SI Appendix for

Shear rate sensitizes bacterial pathogens to H_2O_2 stress

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This includes:

Materials and Methods

Strains, plasmids, and growth conditions

The bacterial strains used in this paper are described in the Supplementary Table 1. Primers are listed in Supplementary Table 2, and plasmids used are described in Supplementary Table 3. *P. aeruginosa* cultures were grown in liquid LB on a roller drum, and on LB plates (1.5% Bacto Agar) at 37 °C. *S. aureus* cultures were grown in liquid LB supplemented with chloramphenicol (10 μg ml^{-1}), and on LB plates with chloramphenicol at 37 °C. LB was prepared using premix Miller LB Broth (BD Biosciences) using standard LB preparation protocols. M9 minimal media with casamino acids was prepared using M9CA broth premix (VWR) and supplemented with 1 M magnesium sulfate (Sigma), 1 M calcium chloride (Sigma) and 20% glucose (VWR). Sodium hydroxide (NaOH) was used to pH solution to 7.4.

Generation of *P. aeruginosa* mutants

Gene deletions were generated using the lambda Red recombinase system as previously described[13,46]. The deletion construct was Gibson-assembled from three PCR products. First, approximately 500 bp upstream of the target insertion site was amplified from PA14 genomic DNA. Second, a fragment containing *aacC1* ORF flanked by FRT sites was amplified from pAS03. Third, approximately 500 bp downstream of the target insertion site was amplified from PA14 genomic DNA. The Gibson- assembled product was transformed into PA14 cells expressing the plasmid pUCP18-RedS. The colonies were selected on 30 μg ml^{-1} gentamycin, and then the mutants of interest were counter-selected on 5% sucrose, and pFLP2 was used to flip out the antibiotic resistance gene. pUCP18-RedS and pFLP2 were selected for using 300 μg ml^{-1} carbenicillin. The double and triple mutants were created by subsequent deletions performed similarly.

Generation of *S. aureus* strains

USA300 Δ*ahpCF* was generated by amplifying the 5' and 3' flanking regions (~1 kb up- and downstream) of *ahpCF* using the indicated primers (Table S2). 5' and 3' fragments were cloned into the pKOR1 knockout vector via site-specific recombination. The deletions were created using allelic replacement, as described previously[47]. USA300 JE2 *katA*:erm was obtained from the Nebraska library[48]. USA300 JE2 *katA*:erm Δ*ahpCF* was generated by transducing the *katA*:erm allele via Φ85 phage from USA300 JE2 *katA*:erm. To create the *ahpCF* reporter construct, the *ahpCF* promoter was cloned into the pAH5 vector[49] via the indicated primers (Table S2). All constructs were verified by sequencing and all mutant strains were confirmed to be hemolytic by growth on TSA blood agar plates.

Bacterial Conditioning of LB Media and H_2O_2 treatment

P. aeruginosa and *S. aureus* were used to condition media for experiments involving their respective bacteria. Media were conditioned by diluting 50 μL of bacteria from an overnight culture into a 5 mL tube (or scaled up at the same ratio) and allowing it to sit for a defined period at 22 °C. Bacterial cells were then filtered out using a Steriflip sterile filter unit (0.22 μm pore size). To generate LB with defined H_2O_2 concentrations, LB was first conditioned, and then defined concentrations of H_2O_2 were added. For experiments using purified catalase, 8 mg/ml of bovine liver catalase (Sigma) was used.

Fabrication of microfluidic devices

Microfluidic devices were created and fabricated using soft lithography techniques. Devices were designed on Illustrator (Adobe Creative Suite) and masks were subsequently printed by CAD/Art Services. Molds were produced on 100mm silicon wafers (University Wafer) and then spin coated with SU-8 3050 photoresist (MicroChem). Polydimethylsiloxane (PDMS) chips were

plasma-treated for bonding to glass slides at least 24 hours before experiments. The devices used in all other experiments discussed above using the *fro* reporter and P_{ahp} reporter contained 7 parallel channels 500 µm wide x 50 µm tall x 2 cm long. Long channel experiments used device that were 500 µm wide x 50 µm tall x 27 cm long. Each channel individually contained an inlet and an outlet. These chips were plasma bonded to a 60 mm x 35 mm x 0.16 mm superslip micro cover glass (Ted Pella, Inc.).

P. aeruginosa in microfluidic devices

Experiments involving the *fro* reporter were conducted using microfluidic channels seeded with cells from a mid-log culture with an optical density of approximately 0.5, unless otherwise specified. For Figure S11, cells were seeded into the channel from either a low cell density (OD ~ 0.2) or a medium cell density (OD ~ 0.5) culture. All microfluidic experiments in the paper were performed at ~ 22 °C. Cells were injected into the microfluidic device using a pipette and were allowed to settle in the device for 10 minutes prior to exposure to flow. The device set-up involves the use of plastic 5 mL syringes (BD) with attached tubing connecting the needle to the inlet of the device (BD Intramedic Polyethylene Tubing; 0.38 mm inside diameter, 1.09 mm outside diameter). These syringes were situated on a syringe pump (KD Scientific Legato 210) which was used to produce fluid flow. The outlet of the device employed the same tubing and vacated into a bleach-containing waste container. The syringe pump was used to generate flow rates of 0.1-10 µL/min, which correspond to shear rates of 8 – 800 s^{-1}.

S. aureus in microfluidic devices

Experiments measuring P_{ahp} expression in *S. aureus* reporter use cells from mid-log phase culture. Flow media was conditioned in *S. aureus* cells for at most 1 hour. Conditioned LB was filtered of cells and supplemented with 8 µM H_2O_2 before loading into 5 mL syringe. Cells were injected directly into the flow chamber inlet with a pipette and allowed to settle for 10 min. Flow devices were setup with exact same methods as described in *P. aeruginosa* in microfluidic devices. For no flow conditions, injected cells in flow chambers were given fresh conditioned LB with 8 µM H_2O_2 for 2 min.

Shear rate calculations

The shear rate experienced in the microfluidic devices was calculated using the equation:

$$\frac{6Q}{wh^2}$$

Where Q is flow rate, w is channel width, and h is the channel height.

Phase contrast and fluorescence microscopy

Images were obtained on a Nikon Eclipse Ti-2 microscope using the NIS Elements interface. All images were taken with Nikon 40x Plan Apo Ph2 0.95 NA objective, a Hamamatsu Orca-Flash4.0LT camera, and Lumencor Sola Light Engine LED light source.

Quantification of *fro*/*ahpCF* expression

To quantify reporter intensity during flow treatment, we optimized and employed a MATLAB-based program[50] to identify cells and quantify single cell fluorescence intensity (Figure S1). The image analysis pipeline employs a readily available quantification program (OUFTI) as well as novel code written in MATLAB (Mathworks). Using OUFTI, cell meshes were developed and then used to quantify fluorescence on the YFP and a constitutive mCherry. Expression of mCherry is driven by a $P_{A1/04/03}$ promoter that has been shown previously to be constitutive[13,51]. In the case of the *S. aureus* experiments, only YFP was used. These computed values were then extracted and averaged in MATLAB to yield the per-frame average fluorescence intensity of all cells meshed (>100 per frame) across 3 technical replicates. The ratio of YFP/mCherry or

YFP/YFP were then taken to obtain a representative induction value of *P. aeruginosa fro* expression and *S. aureus* P_{ahp} expression.

Mathematical simulations

To simulate the advection-diffusion of H_2O_2 molecules in the microfluidic channel, we combined the laminar transport due to flow with a Brownian dynamics simulation to capture the diffusive behavior. Initially, the simulated channel was seeded with molecules at random positions according to the concentration *c*. Flow was then modeled with a parabolic flow speed profile $v(y) = v_0\left(1 - \left(\frac{y-\frac{h}{2}}{\frac{h}{2}}\right)^2\right)$ according to the Hagen-Poiseuille equation, with the channel height *h* and width *w* and maximum velocity $v_0 = 1.7 \cdot 10^{-11}\frac{3}{2hw}Q$ in the center of the channel. In time steps of Δt = 1 ms, each particle was then displaced along the channel according to its lateral position *v*(*y*). In addition, each particle was allowed to diffuse the distance $\frac{\Delta x/y}{\Delta t} = n_0\sqrt{\frac{24D_h}{\Delta t}}$ lateral (y) and along (x) the channel, where n_0 is a random number drawn from a uniform distribution in the interval $-0.5 \leq n_0 \leq 0.5$ and $D_h = 1.5 \cdot 10^{-9}\frac{m^2}{s}$ is the diffusion coefficient of H_2O_2.

For the simulations, we estimated that 1 of every 100 molecules that reached the channel bottom (where cells are located) were removed. Based on the simulated results, we confirmed that our estimate was reasonable based on the following logic: Figure 3 shows that cells at OD of ~0.5 remove a majority of H_2O_2 in 30 seconds, our calculations show that cells in microfluidic channels are also at an OD of ~0.5, our calculations show that media flowing at 80 sec^{-1} resides in the channel for ~30 seconds, and our simulation demonstrates that a shear rate of 80 sec^{-1} results in scavenging of a majority of H_2O_2 molecules using our 1 in 100 estimate.

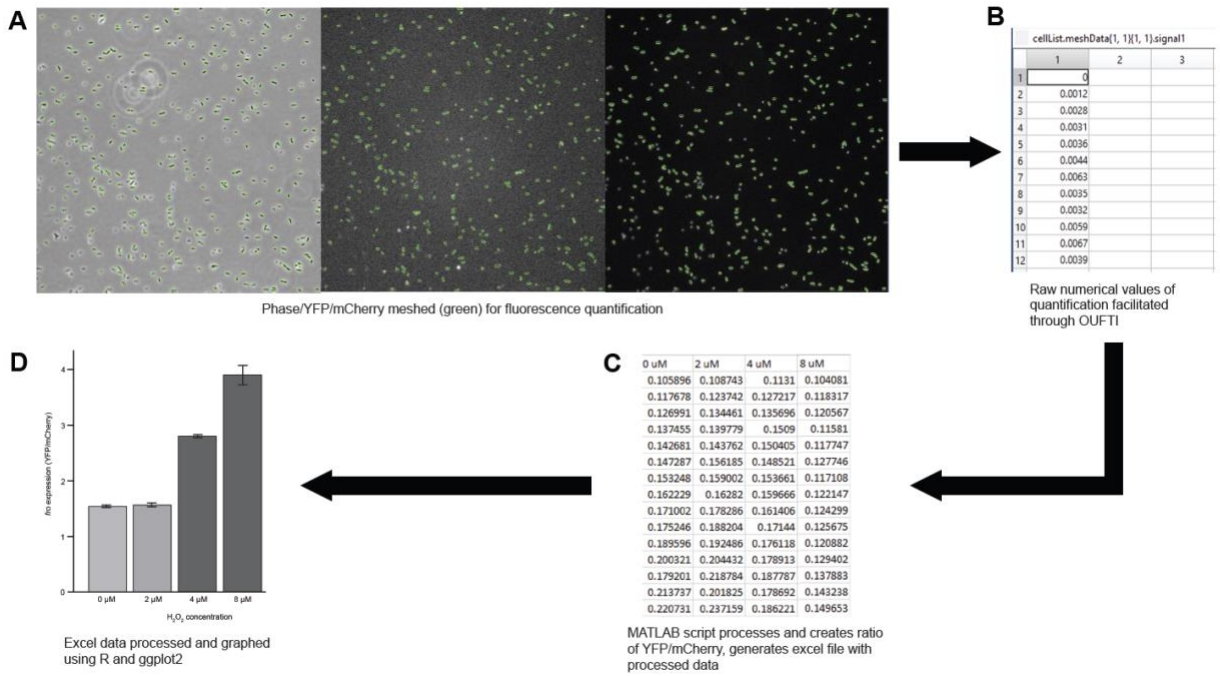

Figure S1: Computational workflow for fluorescence based single cell microscopy quantification. (A) Raw microscopy images demonstrating meshes generated through OUFTI from phase image. Meshes overlayed over YFP and mCherry fluorescent images. **(B)** Raw MATLAB output of quantified fluorescent values post-processing with OUFTI. **(C)** Data post-processing with in-lab MATLAB code to yield final values. **(D)** Representative plot generated using data yielded from **C**.

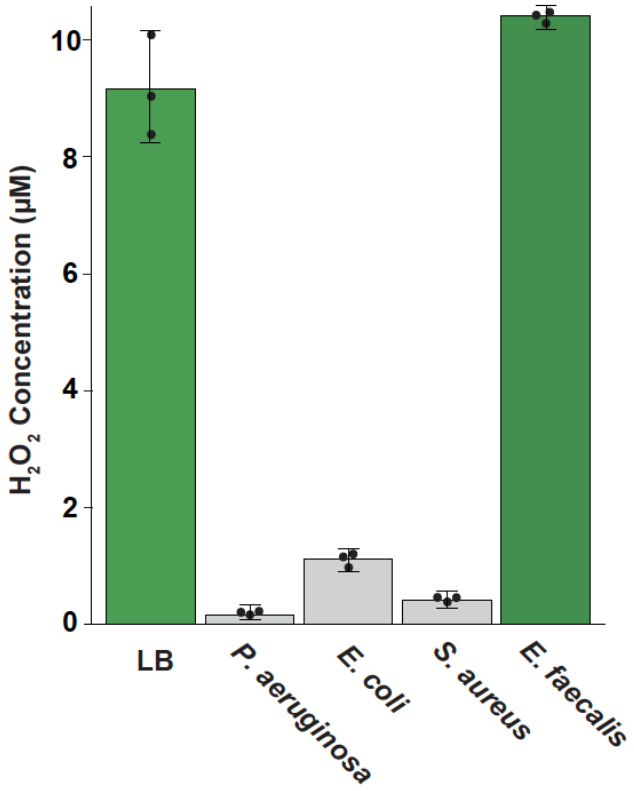

Figure S2: Bacteria scavenging of H_2O_2 is conserved, but not universal. H_2O_2 concentration of LB when untreated, or treated with ~0.5 OD wild-type *P. aeruginosa*, *E. coli*, *S. aureus,* or *E. faecalis* for 30 minutes. H_2O_2 concentrations were measured using a peroxidase assay[21]. Quantification shows the average and standard deviation of three biological replicates.

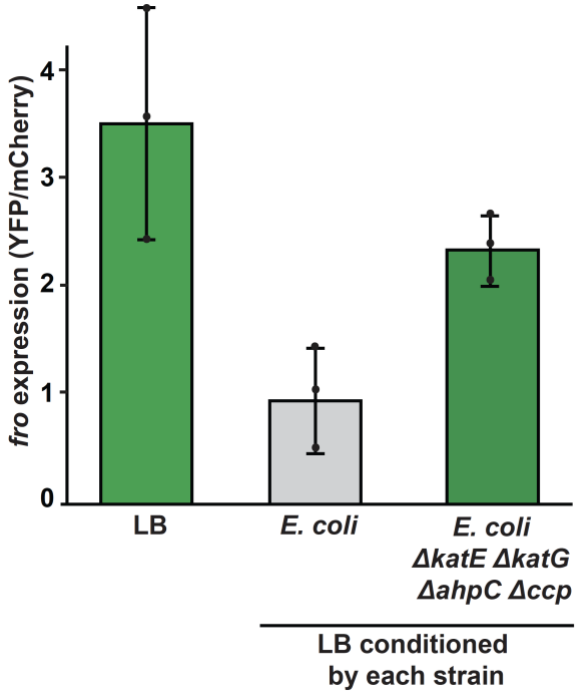

Figure S3. *E. coli* scavenging enzymes are required to condition media. *P. aeruginosa fro* expression after 180 minutes in flow (at a shear rate of 800 s^{-1}) imaged 1 cm into the channel. Cells were exposed to flow of LB media, media conditioned with ~0.5 OD wild-type *E. coli*, or media conditioned with ~0.5 OD *E. coli* Δ*ahpC* Δ*katG* Δ*katE* Δ*ccp* mutant[20] for 60 minutes. Quantification shows the average and standard deviation of three biological replicates.

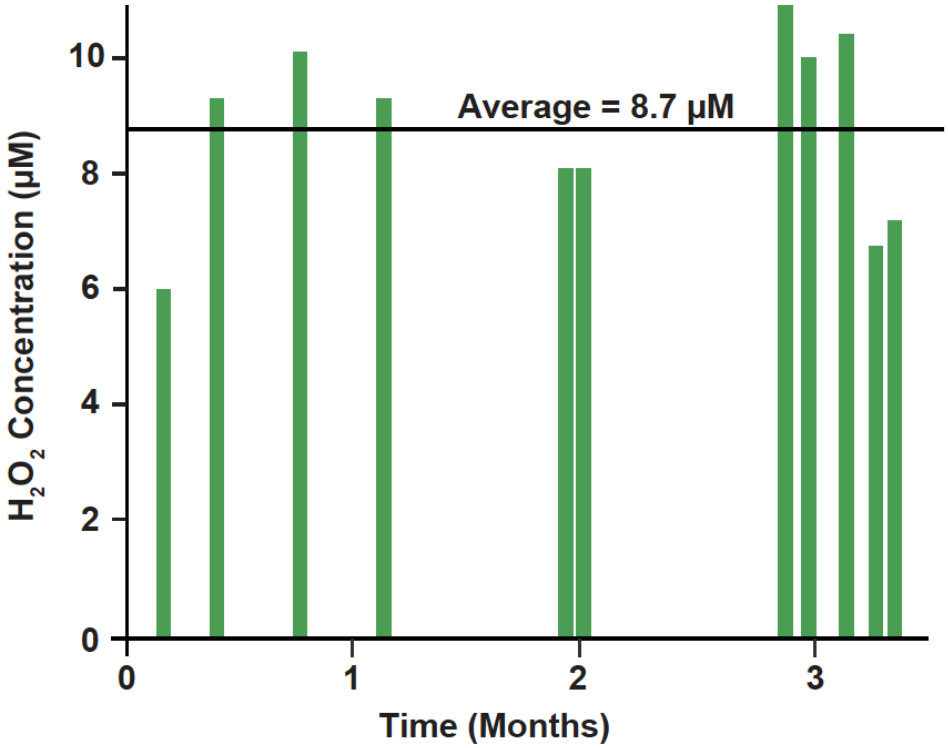

Figure S4: LB contains variable amounts of H_2O_2 in standard laboratory conditions. H_2O_2 concentration of LB from laboratory storage measured over a period of months. H_2O_2 concentrations were measured using a peroxidase assay[21].

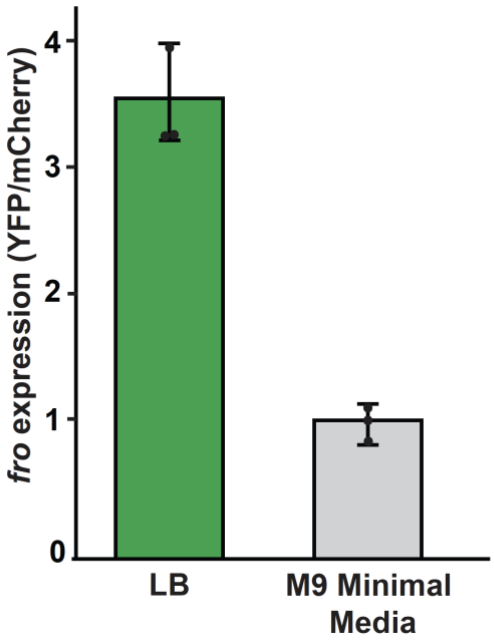

Figure S5: M9 minimal media lacking H_2O_2 does not induce *fro* expression. *P. aeruginosa* *fro* expression after 180 minutes in flow (at a shear rate of 800 s^{-1}) imaged 1 cm into the channel. Cells were exposed to flow of LB media or M9 minimal media. Quantification shows the average and standard deviation of three biological replicates.

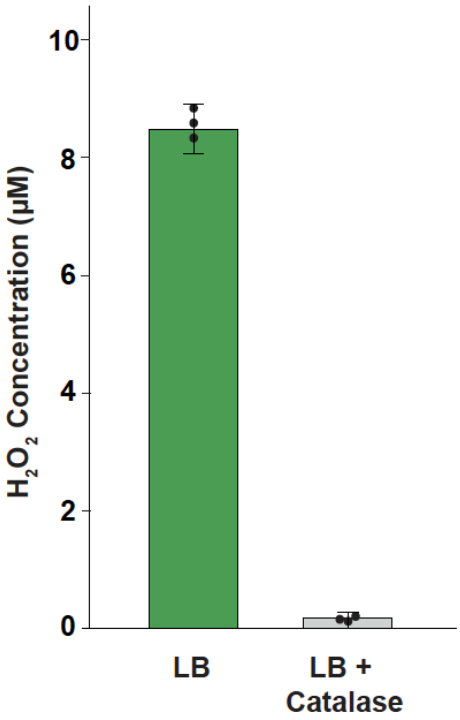

Figure S6: Addition of purified catalase to LB media depletes H_2O_2. H_2O_2 concentration of LB when untreated or treated with purified catalase. H_2O_2 concentrations were measured using a peroxidase assay[21]. Quantification shows the average and standard deviation of three biological replicates.

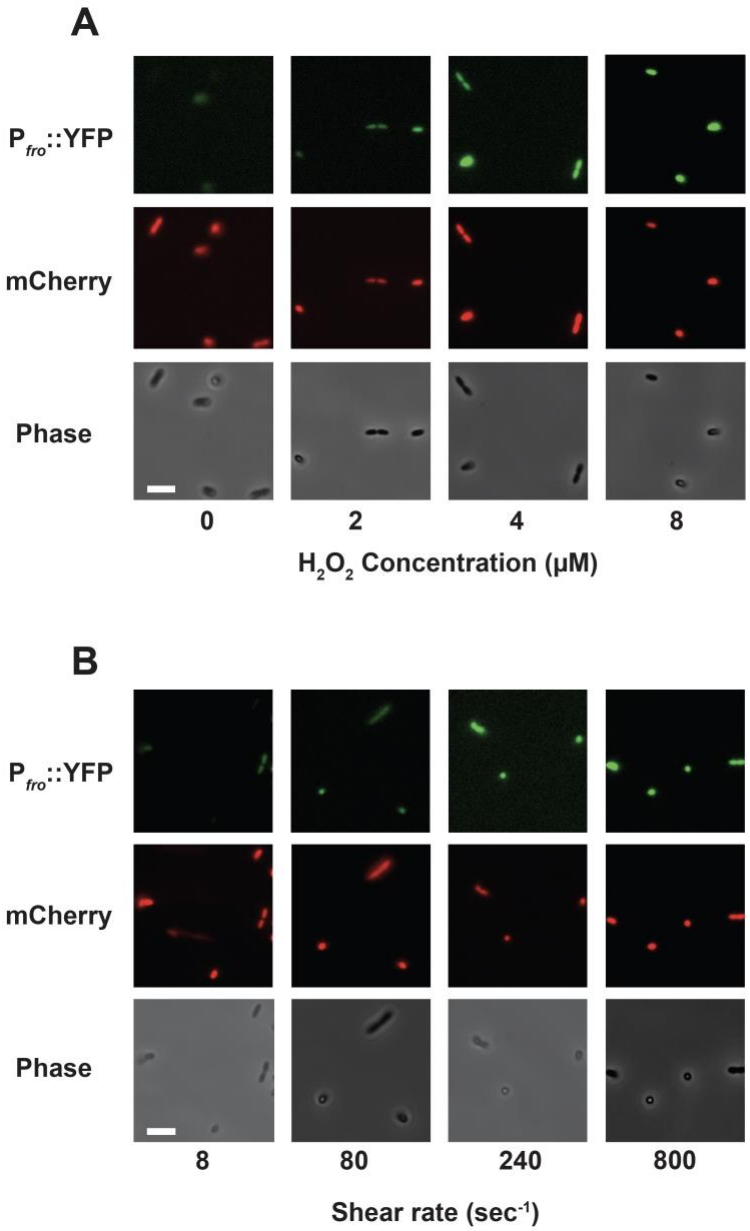

Figure S7: H_2O_2 concentration and shear rate tune flow-sensitive gene expression **(A)** YFP fluorescence, mCherry fluorescence, and phase contrast images representative of three biological replicates showing *P. aeruginosa* cells imaged 1 cm into the channel treated with 800 sec^{-1} flow and LB with varied H_2O_2 concentrations. **(B)** YFP fluorescence, mCherry fluorescence, and phase contrast images representative of three biological replicates showing *P. aeruginosa* cells treated with 8 μM H_2O_2 and varied shear rates. Scale bars, 5 μm. Channels are 50 μm tall x 500 μm wide.

$Pe < 1$ indicates diffusion is dominant transport phenomenon

$Pe > 1$ indicates shear rate is the dominant transport phenomenon

$$Pe = \frac{\text{advective transport}}{\text{diffusive transport}}$$

$$Pe = \frac{\text{shear rate} * \text{cell length}^2}{\text{diffusion coefficient}}$$

Here we set $\text{Pe} = 1$, to solve for the critical shear rate.

$$\text{Critical shear rate} = \frac{\text{diffusion coefficient}}{\text{cell length}^2}$$

$$\text{Critical shear rate} \approx \frac{1.5 * 10^{-9} \frac{\text{m}^2}{\text{sec}}}{(3.0 * 10^{-6}\ \text{m})^2} \approx 166.\overline{6}\ \text{sec}^{-1}$$

Figure S8: Péclet number calculation. Critical shear rate was calculated by setting the Péclet number = 1. The diffusion coefficient of H_2O_2 was estimated to be 1.5 x 10^{-9} m^2/sec[37]. The cell length of *P. aeruginosa* was estimated to be 3 μm. Cell length is used a characteristic length that defines the relevant scale of the system.

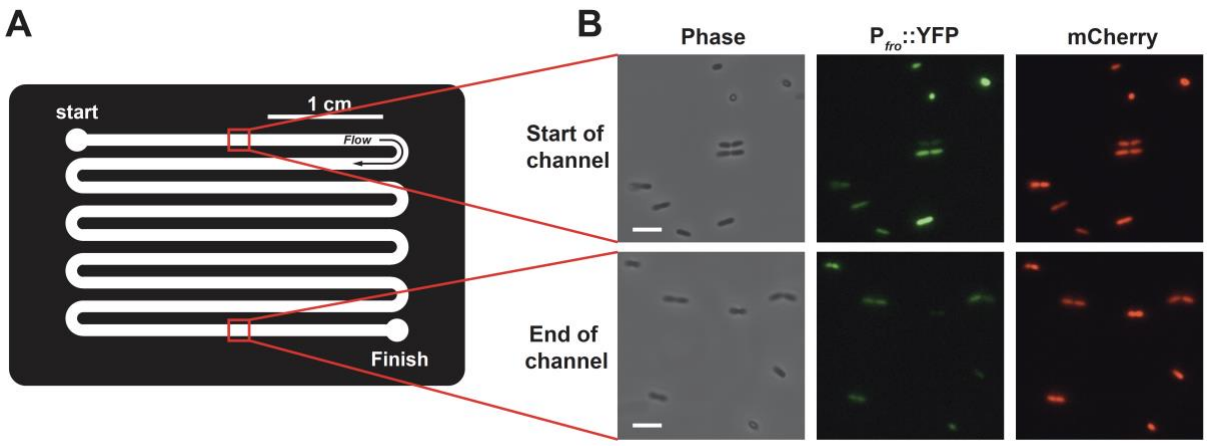

Figure S9: Flow generates gradients of H_2O_2 and gene expression
(A) Schematic showing top-down view of the long channel microfluidic device used in Figure 4E and in panel B. **(B)** Phase contrast, YFP fluorescence, and mCherry fluorescence images representative of three biological replicates showing *P. aeruginosa* cells treated with 240 sec^{-1} flow and LB with a H_2O_2 concentration of 8 µM. Images are from the middle of lane 1 ("start of channel", 1.5 cm) and the middle of lane 9 ("end of channel", 25.5 cm). Scale bars, 5 µm. Channels are 50 µm tall x 500 µm wide x 27 cm long.

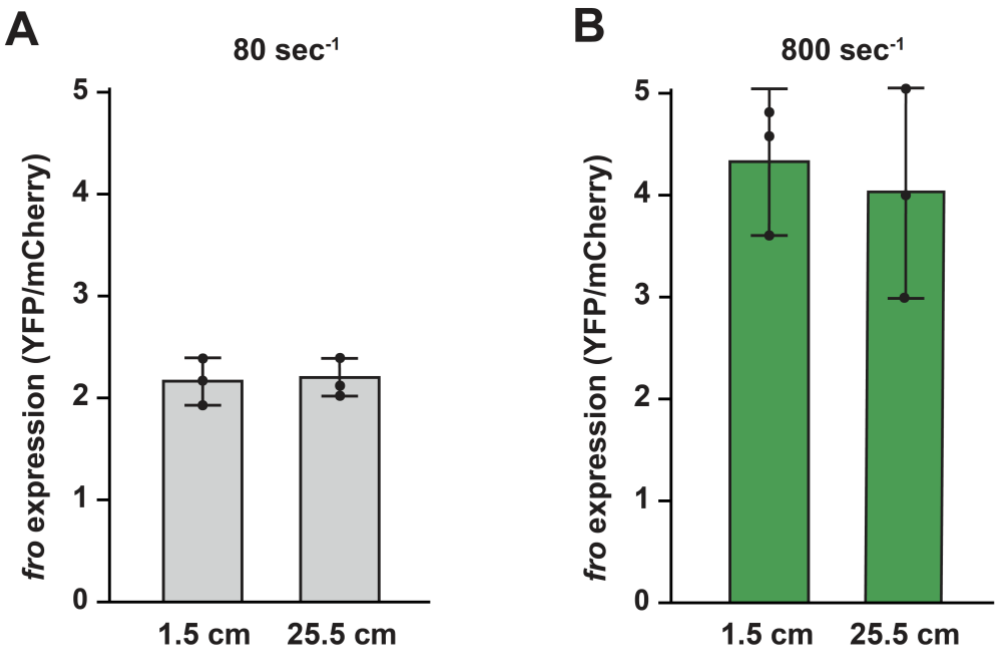

Figure S10: Long distance transport of H_2O_2 is dependent on shear rate. (A) *P. aeruginosa fro* expression after 180 minutes in flow (at a shear rate of 80 sec^{-1}) using LB with a H_2O_2 concentration of 8 µM imaged at 1.5 cm and 25.5 cm in a 27 cm channel. **(B)** *P. aeruginosa fro* expression after 180 minutes in flow (at a shear rate of 800 sec^{-1}) with 8 µM H_2O_2 LB imaged at 1.5 cm and 25.5 cm in a 27 cm channel. Quantification shows the average and standard deviation of three biological replicates.

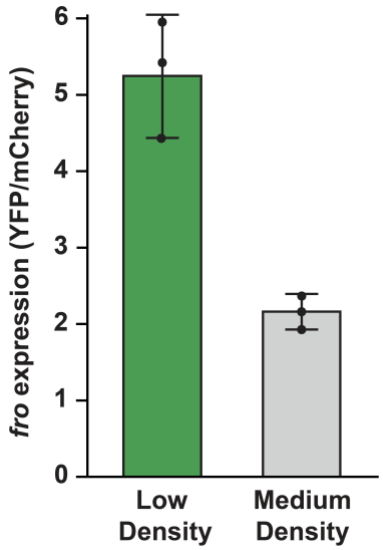

Figure S11: *P. aeruginosa fro* induction is dependent on cell density in flow. *P. aeruginosa fro* expression after 180 minutes in flow using LB with a H_2O_2 concentration of 8 µM imaged at 1.5 cm into a channel. Cells were seeded into the channel from a low cell density culture (~0.2 OD) or a medium density culture (~0.5 OD). Quantification shows the average and standard deviation of three biological replicates.

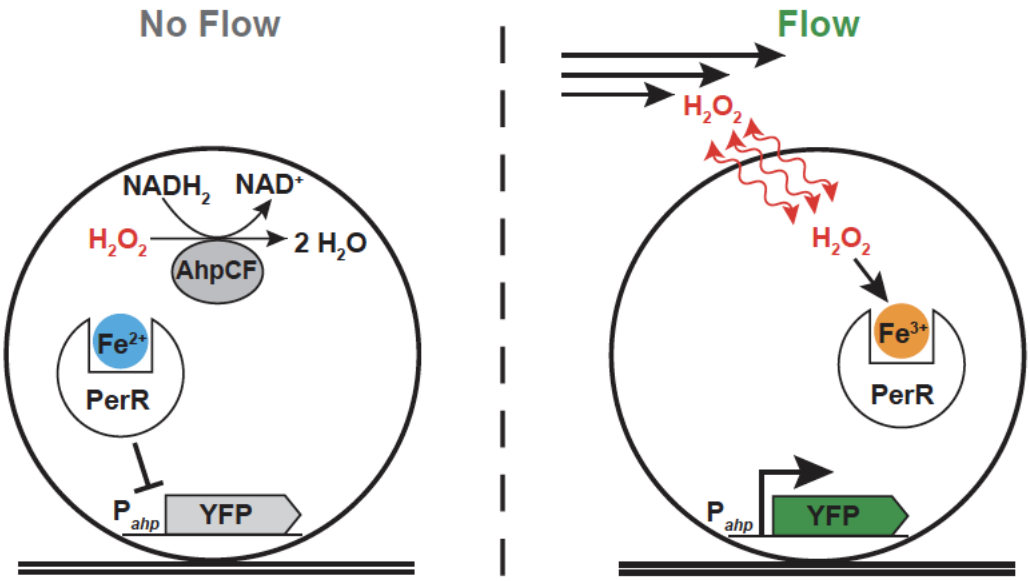

Figure S12: Regulatory model for flow-sensitive gene expression in *S. aureus*. In conditions without flow, H_2O_2 is removed from the intracellular environment by the enzymes KatA (not shown here) and AhpCF. With low intracellular H_2O_2, the transcriptional regulator PerR is bound to Fe^{2+} and represses transcription from the *ahp* promoter. In conditions with flow, H_2O_2 is replenished in the extracellular environment, which leads to a diffusion-driven accumulation of H_2O_2 in the cell. With high intracellular H_2O_2, Fe^{2+} is oxidized to Fe^{3+}. When the transcriptional regulator PerR is bound to Fe^{3+}, the *ahp* promoter is derepressed. Thus, flow leads to an induction of *ahpCF* transcription, which we observe as an increase in cytoplasmic YFP.

Table S1: Strains used in this study

Strain	Description	Source
P. aeruginosa		
PA14	wild-type; clinical isolate from burn wound	52
JS16	*froA*::YFP FRT *attB*::[$P_{A1/04/03}$-mCherry FRT]	13
JS230	Δ*katB*::*aacC1*	This paper
JS231	Δ*katB*::FRT	This paper
JS232	Δ*ahpCF*::*aacC1*	This paper
JS233	Δ*ahpCF*::FRT	This paper
JS234	Δ*ahpCF*::FRT pUCP18-RedS	This paper
JS235	Δ*ahpCF*::FRT Δ*katB*::*aacC1*	This paper
JS236	Δ*ahpCF*::FRT Δ*katB*::FRT	This paper
JS253	Δ*katA*::FRT	This paper
JS254	Δ*katB*::FRT Δ*katA*::FRT	This paper
JS255	Δ*ahpCF*::FRT Δ*katA*::FRT	This paper
JS256	Δ*ahpCF*::FRT Δ*katB*::FRT Δ*katA*::FRT	This paper
S. aureus		
USA300 JE2	wild-type; used for peroxide conditioned media experiments.	48
JS244	∆*ahp*	This paper
JS251	*katA*::*erm*	This paper
JS252	∆*ahp*, *katA*::*erm*	This paper
JS258	pAH5:*ahp*	This paper
E. coli		
MG1655	wild-type; used for peroxide scavenging experiments	*E. coli* Genetic Stock Center
AG75	Hpx- ∆*ccp*; Δ*ahpF*::kan, Δ(*katG17*::Tn10)1, Δ(*katE12*::Tn10)1, Δ*ccp*	20
E. faecalis		
OG1RF	wild-type; used for peroxide conditioned media experiments.	ATCC 47077

240 **Table S2: Primers used in this study.**

Primer	Sequence (5’ to 3’)
ahpCF-1	caccctggcggtgctcttc
ahpCF-2	cagtcgttcctctctcagttga
ahpCF-3	cgatttgaaacaccccattcaactgagagaggaacgactgattccggggatccgtcgacc
ahpCF-4	cgcgctccgggcgtccctggttcgacggtcgctggccggggtgtaggctggagctgcttc
ahpCF-5	ggccagcgaccgtcgaac
ahpCF-6	catgctgagcaacagcggca
katA-1	tcaccctggccaaggaagac
katA-2	ttactctctcctcaacggctaac
katA-3	gcttcgcttcatagcacgttagccgttgaggagagagtaaattccggggatccgtcgacc
katA-4	ccccttcctaggaaggggccgggggcctcatcaggccatgtgtaggctggagctgcttc
katA-5	tggcctgatgaggcccccgg
katA-6	tcaggccaagctgttgctcca
katB-1	cgctaataggtaaaggcagtct
katB-2	ggaagagctcctaatggcttg
katB-3	ggcgcaacaagacgcctgacaagccattaggagctcttccattccggggatccgtcgacc
katB-4	gatagaggaagggcggcctggatggccgccccggttgcgagtgtaggctggagctgcttc
katB-5	tcgcaaccggggcggc
katB-6	gacagcgcctggagcagctc
ahp 5' KO_fwd	tacgactcactatagggatAGCGTGGCTTGGCTGCAC
ahp 5' KO_rev	atcaatcacaGTAACTTCTTTAAATTGATCTTTTTTTGGATCGAAAGCTTGC
ahp 3' KO_fwd	aagaagttacTGTGATTGATCGTAACAATAATAC
ahp 3' KO_rev	agcgagtcagtgagcgaggaATTAACATCACCGGCATATTTTG
ahp prom F	gatcggatccacaccattatcaata
ahp prom R	gatcggtaccataaatatcttcct

Table S3: Plasmids used in this study.

Plasmid	Description	Reference
pAH5	Plasmid for transcriptional reporter on *ahpCF* in *S. aureus*	49
pAS03	Plasmid for generating deletion mutants in *P. aeruginosa*	53
pFLP2	Plasmid expressing FLP2 to recombine FRT sites	54
pUCP18-RedS	Lambda red recombineering vector	53

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