Table S1. Strains and plasmids

Strain or Plasmid	Genotype and relevant characteristics	Source or Reference
Strains		
Pseudomonas aeruginosa PAO1		(1)
P. aeruginosa PAO1 Δ*hpf*	*P. aeruginosa* with markerless *hpf* deletion	(2)
P. aeruginosa PAO1 Δ*rmf*	*P. aeruginosa* with markerless *rmf* deletion	(2)
P. aeruginosa PAO1 Δ*hpf*/Δ*rmf*	*P. aeruginosa* with markerless *hpf* and rmf deletions	(2)
Plasmids		
pMF54	P_{trc} exression vector pKK233-2 with $oriV_{SF}$ *oriT lacI*^{q}; Ap^{r}	(3)
pES43	pMF54 with wild-type *hpf* PCR product in *Nco*I-*Hin*DIII site	This study
pES44	pMF54 with *hpf* R83A	This study
pES45	pMF54 with *hpf* K87A	This study
pES49	pMF54 with *hpf* R29A	This study
pES50	pMF54 with *hpf* R26A	This study
pES53	pMF54 with *hpf* Y71S	This study
pES54	pMF54 with *hpf* Y71F	This study
pES55	pMF54 with *hpf* L27R	This study
pES56	pMF54 with *hpf* L81A	This study
pES57	pMF54 with *hpf* L85A	This study
pES58	pMF54 with *hpf* K23A	This study
pES59	pMF54 with *hpf* L24A	This study
pES60	pMF54 with *hpf* L27A	This study
pES61	pMF54 with *hpf* M70K	This study
pES62	pMF54 with *hpf* K80A	This study
pES69	pMF54 with *hpf* H8A mutation	This study
pES70	pMF54 with *hpf* L10A	This study
pES71	pMF54 with *hpf* I56A	This study
pES72	pMF54 with *hpf* I74A	This study
pMF496	pMF54 with *hpf* Y71F/R83S	This study
pMF497	pMF54 with *hpf* Y71F/K23A	This study
pMF498	pMF54 with *hpf* Y71F/R29A	This study
pMF499	pMF54 with *hpf* Y71F/K80A	This study
pMF501	pMF54 with *hpf* Y71F/R26A	This study
pMF503	pMF54 with *hpf* Y71F/R26A/K80A	This study
pMF504	pMF54 with *hpf* Y71F/R26A/R83A	This study
pMF505	pMF54 with *hpf* Y71F/R29A/K80A	This study
pES74	pMF54 with C-terminal truncated *hpf* (95 aa)	This study
pES77	pMF54 with *hpf* from *Escherichia coli*	This study
pES78	pMF54 with *yfiA* from *E. coli*	This study
pES79	pMF54 with long-*hpf* from *Staphylococcus aureus* USA300	This study
pES80	pMF54 with long-*hpf* (CD0142) from *C. difficile* VPI 10463	This study
pES81	pMF54 with long-*hpf* (CD2444) from *C. difficile* VP1 10463	This study

Table S2: Primers Sequences used in this study

Name	Sequence
5'_NcoI_hpf_inframe	TTACCATGGAAGTCAACATCAGTGGC
HPF_3'_HindIII	GGTCAAGCTTTGCTCAAGTCGGATCATAGGGGA
5'_NcoI_hpf_inframe_H8A_FW	TTACCATGGAAGTCAACATCAGTGGCGCTCAACTGGAT
5'_NcoI_hpf_L10A_FW	TTACCATGGAAGTCAACATCAGTGGCCATCAAGCGGATGTGACC
HPF_K23A_Down_FW	TATGTCGGCGAGGCACTGAGCCGTCT
HPF_K23A_Up_REV	AGACGGCTCAGTGCCTCGCCGACATA
HPF_L24A_Down_FW	GTCGGCGAGAAAGCGAGCCGTCTGGA
HPF_L24A_UP_REV	TCCAGACGGCTCGCTTTCTCGCCGAC
HPF_R26A_Down_for	GGCGAGAAACTGAGCGCTCTGGAGCGCCATTTC
HPF_R26A_Up_rev	GAAATGGCGCTCCAGAGCGCTCAGTTTCTCGCC
HPF_L27A_Down_FW	AAACTGAGCCGTGCGGAGCGCCATTT
HPF_L27A_Up_REV	AAATGGCGCTCGCGACGGCTCAGTTT
HPF R29A_Down_for	CTGAGCCGTCTGGAGGCCCATTTCGACAAGATC
HPF R29A_Up_rev	GATCTTGTCGAAATGGGCCTCCAGACGGCTCAG
HPF_I56A_Up_REV	TCGCCACCGGCGGCGCGCAGGGTG
HPF_I56A_Down_FW	CACCCTGCGCGCCGCCGGTGGCGA
HPF_M70A_Down_FW	GAACATGAGGACGCGTACGCGGCCAT
HPF_M70A_Up_REV	ATGGCCGCGTACGCGTCCTCATGTTC
HPF Y71S_Up_Rev	GTCGATGGCCGCGGACATGTCCTCATG
HPF Y71S_Down_for	CATGAGGACATGTCCGCGGCCATCGAC
HPF Y71F_Down_for	CATGAGGACATGTTCGCGGCCATCGAC
HPF Y71F_Up_Rev	GTCGATGGCCGCGAACATGTCCTCATG
HPF_I74A_Down_FW	CATGTACGCGGCCGCCGACCTGCTGATCG
HPF_I74A_Up_REV	CGATCAGCAGGTCGGCGGCCGCGTACATG
HPF_K80A_Down_FW	CTGCTGATCGACGCACTCGACCGTCA
HPF_K80A_Up_REV	TGACGGTCGAGTGCGTCGATCAGCAG
HPF_L81A_Down_FW	TGCTGATCGACAAAGCCGACCGTCAACTGA
HPF_L81A_Up_REV	TCAGTTGACGGTCGGCTTTGTCGATCAGCA
HPF R83A_Down_for	ATCGACAAACTCGACGCTCAACTGATCAAACAC
HPF R83A_Up_rev	GTGTTTGATCAGTTGAGCGTCGAGTTTGTCGAT
HPF_L85A_Down_FW	ACTCGACCGTCAAGCGATCAAACACAAAG
HPF_L85A_Up_REV	CTTTGTGTTTGATCGCTTGACGGTCGAGTT
HPF_K87A_Down_for	GACCGTCAACTGATCGCACACAAAGAGAAGTAT
HPF K87A_Up_rev	ATACTTCTCTTTGTGTGCGATCAGTTGACGGTC
3'_hpf_trunc95_HindIII_REV	GATAAGCTTTCACCGTTCGAGATACTTCTCTTTG
3'_hpf_trunc97_HindIII_REV	GATAAGCTTTCATTGCTGCCGTTCGAGATACT
3'_hpf_trunc99_HindIII_REV	GATAAGCTTTCATACGCCTTGCTGCCGT
5'_NcoI_hpf_EC	TTACCATGGAGCTCAACATTACCGG
3'_hpf_EC_HindIII	GCAAAGCTTCCGCCATGCACATGCTAAT
5'_NcoI_yfiA_EC	TTACCATGGCAATGAACATTACCAGCAA
3'_yfiA_EC_HindIII	ATCAAGCTTCGTTGGCGATACACTCAATA
5'_NcoI_CD0142	TATCCATGGACATAATTATATCTGGAAAACAAA
3'_CD0142_HindIII	TCCAAGCTTTATCGATAGCTAATATGATTAAATCTAAATTG
5'_NcoI_CD2444	TTTCCATGGAAATAATAGTATCTGGGAGACA
3'_CD2444_HindIII	ATCAAGCTTTCTTATATAAAATCTAAACGTATACAATTTTTATT
5'_NcoI_yfiA_SA	TTACCATGGTTAGATTTGAAATTCATGGAGATAACC
3'_yfiA_SA_HindIII	TGGAAGCTTAAAAAGCACTTGTGCAAAAACA

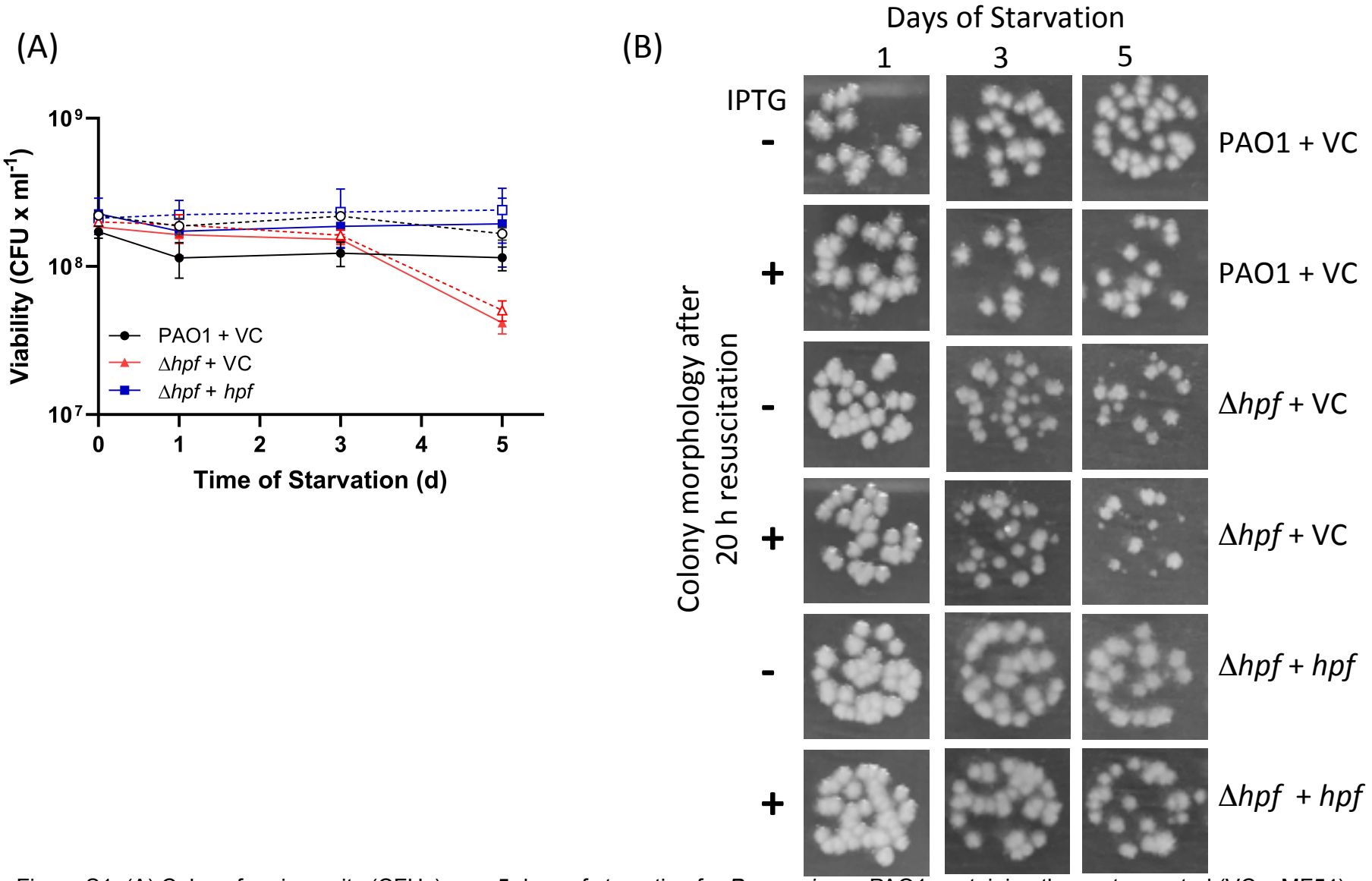

Figure S1. (A) Colony forming units (CFUs) over 5 days of starvation for *P. aeruginosa* PAO1 containing the vector control (VC, pMF54) (circles), *P. aeruginosa* Δ*hpf* + vector control (triangles), and *P. aeruginosa* Δ*hpf* complemented with IPTG inducible *hpf* (squares). Bacteria were cultured to stationary phase (20 h) in TSB with IPTG (solid lines) or without IPTG (dashed lines), washed in PBS, then incubated in PBS at 37C for 5 d. Aliquots were plated and CFUs were used to determine the total number of recoverable cells following starvation. Error bars represent the mean and standard error for three independent biological replicates. (B) Colony morphologies of *P. aeruginosa* PAO1 and the *P. aeruginosa* Δ*hpf* mutant strains over the course of starvation. *P. aeruginosa* Δ*hpf* shows colony heterogeneity, including small colony variants, after starvation. The wild-type strain and the *hpf* complemented strain show homogeneous large colony morphology over the course of starvation. Induction of *hpf* with IPTG did not affect colony morphology.

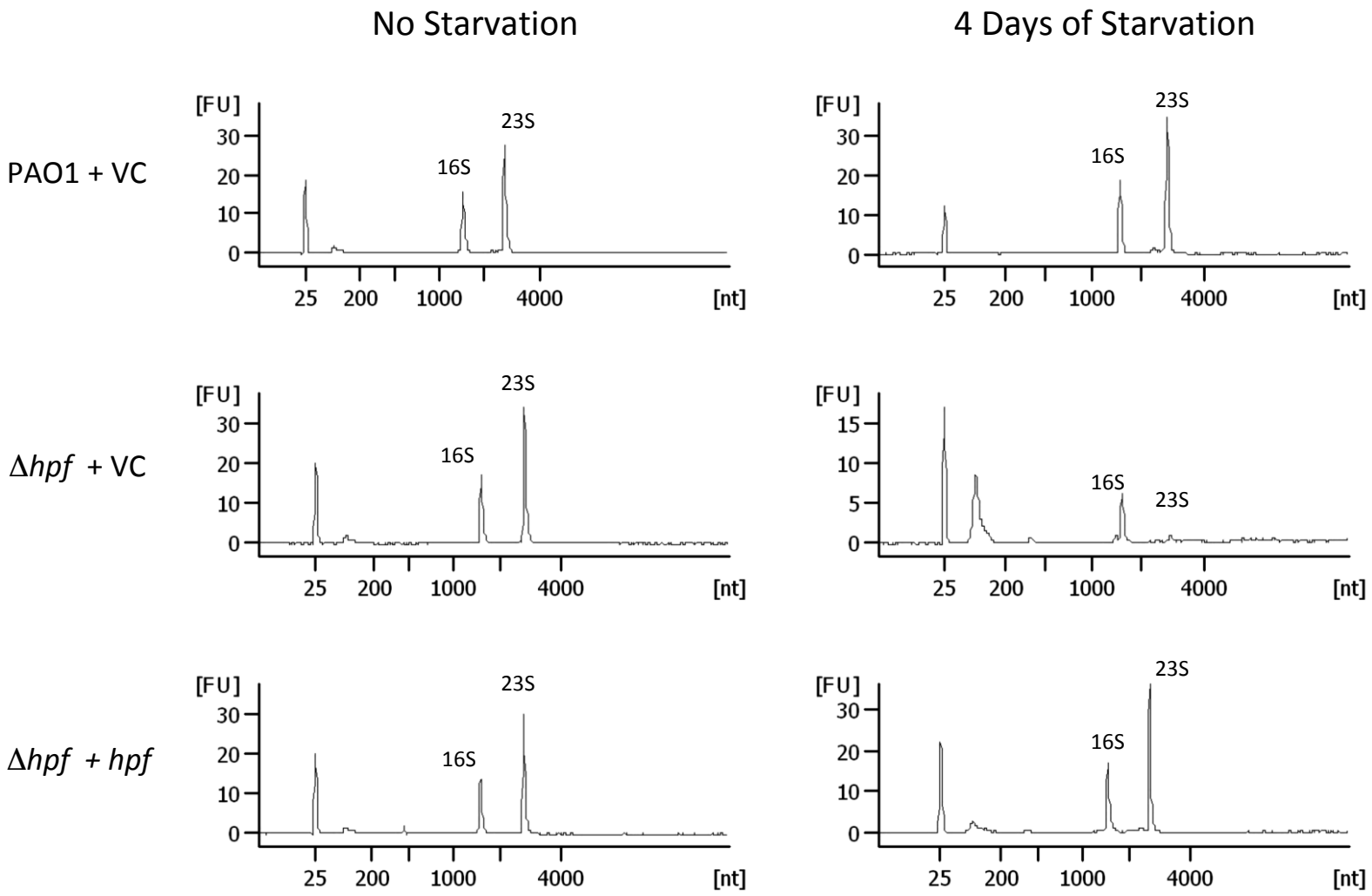

Figure S2. Analysis of 23S and 16S rRNA using the Agilent Bioanalyzer. *P. aeruginosa* PAO1 with the empty vector control showed 23S to16S rRNA ratios of approximately 1.5 prior to starvation and after 4 days of starvation. *P. aeruginosa Δhpf* + vector control had similar 23S/16S rRNA ratios to the wild-type strain prior to starvation, but the 23S rRNA band was almost completely lost after four days of starvation. The Δ*hpf* strain complemented with wild-type *hpf* expressed *in trans* had 23S/16S rRNA ratios that were similar to the wild-type strain before and after four days of starvation.

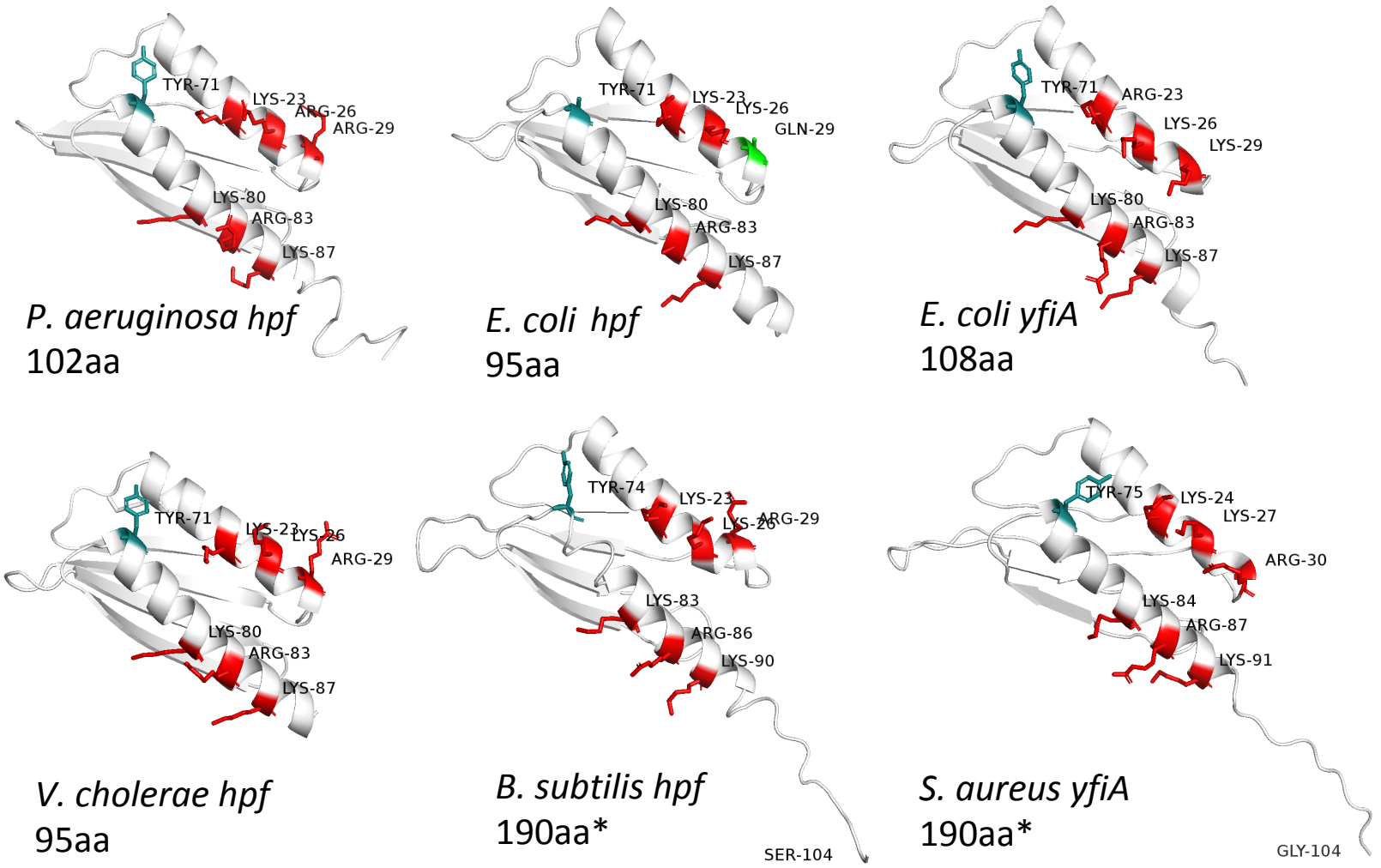

Figure S3: Structures of HPF homologs obtained from the protein database (PDB) and the predicted structure of HPF from *P. aeruginosa* using iTASSER. Highlighted are conserved positively charged amino acids (red) and a conserved tyrosine (Y71) residue. *PDB files for long-HPFs of *B. subtilis* and *S. aureus* were truncated to highlight the conserved N-terminal domain. PDB files: *E. coli* HPF (4V8H) (4), *E. coli* YfiA (4V8I) (4), *V. cholerae* HPF (4HEI) (5), *B. subtilis* HPF (5NJT) (6), *S. aureus* HPF (5ND9) (7).

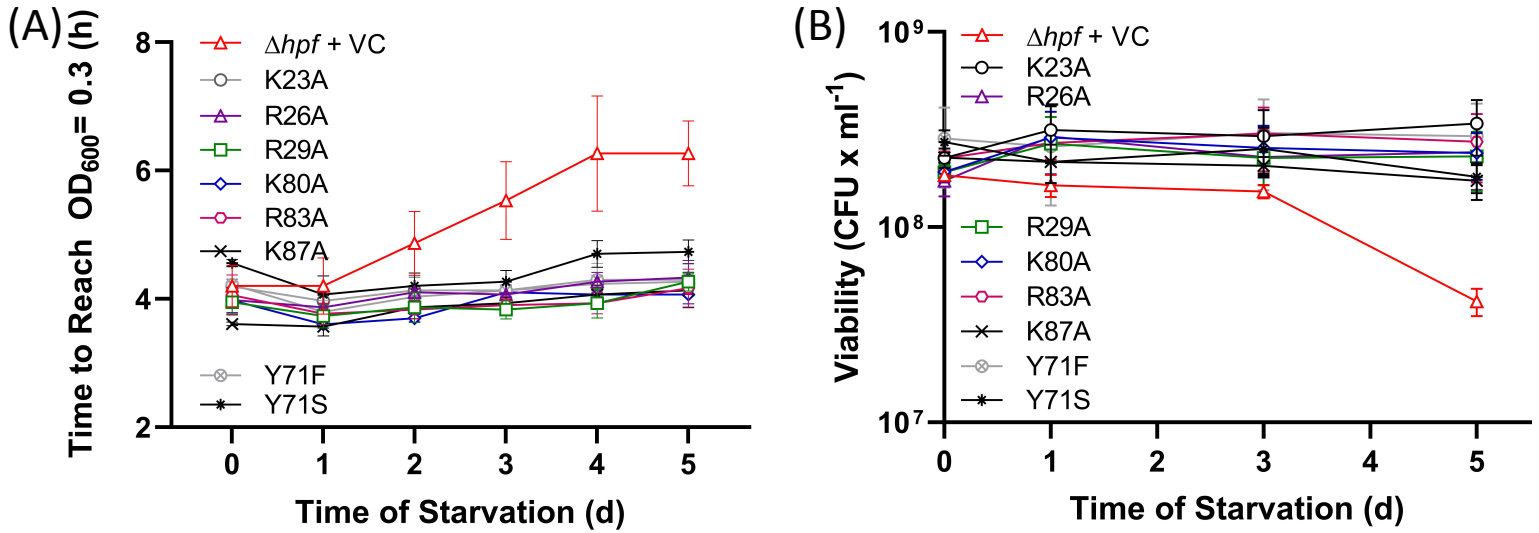

Figure S4. (A) Resuscitation of *P. aeruginosa* Δ*hpf* with mutant forms of HPF measured as time to reach $O.D._{600}$ = 0.3, for strains pre-cultured with 1 mM IPTG. (B) Resuscitation of *P. aeruginosa*, as indicated by colony forming units (CFUs) following starvation. Strains were precultured in the presence of 1 mM IPTG. Red triangles show impaired resuscitation of the *P. aeruginosa* Δ*hpf* mutant, but restoration of survival during starvation for strains complemented with *hpf* containing point mutations. The symbols are the same as in Fig 3. Data show the mean and standard error of the mean for three biological replicates.

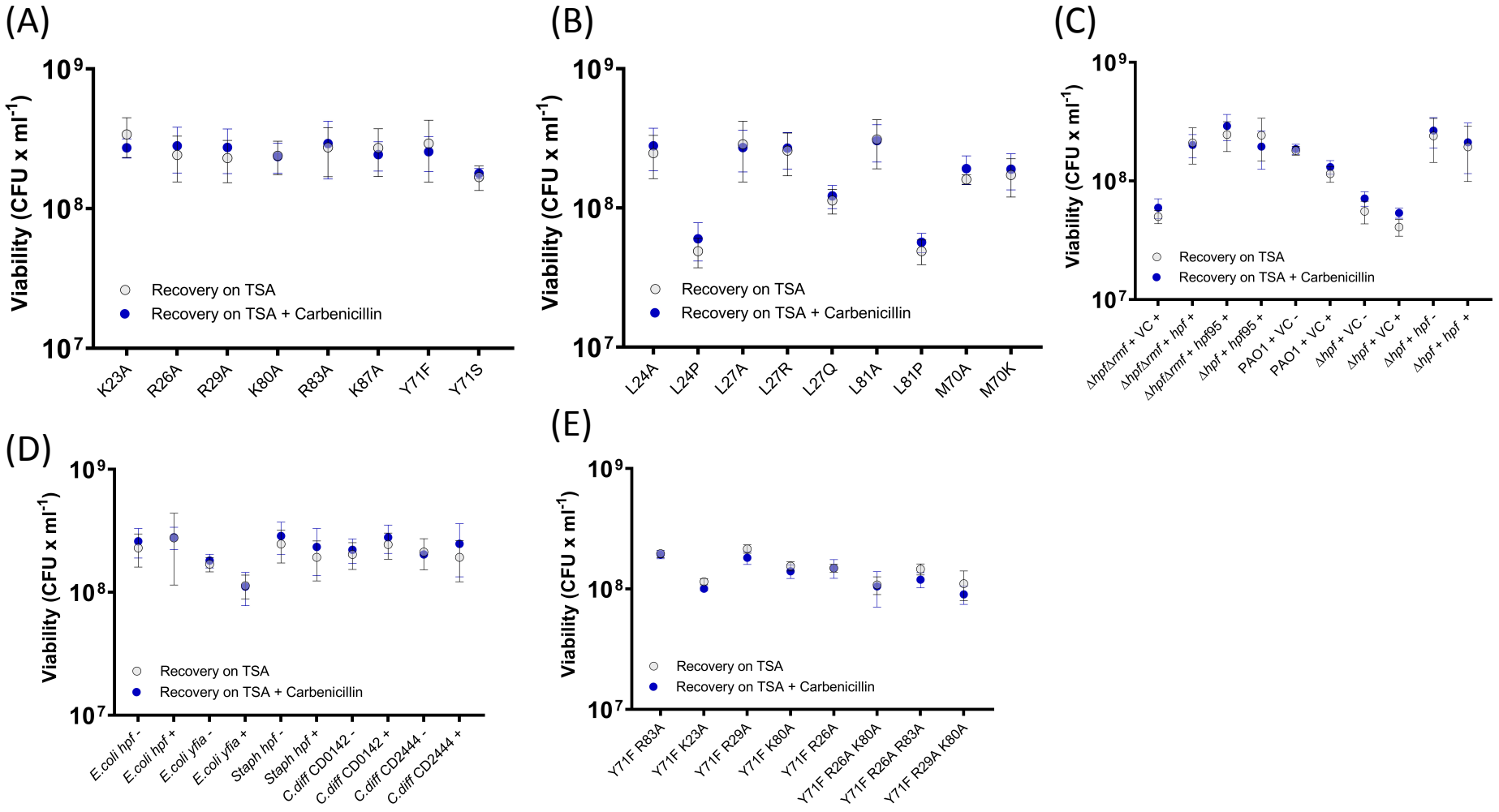

Figure S5: Comparison of CFUs after 5 d of starvation with cells plated on TSB (blue circles) and on TSB with carbenicillin (gray circles). No significant difference was observed in the CFU numbers comparing with and without carbenicillin, indicating that all strains maintained plasmids for 5 d of starvation. Error bars represent the mean and standard error for three independent biological replicates.

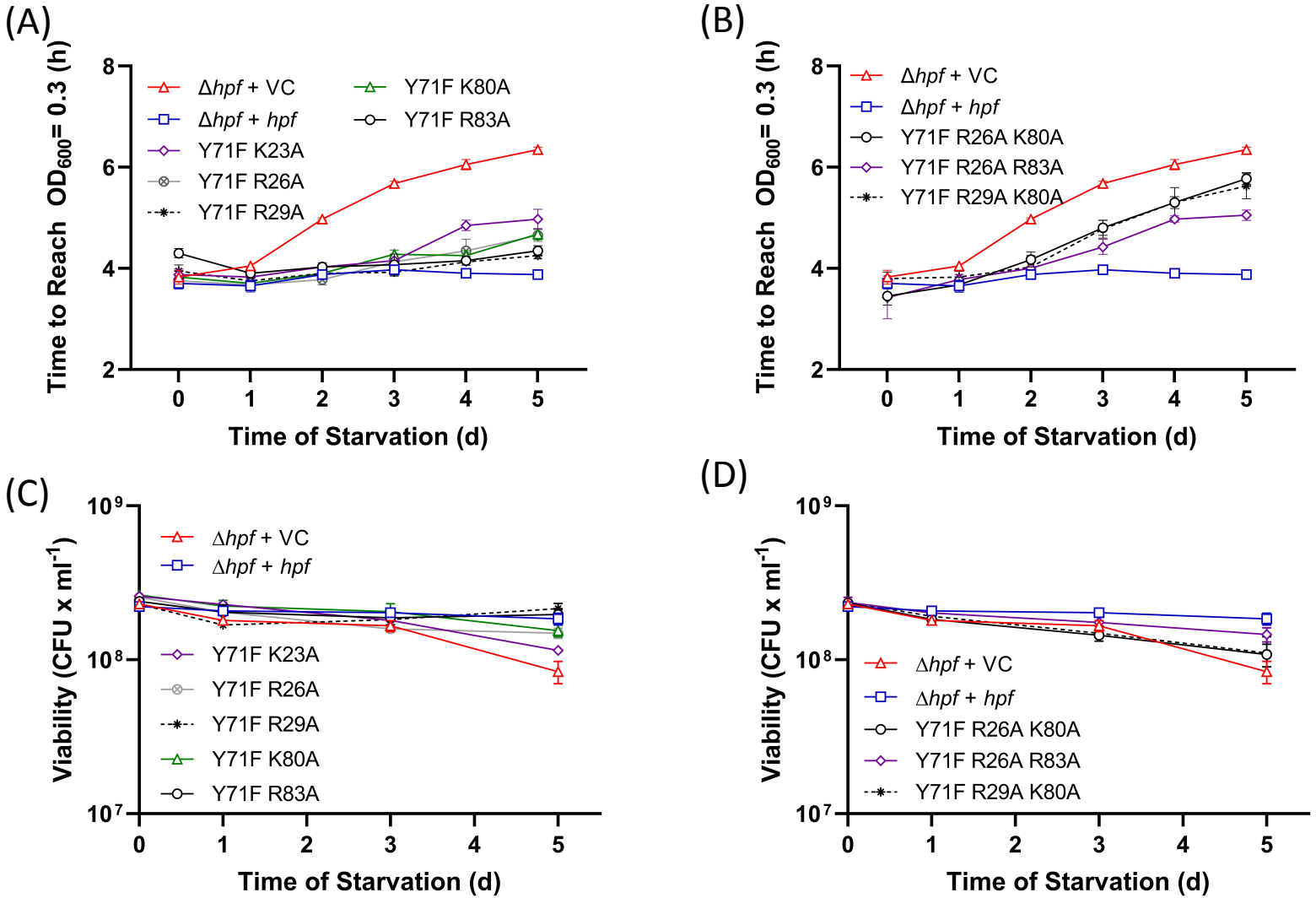

Figure S6 Effect of double and triple point-mutantions on regrowth over the course of starvation when the strains were pre-cultured with 1 mM IPTG added to the medium. (A) Time to reach O.D.600 = 0.3 over the course of starvation, for the double point-mutants. (B) Time to reach O.D.600 = 0.3 over the course of starvation, for the triple point-mutants. (C) Effect of double point-mutations in HPF on regrowth of *P. aeruginosa* Δ*hpf* over the course of starvation, measured as CFUs. (D) Effect of triple point-mutations in HPF on regrowth of *P. aeruginosa* Δ*hpf* over the course of starvation, measured as CFUs. All data show the mean and standard error of the mean for three biological replicates.

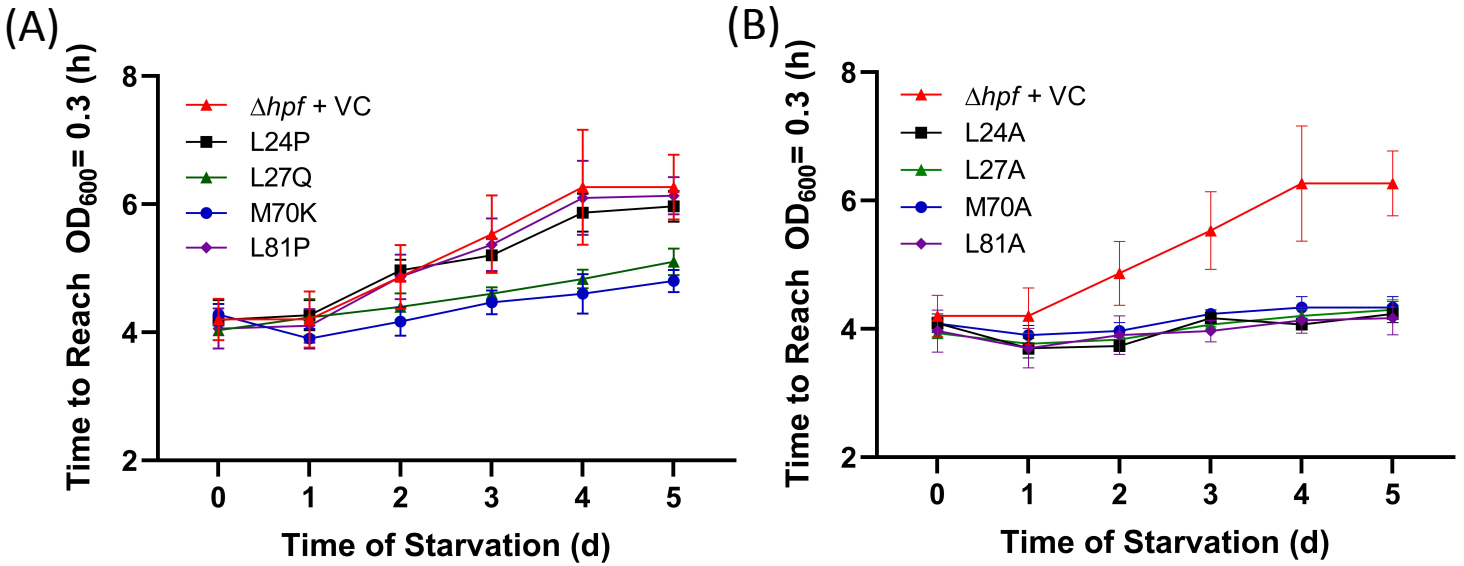

Figure S7. (A) Regrowth following starvation of *P. aeruginosa* Δ*hpf* containing *hpf* with point mutations obtained by error-prone PCR and screening for a resuscitation defect. Strains were pre-cultured in the presence of 1 mM IPTG. Red triangles indicate *P. aeruginosa* Δ*hpf* containing the vector control. (B) Regrowth following starvation of *P. aeruginosa* Δ*hpf* complemented with *hpf* where the mutations shown in panel A were replaced by alanines. Strains were pre-cultured in the presence of 1 mM IPTG. All data show the mean and standard error of the mean for three biological replicates.

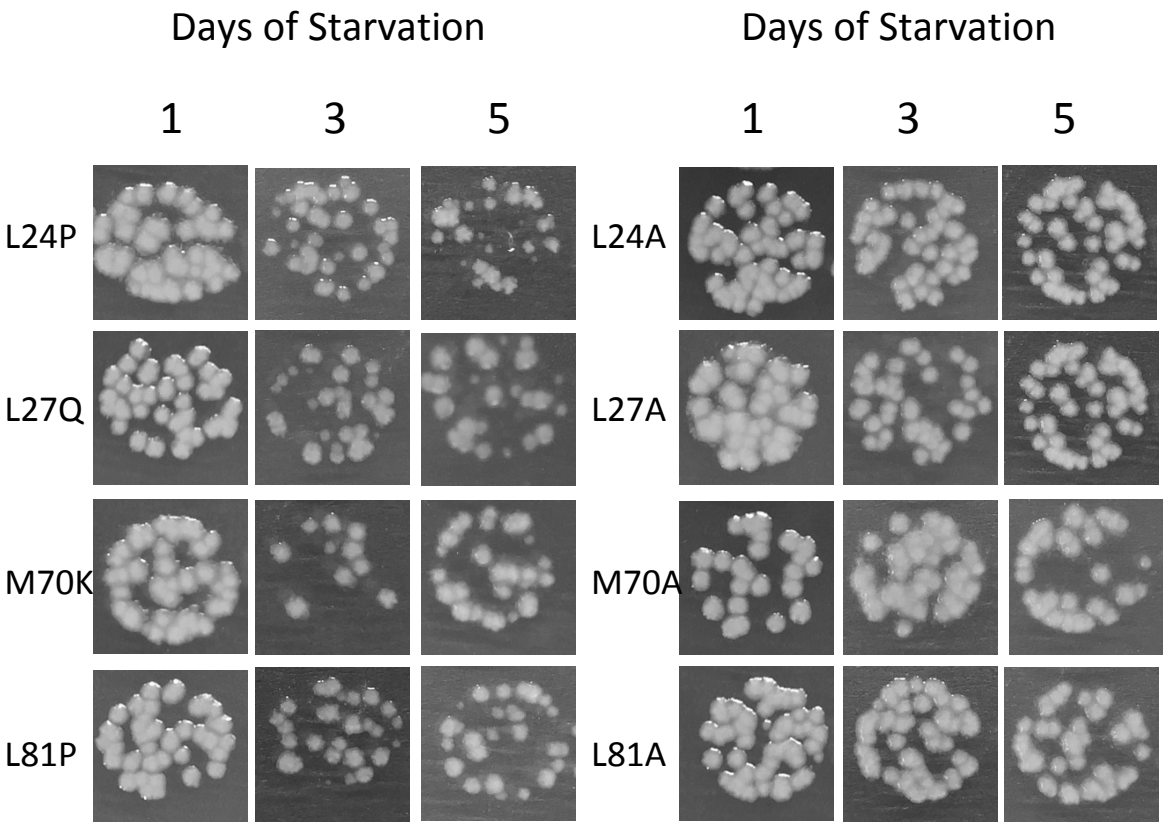

Figure S8. Colony morphologies of *P. aeruginosa* Δ*hpf* containing mutant *hpf* alleles over the course of starvation. *P. aeruginosa* Δ*hpf* containing *hpf* with random mutations show increased colony heterogeneity over the course of starvation, whereas alanine substitutions in equivalent positions restore wild-type colony morphologies.

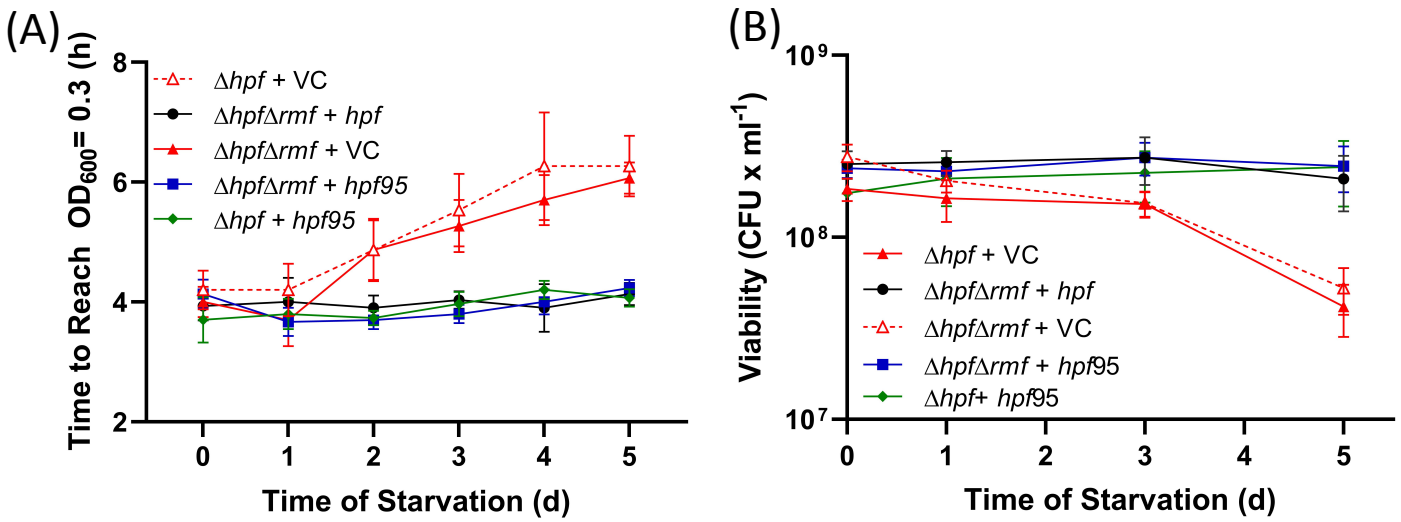

Figure S9. Resuscitation over the course of starvation for strains with C-terminal truncation of HPF. Strains were pre-cultured in the presence of 1 mM IPTG. (A) Resuscitation determined as time to reach O.D.=0.3 Red triangles show the Δ*hpf* mutant with vector control (dashed lines) and the Δ*hpf*/Δ*rmf* double mutant with vector control (solid lines). The *hpf*95 truncated gene complemented both the Δ*hpf* mutant (green diamonds) and the Δ*hpf*/Δ*rmf* double mutant (blue squares). (B) Resuscitation of the Δ*hpf* mutant, and the Δ*hpf*/Δ*rmf* double mutant containing truncated *hpf*, quantified as CFUs, over the course of starvation. All data show the mean and standard error of the mean for three biological replicates.

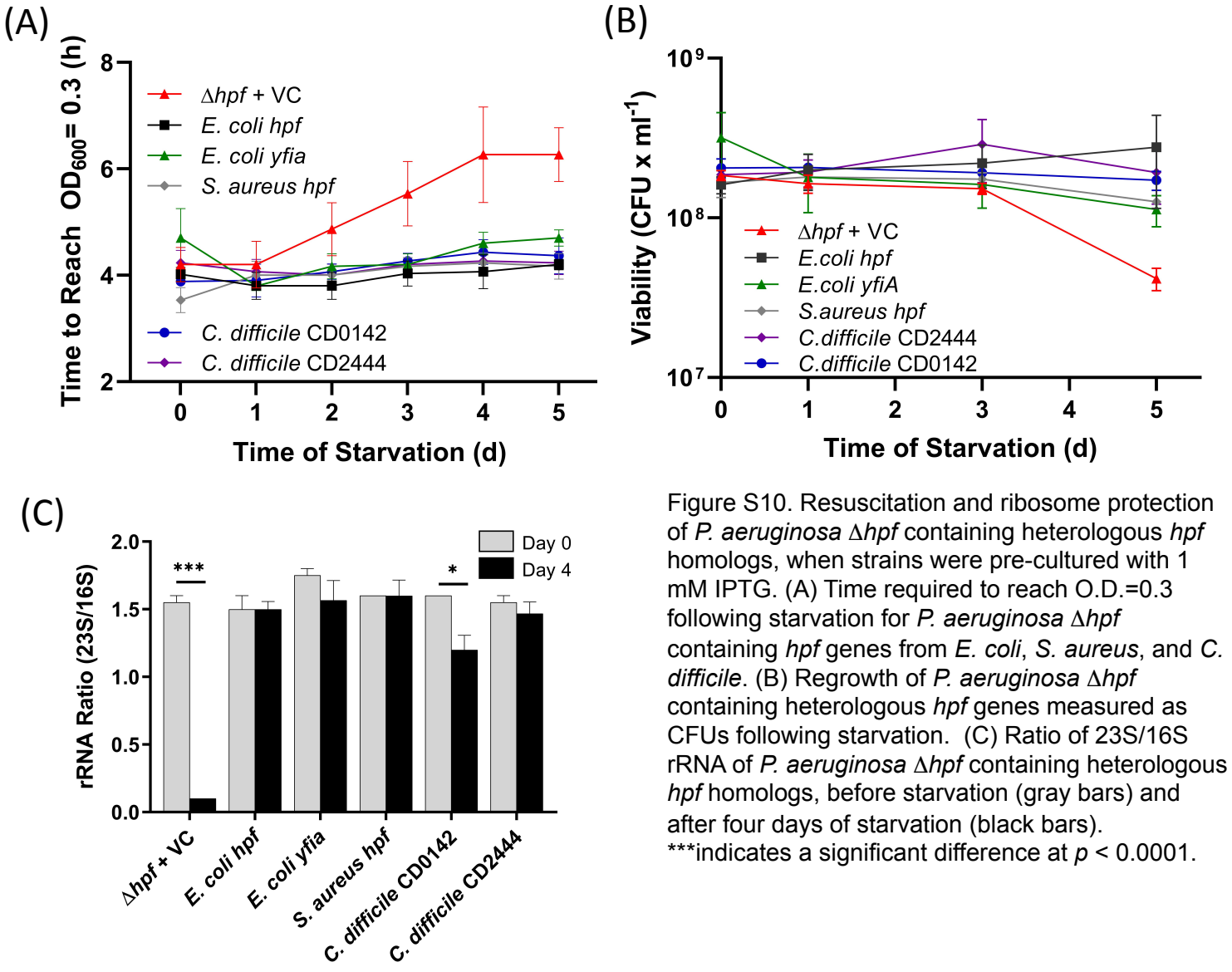

Figure S10. Resuscitation and ribosome protection of *P. aeruginosa* Δ*hpf* containing heterologous *hpf* homologs, when strains were pre-cultured with 1 mM IPTG. (A) Time required to reach O.D.=0.3 following starvation for *P. aeruginosa* Δ*hpf* containing *hpf* genes from *E. coli*, *S. aureus*, and *C. difficile*. (B) Regrowth of *P. aeruginosa* Δ*hpf* containing heterologous *hpf* genes measured as CFUs following starvation. (C) Ratio of 23S/16S rRNA of *P. aeruginosa* Δ*hpf* containing heterologous *hpf* homologs, before starvation (gray bars) and after four days of starvation (black bars). ***indicates a significant difference at $p < 0.0001$.

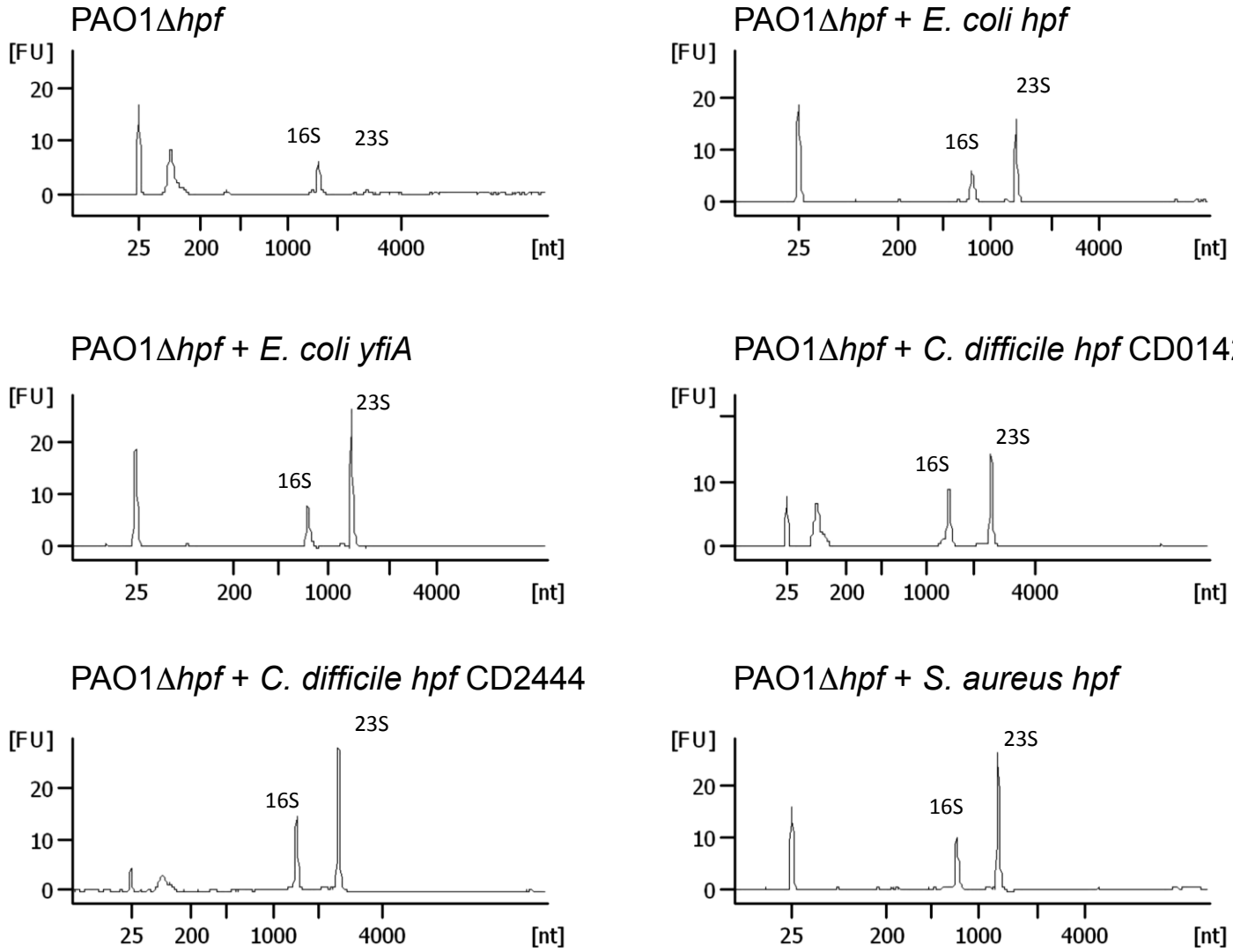

Figure S11. Representative Bioanalyzer traces showing 16S and 23S rRNA from *P. aeruginosa* Δ*hpf* containing heterologous HPFs after 4 d of starvation. HPFs were expressed from plasmids under control of the P_{trc} promoter, induced with 1 mM IPTG. Following growth in TSB + IPTG, cells were starved for 4d in PBS. RNA was extracted from the starved cultures and run on an Agilent Bioanalyzer. The heterologous *hpf* genes included *hpf* and *yfiA* from *E. coli*, long HPFs from *C. difficile* CD0142 and CD2444, and long HPF from *S. aureus*. The results show that after starvation, the 23S rRNA is present in the *P. aeruginosa* Δ*hpf* mutant complemented with all of the heterologous HPFs, but not in the vector control strain.

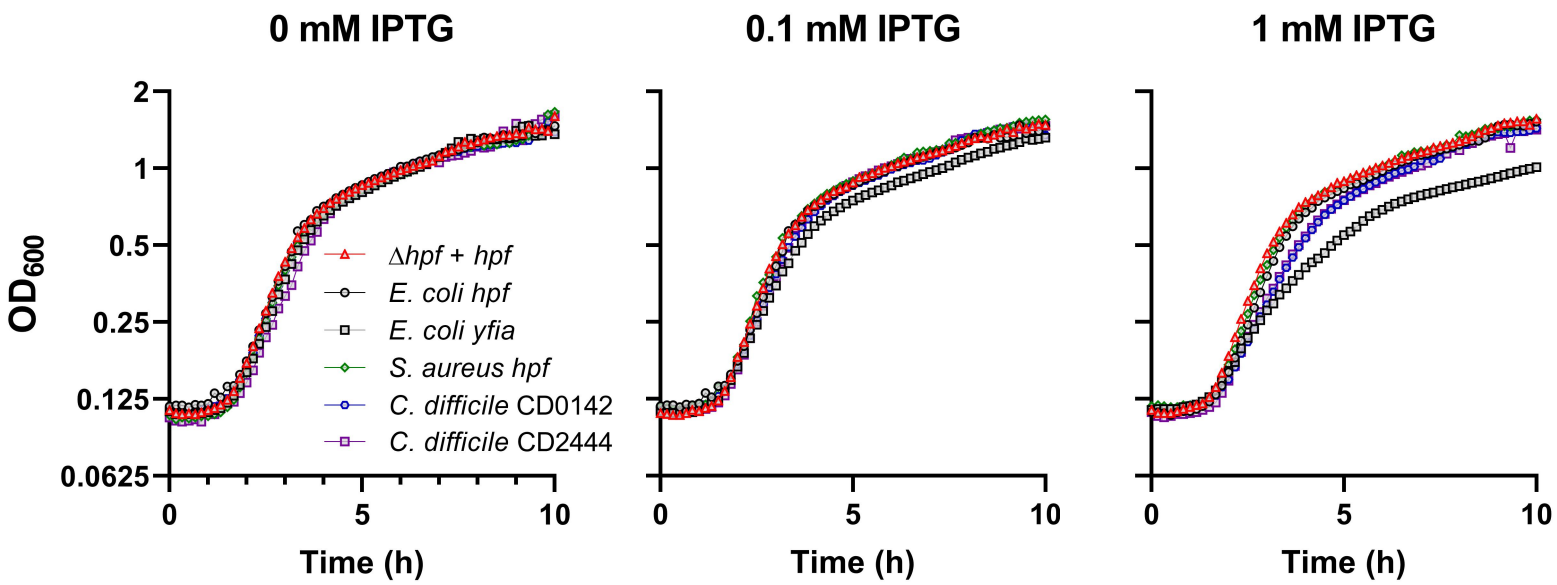

Fig S12. Growth curves of *P. aeruginosa* Δ*hpf* complemented with heterologous *hpf* homologs, when no IPTG or 0.1 mM or 1 mM IPTG was added to the medium. When overexpressed with 1 mM IPTG, the *P. aeruginosa* HPF (red), *E. coli* HPF (gray circles), and *S. aureus* longHPF (green) did not affect growth. When overexpressed, *E. coli yfiA* (black squares) inhibited the growth rate of *P. aeruginosa* Δ*hpf*. The *C. difficile* long-*hpf* 0142 (blue) and *C. difficile* long-*hpf* CD2444 (purple) had a minor influence on cell growth when overexpressed.

Literature cited for supplementary tables and figures

1. Stover CK, Pham XQ, Erwin AL, Mizoguchi SD, Warrener P, Hickey MJ, Brinkman FS, Hufnagle WO, Kowalik DJ, Lagrou M, Garber RL, Goltry L, Tolentino E, Westbrock-Wadman S, Yuan Y, Brody LL, Coulter SN, Folger KR, Kas A, Larbig K, Lim R, Smith K, Spencer D, Wong GK, Wu Z, Paulsen IT, Reizer J, Saier MH, Hancock RE, Lory S, Olson MV. 2000. Complete genome sequence of *Pseudomonas aeruginosa* PA01, an opportunistic pathogen. Nature **406:**959-964.

2. Williamson KS, Richards LA, Perez-Osorio AC, Pitts B, McInnerney K, Stewart PS, Franklin MJ. 2012. Heterogeneity in *Pseudomonas aeruginosa* biofilms includes expression of ribosome hibernation factors in the antibiotic-tolerant subpopulation and hypoxia-induced stress response in the metabolically active population. J Bacteriol **194:**2062-2073.

3. Franklin MJ, Chitnis CE, Gacesa P, Sonesson A, White DC, Ohman DE. 1994. *Pseudomonas aeruginosa* AlgG is a polymer level alginate C5-mannuronan epimerase. J. Bacteriol. **176:**1821-1830.

4. Polikanov YS, Blaha GM, Steitz TA. 2012. How hibernation factors RMF, HPF, and YfiA turn off protein synthesis. Science **336:**915-918.

5. De Bari H, Berry EA. 2013. Structure of *Vibrio cholerae* ribosome hibernation promoting factor. Acta Crystallogr Sect F Struct Biol Cryst Commun **69:**228-236.

6. Beckert B, Abdelshahid M, Schafer H, Steinchen W, Arenz S, Berninghausen O, Beckmann R, Bange G, Turgay K, Wilson DN. 2017. Structure of the *Bacillus subtilis* hibernating 100S ribosome reveals the basis for 70S dimerization. EMBO Journal **36:**2061-2072.

7. Khusainov I, Vicens Q, Ayupov R, Usachev K, Myasnikov A, Simonetti A, Validov S, Kieffer B, Yusupova G, Yusupov M, Hashem Y. 2017. Structures and dynamics of hibernating ribosomes from *Staphylococcus aureus* mediated by intermolecular interactions of HPF. EMBO Journal **36:**2073-2087.