Bacteria detect neutrophils via a system that responds to hypochlorous acid and flow

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ABSTRACT

Neutrophils respond to the presence of bacteria by producing oxidative molecules that are lethal to bacteria, including hypochlorous acid (HOCl). However, the extent to which bacteria detect activated neutrophils or the HOCl that neutrophils produce, has not been understood. Here we report that the opportunistic bacterial pathogen *Pseudomonas aeruginosa* upregulates expression of its *fro* operon in response to stimulated neutrophils. This operon was previously shown to be activated by shear rate of fluid flow in the environment. We show that *fro* is specifically upregulated by HOCl, while other oxidative factors that neutrophils produce including H_2O_2, do not upregulate *fro*. The *fro*-dependent response to HOCl upregulates the expression of multiple methionine sulfoxide reductases, which relieve oxidative stress that would otherwise inhibit growth. Our findings suggest a model in which the detection of shear rate or HOCl activates the *fro* operon, which serves as an early and sensitive host-detection system for *P. aeruginosa* that improves its own survival against neutrophil-mediated host defenses. In support of this model, we found that the *fro* operon is activated in an infection model where flow and neutrophils are present. This response could promote the bacterium's pathogenicity, colonization of tissue, and persistence in infections.

16 **INTRODUCTION**

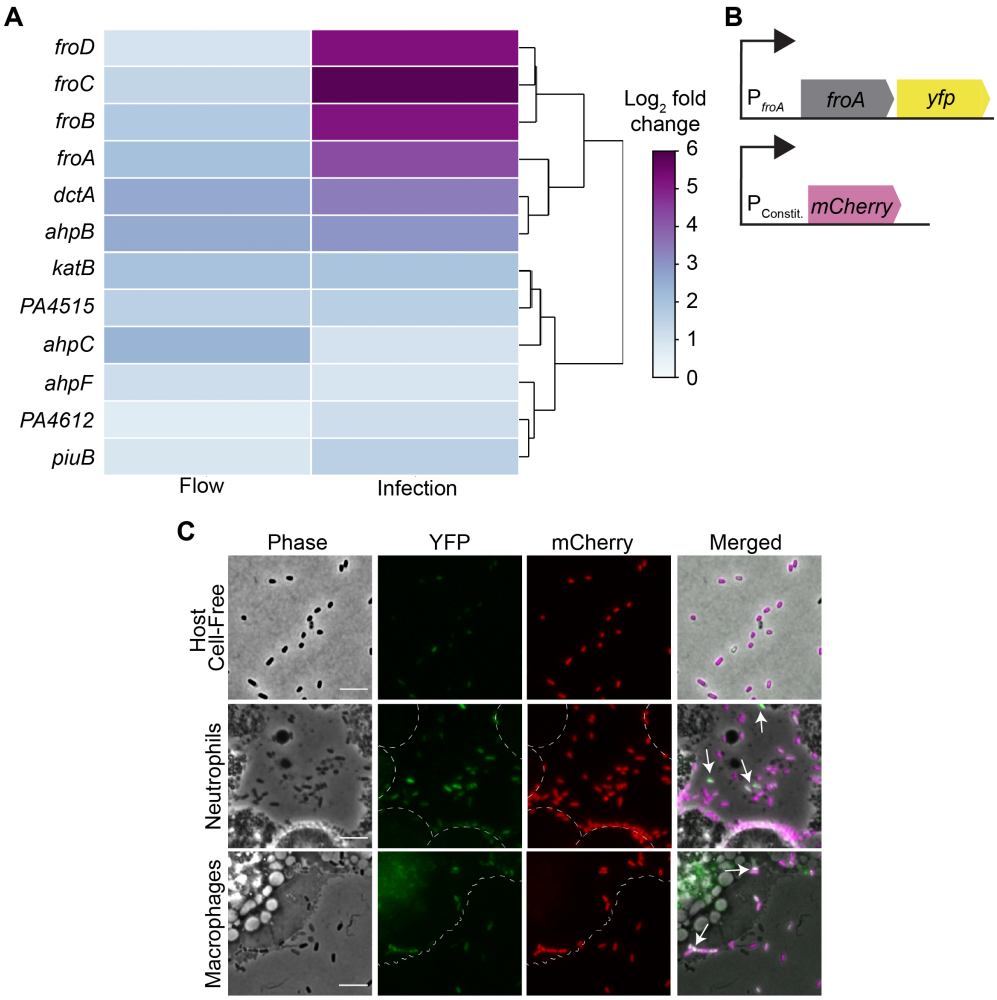
17 *P. aeruginosa* is a Gram-negative bacterium that is a major cause of hospital-acquired infections
18 and is a significant antibiotic resistance threat [1]. To survive in a broad range of conditions, this
19 bacterium has evolved signaling pathways that relay information about changes in nutrients, chemical
20 gradients, and mechanical forces such as those generated by fluid flow, and activates responses that
21 promote its survival [2,3]. Understanding how extracellular-sensing pathways detect and respond to host
22 cues is critical for improving treating bacterial infections.

23 Mammalian host immune systems respond to the presence of bacterial pathogens by recruiting
24 immune cells that migrate rapidly through circulating blood to sites of infection [4]. Neutrophils are the first
25 and most abundant immune cells recruited to inflamed or damaged tissues [5] and are stimulated into an
26 activated state by bacterial components including the membrane constituent lipopolysaccharide.
27 Activated neutrophils produce multiple products that are toxic to bacteria including reactive oxygen
28 species (ROS) and reactive chlorine species (RCS) [6–9]. While the toxic effects of ROS on bacteria are
29 understood, far less is known about how bacteria are affected by RCS [10,11]. Hypochlorous acid (HOCl)
30 is a major neutrophil-produced RCS that is formed through the activity of myeloperoxidase with H_2O_2 and
31 chlorine ions [6,7,12] and is strongly bactericidal [13–15]. *P. aeruginosa* is a potent activator of neutrophil
32 respiratory bursts [16] but whether and how the bacteria detect and respond to these respiratory bursts, is
33 not understood.

34 *P. aeruginosa* detects shear rate generated by the flow of fluids via the *fro* operon [3,17], which is
35 upregulated in the bacterium during infection in humans [3,18] (Fig. 1A). In addition, *fro* is necessary for
36 successful colonization of the gastrointestinal tract and lung infections [19,20], although the reasons for
37 its necessity have been unclear. Because flow is ubiquitous in the pulmonary and circulatory systems, in
38 principle the *fro* operon could be upregulated in flow-associated host environments. Given its important
39 role in infection, it is possible that Fro could also respond to other immune-associated cues, such as the
40 presence of neutrophils. The *fro* operon consists of *froABCD* (*PA14_21570*, *PA14_21580, PA14_21590,*
41 *PA14_21600*), with FroC and FroD predicted to localize to the inner membrane, and FroB predicted to
42 localize in the cytoplasm [21]. Expression of the operon is regulated by the sigma and anti-sigma factors
43 *froR* (*PA14_21550)* and *froI* (*PA14_21560*), respectively [22]. Overexpression of *froR* upregulates *fro,*

44 whereas overexpression of *froI* downregulates *fro* [3]. Flow activates FroR-dependent transcription of

45 *froABCD* through an unknown mechanism [3].



46
47 **Figure 1. Activation of *fro* promoter transcription by flow and during infection of human tissue.**
48 **(A)** RNA-seq heatmap of *P. aeruginosa* genes that are upregulated by at least 3-fold by flow and during
49 infection in human wounds. Datasets are from [3,18]. **(B)** Schematic of a *P. aeruginosa* fluorescent
50 reporter strain that contains a transcriptional fusion of *yfp* to the *fro* promoter and expresses mCherry
51 under a constitutive promoter. **(C)** Representative phase contrast, fluorescence, and merged images of
52 the *P. aeruginosa* reporter strain co-incubated with neutrophils, macrophages, or host cell-free medium.
53 Dashed lines indicate boundaries of the host cells and white arrows indicate *P. aeruginosa* with high
54 YFP/mCherry ratios. Images are representative of three independent experiments. Scale bars represent
55 5 μm.
56

57 We show that the *fro* operon responds directly to HOCl, which is produced by neutrophils during

58 immune response. Using a fluorescent reporter system in live cells and through quantitative

59 measurements of transcription, we show that *fro* expression is upregulated in a mouse model of corneal

60 infection and by activated neutrophils. Through transcriptional profiling, we determine that *fro* upregulation

61 mitigates the toxic effects of HOCl by activating methionine sulfoxide reductases that relieve oxidative

62 stress in bacteria. These data suggest a novel mechanism by which bacterial defense against host innate
63 immunity is primed by a single system to detect host defense cues through both flow and the presence of
64 the highly potent oxidative factor HOCl.
65
66 **RESULTS**

67 **Host cells stimulate *fro* expression in *P. aeruginosa* under non-flow conditions**

68 We investigated whether the *fro* operon is activated directly by immune cells. To quantify *fro*
69 expression, we used a *P. aeruginosa* strain that co-expresses the yellow fluorescent protein (YFP) under
70 the transcriptional control of the *fro* promoter, and mCherry under the control of a constitutive promoter
71 (Fig. 1B and [3]). While *fro* expression increases under flow (Fig. S1A in the Supplementary Information
72 (SI) and [3]), experiments in this study were performed in the absence of flow in order to deconvolve the
73 effects of flow from potential activation of *fro* by immune cells. Neutrophils and macrophages are among
74 the first immune cells that are recruited to sites of infection. We co-incubated *P. aeruginosa* with
75 neutrophils or macrophages for 30 minutes to determine whether they would elicit a response in the *fro*
76 operon. Relative to *P. aeruginosa* that were incubated in host cell-free media, *P. aeruginosa* in close
77 proximity to human neutrophils or that were engulfed in mouse macrophages appeared to increase *fro*
78 expression (Fig. 1C), suggesting that close proximity to or direct interaction with these host cells stimulate
79 *fro* expression in *P. aeruginosa*.
80

81 **Activated neutrophils induce *fro* expression**

82 We focused on the potential activation of *fro* expression by neutrophils because *P. aeruginosa*
83 triggers respiratory bursts in neutrophils [16], which generates ROS and RCS. We hypothesized that *fro*
84 activation could be a response to respiratory burst products. *P. aeruginosa* were incubated in conditioned
85 medium in which respiratory bursts were induced in human neutrophils using phorbol myristate acetate
86 (PMA) [23,24] (Fig. 2A). The expression of *fro* was quantified by averaging the ratios of YFP to mCherry
87 fluorescence for at least one hundred individual *P. aeruginosa*. In support of our hypothesis, conditioned
88 medium from PMA-stimulated neutrophils increased expression of *fro* expression by 30-fold compared to
89 conditioned medium from unstimulated neutrophils (Fig. 2B). The increased *fro* expression was not due to

PMA alone, as PMA-treated medium without neutrophils had no effect on *fro* expression (Fig. 2B). Conditioned medium from unstimulated neutrophils had no significant effect on *fro* expression compared to medium alone (Fig. 2B), suggesting that the activation of *fro* is due to the products of respiratory bursts.

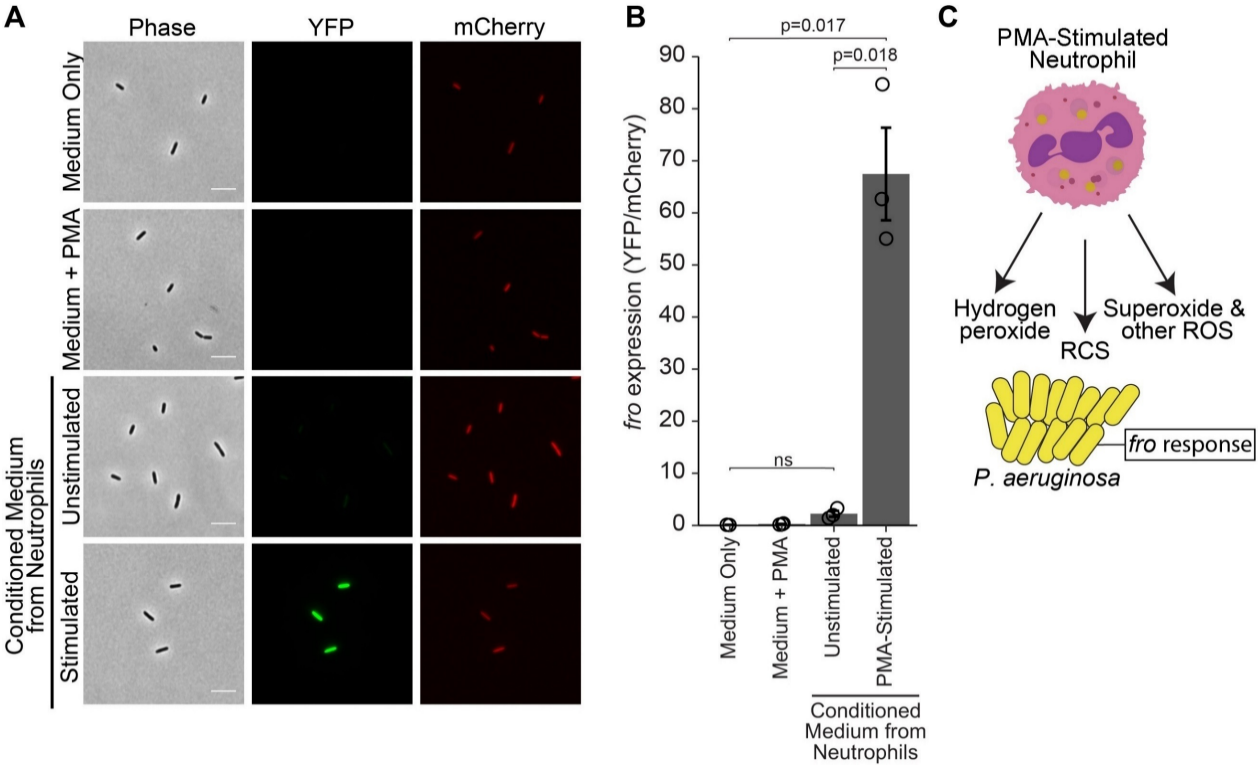


Figure 2. Conditioned medium from stimulated neutrophils induces *fro* expression. **(A)** Representative phase-contrast and fluorescence images and **(B)** *fro* expression as determined by ratios of YFP to mCherry fluorescence, of *P. aeruginosa* that were cultured in medium only, medium with PMA, conditioned medium from PMA-stimulated neutrophils, or conditioned medium from unstimulated neutrophils. Scale bars represent 5 µm. Data points indicate an average from at least one hundred individual *P. aeruginosa*. Gray columns indicate the mean of at least three independent experiments and error bars represent the standard error of the mean (SEM). P-values were obtained using two-tailed t-tests with unequal variances and values of p>0.05 are denoted as ns (nonsignificant). **(C)** Schematic depicting that PMA-stimulated neutrophils produce respiratory burst products that could activate *fro* expression.

HOCl but not H_2O_2 or HNO_3 induces *fro* expression

Activated neutrophils generate ROS and RCS through respiratory bursts (Fig. 2C), which have an inhibitory effect on bacterial growth [6,7,10,13,14]. To explore the hypothesis that these reactive species induce *fro* expression, we measured the effects of NaOCl, H_2O_2, and HNO_3 on *fro* expression at concentrations just below the minimum inhibitory concentrations (MICs) (Fig. 3A-B). NaOCl increased *fro* expression by up 74-fold at 1 µM (Fig. 3B). At 2 µM, NaOCl caused only a 3-fold increase in *fro*

expression but these bacteria were noticeably smaller in size (Fig. S1B in the SI), raising the possibility that the treatment could have interfered with transcription. The activation of *fro* transcription by NaOCl was further assessed using reverse transcription quantitative PCR (RT-qPCR). In support of the *fro-yfp* reporter findings, a 200-fold increase in *fro* transcription was observed using 1 μM NaOCl (Fig. 3C).

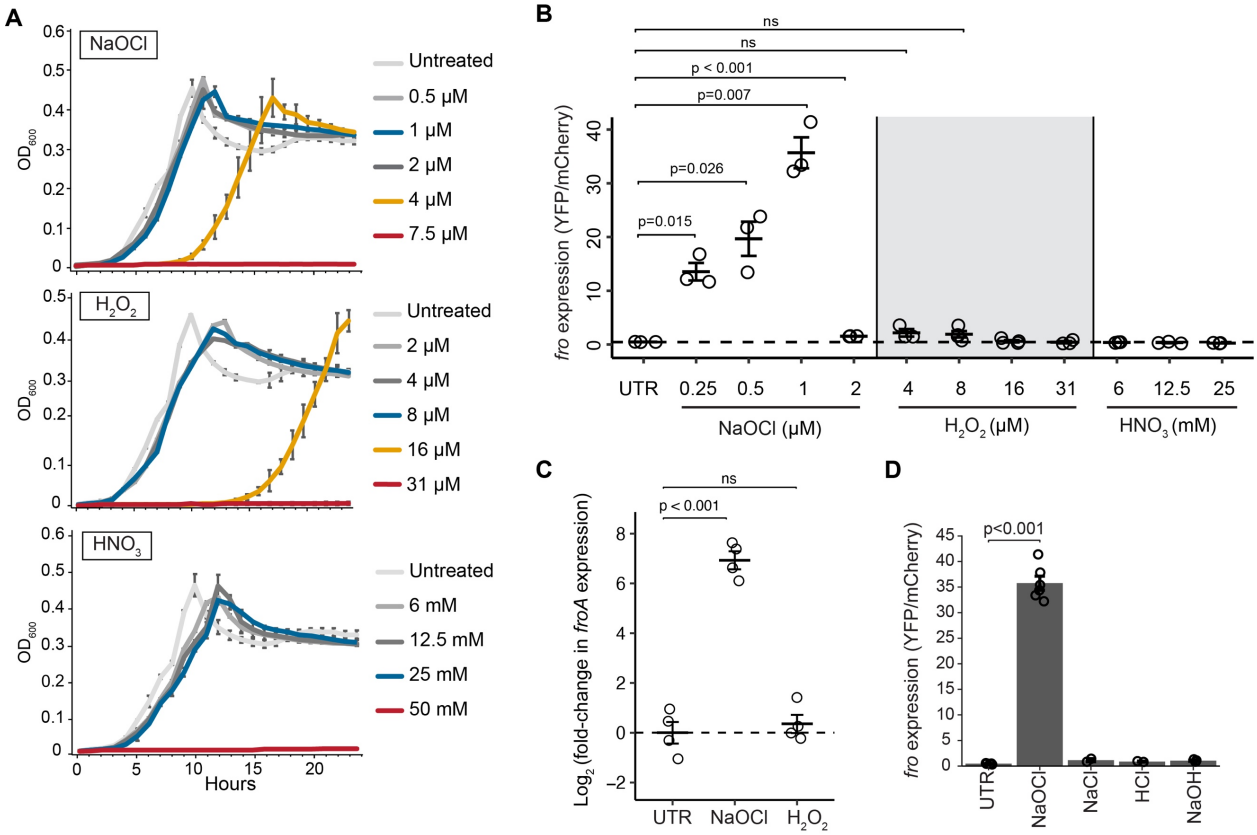


Figure 3. NaOCl but not H_2O_2 or HNO_3 induces *fro* expression.
(A) Growth profiles as measured by optical density (OD_{600}) of *P. aeruginosa* treated with NaOCl, H_2O_2, HNO_3, or no treatment. Data points indicate a mean of at least two independent experiments and error bars indicate SEM. **(B)** *fro* expression as determined by ratios of YFP to mCherry fluorescence in *P. aeruginosa* treated with NaOCl, H_2O_2, or HNO_3, or no treatment (UTR). Horizontal bars indicate the mean of at least three independent experiments and error bars indicate SEM. The dashed line indicates the average YFP/mCherry ratio from the untreated condition. **(C)** Abundance of *fro*A transcripts in *P. aeruginosa* following treatment with 1 μM NaOCl, 4 μM H_2O_2, or no treatment (UTR), as measured by RT-qPCR. Measurements were normalized to 5S ribosomal RNA and the logarithm (base 2) of the fold-change in transcription was computed relative to the untreated condition. Horizontal bars indicate the average of four independent experiments and error bars indicate SEM. **(D)** *fro* expression, measured by YFP to mCherry ratio, in *P. aeruginosa* after treatment with 1 μM NaOCl, 1 μM NaCl, 200 μM HCl, 6 mM NaOH, or no treatment (UTR). Error bars indicate SEM. In panels B and D, data points indicate the average from at least one hundred individual *P. aeruginosa*. P-values were obtained using two-tailed t-tests with unequal variances, with values of p>0.05 denoted as ns.

The increased *fro* expression was not due to sodium or chloride ions alone, as neither NaCl nor HCl affected *fro* expression in the absence of NaOCl (Fig. 3D). The *fro* response to NaOCl was not due to

134 basic pH, as treatment with up to 6 mM of NaOH had no significant impact on *fro* activation (Fig. 3D). As
135 *fro* activation is not due to sodium or chloride ions, nor changes in pH, we attributed the effects to
136 hypochlorite (OCl^-), which is in equilibrium with the hypochlorous acid species (HOCl) at the pH of 7.1
137 used in our experiments.

138 Changes in *fro* expression were assessed using H_2O_2, a strong oxidizer. Small increases in *fro*
139 expression were observed using the subinhibitory concentrations of 4 and 8 μM but these changes were
140 not statistically significant compared to untreated cultures (Fig. 3A-B), and no significant changes in *fro*
141 transcript abundance were observed using qRT-PCR (Fig. 3C). The *fro* response was also assessed
142 using subinhibitory concentrations of HNO_3, for which no change in expression was detected up to near-
143 MIC concentrations (Fig. 3A-B). Together, these results show that Fro expression is activated by HOCl
144 and suggest that HOCl produced by stimulated neutrophils could activate *fro* expression.
145

146 **Corneal infection activates *fro* expression**

147 *P. aeruginosa* is a major cause of corneal ulcers and keratitis, which can cause visual impairment
148 and blindness. Neutrophils are recruited within hours to infected corneal epithelia with neutrophil cell
149 density peaking at 18 hours [25]. We tested the hypothesis that *fro* expression is upregulated during
150 infection using a previously-established murine model of corneal infection in which *P. aeruginosa* strain
151 PAO1F is inoculated at the site of a corneal abrasion [26]. Mice corneas were harvested 2 or 20 hours
152 post-infection and *fro* transcripts were quantified using RT-qPCR. Transcription was normalized for
153 number of *P. aeruginosa* using 5S ribosomal RNA. We confirmed that this strain of *P. aeruginosa*
154 upregulates its *fro* transcription in response to NaOCl but not H_2O_2 (Fig. S1B in the SI), consistent with
155 our previous results. In support of the hypothesis, *fro* expression increased significantly by 20 hours post-
156 infection (Fig. 4A). Expression levels showed no significant change within the first two hours following
157 infection, which we attribute to the low level of immune cell recruitment during this period.

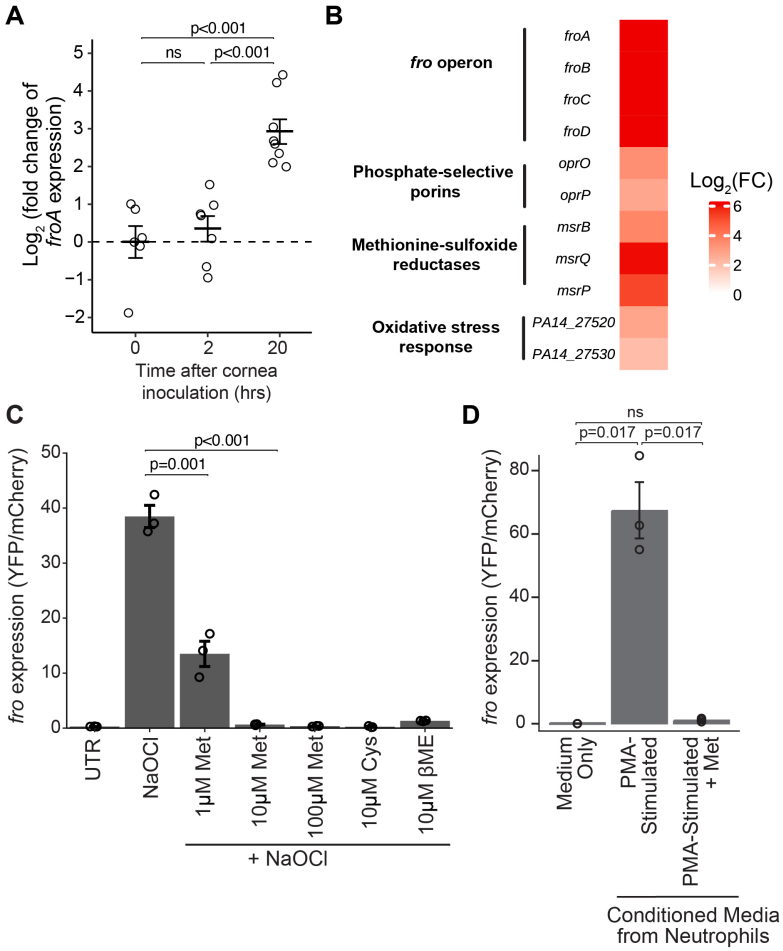


Figure 4. The expression of *fro* is activated during corneal infection and inhibited by methionine and antioxidants.
(A) Abundance of *fro*A transcripts in *P. aeruginosa* 0, 2, or 20 hours after being inoculated on a mouse corneal abrasion, as measured by RT-qPCR. Measurements were normalized to 5S ribosomal RNA and the logarithm (base 2) of the fold-change in transcription was computed relative to the initial inoculum. Data points represent individual experiments. Horizontal bars indicate the average of at least six independent experiments and error bars represent SEM. **(B)** Heatmap showing *P. aeruginosa* gene transcripts that were upregulated by at least 4-fold in wild type compared to the Δ*froR* mutant after treatment with 1 μM NaOCl and that had p-values less than 0.05 (n≥3), sorted by genomic locus. Raw values are in Table S1 in the SI. Log_2(FC) indicates the log_2 of the fold-change. **(C)** *fro* expression, determined by ratios of YFP to mCherry, in *P. aeruginosa* with no treatment (UTR), or treated with 1 μM NaOCl, or 1 μM NaOCl with methionine (Met), cysteine (Cys), or β-mercaptoethanol (βME). **(D)** *fro* expression, determined by ratios of YFP to mCherry, in *P. aeruginosa* after incubation with conditioned medium only (replotted from Fig. 2B), conditioned medium from stimulated neutrophils, or in conditioned medium from stimulated neutrophils with 100 μM methionine. Data points indicate an average from at least one hundred individual *P. aeruginosa*. Gray columns indicate the mean of at least three independent experiments and error bars represent SEM. P-values were obtained using two-tailed t-tests with unequal variances and values of p>0.05 are denoted as ns.

Methionine sulfoxide reductase upregulation requires FroR

To understand how the HOCl response is regulated by *fro*, we performed transcriptional profiling of wild-type (WT) *P. aeruginosa* and a strain containing a deletion of FroR, which is the sigma factor that

180 is required for *fro* activation [3]. The genes that were most upregulated by NaOCl in WT compared to the
181 Δ*froR* mutant were in the *fro* operon, the phosphate-specific and pyrophosphate-specific outer membrane
182 porin genes *oprO* and *oprP,* and the methionine sulfoxide reductase genes *msrB, msrQ* and *msrP* (Fig.
183 4B). The methionine sulfoxide reductases (MSRs) have an essential role in bacterial survival and defense
184 against RCS stress. HOCl oxidizes methionine residues 100-fold more rapidly than other cellular
185 components, forming methionine sulfoxide, which impairs protein function [27–31]. MSRs restore proper
186 protein function by reducing methionine sulfoxide back to methionine [11,32]. Our data suggest that *froR*
187 is required for the upregulation of MSRs in response to HOCl stress. It is possible that oxidized
188 methionine increases *fro* expression, which in-turn increases MSR expression.
189 We reasoned that if oxidized methionine upregulates *fro*, an excess of methionine should inhibit
190 *fro* activation. In support of our interpretation, methionine co-treatment with NaOCl suppressed *fro*
191 expression (Fig. 4C). We hypothesized that if *fro* expression is activated by HOCl oxidation, antioxidants
192 such as cysteine and β-mercaptoethanol (βME) should suppress *fro* activation by functioning as
193 alternative oxidation targets. Indeed, co-treatment of NaOCl with either cysteine or βME significantly
194 suppressed NaOCl-activated *fro* expression (Fig. 4C and Fig. S2 in the SI).
195 We considered how *fro* could be upregulated by stimulated neutrophils in the context of these
196 findings. RCS produced by neutrophils could oxidize methionine in *P. aeruginosa*, resulting in *fro*
197 upregulation. Under this interpretation, supplying excess methionine should inhibit activation. Indeed,
198 supplementing conditioned media from PMA-stimulated neutrophils with methionine completely
199 suppressed *fro* expression (Fig. 4D). Together, these data suggest a model in which *fro* responds to
200 HOCl-induced oxidation of methionine by upregulating MSRs.
201

202 **FroR protects *P. aeruginosa* against HOCl**

203 The upregulation of MSRs is expected to improve bacterial growth against HOCl stress. We
204 measured the growth profiles of WT and Δ*froR* strains of *P. aeruginosa*. Treatment with 16 μM NaOCl
205 significantly inhibited the growth of the wild-type strain (Fig. 5A) but resulted in even greater growth
206 inhibition in the Δ*froR* strain. This observation suggests that the *fro* operon improves *P. aeruginosa*
207 tolerance to HOCl stress.

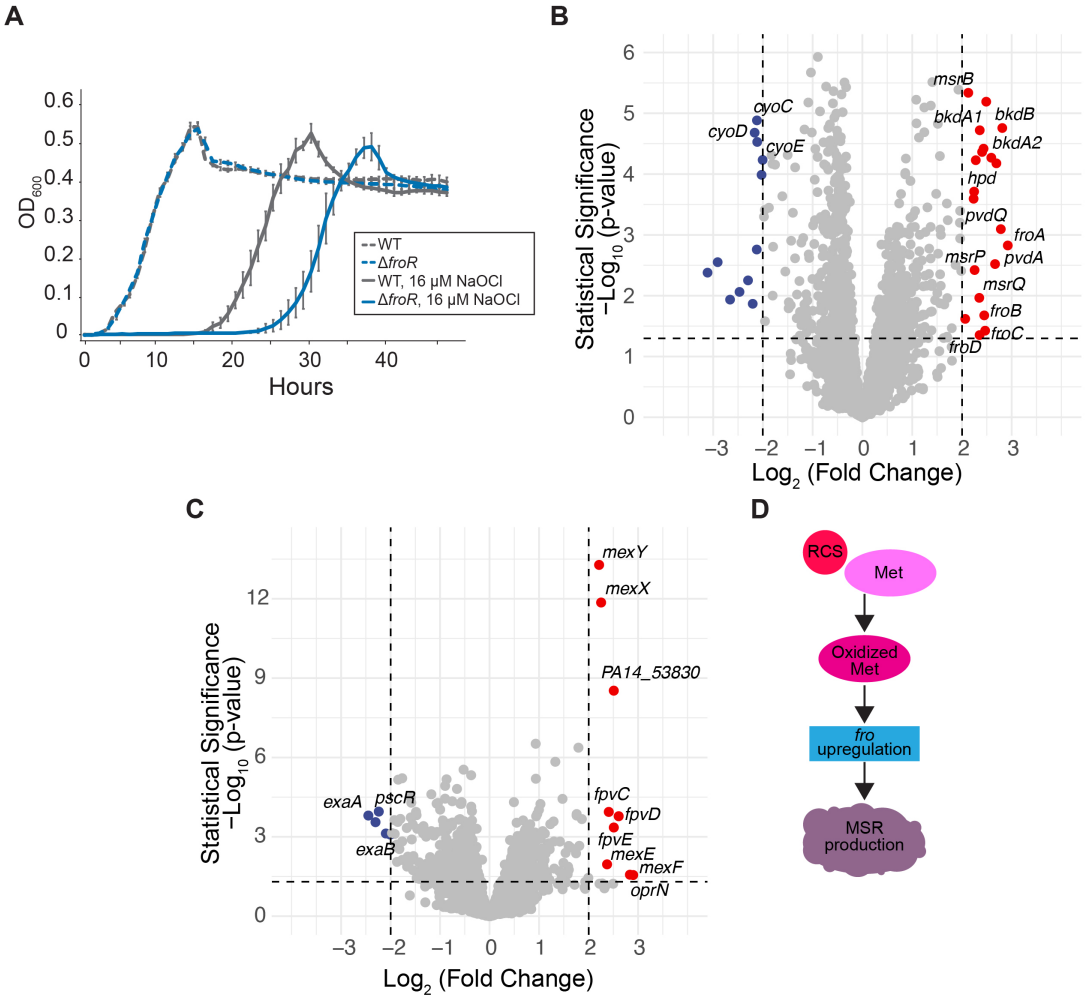


Figure 5. FroR improves *P. aeruginosa* growth against HOCl stress and regulates transcription of genes involved in antioxidant defense.
(A) Growth profiles of wild-type *P. aeruginosa* (WT) and Δ*froR* mutants treated with 16 μM NaOCl or untreated. Data points represent an average of at least three independent experiments and error bars indicate SEM. **(B-C)** Statistical significance as a function of fold-change in transcript abundance in **(B)** WT and **(C)** *ΔfroR P. aeruginosa* after treatment with 1 μM NaOCl (n≥3). Genes in red (upregulated) and blue (downregulated) were altered by at least four-fold (raw values in Table S2-S3 in the SI). The unlabeled genes have not been previously annotated. P-values were determined using the Wald test. Greater values along the vertical axis indicate greater statistical significance and the dashed horizontal line indicates a p-value of 0.05. **(D)** Schematic that depicts a model in which reactive chlorine species (RCS) reacting with methionine (Met) produces oxidized methionine, which upregulates *fro* and in-turn increases the production of methionine sulfoxide reductases.

We considered other genes which may improve WT *P. aeruginosa* tolerance to HOCl. HOCl increased the expression of iron uptake genes (*pvdA* and *pvdQ*) and downregulated the expression of oxidative phosphorylation genes (*cyoCDE*) (Fig. 5B and Table S2 in the SI). These changes suggest that HOCl causes an iron deficiency and decreases oxidative phosphorylation. In order to compensate for decreased oxidative phosphorylation, *P. aeruginosa* may utilize amino acid catabolism as an alternative

227 energy source, as evidenced by a concomitant upregulation of branched chain amino acid catabolism
228 genes (*hpd, bkdA1, bkdA2,* and *bkdB*) by HOCl (Fig. 5B and Table S2 in the SI).
229 While the Δ*froR* strain was sensitive to HOCl, the strain was tolerant (Fig. 5A), suggesting that
230 additional mechanisms relieve HOCl stress. In the Δ*froR* mutant, HOCl upregulated genes encoding
231 multidrug efflux pumps (*mexEF-oprN* and *mexXY*) (Fig. 5C and Table S3 in the SI). While these pumps
232 can relieve oxidative stress [33,34], they were not activated in the WT strain. This observation suggests
233 that the response to HOCl may be hierarchical, in which Fro-regulated and Mex pathways could be first-
234 line and second-line responses to HOCl stress, respectively. Supporting this interpretation, it has been
235 reported that treatment of the WT strain using significantly higher (millimolar) HOCl concentrations
236 upregulates *mexEF-oprN* [35] and the *mexXY* regulator *mexT* [36].
237
238 **DISCUSSION**
239 Neutrophils are activated by the presence of bacteria that infect host tissue. In their activated
240 state, neutrophils generate respiratory bursts that produce an abundance of HOCl. Whether and how
241 bacteria respond to activated neutrophils has not been clear. We have demonstrated that *P. aeruginosa*
242 responds to activated neutrophils by upregulating the transcriptional expression of the *fro* operon, which
243 relieves HOCl stress. Our data suggest that the upregulation of *fro* could function as a bacterial defense
244 mechanism against neutrophil attack, thus improving *P. aeruginosa* survival and colonization of human
245 tissue during infection.
246 One of the striking features of the *fro* system is that it is finely tuned to activate at sub-lethal
247 concentrations (1-2 μM) of NaOCl. Activated neutrophils produce HOCl concentrations as high as 50 μM
248 [24]. The activation of *fro* at relatively low concentrations suggests that the system functions as an early
249 and sensitive detector of HOCl. In hosts, *fro* may be upregulated in environments where HOCl is present
250 in low concentrations, such as during early stages of neutrophil recruitment in the cornea or when *P.*
251 *aeruginosa* is spatially distant from activated neutrophils. The early activation of *fro* could benefit *P.*
252 *aeruginosa* by triggering a protective mechanism before HOCl reaches lethal concentrations. The ability
253 of the Fro system to respond to the presence of fluid flow provides an additional layer of early activation,
254 such as in the case of host circulatory systems. Shear rates that activate *fro* [3] could prime *P. aeruginosa*

255 defenses against circulating neutrophils. Consistent with this interpretation, shear rate sensitizes Fro to
256 H_2O_2 [17], which is also produced by neutrophils in response to the presence of bacteria [6].
257 Based on the observations that multi-drug efflux pumps only activate in the absence of FroR and
258 at high HOCl concentrations [35], and that multiple systems repair RCS damage [1–3], the HOCl
259 response is likely to follow a hierarchy of activation. Our data suggest a model in which the *fro* system is
260 the first-line response that suppresses the toxic effects of HOCl towards methionine (Fig. 5D). FroR
261 activates two distinct classes of reductases that repair RCS-oxidized methionine: MsrB reduces
262 methionine sulfoxide with electrons provided by the thioredoxin system [37,38], and MsrPQ uses
263 electrons from the respiratory chain [39]. The activation of both reductase classes enables *P. aeruginosa*
264 to limit RCS stress in both the cytoplasmic and periplasmic spaces by utilizing multiple electron donor
265 sources.
266 Bacteria that are exposed to low concentrations of HOCl have higher rates of horizontal gene
267 transfer and can acquire antibiotic resistance genes more rapidly [40,41]. The *fro* system could thus
268 facilitate antibiotic resistance through increasing *P. aeruginosa* tolerance to HOCl. Our findings thus
269 suggest that the *fro* pathway could be an important defense mechanism to target in the development of
270 novel antibiotic therapeutics. Inhibition of this pathway could increase *P. aeruginosa* susceptibility to
271 neutrophil-mediated killing while minimizing antibiotic resistance.
272
273 **MATERIALS AND METHODS**

274 **Strains, Growth Conditions, and Reagents**

275 Experiments were performed using the *P. aeruginosa* strain AL143, which is strain PA14 strain
276 that contains the *yfp* gene integrated between *froA* and *froB* and a constitutively-expressed *mCherry* gene
277 [3], Δ*froR* and Δ*froI* mutant strains that were previously constructed [3], and *P. aeruginosa* strain
278 PAO1F[42,43]. Strains were streaked onto LB-Miller (BD Biosciences, Franklin Lakes, NJ) petri dishes
279 containing 2% Bacto agar (BD Biosciences, Franklin Lakes, NJ), incubated overnight at 37 °C, and single
280 colonies were inoculated into 2mL of modified MinA minimal medium (60.3mM K_2HPO_4, 33.0mM
281 KH_2PO4, 7.6mM $(NH_4)_2SO_4$, 1.0mM $MgSO_4$,) [44] containing 0.2% (w/v) sodium citrate as the carbon
282 source. Strains were cultured in MinA minimal medium for 18 hours in a roller drum spinning at 200 rpm

283 at 37 °C, diluted 1:1000 into fresh MinA minimal medium supplemented with oxidative or antioxidative
284 agents, or were not supplemented and imaged using fluorescence microscopy. *P. aeruginosa* cultures
285 were incubated with NaOCl, H_2O_2, HNO_3 for 3 hours unless otherwise stated at 37 °C before imaging.
286 Neutrophils were isolated from blood samples from healthy donors between 18 to 65 years old
287 that were collected at the Institute for Clinical and Translational Science at the University of California,
288 Irvine, through approved protocols with the UCI Institutional Review Board HS#2001-2058, and informed
289 consent was obtained from all donors. Neutrophils were prepared as described in [45]. Briefly, 3%
290 dextran (from Leuconostoc spp, M_r 450,000-650,000, Sigma-Aldrich, St. Louis, MO) in PBS (Gibco,
291 ThermoFisher, Waltham, MA) was used to separate red blood cells (RBCs) from whole blood. Neutrophils
292 were purified from the remaining cells by overlaying on a Ficoll density gradient (GE Healthcare, Chicago,
293 IL) following centrifugation in Ficoll-Paque Centrifugation Media (GE Healthcare, Chicago, IL) for 25
294 minutes at 500 × g. The remaining RBCs were lysed using RBC Lysis Buffer (Fisher Scientific, Hampton,
295 NH), and neutrophils were resuspended in RPMI 1640 medium (ATCC, Manassas, VA) and purity was
296 assessed by flow cytometry using the ACEA NovoCyte Flow Cytometer and fluorescent antibodies APC-
297 CD11b, FITC-CD16 and PE-CD66b (eBioscience, San Diego, CA). Neutrophils were immediately used
298 for neutrophil stimulation or co-incubation experiments with *P. aeruginosa*. This procedure routinely
299 yielded greater than 95% CD11B-positive neutrophil populations.
300 J774.1 mouse macrophages (ATCC) were cultured in 10 mL DMEM (Gibco, ThermoFisher) in
301 T75 flasks (VWR, Radnor, PA) at 37 °C with 5% CO_2. Macrophages were passaged every three days
302 through scraping and were passaged every 5 days through trypsinization.
303 For *P. aeruginosa* size analysis experiments, cultures were incubated at 37 °C in 250 mL flasks
304 for 3 hours with shaking at 225 rpm following the 1:1000 dilution. For methionine and antioxidant
305 treatments, following the 1:1000 dilution, cultures were supplemented with NaOCl and the addition of
306 methionine, β-mercaptoethanol (βME) or cysteine. 50 mL of each bacterial culture was incubated at 37 °C
307 in 250 mL flasks for 3 hours with shaking at 225 rpm, imaged, and analyzed as described in the
308 fluorescence microscopy section.

309 **Fluorescence and Phase Contrast Microscopy**

310 Bacteria were immobilized on 1% agarose pads containing minimal medium and imaged
311 immediately. Cultures containing densities below 10 *P. aeruginosa* per frame were concentrated using a
312 syringe filter with 0.2 or 0.8 μm pore sizes (Millipore, Burlington, MA). Images were acquired using a
313 Nikon Eclipse Ti-E microscope (Nikon, Melville, NY) containing a Nikon 100X Plan Apo (1.45 N.A.)
314 objective, a Nikon Ph3 phase contrast condenser annulus, a Sola light engine (Lumencor, Beaverton,
315 OR), an LED-DA/FI/TX filter set (Semrock, Rochester, NY) for visualizing the mCherry fluorescence
316 spectrum containing a 409/493/596 dichroic and 575/25 nm filters for excitation and 641/75 nm filters for
317 emission, an LED-CFP/YFP/MCHERRY filter set (Semrock) for visualizing YFP fluorescence containing a
318 459/526/596 dichroic and 509/22 nm filters for excitation and 544/24 nm filters for emission, and a
319 Hamamatsu Orca Flash 4.0 V2 camera (Hamamatsu, Bridgewater, NJ).
320 Images were acquired using Nikon NIS-Elements and analyzed using custom built software
321 written previously [46] in Matlab (Mathworks, Natick, MA). See the "Data, code, and materials availability"
322 section below to download code. Briefly, *P. aeruginosa* masks were determined from phase contrast
323 images using an edge-detection algorithm. For size analysis, the total pixel area of each individual *P.*
324 *aeruginosa* was determined by computing the mask area and converting from pixels to μm^2 by multiplying
325 the mask area by a factor of 0.004225 μm^2/pixel to account for the microscope camera pixel size and
326 objective magnification. *Fro* expression was quantified by averaging computed ratios of YFP to mCherry
327 fluorescence for at least one hundred individual *P. aeruginosa*. In each experiment, a minimum of 100 *P.*
328 *aeruginosa* were imaged and used for analysis in which ratios were computed from the masked areas. To
329 image *P. aeruginosa* under flow, microfluidic devices were fabricated using standard soft lithography
330 techniques as established previously [47] with channel dimensions of 100 μm x 50 μm (width x height)
331 and a flow rate of approximately 5 μL/min.

332 **Co-incubation of Mammalian Cells with *P. aeruginosa***

333 *P. aeruginosa* were cultured for 18 hours in minimal medium, centrifuged at 4600 x g for 2
334 minutes and resuspended in DPBS (Gibco, ThermoFisher) three times, and diluted to a final OD_{600} of 0.2.
335 Macrophages that were harvested from growth flasks or freshly prepared neutrophils were washed three
336 times by centrifuging at 300 x g for 5 minutes and resuspending in DPBS, and 1 mL aliquots containing
337 $4x10^5$ cells/mL were transferred into flat-bottom dishes. *P. aeruginosa* culture was added to the cells at a

338 multiplicity of infection of 10 and incubated at 37 °C with CO_2 for 30 minutes. Dishes were aspirated until
339 approximately 20 µL of medium remained and pads consisting of 1% agarose containing DPBS were
340 placed on top of the samples in the dishes to immobilize bacteria and cells. Samples were imaged
341 immediately using microscopy.

342 **Conditioned Medium from Neutrophils**

343 Approximately $2x10^6$/mL of freshly harvested neutrophils were incubated at 37 °C in a roller drum
344 spinning at a speed of 4 rotations per minute in PBGT medium (10 mM phosphate buffer pH 7.4 (2.46
345 mM monobasic with 7.54 mM dibasic sodium phosphate) containing 140 mM sodium chloride, 10 mM
346 potassium chloride, 0.5 mM magnesium chloride, 1 mM calcium chloride, 1 mg/mL glucose, and 5 mM
347 taurine [24]). To activate neutrophils, cultures were supplemented with 100 ng/mL of PMA for 1 hour and
348 were separated from the supernatant by centrifugation for 5 minutes at 4,600 x g and 25 °C. The
349 supernatant was isolated and used as conditioned medium. *P. aeruginosa* cultures were incubated for
350 3 hours in conditioned medium at 37 °C before imaging.

351 **Growth Curves**

352 Growth curve experiments were performed using a Synergy HTX multi-mode plate reader and
353 sterile, tissue-culture treated, clear bottom, black polystyrene 96-well microplates (Corning, Corning, NY)
354 containing 200 µL of culture in each well. The temperature set point was 37 °C and preheated before
355 beginning measurements. For experiments performed with stationary phase bacteria, overnight cultures
356 of bacteria were grown for 18 hours at 37 °C in the roller drum at 200 rpm to saturation, diluted into
357 minimal medium to an optical density at 600 nm (OD_{600}) of 0.01 containing NaOCl, H_2O_2, HNO_3 or no
358 supplement. Plates were incubated at 37 °C with continuous orbital shaking with an amplitude of 3 mm at
359 the frequency of 180 cycles per minute and measured for OD_{600} every 20 minutes.

360 **Reverse Transcription Quantitative PCR (RT-qPCR)**

361 Strains were cultured overnight, diluted 1:1000 into MinA medium, incubated in a shaker at 225
362 rpm at 37°C for 2.5 hours, treated with 1 µM NaOCl, 4 µM H_2O_2, or untreated for 30 minutes,
363 concentrated using syringe filters, and centrifuged at 13,000 × g for 4 minutes to pellet *P. aeruginosa*.
364 Pellets were either snap-frozen in liquid nitrogen or processed immediately for RNA extraction using the

365 NucleoSpin RNA kit (Macherey-Nagel, Allentown, PA). cDNA was prepared using the High-Capacity
366 cDNA Reverse Transcriptase Kit (ThermoFisher). Quantitative PCR was performed using the
367 SsoAdvanced Universal SYBR Green Supermix (BioRad, Hercules, CA) using the C1000 Thermal Cycler
368 with a CFX96 real-time detection system (Bio-Rad). *froA* transcripts were quantified using the primers 5'-
369 TTTCCCTCGCTTCCTCCGTC-3' and 5'-ACCTTCCTTGGCCTTCTCGG-3', which target PA14_21570 or
370 PA3284 in PA14 or PAO1, respectively. The transcript abundance for each sample was normalized by 5S
371 rRNA abundance, which was determined using the primers 5'-TAGAGCGTTGGAACCACCTG-3' and 5'-
372 GAGACCCCACACTACCATCG-3' [48], yielding normalized count thresholds (Cts). The average fold
373 change in transcript abundance was determined by computing the ratio of the averaged normalized Cts
374 and raising by the power of 2, as performed previously [49].

375 **Murine Model of Corneal Infection**

376 The expression of *froA* was assessed in a murine model of corneal infection as described
377 previously[50,51]. Briefly, *P. aeruginosa* strain PAO1F was cultured to mid-exponential phase in modified
378 MinA minimal medium containing 0.05% citrate, 0.1% casamino acids (Gibco) and 0.2% glucose as the
379 carbon sources and resuspended in PBS (Gibco). Corneal epithelia of C57BL/6J mice aged 6-8 weeks
380 (Jackson Laboratory, Bar Harbor, ME) were abraded with 3x5 mm scratches using a 25 g needle, and 2
381 µL of PBS containing $5x10^4$ of *P. aeruginosa* strain PAO1F was applied topically. Comparable numbers of
382 male and female mice were used in each experimental group. Mouse eyeballs were collected and
383 homogenized in 1 mL PBS at 2 hours post infection or 20 hours post infection. The homogenate was
384 centrifuged for 5 minutes at 150 x g to remove corneal materials. Supernatant was centrifuged for 4
385 minutes at 13,000 x g. Pellets were suspended in lysis solution (10 mM Tris-HCl, 1 mM EDTA pH 8.0, 0.5
386 mg/mL lysozyme, 1% SDS) [49]. RNA was prepared using the NucleoSpin RNA kit (Macherey-Nagel) and
387 assessed for mRNA expression using RT-qPCR.

388 **RNA-seq Library Preparation and Data Analysis**

389 *P. aeruginosa* was cultured and RNA was prepared as described in the RT-qPCR section. RNA
390 yield was measured using a Nanodrop 2000 (Thermo Fisher, Waltham, MA). Samples were depleted of
391 ribosomal RNA using the NEBNext rRNA Depletion kit (New Enland Biolabs, Ipswich, MA), from which
392 cDNA libraries were constructed using the NEBNext Ultra II Directional Library kit (NEB), which were

393 sequenced by the UC Irvine Genomics Research & Technology Hub (Irvine, CA) using an Illumina
394 NovaSeq X Plus (Illumina, San Diego, CA) using paired-end 150 bp reads at a depth of approximately 10
395 M read per sample. Raw reads were checked with fastQC [52] (version 0.21.1), and trimmed and filtered
396 into paired and unpaired reads using Trimmomatic [53] (version 0.39) in Java (1.8.0) using the 'PE'
397 setting. Paired and unpaired reads were aligned to the PA14 genome (NCBI accession NC_008463.1)
398 using Bowtie2 [54] (version 2.5.4) using the default 'sensitive' mode, were counted using the
399 featureCounts program in Subread [55] (version 2.0.8) with the 'countReadPairs' option enabled for
400 paired-end reads, and were fit into a negative binomial model to compute fold-change in gene expression
401 using DeSeq2 [56] (version 1.40.2). The statistical significance of the changes were computed using the
402 Wald test in DeSeq2. Gene Ontology enrichment analysis for Fig. 1A was performed using
403 GOEnrichment [57] (version 2.0.1). The *msrQ* (PA14_62100), *msrP* (PA14_62110) genes were annotated
404 at Pseudomonas.com [21] as *yedZ* and *yedY*, respectively, but are referred to here with their updated
405 names [58].

406 **Statistical Analysis**

407 Statistical analysis and figures were generated in R (The R Foundation, Vienna, Austria) (version
408 4) and RStudio (Posit Software, Boston, MA).

409 **Data, Code and Materials Availability**

410 Raw data for all figures that contain statistical analyses are available in the Supplementary
411 Information Source Data file. RNA-seq data is available at the National Center for Biotechnology
412 Information Gene Expression Omnibus (GEO) (accession number GSE290217, *accessible during review*
413 *period using the token: whcbicwsvnmjdqj*). The custom Matlab scripts used to process and analyze
414 fluorescence microscopy data are freely available at https://github.com/asirya/AVIassembleGUI.
415

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