCTCGCGTACACAAGTG		
PBM_0374	CTTGTGCGGATGTTGCACACA		
PBM_0371	CTCCACCGGAGGCTATGGGCCAGCCGTTATGCGGCA	ermF	
PBM_0372	CACTTGTGTACGCGAGGGTCCACCGCGTTGTCTCTCTTT		
PBM_0381	ACCGATGCCGGCCGTG	husB/PGA7_RS10005	Verification of the construction of ΔhusAB mutant strain (this study)
PBM_0382	CGGCTGCTATCCGGGAAAAG		
F_ermF	TGCCGCATAACGGCTGGC	ermF	
R_ermF	CCACCGCGGTTGTCTCTCTTT		
16SrRNA-F	CTTGACTTCAGTGGCGGCAG	16S rRNA/PGA7_RS00460	RT-PCR and qPCR analyses [68]
16SrRNA-R	AGGGAAGACGGTTTTACCA		
HYq4_F	GCTTCGAAATACGAAACGTG	hmuY/PGA7_RS02055	qPCR analysis [69]
HYq4_R	TATATCCGTCTGTGCGGAACG		
PBM_0747	CTACCGACACCATCGTATCC	hmuR/PGA7_RS02050	qPCR analysis [70]
PBM_0748	CATTGAGCTGATCTCTGGAAC		
PBM_0073	CTTTTTCAGCAGGCAATAAGC	hmuS/PGA7_RS02045	qPCR analysis [70]
PBM_0074	GTCTTCCAACCAAGTCTCTC		
PBM_0838	GCCTTATTGGCTACGCTCAAG	hmuT/PGA7_RS02040	qPCR analysis [70]
PBM_0839	GCGTACATATATATACGACGTACAG		
PBM_0840	TAGTCGCCGGACTCATCTATC	hmuU/PGA7_RS02035	qPCR analysis [70]
PBM_0841	AATCTGCATCGAGGACTTCG		
PBM_0842	GCAAGGACAACATGGAAATC	hmuV/PGA7_RS02030	qPCR analysis [70]
PBM_0843	CCGGCACGTAGATGATTTG		
PBM_0745	ATCGGCTATGCGAAGAAGC	husA/PGA7_RS10010	RT-PCR and qPCR analyses [25]
PBM_0746	GAAGTAGGCTCGCGGATTAG		
PBM_0749	GGACGATATGGAACCCGACT	husB/PGA7_RS10005	RT-PCR and qPCR analyses [25]

PBM_0750	AGCCATCCCGAATCCATAGG		
PBM_0681	CGCTGCTCATGTCCGAATTG	<i>husC</i> /PGA7_RS10000	RT-PCR and qPCR analyses (this study)
PBM_0682	GTGGCTCTACAGGTGGTACG		
PBM_0683	TTTGCCACGAGTCTGCTGAT	<i>husD</i> /PGA7_RS09995	RT-PCR and qPCR analyses (this study)
PBM_0684	TGCCGTAACATTCCAGTCCGA		
PBM_0799	CGGGCACCTATTCTCTCCTT	<i>feoB</i> /PGA7_RS04380	qPCR analysis [70]
PBM_0800	TCGGCTTCGTCCATTAGATT		
PBM_0771	CTATCCTGGTGGGACTGCATC	<i>fimA</i> /PGA7_RS09555	qPCR analysis [71]
PBM_0772	ACCAAAGAATTGCCGAAAATC		
PBM_0769	GCCGAATTGTTTGTGCGATAG	<i>sodB</i> /PGA7_RS02075	qPCR analysis [71]
PBM_0770	AATTCCACCACGGTAAGCACC		
PBM_0759	GAATGGTGCGGTTACTGTCC	<i>hbp35</i> /PGA7_RS06285	qPCR analysis [25]
PBM_0760	GAATGCCCAATGTTTGATCC		
PBM_0757	AATATCCGTGGCGCAGAG	<i>dps</i> /PGA7_RS00405	qPCR analysis [25]
PBM_0758	CGAGTTCGTGCTCTTCCTTC		
PBM_0751	GAGTGTGGGTGCTAATGCCG	<i>kgp</i> /PGA7_RS08195	qPCR analysis [70]
PBM_0752	CACCAATATGGGTAATATTGCCG		
PBM_0753	CGCTTCCCATTCTATCACGC	<i>rgpA</i> /PGA7_RS09040	qPCR analysis [70]
PBM_0754	CGGATCTTCGTTACGCATAATCAT		
PBM_0755	AATGATAAGCCTTATACTGTAGCTG	<i>rgpB</i> /PGA7_RS06770	qPCR analysis [70]
PBM_0756	GTTTTGTGCTTCGAATACCATGC		
PBM_0640	TCTTCATGTGATTCCCGGCC	<i>ihtB</i> /PGA7_RS0606	qPCR analysis (this study)
PBM_0641	GGTGCCGTGTCCCATGAATA		
PBM_0743	AGGTGTACTTGGCATTCCGTC	<i>hagA</i> /PGA7_RS08180	qPCR analysis [72]
PBM_0744	CGTGTACGTGTAGTCGTTGGA		
PBM_0404	GATGGTTTCAACGAAAGCCC	<i>mfa1</i> /PGA7_RS00800	qPCR analysis (this study)
PBM_0405	GCTGCTGGTAGGAACGAAAC		
PBM_0338	CGAGAGCGTACCTTACCGAC	<i>atpD</i> /PGA7_RS08050	qPCR analysis (this study)
PBM_0339	GAGAGCTTGACACCCTCCAC		
PBM_0398	CGGGTATCGTCCTGCTCATC	PGA7_RS05995	qPCR analysis (this study)
PBM_0399	GAGCAGGGTTATGACGAGGG		

Table S3. Statistical analysis of *P. gingivalis* growth curves shown in Figure 1A. Data with *p*-values <0.05 was considered statistically significant (shown in red).

Time [h]	Hm medium		DIP medium	
	$\Delta hmuYR$ vs. A7436	$\Delta husAB$ vs. A7436	$\Delta hmuYR$ vs. A7436	$\Delta husAB$ vs. A7436
2	>0.9999	>0.9999	>0.9999	>0.9999
4	>0.9999	>0.9999	>0.9999	>0.9999
6	0.9995	>0.9999	0.9993	>0.9999
8	0.9809	>0.9999	0.5894	>0.9999
10	0.9225	0.9998	0.0335	0.9994
12	0.8634	>0.9999	0.0015	0.9987
14	0.8750	>0.9999	0.0004	>0.9999
16	0.9385	>0.9999	0.0003	>0.9999
18	0.9957	>0.9999	0.0015	>0.9999
20	>0.9999	>0.9999	0.0076	>0.9999
22	>0.9999	>0.9999	0.0213	>0.9999
24	>0.9999	>0.9999	0.0506	>0.9999
26	>0.9999	>0.9999	0.1260	>0.9999
28	>0.9999	>0.9999	0.2572	>0.9999
30	>0.9999	>0.9999	0.4282	>0.9999
32	>0.9999	>0.9999	0.6560	>0.9999
34	>0.9999	>0.9999	0.7503	>0.9999
36	>0.9999	>0.9999	0.7791	>0.9999
38	>0.9999	>0.9999	0.8229	>0.9999
40	>0.9999	>0.9999	0.8495	>0.9999
42	>0.9999	>0.9999	0.8509	>0.9999
44	>0.9999	>0.9999	0.9120	>0.9999
46	>0.9999	>0.9999	0.9344	>0.9999
48	>0.9999	>0.9999	0.9398	>0.9999

Table S5. Statistical analysis of *P. gingivalis* growth curves shown in Figure 6A. Data with *p*-values <0.05 was considered statistically significant (shown in red).

Time [h]	no Hm vs 2.5 μ M Hm			2.5 μ M Hm + 5 μ M HmuY vs 2.5 μ M Hm			2.5 μ M Hm + 5 μ M HusA vs 2.5 μ M Hm		
	A7436	$\Delta hmuYR$	$\Delta husAB$	A7436	$\Delta hmuYR$	$\Delta husAB$	A7436	$\Delta hmuYR$	$\Delta husAB$
2	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999
4	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999
6	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999
8	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999
10	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999
12	>0.9999	>0.9999	0.9992	>0.9999	>0.9999	0.9747	>0.9999	>0.9999	>0.9999
14	>0.9999	>0.9999	0.9461	0.9965	>0.9999	0.5674	>0.9999	>0.9999	>0.9999
16	>0.9999	>0.9999	0.5692	0.9471	>0.9999	0.0635	>0.9999	>0.9999	0.9970
18	0.9953	>0.9999	0.1018	0.7091	>0.9999	0.0029	>0.9999	>0.9999	0.9685
20	0.9322	>0.9999	0.0064	0.3558	>0.9999	<0.0001	>0.9999	>0.9999	0.9008
22	0.6838	>0.9999	0.0002	0.1695	>0.9999	<0.0001	0.9996	>0.9999	0.7447
24	0.3024	>0.9999	<0.0001	0.1068	>0.9999	<0.0001	0.9975	>0.9999	0.6271
26	0.1320	0.9995	<0.0001	0.1011	>0.9999	0.0002	0.9960	>0.9999	0.4744
28	0.0479	0.9977	<0.0001	0.1249	>0.9999	0.0019	0.9933	>0.9999	0.7883
30	0.0330	0.9789	<0.0001	0.2200	>0.9999	0.0102	0.9966	>0.9999	0.9333
32	0.0257	0.8954	<0.0001	0.2968	>0.9999	0.0368	0.9970	>0.9999	0.9932
34	0.0343	0.7083	<0.0001	0.3589	>0.9999	0.0882	0.9941	0.9996	0.9996
36	0.0473	0.5007	<0.0001	0.4275	>0.9999	0.0888	0.9943	0.9994	0.9999
38	0.0797	0.2997	<0.0001	0.4185	>0.9999	0.1200	0.9938	0.9993	>0.9999
40	0.1068	0.2096	<0.0001	0.4094	>0.9999	0.1662	0.9976	0.9985	>0.9999
42	0.1397	0.1383	<0.0001	0.4014	>0.9999	0.2879	0.9979	0.9988	>0.9999
44	0.1852	0.1004	<0.0001	0.4187	>0.9999	0.3137	0.9973	0.9989	>0.9999
46	0.2922	0.0711	<0.0001	0.4269	>0.9999	0.3255	0.9966	0.9987	>0.9999
48	0.3745	0.0436	<0.0001	0.4269	>0.9999	0.3600	0.9966	0.9996	>0.9999

Table S6 Statistical analysis of *P. gingivalis* growth curves shown in Figure 6C. Data with *p*-values <0.05 was considered statistically significant (shown in red).

Time [h]	no PPIX vs 2.5 μ M PPIX			2.5 μ M PPIX + 5 μ M HmuY vs 2.5 μ M PPIX			2.5 μ M PPIX + 5 μ M HusA vs 2.5 μ M PPIX		
	A7436	$\Delta hmuYR$	$\Delta husAB$	A7436	$\Delta hmuYR$	$\Delta husAB$	A7436	$\Delta hmuYR$	$\Delta husAB$
2	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999
4	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999
6	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999
8	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999
10	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999
12	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999
14	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999
16	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999
18	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999
20	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999
22	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999
24	>0.9999	>0.9999	0.9968	>0.9999	>0.9999	0.9991	>0.9999	>0.9999	>0.9999
26	>0.9999	>0.9999	0.9861	0.9998	>0.9999	0.9988	>0.9999	>0.9999	>0.9999
28	>0.9999	>0.9999	0.9693	0.9994	>0.9999	0.9911	>0.9999	>0.9999	>0.9999
30	0.9972	0.9994	0.9519	0.9989	>0.9999	0.9915	>0.9999	>0.9999	>0.9999
32	0.9771	0.9998	0.7967	0.9984	0.9958	0.9714	>0.9999	>0.9999	>0.9999
34	0.8993	0.9990	0.7638	0.9998	0.9707	0.9660	>0.9999	>0.9999	>0.9999
36	0.9048	0.9854	0.5947	0.9989	0.8736	0.9787	>0.9999	>0.9999	>0.9999
38	0.8396	0.7684	0.6469	0.9988	0.8050	0.9831	>0.9999	>0.9999	>0.9999
40	0.8448	0.5082	0.6415	0.9990	0.4537	0.9812	>0.9999	0.9978	>0.9999
42	0.8337	0.0337	0.8166	0.9986	0.3598	0.9808	>0.9999	0.9087	>0.9999
44	0.7812	0.0070	0.8302	0.9996	0.1222	0.9747	>0.9999	0.9091	>0.9999
46	0.8499	0.0004	0.8806	0.9996	0.1202	0.9775	>0.9999	0.8096	>0.9999
48	0.8945	<0.0001	0.9468	0.9995	0.1453	0.9796	0.9999	0.7975	>0.9999

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